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

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Brain neurotransmitters, stress, innate immunity, Pikeperch, fractional factorial design.

Abstract

High mortality and impairment in growth rate during pikeperch (Sander lucioperca) ongrowing are among the major bottlenecks for its development in aquaculture. These failures may be related to high stress responsiveness since the rearing conditions are not yet optimized for this species. The objectives were to characterize the stress and immunological responses of pikeperch to major aquaculture modalities, and to identify the optimal aquaculture conditions for improving its welfare status. In a screening experiment, eight factors considered as relevant for the welfare of pikeperch were compared in two modalities using a fractional multifactorial design (28-4). Each experimental unit represented a combination of 8 factors in two modalities including grading, stocking density (15 vs 30 kg•m3), feed type (sinking vs mid-floating), light intensity (10 vs 100 lux), light spectrum (red vs white), photoperiod (long vs short), dissolved oxygen (60 vs 90%) and temperature (21 vs 26 °C). Fish sampling occurred on days 36 and 63. Stress markers (glucose, cortisol and brain serotonergic activity), innate immune parameters (plasma lysozyme and complement activities) and expression of some immune genes were assessed. Light intensity and the type of feed clearly appeared as directive factors for pikeperch culture. A strong effect of the feed type was observed on growth parameters while survival was impacted by high light intensity. Light characteristics (intensity, spectrum and photoperiod) and temperature were identified as determining factors for physiological and immune markers. No obvious relation was established between stress status and growth parameters and further investigations are needed to improve management strategies of pikeperch culture and knowledge on the relations between environmental conditions, stress and immunity in percid fish.

1. Introduction

Over the last decade, European inland aquaculture has shown weak increase in productivity despite the increase in the demand for fish products throughout the world (FAO, 2014). This low performance in fish production might be attributed to the low number of fish species that are cultivated in Europe. Indeed, European aquaculture is mainly focused on 5 fish species: Atlantic salmon (Salmo salar), rainbow trout (Oncorhynchus mykiss), common carp (Cyprinus carpio), European seabass (Dicentrarchus labrax) and gilthead seabream (Sparus aurata). Therefore, itis essential to diversify the fish species pool inorder to increase fish productivity throughout Europe and to tap into new niche markets. Pikeperch (Sander lucioperca) is one of the most promising freshwater fish species for diversification and an attractive alternative for inland aquaculture species according to its relatively fast growth compared to other, its high quality flesh

and a favorable market acceptance leading to high economical expectations species (Hilge and Steffens, 1996; Barry and Malison, 2004; Wang et al., 2009; Dalsgaard et al., 2013).

However, the culture of pikeperch is still limited by an unpredictable high mortality rate and impairment in growth rate during both larval and juvenile stages with survival rate estimated between 8 and 30% (Kestemont et al., 2007; Szkudlarek and Zakes, 2007; Dalsgaard et al., 2013). A low welfare related to high stress level may be one of the major causes of high mortality rate observed for young pikeperch. While intensive aquaculture is well mastered for salmonid species such as Atlantic salmon and rainbow trout, aquaculture technology for percid fishes is not yet optimal. It has also been demonstrated that percid fish such as Eurasian perch (Perca fluviatilis) are more sensitive to some aquaculture stressors such as emersion and handling (Jentoft et al., 2005). For the latter species, it was also demonstrated that moderate hypoxia may considerably impair the behavior but also the physiological status such as the immune functions (Strand et al., 2007; Douxfils et al., 2014). Stress responsiveness has received little attention in pikeperch whatever its developmental stage. It has been reported that light characteristics such as light intensity and light spectrum have a huge impact on the pikeperch physiology and behavior due to some retinal histo-anatomical features (presence of tapetum lucidum and macroreceptors) which improve their vision in dim environment (Kozlowski et al., 2010; Luchiari et al., 2009; Sarameh et al., 2012). It was especially observed that pike-perch exhibited higher growth rate and food conversion rate under high light intensity (100 lux) of red spectrum than under white spectrum (Luchiari et al., 2009). But there is no clear information about the optimal intensity or light spectrum. Luchiari et al. (2006) also showed a clear preference of pikeperch for low light intensities. These findings are in agreement with the behaviour of juvenile and adult pikeperch in natural environments since this species is a crepuscular predator that is actively feeding during dusk and night (Luchiari et al., 2006; Zingel and Paaver, 2010; Dalsgaard et al., 2013). The effects of temperature conditions on pikeperch stress physiology are also reported inconsistently. It was reported that the optimal temperature for pikeperch is in the range from 10 to 27 °C (Frisk et al., 2012), but fish size should be considered when optimizing temperature level in aquaculture production. According to some authors, better growth and feed utilization for ongrowing pikeperch are achieved under high temperature conditions ranging between 25 and 28 °C (Rónyai and Csengeri, 2008; Wang et al., 2009; Dalsgaard et al., 2013). Nonetheless, Frisk et al. (2013) reported that a smaller fraction of metabolic scope was utilized for digestion at 19 °C compared to 25 °C, indicating that low temperature conditions may be more suitable for 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 manipulations should be appropriate to the feeding behaviour of targeted fish species to avoid a possible stress side effect.

