Deliverable Report

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WP Title:	Growth out husbandry - pikeperch			
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Task Title:	Effect of husbandry practices and environmental factors on pikeperch growth, immune and physiological status			
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I. Objectives

High mortality and unpredictable growth rates during the early developmental stages are major bottlenecks for the development of pikeperch (*Sander lucioperca*) aquaculture. These failures may be related to high stress responsiveness since they are often observed after procedures of aquaculture management. A previous study demonstrated that juveniles of Eurasian perch *Perca fluviatilis*, a percid species close to pikeperch, are more sensitive to aquaculture management than species with a long domestication history, such as rainbow trout (Jentoft et al., 2005). Moreover, several studies have demonstrated that high physiological stress may suppress the immune competence in fish and decrease their disease resistance (Barton, 2002; Stolte et al., 2008; Milla et al., 2010; Tort et al., 2011; Mathieu et al., 2014; Soltanian et al., 2014; Liu et al., 2016). Therefore, the objectives of this deliverable were:

(1) To characterize the effects of major husbandry and environmental factors on growth related parameters as well as on physiological and immune responses of cultured pikeperch.

(2) To identify the optimal husbandry and environmental conditions for improving the growth and the survival rates as well as the welfare of pikeperch in intensive culture.

These objectives were achieved through two in vivo experimental settings: (1) a multifactorial protocol comparing 16 combinations of husbandry and environmental modalities in order to identify the best culture conditions in which pikeperch exhibit the best immune defense capacity and thereby a good welfare status, (2) a confirmation experiment in which the best combinations characterized as being the less stressful modalities were compared to standard rearing modalities and in order to emphasize the immune defense capacity the reared pikeperch groups were subjected to an effective bacterial challenge. Before those two main experiments, preliminary assays were conducted in order to fine-tune some methodological aspects.

II. Background

During their young developmental stages, pikeperch are submitted to frequent grading procedures, about every two weeks, to avoid high size heterogeneity, which induces high mortality by increasing intra-cohort cannibalism. Despite that positive effect, grading manipulations may be stressful for the

overall juvenile populations since fish are submitted to netting and partial air exposure for about 1 min. 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, including changes in some key immune gene proteins such as lysozyme, TNFα and apolipoprotein A (Milla et al., 2010; Douxfils et al., 2014). Other stressors related to aquaculture husbandry such as frequent tank cleaning and moderate hypoxia may also impair significantly growth related parameters and various physiological pathways, including changes in some immune functions for Eurasian perch (Strand et al., 2007; Douxfils et al., 2012). However, the relationship between stress response and immune system has received little attention for pikeperch in intensive culture conditions.

Unsuitable light characteristics may induce high stress intensity, which may negatively affect growth processes in fish. It was especially 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).

The effects of photothermal conditions on pikeperch stress physiology are also reported inconsistently. Based on various metabolic indicators, it was reported that the optimal temperature for pikeperch is in the range between 10 and 27 °C (Frisk et al., 2012), but fish size should be considered when optimizing temperature levels in aquaculture production. According to some authors, better growth and feed utilization for ongrowing pikeperch are obtained under high temperature conditions ranging between 25-28°C (Rónyai and Csengeri, 2008; Wang et al., 2009; Dalsgaard et al., 2013). However, 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 pikeperch 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 pikeperch. Photoperiod manipulation should be appropriated to the feeding behavior 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; Yarahmadi et al., 2016) but limited information is available for pikeperch. Preliminary observations reported that high stocking density has no marked effects on growth and food utilization of young pikeperch, and that pikeperch juveniles can be kept at high densities ranging between 30–60 kg m^{-3} without any increase in physiological stress response (Molnar et al., 2004; Steenfeldt et al., 2010; Dalsgaard et al., 2013). However, another study reported that high density can increase the susceptibility to diseases for pikeperch juveniles (Jensen et al., 2011).

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. As an example, 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, Volkaert 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 related to aquaculture management and the immune competence is a relevant step for improving the performance of this species in intensive production systems.

III. Preliminary experiments

A. Stress response to emersion stress in pikeperch juveniles

Since there is limited information on stress and immune responses for pikeperch, it was necessary to standardize some methodological aspects before the actual start of the multifactorial experiment.

The objectives of the first refinement experiment were: (a) to determine the sensitivity of pikeperch to a single or repeated emersion stress, (b) to test whether dietary tryptophan may mitigate the primary stress response, and mitigate the overall effects of emersion stress.

Methodology: The fish used were juveniles of 10-12 g from Excellence Fish farm, Netherlands that were transferred to Namur on May 28, 2014. They were acclimatized to RAS and to the experimental feeds for about 3 weeks before being transferred to the experimental tanks (in triplicate), each stocked with 42 fish.

The experimental protocol included (a) complete removal of tank water for + 30 s - emersion (mimicking grading) once per week and (b) two experimental diets containing L-tryptophan (TRP).

Thus, four experimental variants were created:

- CT: control groups without any stress and feed additive,
- CTs: groups submitted to emersion stress but without any feed additive,
- 3TRPs: groups receiving 3-times more TRP in their diet than the control group (CT = 0.59% of tryptophan vs 3TRPs = 1.77%) and submitted to emersion stress,
- 6TRPs: groups receiving 6-times more TRP in their diet than the control group (CT = 0.59% of tryptophan vs 6TRPs = 3.54%) and submitted to emersion stress.

Six fish per tank were sampled on D0 (when also TRP feeding started), D7 (after the 1st emersion stress), D37 and D91 (after 5 or 15 emersion stress). For stressed fish, samplings (plasma, spleen, liver, brain and head kidney) were done 1h after the emersion manipulations. Fish from control groups were also sampled at the same time.

Plasma cortisol was assayed in duplicate using a cortisol ELISA kit (DRG, EIA-1887) and following the manufacturer's instructions (BioSource, Belgium). Cortisol was determined in one assay; the intra-assay coefficient of variation was 3.6% and the assay dynamic range was between 0 and 800 $ng \cdot ml^{-1}$. Plasma glucose was determined calorimetrically based on a glucose oxidase/peroxidase method described by Trinder (1969). Lysozyme activity serum samples were used for lysozyme assay according to the method of Siwicki and Studnicka (1987) adapted by Fatima et al. (2007).

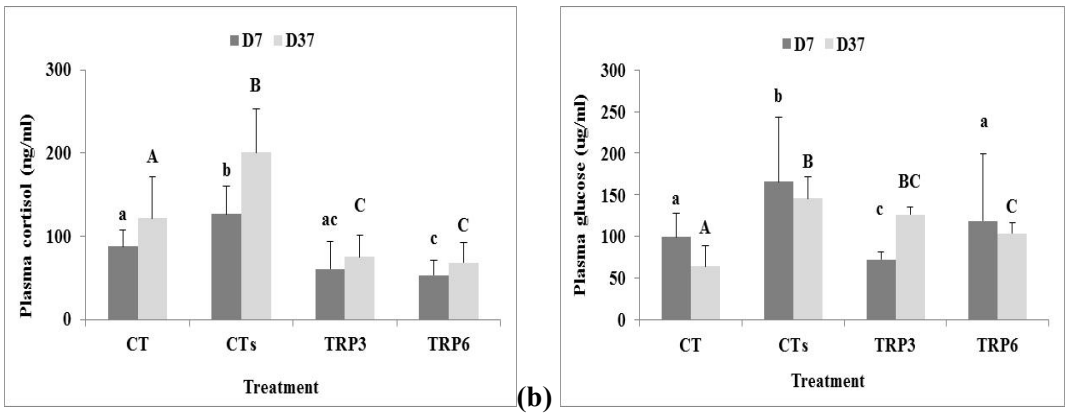
Data are expressed as the mean ± standard deviation (SD). Replicate tank was considered as the statistical unit (n = 3). Data were analyzed using a fixed two-way ANOVA. Normality and variance homogeneity were tested according to Kolmogorov–Smirnov and Bartlett tests respectively. “Ln” transformation was performed for cortisol and glucose values in order to fit with variance homogeneity requirements. Calculations were done using Statistica software (StatSoft, Tulsa, OK, USA).

Results and discussion

(a) Physiological and immune responses:

- *Cortisol response:* Control fish displayed high cortisol levels in plasma (CT = 88-122 ng ml^{-1}) (**Figure 22.1a**) compared to salmonids such as rainbow trout (<25 ng/ml, Jentoft et al., 2005) confirming a higher responsiveness or sensitivity of pikeperch to captive environmental conditions, possibly related to lack of domestication. Emersion stress induced a significant increase in plasma cortisol both after a single stress ($F=23.36$, $p=1.51*10^{-10}$) or repeated stress ($F=14.09$, $p=4.60*10^{-7}$). Dietary TRP supplementation induced a significant decrease in cortisolemia in a dose related manner, indicating that TRP may interfere with the primary stress response in pikeperch as already demonstrated for some salmonids (Basic et al., 2013).

- *Glucose response:* Emersion stress induced a significant increase in plasma glucose (**Fig. 22.1b**) both after a single stress ($F=15.96$, $p=1.50*10^{-7}$) or repeated stress ($F=7.71$, $p=0.00019$). Dietary TRP showed a trend of decrease after a single emersion on D7 of TRP supplementation but long-term dietary treatment did not seem effective in reducing stress-inducing glycaemia.



(a) **(b)**

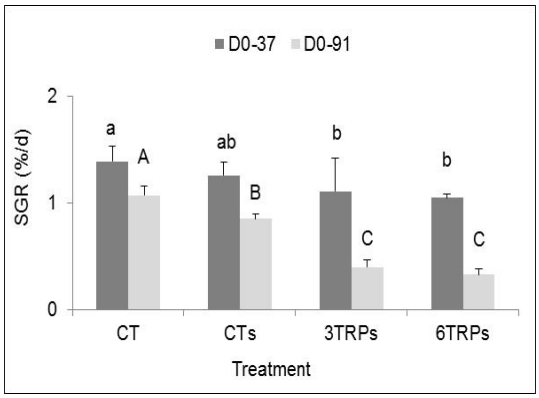
Figure 22.1a-b: Mean (±SD) plasma cortisol (a) and glucose (b) of pikeperch juveniles submitted once a week to emersion stress, D7: single stress, D37: repeated stress. CT or CTs = fish receiving the control diet or receiving the control diet and submitted to stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times the amount of TRP in the control diet, respectively.

(b) Growth and immune response to emersion stress:

Emersion stress once a week did not show marked effect on the growth rate (**Fig. 22.2.a**), neither on feed intake nor the feed conversion ratio (FCR, **Fig. 22.2.b**) at D37. However, after a long-term application of stress a significant decrease in growth was observed at D91 ($F=84.46$, $p=2.13*10^{-6}$) associated to low food utilization ($F=56.91$, $p=8.45*10^{-5}$).

TRP dietary supplementation induced a significant decrease in growth whatever the dose, more specifically after a long-term treatment at D91. The depression in growth rate by the long-term TRP supplementation was associated to a decrease in daily feed intake and to negative feed utilization for both doses.

(a)



(b)

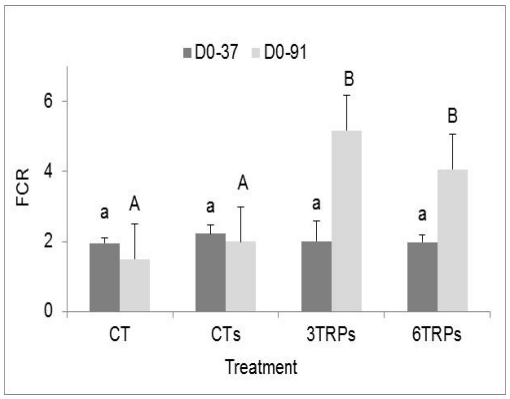


Figure 22.2: Mean (±SD) specific growth rate (SGR) (a) and food conversion ratio (b) of pikeperch juveniles submitted to emersion stress once a week during three months. CT or CTs = fish receiving the control diet or control diet and submitted to stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times the amount of TRP in the control diet, respectively.

Plasma lysozyme activity, a relevant indicator for innate immune system in fish (Sahoo et al., 2008) was used to determine the long-term effect of emersion stress on pikeperch juveniles. Emersion stress applied once a week during 3 months was not associated to significant decrease in plasma lysozyme activity indicating no marked effect on the innate immune system (**Fig. 22.3**). In contrast to what was expected, dietary TRP treatments highly ($P < 0.01$) decreased plasma lysozyme activity, as observed for growth rate. Previous studies reported that the effects of cortisol reduction by TRP supplementation are not consistent and may depend on fish species and experimental conditions (Basic et al, 2013; Machado et al, 2015).

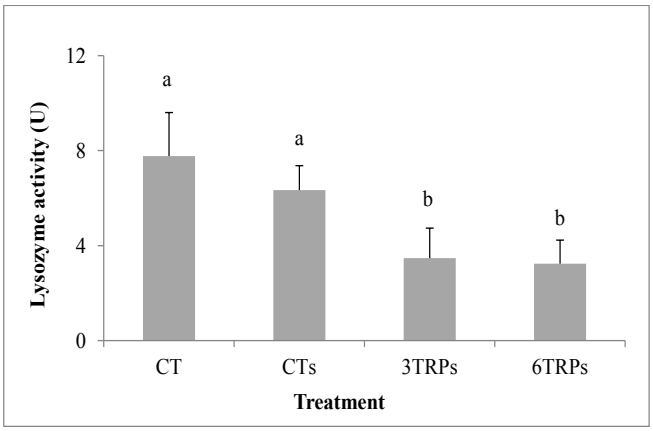


Figure 22.3: Mean (±SD) plasma lysozyme activity of pikeperch juveniles after 91 days of emersion stress challenge. CT or CTs = fish receiving the control diet or control diet and submitted once a week to emersion stress. TRP3 or TRP6 = fish submitted to stress and supplemented with 3 or 6 times the amount of TRP in the control diet, respectively.

In conclusion, the results seem to indicate that pikeperch juveniles are highly sensitive to aquaculture manipulations such as grading. They also indicate that long-term application of grading manipulations may depress growth and some innate components.

B. Resilience response of emersion stress in pikeperch

- *The objective* was to determine the optimal time for sampling in order to minimize as much as possible the β error during the multifactorial experiment since it was planned to evaluate at the same time stress indicators from 16 modality combinations. In this regard, our previous study demonstrated that for evaluation of stress-induced cortisol in Eurasian perch fish, sampling should be done within 5 min in order to avoid extra secretion artifacts related to manipulations.
- *Methodology:*

- *Experimental design:*

. About 370 pikeperch juveniles (100 g) from Asialor were transferred from France to Namur on February 16, 2015.

. Two experimental groups were compared in triplicate tanks of 30 fish each:

(a) Control treatment without emersion during all the experiment,

(b) Fish submitted to emersion stress: once a week for 30 sec (D7 and D15).

. Fish allocated to the two treatments were reared in separate RAS to avoid any release of stress components through the water tank.

- *Sampling schedule:*

. Emersion stress challenges: D0, D7, and D15

. Serial samplings: on D15, hours post-stress 0, 0.5, 1, 3 and 8.

- *Analytical methods*: Levels of plasma cortisol, glucose and lysozyme activity, as well as statistical analyses, were determined as done for the 1st preliminary essay.

Results and discussion

- *Plasma cortisol and glucose:*

As for the first preliminary essay, stress indicators confirmed a high sensitivity of pikeperch juveniles to emersion stress. Moreover, the serial sampling model showed a transient primary response in terms of cortisol profile (**Fig. 22.5.a**) in stressed fish culminated 30 minutes after emersion, and the recovery was observed just after 1h. However, in terms of plasma glucose (**Fig. 22.5.b**) it seemed that the stress response was sustained more than 3 h indicating the interest for using various stress indicators to account for the stress responsiveness in pikeperch.

- *Innate immune response:*

As observed during the first preliminary experiment, emersion stress did not affect significantly the level of lysozyme activity (**Fig. 22.6**) after 15 days of application, but other immune parameters should be considered to have a large overview concerning the immune defense capacity.

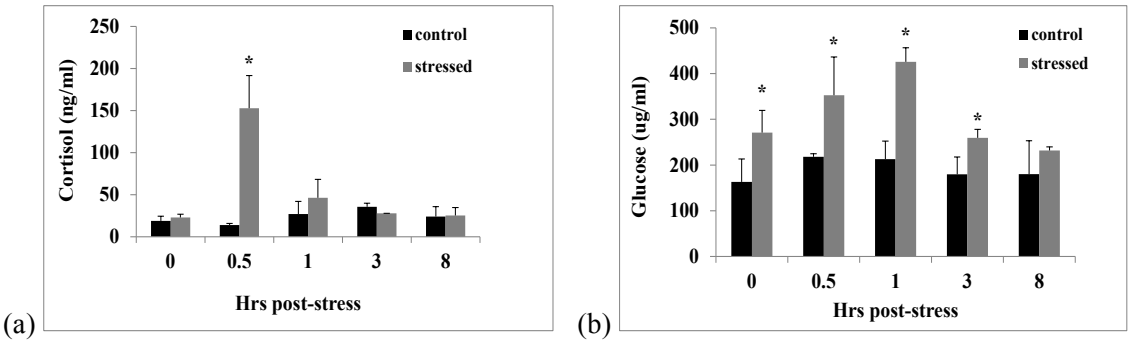


Figure 22.5.a-b: Mean (±SD) plasma cortisol (a) and glucose (b) of pikeperch juveniles submitted to serial samplings after 2 weeks of emersion challenge (30 sec, once a week).

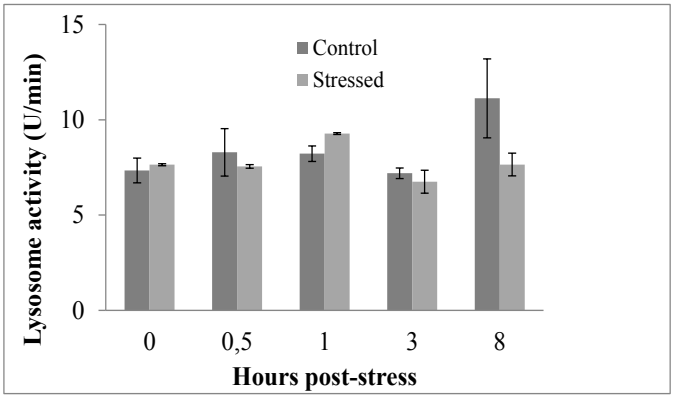


Figure 22.6: Mean (±SD) plasma lysozyme activity of pikeperch juveniles submitted to serial samplings after 2 weeks of emersion challenge (30 sec, once a week).

IV. Multifactorial experiment

A. Materials and methods

1. Experimental design

A multifactorial approach based on a fractional factorial design was used to identify the most suitable husbandry and environmental conditions for pikeperch. Such experimental design has been validated as a powerful approach screening for various purposes and has various advantages, such as: (a) evaluating the possible interactions between tested factors, (b) classifying the relative importance of all factors (main effects and interaction groups), and (c) determining the combinations of factors that would be required to improve the rearing system (Hamre et al., 2004; Gardeur et al., 2007; Teletchea et al., 2009).

For this multifactorial protocol, 8 factors (**Table 22.1**) were selected taking into account the current practices in various pikeperch farms, and the few available results concerning the stress sensitivity in percid fish. Two modalities considered as high or low level were applied (**Table 22.2**) for each factor, so 16 experimental conditions were compared (**Table 22.2**). Rather than the 256 runs that would be required for a full 2^8 factorial experiment, this fractional factorial design required only 16 experimental units.

Table 22.1: Selected factors and modalities

Factors	Levels
Photoperiod (h)	10 : 14
	24 : 00
Light intensity (lux)	10
	100
Spectrum	White
	Red
Density (kg/m^3)	15
	30
Temperature (°C)	21
	26
Oxygen saturation (%)	60
	90
Food	Floating
	Sinking
Grading	Yes
	No

The experimental units were separated between each other since each experimental combination represented a unique variant rearing system. So, the preparation of the experimental systems took more time than expected, so it was not possible to start the multifactorial experiment at the time indicated in the DOW.