The relationships between population parameters such as stocking density and physiological stress status or immune competence have been described in various fish species (Barton, 2002; Pankhurst, 2011) whereas limited information is available for pike-perch. Preliminary observations reported that high stocking density has no marked effects on growth and food utilization of young pikeperch, and that pikeperch juveniles can be maintained at high densities comprised between 30 and 60 kg•m-3 without any increase in physiological stress response (Molnár et al., 2004; Steenfeldt et al., 2010; Dalsgaard et al., 2013). On the contrary, another study reported that high density may increase the susceptibility to diseases for pikeperch juveniles (Jensen et al., 2011). According to our observations, juveniles of pikeperch in intensive culture conditions are found to feed both on the ground and in the water column. Mid-floating feed has lower sinking velocity and better dispersion in the water which can improve feeding behavior in some fish species. No clear data are available on juvenile pikeperch and current practices are not yet standardized.

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

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

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

2. Material and Methods

2.1. Experimental design

Taking into account current practices in major European pike-perch intensive farms and available data in the literature (Luchiari et al., 2006; Teletchea et al., 2009; Wang et al., 2009; Sarameh et al., 2012; Dalsgaard et al., 2013), eight environmental factors at two levels (Table 1) were selected and tested in a multifactorial experiment based on a 2(8-4) reduced factorial design (Kobilinsky, 2000; Torstensen et al., 2001; Hamre et al., 2004; Gardeur et al., 2007; Mairesse et al., 2007; Blanchard et al., 2008). With a full factorial design study of 8 factors at 2 levels, 256 (28) treatments would be tested resulting in the calculation of the main effects and all the interactions. With a fractional factorial design study, the number of combinations (treatments) is reduced from 256 (28) to 16 (24). To generate such a design, an alias structure was selected (Table 2), determining which effects are confounded with others (Hamre et al., 2004, Trabelsi et al., 2011). Thus, itis possible to calculate main effects separated from each other and from the effects of two-factor interactions (Kobilinsky, 2000; Hamre et al., 2004; Gardeur et al., 2007). The main advantage of this approach is that it considers simultaneously the impact of a large number of interrelated aquaculture factors using a limited number of experimental units. With a 2(8-4) reduced factorial design, each of the 16 combinations was tested once but each level of every factor was repeated eight times (Table 3).

2.2. Animals and rearing conditions

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

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

2.3. Sampling procedure

Fish were sampled on days 36 and 63 and were starved one day before. Six fish were removed randomly from each tank and anesthetized with MS-222 (150 mg/L). Blood was quickly collected by caudal vein puncture with heparinized syringes within 5 min and centrifuged at 7500g during 10 min at 4 °C. Plasma was stored at -80 °C until assayed. Fish were then rapidly killed by cervical dislocation to collect the whole brain and the anterior kidney. These organs were directly frozen in liquid nitrogen and stored at -80 °C.

2.4. Husbandry variables

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

2.5. Stress indicators

2.5.1. Cortisol and glucose assays

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

2.5.2. Brain neurotransmitters

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

For each fish, the whole brain tissue was weighed out and homogenized for 6 min in perchloric acid 4% (250 µL per 50 mg of tissue) containing 4 µM 2–3 dihydroxybenzoic acid (DHBA) as internal standard. The homogenate was sonicated for 20 s and then centrifuged at 21.000g 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, Sigma-Aldrich) and 10% MeOH adjusted to pH 3) and centrifuged at 21.000g 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 (0.80 V, Ag/AgCl – P/N 061677). The mobile phases were all degassed with helium. ChromeleonTM 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.) C18 analytical Kinetex column at 1 ml/min. The

mobile phase consisted of 75 mM NaH2PO4, 7 mM octane sulfonic acid (OSA, Sigma-Aldrich) and 10% MeOH adjusted to pH 3.0. The column was reconditioned by washing with 95% MeOH during 10 min and then re-equilibrated with buffer during 20 min. The column was kept at 25 °C.

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

2.6. Immune variables

2.6.1. Plasma lysozyme activity

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 10 µl of Na2HPO4 0.05 M pH 6.2 and 130 µl Micrococcus lysodeikticus (Sigma-Aldrich) solution (0.6 g/L). This assay was performed in triplicate. Absorbance was measured at 450 nm every 5 min during 30 min at room temperature. Lysozyme activity (units) is defined as the amount of enzyme decreasing the turbidity of 0.001 OD per min.