Table 22.2: Experimental factors-modalities (= C experimental conditions)

Exp. Conditions (n°)	Light intensity (lux)	Density (kg.m^{-3})	Light spectrum	Photoperiod (h)	Water temperature (°C)	Feed Type	Grading	Oxygen saturation (% O_2)
1	10	30	white	24	21	sinking	Y	90
2	100	15	red	10	26	floating	-	60
3	100	15	white	24	21	sinking	-	60
4	100	30	red	10	21	sinking	-	90
5	10	15	red	10	21	sinking	Y	60

6	10	15	white	10	21	floating	-	90
7	100	15	red	24	21	floating	Y	90
8	10	15	white	24	26	floating	Y	60
9	100	15	white	10	26	sinking	Y	90
10	100	30	white	10	21	floating	Y	60
11	100	30	white	24	26	floating	-	90
12	10	30	red	10	26	floating	Y	90
13	100	30	red	24	26	sinking	Y	60
14	10	30	red	24	21	floating	-	60
15	10	30	white	10	26	sinking	-	60
16	10	15	red	24	26	sinking	-	90

2. Rearing conditions

A stock of 3200 pikeperch juveniles (20-30g) were provided by the Asialor farm and transferred to the URAFPA facilities (University of Lorraine). Fish were first reared in 6 large tanks for acclimatization and ongrowing until they reached 70 ± 30 g body weight before the multifactorial experiment started. They were maintained in constant conditions (temperature: 23°C; light intensity: 10 lux; photoperiod: 12h-12h) and were fed twice daily at 1.5 % biomass.

The multifactorial protocol started on mid-June 2015 with 1680 juveniles. They were allocated to the 16 experimental combinations of modalities taking into account the stocking density, so the numbers of fish (n = 60 to 140) varied between the experimental units but the average body weight was comparable. Other factors-modalities were set as indicated in the tables 22-1 and 22-2.

During the experiment, temperature, light intensity and oxygen saturation were checked once daily, while pH, nitrite and nitrate concentrations were measured once a week. Fish were fed with either a mid-floating or a sinking feed (D-D Optibream 2P or 2P-Optobream, 4 mm, Skretting) containing the same contents of crude proteins (46%) or lipids (16%). Food at 1.5% biomass was distributed during a photophase of either 24h or 10h as actually practiced at Aquapri farm in Denmark or Asialor farm in France.

3. Samplings and output variables

The experiment lasted for 63 days, and growth parameters were measured on days 0, 36 and 63, while final survival rate was estimated only on D63.

On days 36 and 63, 6 fish were removed randomly from each tank and anesthetized with MS222 (150 mg l^{-1}). Then, blood samplings were done within 5 min in order to avoid an eventual extra stress artifact. Heparinized blood was immediately centrifuged at 7500 g min^{-1} for 10 min at 4°C, and plasma was stored at -80°C until analyzed. After blood samplings fish were killed by cervical dislocation, and then dissected for various organs: brain, kidneys, liver, spleen and gonads.

Output variables:

(a) Husbandry parameters

Survival rate (SR) and Relative Growth Rate (RGR) were estimated for each tank according to the following formulas:

$SR = N_{(D63)}*100/(N_{(D0)}-N_{(sampled\ fish)})$

RGR = (final average weight – initial average weight) *100 / initial average weight

(b) Stress indicators:

Plasma cortisol was assayed in duplicate using a cortisol ELISA kit following manufacturer's instructions as described for the preliminary essays.

Plasma glucose levels were determined calorimetrically based on a glucose oxidase/peroxidase method and osmolality was assayed with an osmometer.

Brain neurotransmitters: Some key neurotransmitters possibly associated to the stress response were analyzed in brain tissues: serotonin (5-HT), tryptophan (TRP), 5-HIAA (hydroxyl-indol-acetic acid, serotonin metabolite) and DOPAC (dihydroxy-phenyl acetic acid, dopamine metabolite).

For each fish, the whole brain tissue was weighed out and homogenized for 6 min in perchloric acid 4% (250 µl 50 mg of tissue^{-1}) 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 NaH2PO4, 7 mM octane sulfonic acid (OSA) 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^{TM} 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^{-1}. The mobile phase consisted of 75 mM NaH2PO4, 7 mM octane sulfonic acid (OSA) and 10% MeOH adjusted to pH 3. The column was reconditioned by washing with 95% MeOH for 10 min and then re-equilibrated with buffer for 20 min. The column was kept at 25°C.

Purified serotonin, tryptophan, 5-HIAA and DOPAC were obtained from Sigma. Standard solutions were exactly treated as samples. Concentrations of all compounds were calculated by interpolation of their respective standard curves.

(c) *Immunological parameters*

Lysozyme activity was evaluated in plasma samples. Plasma samples (10 µl) were mixed with 10 µl of Na_2HPO_4 0,05M and 130 µl *Micrococcus lysodeikticus* (Sigma-Aldrich) solution (0.6 g l^{-1}). This assay was performed in triplicate. Absorbance was measured at 450 nm every 5 min during 30 min at room temperature. Lysozyme activity (Units ml^{-1} min^{-1}) is defined as the amount of enzyme decreasing the turbidity of 0.001.

The activity of the alternative complement pathway (Milla et al., 2010) was assayed by measuring the haemolytic activity in plasma samples against rabbit erythrocytes. In brief, 10 µl of rabbit erythrocytes diluted at 3% in veronal buffer were added to 60 µl of diluted samples (dilutions from 15 to 160 times). This mix was then incubated at 37°C for 100 min and mixed every 20 min. After centrifugation, absorbance at 405 nm was read on supernatants. Spontaneous hemolysis was obtained by adding 60 µl of veronal buffer to 10 µl of rabbit erythrocytes. Total lysis was obtained by mixing 10 µl of rabbit erythrocytes with 60 µl of distilled water.

Expression levels of some immune genes in kidney

Total RNA from kidney was extracted using TRIzol Reagent (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 Reagent. Total RNA was resuspended in 50 µl of DPEC-treated water. RNA concentration and purity was assayed with Nanodrop-1000 (ThermoScientific) at 260 nm. 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 (5µM) and 5 µl of the diluted cDNA. Reaction (95°C 10 min, 40 cycles of 15 s at 95°C followed by 1 min at 60°C) was carried out on a StepOne plus real time PCR machine (Applied Biosystem). Primers sequences are presented in Table 22.3. The mRNA expression levels of complement component 3 (C3) and lysozyme were measured in kidney. Results were normalized using β-actin as reference gene.

Table 22.3: Sequences and melting temperature (Tm) of primers used for gene expression quantification.

Gene	Sens	Sequence (5' to 3')	Tm (°C)
Complement C3	Forward	TGGTGATGTGAGAGGAGCAG	60
	Reverse	GACGTCATGGCAACAGCATA	60.7
Lysozyme	Forward	AGCCAGTGGGAGTCGAGTTA	59.8
	Reverse	CATTGTCGGTCAGGAGCTCA	60
β-actin	Forward	CGACATCCGTAAGGACCTGT	60
	Reverse	GCTGGAAGGTGGACAGAGAG	60

4. Statistical analyses

Statistical analyses were done following the appropriate methods to the fractional factorial design as developed by Kobilinski (2000) and Gardeur et al. (2007). Calculations were done using the Planor-Analys software developed by Kobilinski (2000). The global effects of the experimental combinations on output variables were performed using a principal component analysis (PCA) using R software. The interactions between 3 or more factors are considered insubstantial. When an interaction between two factors was found significant ($P < 0.05$), the potential single effects of these factors were also considered insubstantial. All the significant effects were then tested with the Statistica™ software for windows (Stat Soft, USA) with one- or two-way ANOVA. When significant ($p < 0.05$), means were compared according to the LSD post hoc test.

B. Results

A summary of parameters for which marked effects were calculated is presented in **Table 22.5**. The global effect of the experimental combinations on output variables is summarized in **Fig. 22.7**.

1. Husbandry parameters

After 63 days of multifactorial stress challenges, the lowest *survival* was observed for fish reared in the C9 experimental conditions (59%), and the highest value (97%) in fish reared in the C2 and C15 ones (97%) (**Tables 22.2** and **22.4**, **Fig. 22.7**). So, the statistical analyses also indicated that the C10 experimental condition ("floating feed-white light-100lux-10h-21°C" and high density and grading) was not suitable for a good performance for pikeperch. Mortality was affected by the interactions between high light intensity and low temperature level or high stocking density (**Fig. 22.9**).

Concerning growth, the overall results showed a significant and positive effect of sinking feed (**Fig. 22.8-9**), but significant interactions were calculated between the feed type and red light, low light intensity, low temperature, grading, low temperature and oxygen saturation. The best relative growth rates ranging from 63 to 103% were noticed for 7 experimental treatments and 71% of these treatments were characterized by red light-10 lux-24h-21°C in association with high oxygen saturation for 57% of the cases or without grading for only 42%.

2. Physiological stress response

The interactions of *(a)* density with light spectrum and *(b)* photoperiod with light spectrum, density or temperature significantly influenced plasma cortisol levels on days 36 and 63 (**Fig. 22.10**). Long photoperiod (24L/0D) associated with high density (30 kg m^{-3}), with white light spectrum or with higher temperature (26°C) significantly increased plasma cortisol levels (**Fig. 22.10**).

The type of food, when associated with oxygen saturation, with grading or with light intensity, affected glucose levels on day 36 (**Fig. 22.11**). Sinking food associated with grading, with low light intensity (10 lux) or with low oxygen saturation (60%) increased significantly this parameter. Plasma glucose level was also influenced by the interactions of temperature with light intensity and temperature with density on day 63. Cortisol values decreased between D36 (32 ng ml^{-1}) and D63 (26 ng ml^{-1}).

Light (intensity-spectrum interaction) influenced brain serotonin and tryptophan contents on day 36 with higher concentrations when white light spectrum is associated with low light intensity (10 lux) (**Fig. 22.12**). The concentration of DOPAC hormone in brain was higher at 26°C (**Fig. 22.12**). The levels of those hormones were not influenced by another factor. Statistical analyses also revealed an effect of oxygen-food interaction on 5-HIAA levels on day 36 and an effect of intensity-spectrum interaction on day 63 (**Fig. 22.12**).

3. Immunological parameters

a) Plasma lysozyme and complement activities

The feed type, when associated with oxygen or spectrum, affected lysozyme activity on day 36 (**Fig. 22.13**). Floating feed associated with high oxygen saturation (90%) or with red light spectrum significantly decreased the latter parameter. On day 63, lysozyme values were influenced by the interaction of light intensity and grading. Overall, lysozyme values increased slightly between D36 (15.80 U min^{-1}) and D63 (18.59 U min^{-1}) in parallel to the decrease in plasma cortisol levels.

For plasma alternative pathway complement activity (ACH50), statistics showed an effect of the intensity-oxygen interaction on day 36 and an effect of intensity-grading interaction and photoperiod-spectrum interaction on day 63 (**Fig. 22.14**). Taken together, ACH50 values increased significantly between D36 (124 U) and D63 (232 U), as for lysozyme activity.

b) mRNA expression levels of two key immune genes in kidney

Lysozyme gene expression did not show any difference between experimental conditions. Expression of complement C3 was influenced by the interaction between temperature and grading with significant higher expression at 21°C without grading (**Fig. 22.15**).

Table 22.5: Summary of all the significant effects (single effects and dual interactions) on husbandry, physiological and immunological parameters. RGR: Relative Growth Rate; MR: Mortality Rate; Osmo: osmolality; Lys.: Lysozyme; ACH50: plasma alternative pathway complement activity; TRP: tryptophan; Oxy: oxygen; Temp.: Temperature; Dens: density; Photo: photoperiod; Food: type of food. Fish sampling occurred on days 36 and 63.

Combinations of parameters	Final weight gain	RGR	MR	Cortisol		Glucose		Osmo.	Lys.		ACH50		C3 (mRNA)	Lys. (mRNA)	Serotonin		5-HIAA		DOPAC	TRP	
	D63	D63	D63	D36	D63	D36	D63	D36	D36	D63	D36	D63	D63	D63	D36	D63	D36	D63	D36	D36	D63
Food*oxy.	*					*			*												
Food*grading						*															
Food*spectrum									*												
Food*temp.	*																				
Food*int.	*					*															
Dens.*grading																					
Photo.*temp.	*																				
Food		*																			
Int.*temp.			*				*														
Int.*dens.			*																		
Int.*tri										*		*									
Int.*oxy.											*										
Photo.*spectrum				*	*							*									
Photo.*dens.				*	*																
Photo.*temp.				*																	
Dens.*spectrum				*	*																
Density*temp.							*														
Temp.*grading													*								
Oxy.*food																	*				
Int.*temp.																		*			
Int.*spectrum															*					*	
Temp.																			*		

The directive factors variances are indicated by asterisks (*). For example, the final weight gain was significantly affected by *(a)* the interaction of the feed type and the oxygen saturation, *(b)* the interaction of the type of food and the temperature, *(c)* the interaction of the type of food and the light intensity and *(d)* the interaction of the photoperiod and the temperature. The statistical significance of these interactions is shown in **Fig. 22.7**.

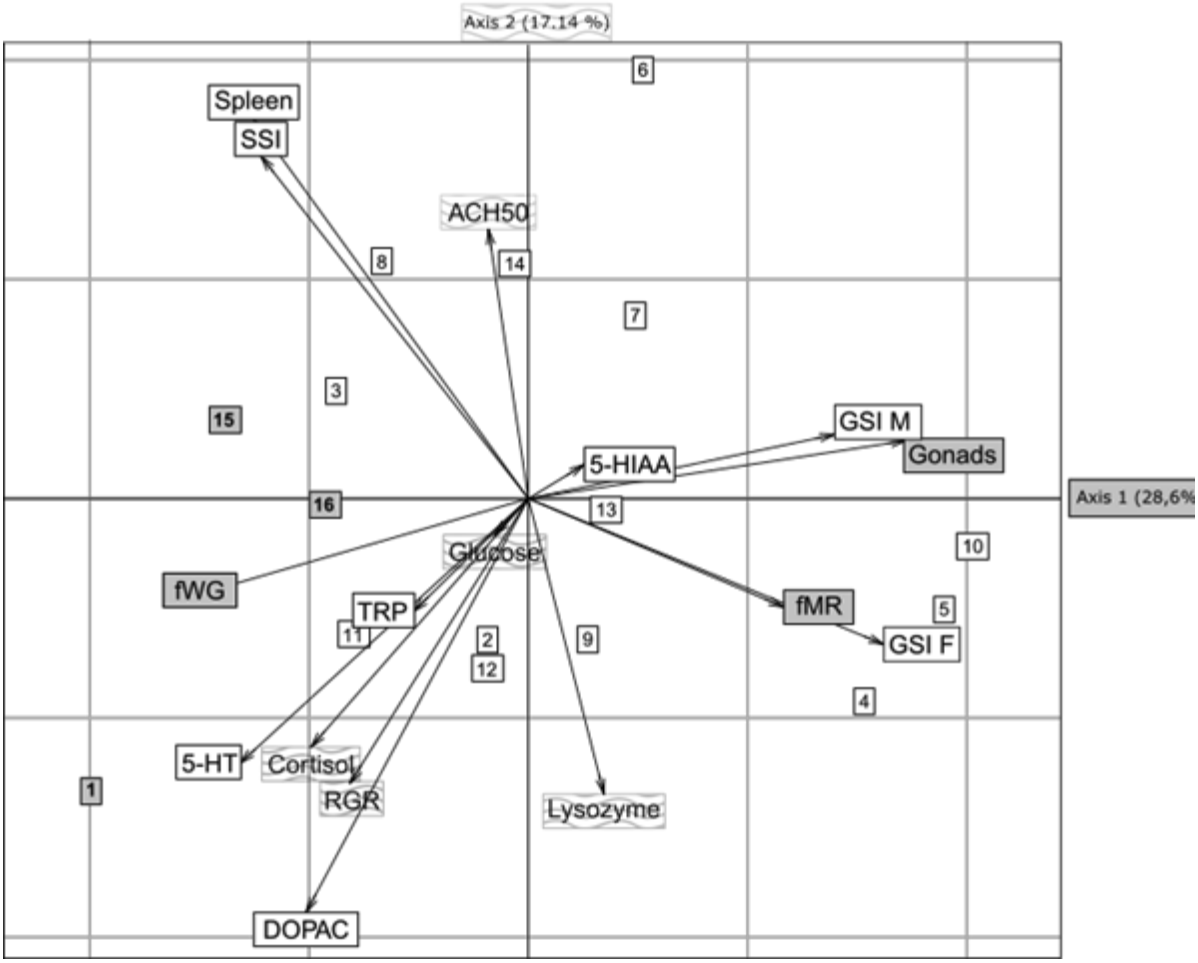


Figure 22.7. Projection of global effect of experimental conditions (C1 to C16) on the plans 1-2 of the principal components analysis (ACP). SSI: Spleno-Somatic Index. fWG: Final Weight Gain. TRP: Tryptophan. 5-HT: Serotonin. RGR: Relative Growth Rate. DOPAC: Dihydroxy-Phenyl Acetic acid (dopamine metabolite). 5-HIAA: Hydroxyl-Indol-Acetic Acid (serotonin metabolite). GSI M: Gonado-Somatic Index Male or Female. fMR: Final Mortality Rate. The plans 1-2 of the ACP explained 45.8% of the inertia (total variance) with axis 1 representing the highest variance. The axis 1 was mainly characterized by higher fWG and lower fMR. So the experimental conditions C15 and C16 were selected as optimal for rearing pikeperch because they induced higher final weight gain and lower mortality, as well as lower stress response (axis 2). The C1 experimental condition was selected to be tested in the confirmation experiment because of high fWG and RGR despite a relatively high stress response.

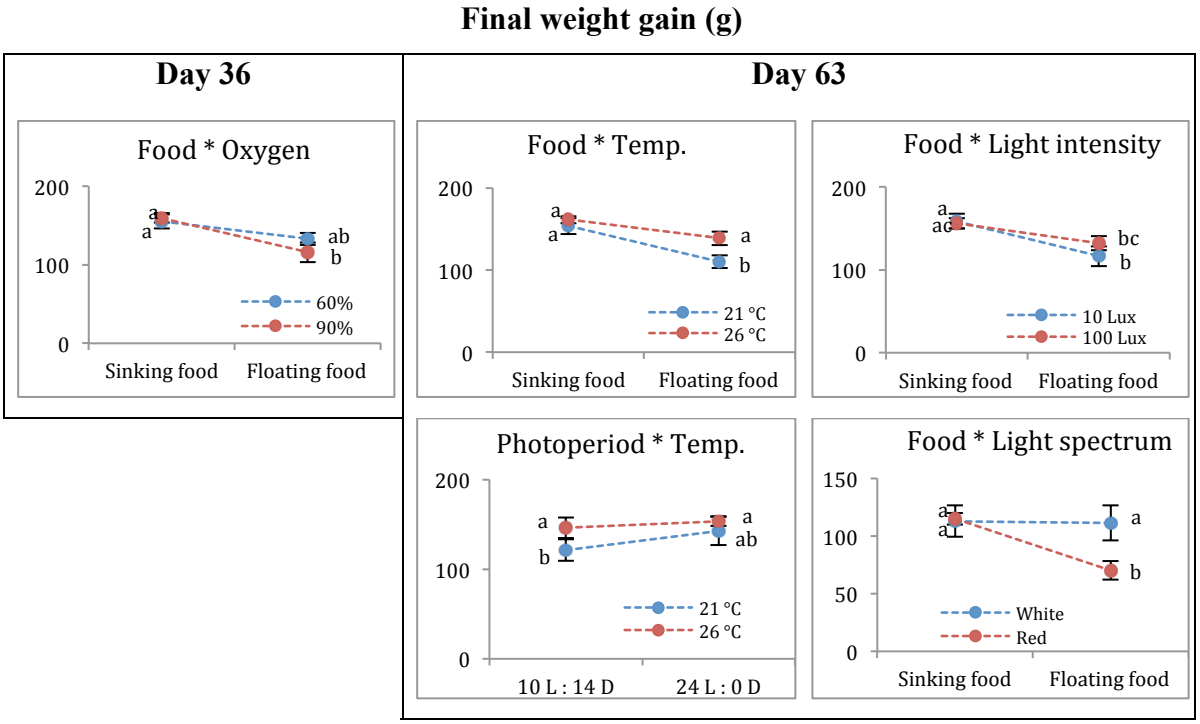


Figure 22.8: Effects of various interaction effects (see graph titles) on the final weight gain of pikeperch on days 36 and 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (n=4). Significant results are indicated by different letters (a and b). Food = type of feed and Temp. = temperature.

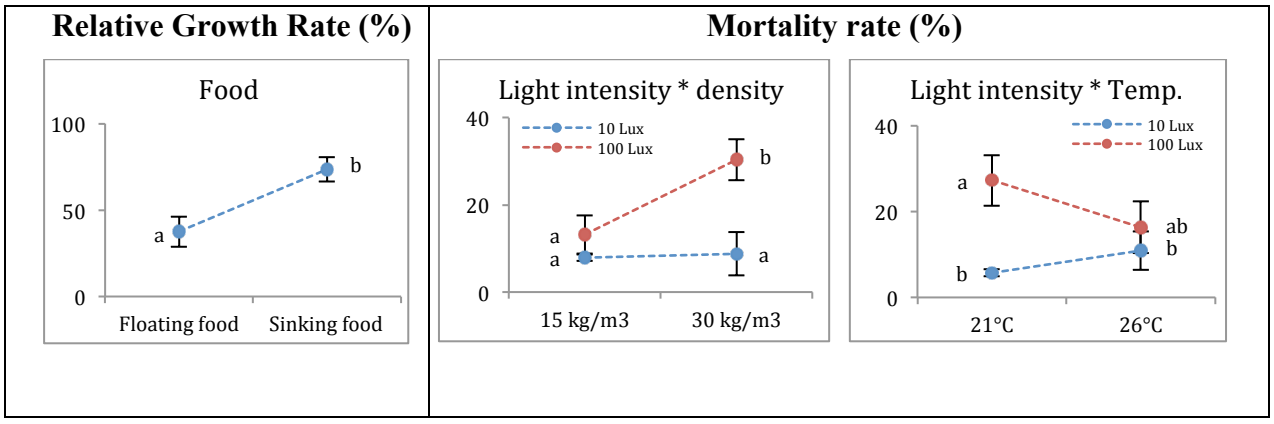


Figure 22.9: Effects of various single and interaction effects (see graph titles) on Relative Growth Rate (%) and Mortality Rate (%) of pikeperch on day 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (single effect: n=8; interaction effect: n=4). Significant results are indicated by different letters (a and b). Food = type of food, and Temp.= temperature.

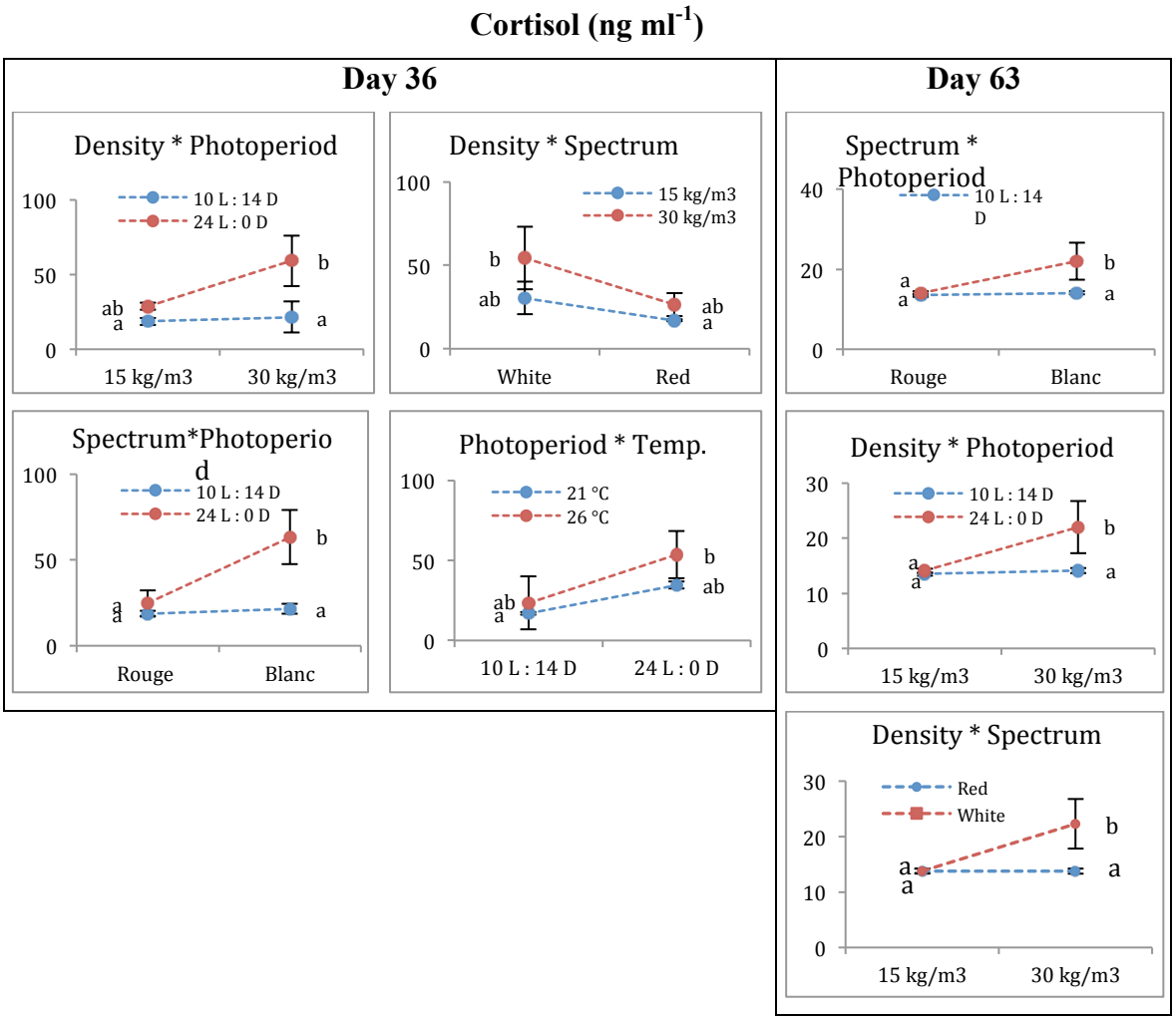


Figure 22.10: Effects of various interaction effects (see graph titles) on plasma cortisol levels (ng ml^{-1}) of pikeperch on days 36 and 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (n=4). Significant results are indicated by different letters (a and b). Temp.= temperature.

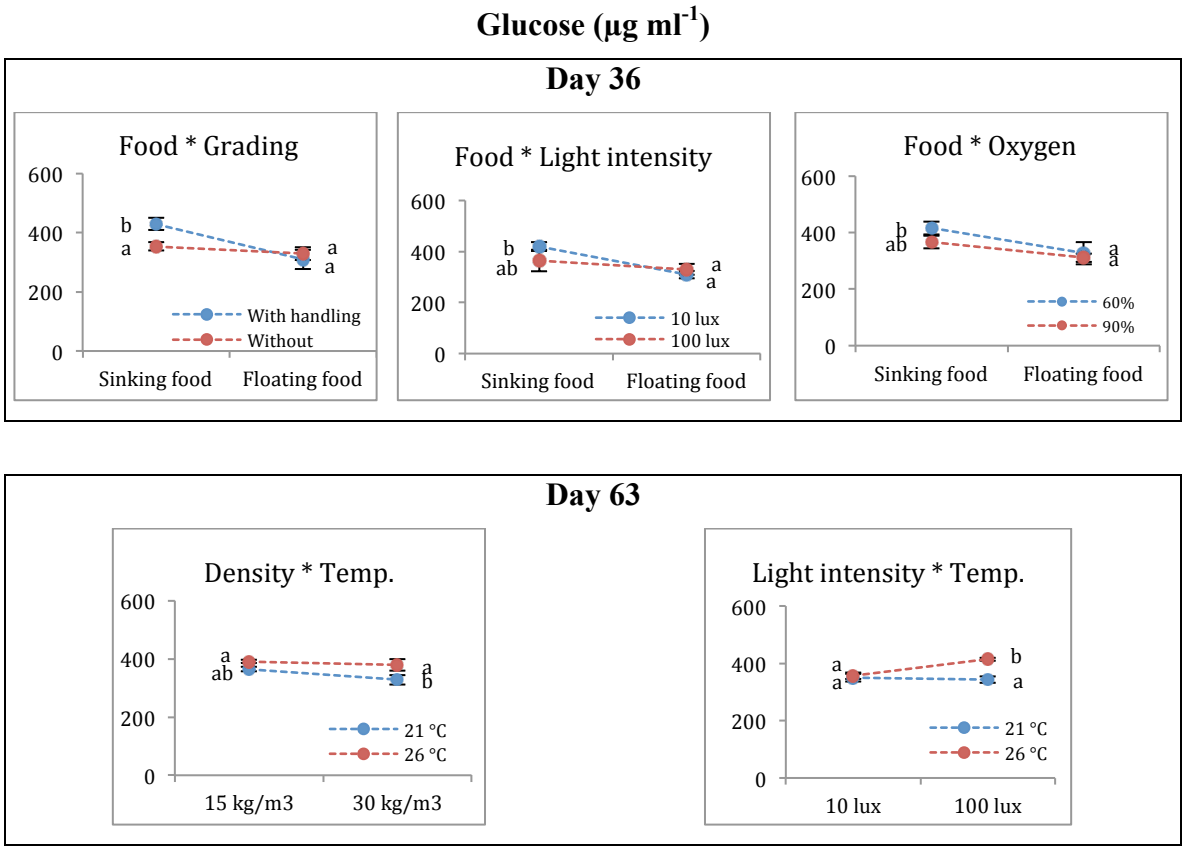


Figure 22.11: Effects of various interaction effects (see graph titles) on plasma glucose level (µg ml^{-1}) of pikeperch on days 36 and 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (n=4). Significant results are indicated by different letters (a and b). Food = type of food and Temp. = temperature.

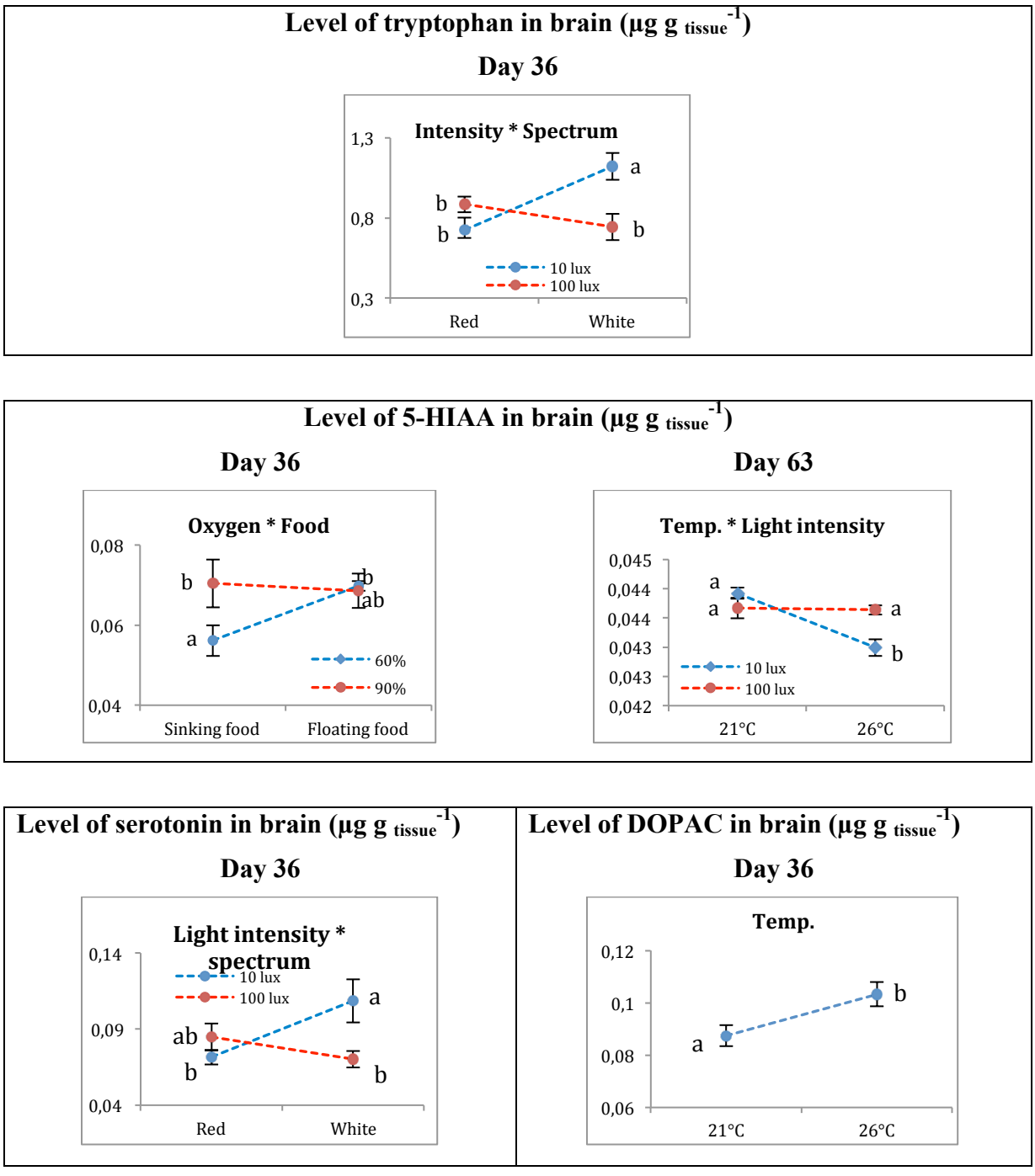


Figure 22.12: Effects of various single and interaction effects (see graph titles) on neurohormone levels (5-HT, 5-HIAA, TRP, DOPAC) in pikeperch brain on days 36 and 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (single effect: n=8; interaction effect: n=4). Significant results are indicated by different letters (a and b). Food = type of food; Temp.= temperature.

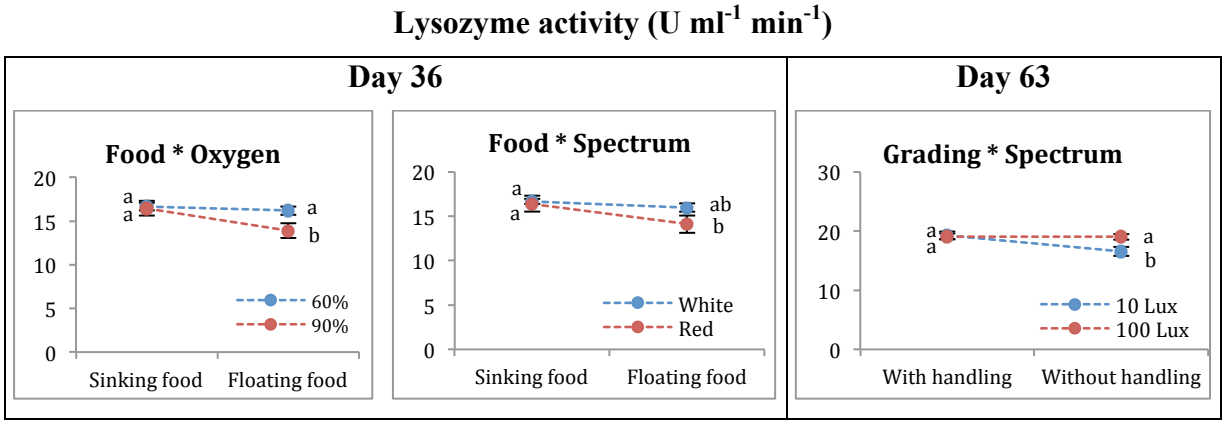


Figure 22.13: Effects of various interaction effects (see graph titles) on plasma lysozyme activity (U ml^{-1} min^{-1}) of pikeperch on days 36 and 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (n=4). Significant results are indicated by different letters (a and b). Temp.= temperature.

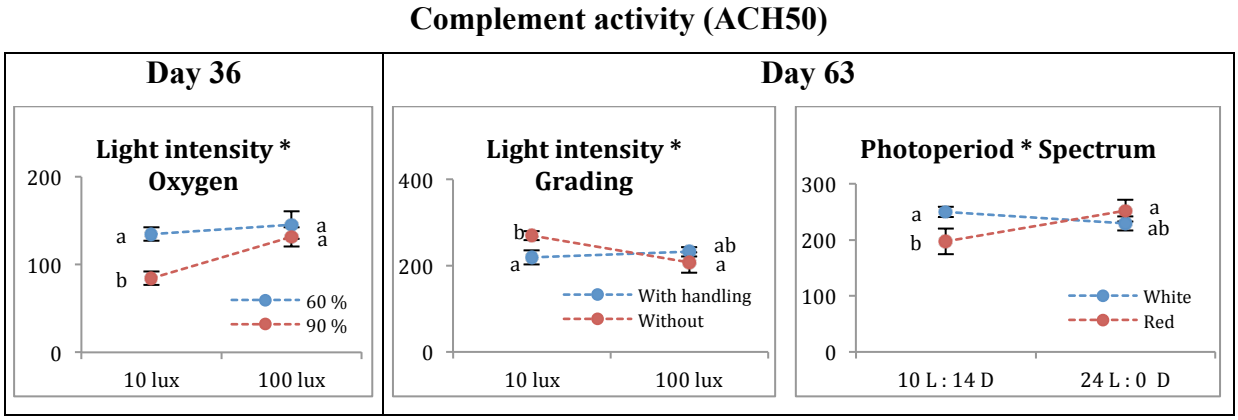


Figure 22.14: Effects of various interaction effects (see graph titles) on plasma alternative pathway complement activity (ACH50) of pikeperch on days 36 and 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (n=4). Significant results are indicated by different letters (a and b). Temp.= temperature.

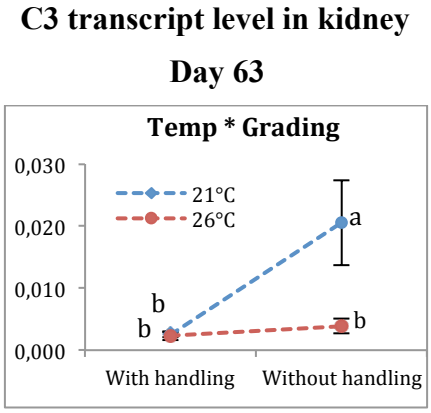


Figure 22.15: Effects of various interaction effects (see graph titles) on C3 transcript level in pikeperch kidney on day 63 of the multifactorial experiment. Only graphs with significant effects are shown. Results are expressed as the Mean ± 1SEM (n=4). Significant results are indicated by different letters (a and b). Temp.= temperature.

C. Discussion

1. Husbandry response

The overall results demonstrated significant and positive effects of low intensity on survival at the end of the multifactorial experiment. Positive interactions were also calculated when light intensity was associated with other factors such as temperature and rearing density. Thus, high mortality rates were observed in experimental conditions composed of high light intensity (100 lux) and low temperature level (21°C). The present results corroborate previous reports that pikeperch prefer low light intensity (Molnár et al., 2004; Luchiari et al., 2006), but do not support the report that this species has a good welfare at high stocking density (Dalsgaard et al, 2013). Indeed, negative interactions between light characteristics and high density were observed even if the density levels tested did not exceed the known limits for pikeperch juveniles (Dalsgaard et al., 2013). At some extent, increasing the stocking density was also found as a positive strategy to decrease the cannibalistic intensity in young Eurasian perch, and thereby decreasing mortality rate (Mélard et al., 1996; Baras et al., 2003).

Growth related parameters were mainly affected by the feed type with positive effects of sinking feed. Positive interactions were calculated with the feed type and red spectrum, low light intensity and low temperature or high oxygen saturation. The impact of other factors-modalities, such as photoperiod, varied greatly between treatments without a conclusive trend. Information about the effect of feed type on husbandry performances is limited in fish, and it is not yet clear whether pikeperch prefers sinking or floating feeds. Some pikeperch famers (such as Aquapri in Denmark) are using floating feeds to facilitate the control of food intake, but it seems that it is necessary to habituate juveniles at an early age to avoid rejection of this type of food later (Dalsgaard et al., 2013). So, it is possible that the feeding strategy interfered slightly with the positive effect obtained in the current experiment since fish were fed with sinking feed before the experiment. Nevertheless, it has been demonstrated that the use of sinking feed has a better effect on food intake and thereby on husbandry performances comparing to floating feed in other fish species such as the Atlantic halibut *Hippoglossus hippoglossus* (Kristiansen and Ferno, 2007).

As far as the sinking feed was considered, the highest growth performances were mainly observed in experimental conditions composed of "red light-10 lux-24h-21°C", indicating the importance of light characteristics for good performances of pikeperch in RAS conditions. Higher growth rates of fish reared under long photoperiod conditions are usually reported in relation to high food intake (Boeuf and Le Bail, 1999; Jourdan et al., 2000; Biswas et al., 2016). Our results demonstrated the same trend for pikeperch if the rearing system includes sinking feed, red spectrum and low light intensity. They also indicate that pikeperch needs specific requirements for light characteristics, perhaps due to the presence of their *tapetum lucidum*, a specific reflective layer of the retina which may amplify the light level, explaining why this species may be highly sensitive to high light intensity (Sandström, 1999).

The results of the present study did not support the hypothesis that high temperature promotes growth rate in pikeperch juveniles (Rónyai and Csengeri, 2008; Wang et al., 2009; Dalsgaard et al., 2013); but corroborate the report that energy is spent for increased metabolic rates over 25°C (Frisk et al., 2012). Indeed, temperature was not found as the main directive factor, and the positive interactions with sinking feed were observed with the treatments which included low temperature of 21°C.

It has been reported that frequent manipulations markedly affect growth rate of young percid fish (Jentoft et al., 2005; Strand et al., 2007) but our results did not clearly demonstrate that grading was a directive impact factor; and good growth performances were obtained in experimental conditions including or not grading manipulations. 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.

2. Physiological and immune responses

In terms of cortisol and glucose, the results in the present study did not indicate a high stress response except for only 4 experimental conditions characterized by white light-24h-26°C-grading. Significant interactions were statistically confirmed between photoperiod or stocking density and light spectrum or temperature; grading showed no marked effect. The impact of light spectrum on cortisol production has been reported for juvenile pikeperch under white light conditions compared to red light (Luchiari et al., 2009). It has been also reported that adult perch reared under conditions of long photoperiod exhibited a high stress response in terms of cortisol release (Montero et al., 1999; Sarameh et al., 2012). The results of the current study showed that the impact of light characteristics may be increased by high stocking density. In other fish species, a decline in stress response related to stocking density was shown with time (Segner et al., 2012) as observed between D36 and D63 in relation to a better acclimation of fish to the experimental rearing conditions, making them less stressed. It also appeared that for experimental conditions including red light, no significant interactions with temperature were observed for cortisol, emphasizing that red spectrum may be less stressful for pikeperch.

Some brain neurotransmitters were analyzed to emphasize the stress responsiveness, and the results indicated that serotonin and tryptophan may be used to confirm the impact of some light characteristics as directive factors-modalities for stress occurrence in pikeperch. Indeed, significant interactions between light intensity and temperature or light spectrum were observed. Tryptophan appeared more relevant as stress indicator since its concentration was higher compared to other neurohormones, perhaps indicating a more active role in this species. Tryptophan is an amino acidic precursor for the synthesis of serotonin and melatonin (Lepage et al., 2002, 2005), and the implication of these two neurohormones in the pathways of physiological stress response has been demonstrated in many fish species (Höglund et al., 2007; Gesto et al., 2016). It has been shown that melatonin manipulation may attenuate stress response in some fish species such as the Senegalese sole, *Solea senegalensis* (López-Patiño et al., 2013; Gesto et al., 2016).

The relationships between physiological status and immune system were demonstrated by some results from the multifactorial experiment. At the end of the experiment (D63), when high husbandry performances were

observed, the stress response was found low in terms of cortisol values of fish reared in 72% of the cases. This confirmed that these modalities mitigated the occurrence of high stress response. Moreover, a trend of decrease was observed in plasma cortisol values on D36 compared to D63, in parallel to an increase in immune parameters, such as plasma lysozyme and ACH50. There are several studies that pointed out the interference of cortisol increase in various pathways of the immune functions (Stolte et al., 2008; Milla et al., 2010; Tort et al., 2011; Mathieu et al., 2014; Soltanian et al., 2014; Liu et al., 2016). Apart from cortisol, the current results may indicate that other stress mediators may participate to such immune trade-off, since cortisol values were generally low while relatively high values of some brain neurotransmitters were observed compared to some fish species such as the Senegalese sole (Gesto et al., 2016).

As for growth related parameters or stress indicators, significant interactions between light intensity and grading or oxygen saturation, as well as between photoperiod and light spectrum affected the variability in lysozyme activity and complement ACH50 confirming the high responsiveness of pikeperch to variation in light characteristics. However such effect was not confirmed by the expressions of their relative genes in the spleen indicating that the sensitivity to light characteristics may vary between lymphoid organs as already shown for the response to handling or emersion stress of Eurasian perch juveniles by our previous studies (Milla et al., 2010; Douxfils et al., 2014).

All in all, the results from the current multifactorial experiment indicate that the C15 and C16 experimental conditions were optimal for increasing the performances and welfare status of pikeperch in intensive culture. These treatments were characterized by low light intensity, sinking feed, high temperature and no grading, but differed by their stocking density, light spectrum and oxygen saturation. It appeared interesting to include the C1 experimental conditions in the confirmation experiment because fish reared under these modalities (high stocking density-grading-high oxygen saturation-low light intensity) exhibited higher husbandry performances in contrast to higher stress response.

V. Confirmation experiment

A. Materials and methods

1. Culture conditions and sampling

The confirmation experiment was conducted at the University of Namur (Research Unit in Environmental and Evolutionary Biology). After one month of acclimatization, fish were exposed to 3 selected experimental conditions for 36 days in 3 separate circulating systems. The comparison was done in 6 replicates of 10-30 fish each depending on the stocking density level. The 3 experimental conditions were the optimal rearing modalities selected from the multifactorial experiment as summarized in **Table 22.6**.

Table 12.6: Experimental conditions (systems 1, 2 and 3 represent C16, C1 and C15 in the multifactorial experiment, respectively).

System	Light intensity (lux)	Density kg m^{-3}	Light spectrum	Photoperiod (h)	Water temperature (°C)	Type of aliment	Grading	Oxygen saturation (% O_2)
1	10	15	Red	24h	26	Sinking	-	90
2	10	30	White	24h	21	Sinking	Yes	60
3	10	30	White	10h	26	Sinking	-	60

After 36 days of experiment, 5 fish from 3 replicates were blood sampled for stress and immune responses. Then, the remaining fish were transferred in the confinement room for bacteria injections on D37. The protocol for disease resistance was conducted in triplicate tanks of 15 fish each. Two days after the bacterial injection (D39), 5 fish of each tank were blood sampled for immune response to bacteria injection, and the 10 remaining fish were followed for mortality for 10 days. Fish were anesthetized in MS-222 (0,15 g l^{-1}), and sampled from the caudal vein within 5 min to avoid the release of grading-induced cortisol (Wang et al., 2004). Blood was then mixed with heparin and kept on ice (Sigma, 5000 U mL^{-1}) before centrifugation (5000 rpm, 10 min) and storage at -80°C.

2. Bacterial challenge

It was necessary to determine the lethal concentration (LC50) of the selected bacteria for the disease resistance because the virulence of bacteria varies greatly between fish species, strains or populations within the same species. *Aeromonas salmonicida achromogenes* was chosen because the culture of such bacteria species is well controlled in our laboratory and it has been shown virulent for Eurasian perch juveniles (Fatima et al., 2007; Jessica et al., 2011; Geay et al., 2015). The bacteria strain was purchased from the Reference Laboratory of Fish Diseases, CER-Groupe, Marloie, Belgium.

The remaining fish from the preliminary experiment were mixed and transferred to a confinement room for bacterial challenge. They were injected intraperitoneally with 3 doses of bacteria in duplicate groups of 12 fish per tank: 10^6, 10^7, 10^8 colony forming units ml $^{-1}$ (CFU ml^{-1}). Mortality was checked for 8 days (**Figure 22.16**), and the LC50 calculated using the probit analysis.

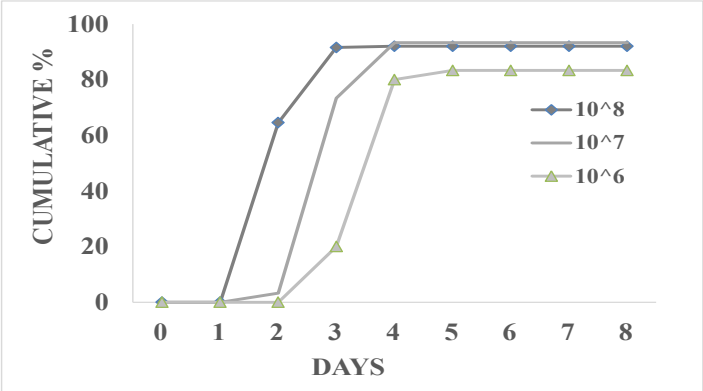


Figure 22.16: Cumulative mortality during the bacterial challenge for the determination of LC50 of *A. salmonicida achromogen* for pikeperch juveniles.

During the confirmation challenge, fish were intraperitoneally injected with the defined LC50 dose of 10^7 CFU 100 g^{-1} of fish. Mortality was daily recorded.

- *Physiological and immunological parameters*

Plasma glucose, cortisol and lysozyme activity levels were assayed as described above.

- *Statistical analysis*

Replicate tank was considered as the statistical unit (n=3). Normality and variance homogeneity were respectively confirmed with Kolmogorov-Smirnov and Bartlett tests. In order to fit normality and variance

homogeneity requirements, “log” and “ln” transformations were applied on values when required. Data were separately analyzed for each sampling time (1 day before and 2 days after bacterial challenge) according to a two-way ANOVA (system as a fixed factor with 3 levels and tank with 3 levels as a random factor). Survival rates between systems were compared with R software with Survival package. All the analyses were performed with R software.

B. Results and discussion

Neither plasma lysozyme activity nor levels of cortisol and glucose were influenced by the tested experimental conditions before or after the bacteria injections (**Fig. 22.17**). Glucose values were found at basal levels as observed at the end of the multifactorial experiment. Moreover, cortisol values were also low except for a higher variability in fish reared under system 1 and 2 experimental conditions. So, it can be considered that the stress sensitivity was low at the end of this confirmation experiment.

With the injected dose (10^7 bacteria 100 g^{-1} of fish) of *A. samonicida achromogenes*, fish started to die after two days and all of them died within 7 days (**Fig. 22.18**). The survival probability curves were the highest for the first rearing system but comparable to those for the rearing system 2, and significantly differed compared to system 3 which showed the lowest disease resistance ($p < 0.01$).

While the innate immune response seemed comparable between the selected 3 rearing systems, fish reared under system 1 including red light, low stocking density, high temperature and oxygen saturation displayed the highest disease resistance to bacteria. It is interesting to note that fish in system 2 with white light, high stocking density and grading displayed comparable disease resistance. The comparison of the 3 rearing systems indicated that light characteristics (low intensity and light duration) may be more important for pikeperch juveniles than the spectrum since all systems were set at 10 lux, and the third system differed by its low photoperiod of 10h, but it is still necessary to validate such hypothesis using large facilities as in pilot farm conditions.

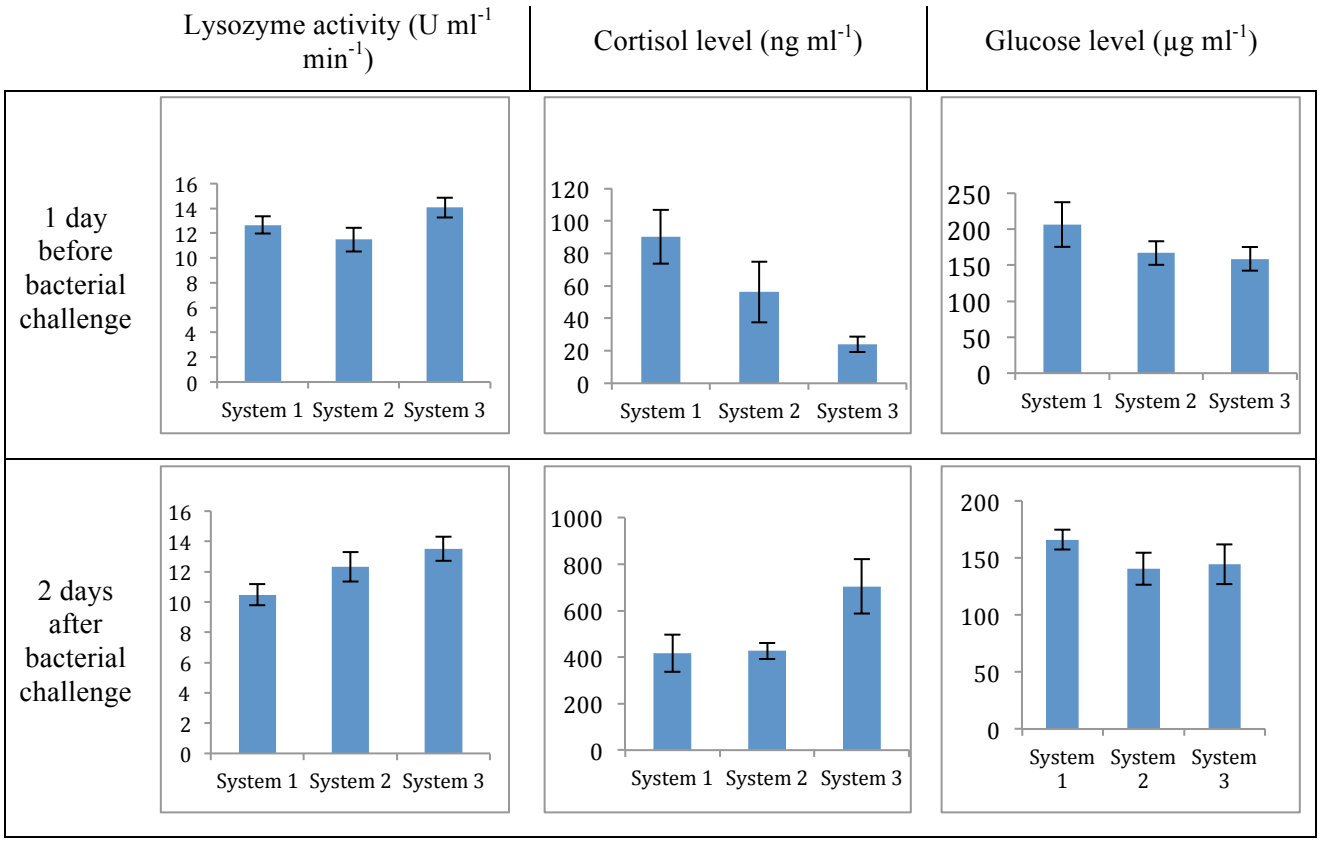


Figure 22.17. Effects of selected combinations of factors (system 1 to 3, see **Table 22.4**) on plasma alternative pathway complement activity (ACH50) and plasma cortisol and glucose levels. Results are expressed as the Mean ± 1SEM (n= 5 as the tank is the experimental unit for statistical tests). Significant results are indicated by different letters (a and b).

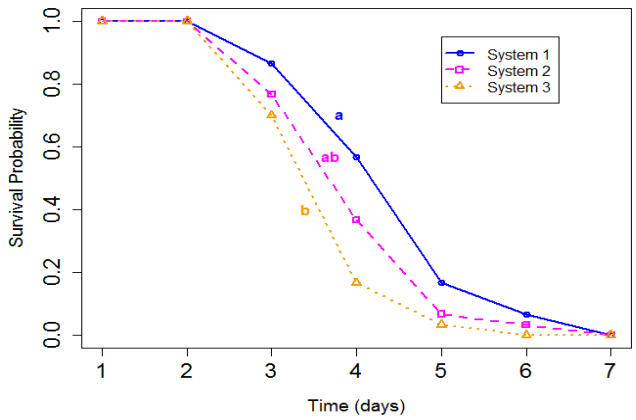


Figure 22.18. Mortality of pikeperch (*Sander lucioperca*) injected with *A. salmonicida achromogen* (10 million of bacteria 100 g^{-1} of fish). Significant differences between curves are indicated by different letters (a and b). Selected combinations of factors for systems 1, 2 and 3 are detailed in **Table 22.4**.

VI. General conclusions

A multifactorial experiment including 16 factors-modalities was conducted in order to select the most suitable husbandry and environmental conditions for improving the performance and welfare status of pikeperch juveniles reared under intensive culture. Various biomarkers, including stress indicators and immune parameters, were used to characterize the stress responses of fish reared under those 16 experimental conditions after two months.

The results from this multifactorial experiment indicated that the type of feed is the main directive factor for the variability in the husbandry performances for pikeperch. Indeed, statistical analyses clearly showed more positive effects of sinking than floating feed. However, strong synergy for variability in growth parameters was shown with feed type and light spectrum, temperature, photoperiod and oxygen saturation levels. Variability in mortality was affected by the interactions between light intensity and temperature or stocking density.

Biomarkers for physiological stress and immune responses were affected by light characteristics (light intensity, photoperiod and light spectrum) in association with stocking density but mostly with temperature level. Neurotransmitters appeared as reliable stress indicators for pikeperch, and were affected mostly by the interactions between light intensity and temperature. Surprisingly, grading did not appear to have a high stress impact, except for its effect on some neurotransmitters such as 5HIAA, a metabolite of serotonin.

The results demonstrated low stress sensitivity in most of experimental conditions in which fish exhibited the highest husbandry performances. Moreover, some results indicated a positive relationship between physiological status and immune competence, namely the decrease in cortisol secretion along the time of the multifactorial experiment was associated to an increase in lysozyme and complement ACH50 activities.

Combining the results on husbandry performances and on stress and immune status, three combination modalities were selected as suitable for sustaining high performances for pikeperch in intensive culture. The validation experiment indicated that these selected experimental conditions had comparable efficiency in terms of low impact on fish stress and immune status. However, fish reared under only two experimental conditions showed higher disease resistance after induced infection by *A. salmonicida*. The latter 2 combinations of husbandry and environmental modalities were selected as optimal rearing conditions for pikeperch.

Since the two selected rearing systems differ by their light spectrum (red vs white) and grading, the validation experiment planned in the DOW (WP22.2) at Asialor will focus mainly on these two modalities.

VII. References

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Deviations:

The deliverable date was delayed because (1) the adaptation of the UL facilities to the multifactorial protocol requirements took more time than expected; (2) it was necessary to perform two preliminary experiments in order to better define some methodological aspects appropriated to the multifactorial stress screening since there is limited information on stress response for pikeperch. More effort was also necessary to analyze the profiles of brain neurotransmitters to further emphasis the physiological stress response. Despite that delay, the results from those preliminary assays facilitated the full achievement of the experiments planned for the task 22.1.

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