2.6.2. . Plasma haemolytic activity of the alternative complement pathway

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

2.6.3. Expression levels of some immune genes in anterior kidney

For further immune markers, the expression level of two immune-related genes was determined in anterior kidney tissues, namely C-type lysozyme and complement component 3 (C3). Primer sequences are presented in Table 5. Total RNA from anterior kidney was extracted using TRIzol Reagant (ThermoFisher Scientific) according to manufacturer's instructions. Tissue samples were homogenized using a SpeedMill PLUS homogenizer (Ana-lytikJena, Germany) in tubes containing ceramic beads and TRIzol Reagant. Total RNA was resuspended in 50 µl of DPEC-treated water. RNA integrity and concentration was checked by denaturat-ing gel electrophoresis (1.2% agarose) and OD260/OD280 and OD260/OD230 nm absorption ratio using Nanodrop-1000 (Thermo-Fisher Scientific). Twelve µg of each RNA sample were treated with FreeDNA kit (Ambion, Austin, TX, USA) to remove genomic DNA. mRNA was then retrotranscribed with Reverse Transcription System kit (Promega, Wisconsin, USA) according to manufacturer's instructions. The cDNA was then 20 times diluted and aliquoted. qPCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystem, Warrington, UK), 2.5 µl of both right and left primers (5 µM) and 5 µl of the diluted cDNA. A four steps experimental run protocol was followed: denaturation (10 min at 95 °C), amplification (40 cycles, 15 s at 95 °C, 1 min at 60 °C), melting curve (40 to 95 °C, heating rate 0.10 °C□s□1)*** and a final cooling step (4 °C) using a StepOne plus real time PCR machine (Applied Biosystem). Relative quantification of the target gene transcript was calculated following the Pfaffl method (Pfaffl, 2001), considering the Ct value and the primer efficiency. The relative expression levels of C-type lysozyme and complement C3 in each sample were normalized with the geometric mean of ef1-a and b-actin calculated by the relative standard curve method.

2.7. Statistical analyses

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

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

3. Results

3.1. Husbandry parameters

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

Further comparison by PCA approach confirmed that only the feed type was the major influencing factor husbandry

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

3.2. Stress and immune parameters

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

While no single factor influenced serotonergic activity in the brain, light spectrum and light intensity appeared clearly to act synergistically since the serotonergic activity increased when the red light was used with low light intensity (combinations c5, c12, c14 and c16) or when white light was combined with high light intensity (combinations c3, c9, c10 and c11) (Fig. 3c, $p < 0.001$). It is interesting to notice that the best three ranked combinations (c15, c1 and c16) in terms of husbandry performances were characterized by low values of serotonergic activity. Moreover, significant negative coefficient of correlation ($R2 = 0.31$; $p < 0.05$) was calculated between values of serotonergic activity on D63 and those of final body weight. So, red light spectrum at low intensity or white light spectrum at high intensity may induce both a higher stress status in pikeperch.

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

4. Discussion

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

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

4.1. Directive factors improving husbandry performances

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

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

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

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

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

Percid fish display sexual growth dimorphism as females grow faster than males (Craig, 2000). Therefore, rearing all-female populations would improve production of these species and this would be achieved by hormonal sex reversal treatment (Rougeot, 2015). The difference of growth performances between mixed-sex families and all-female families reaches 30% in juvenile Eurasian perch after 360 days of rearing (Rougeot and Mélard, 2016). However, the methods for all-female production have not been yet optimized for pikeperch, and since sex-determination in not possible with juvenile pikeperch, a mixed-sex population was used in the present experiment.

4.2. Directive modality interactions for pikeperch physiological and immune status

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

The significant impact of high temperature on plasma glucose observed in the present study may be related to a better feeding behaviour since the feed type influenced glycaemia. Higher temperature conditions ranging between 25 and 28 °C have been described to promote growth and feed utilization in pikeperch juveniles (Rónyai and Csengeri, 2008; Wang et al., 2009, Dalsgaard et al., 2013). However, if this is convenient for juveniles, it cannot be extrapolated to larger pikeperch without investigation since several studies have shown that optimal temperature seems to decrease with fish weight as observed with the African catfish (Clarias gariepinus) or the Atlantic cod (Gadus morhua) (Hogendoorn et al., 1983; Björnsson et al., 2007; Rónyai and Csengeri, 2008; Wang et al., 2009). In the present experiment, while the feeding behavior may have been improved by high temperature, this factor did not significantly affect growth performances. Frisk et al. (2013) reported that a smaller fraction of metabolic scope was utilized for digestion at 19 °C compared to 25 °C, indicating that low temperature conditions are more favorable for pikeperch reared under intensive culture conditions. Even if this was not evidenced in the present study showing low cortisol levels on both D36 and D63, high temperature condition may induce along-term stress that leads to energy reallocation resulting in less energy available for some biological functions such as growth, reproduction or disease resistance (Schreck et al., 2001; Tort, 2011; Segner et al., 2012, Milla et al., 2015).

Serotonergic activity has been described several times as a good indicator of acute and chronic stress in various fish species including the Senegalese sole Solea senegalensis and several salmonids (Winberg and Nilsson, 1993; Gesto et al., 2013, 2016; Conde-Sieira et al., 2014). When considering serotonergic activity level in the presented study, red light spectrum at low intensity or white light spectrum at high intensity induced both a higher stress status. Environmental colors affect the vision of fishes, influencing for example food intake, signals for hierarchical status, reproduction, growth and even survival (Downing, 2002; Politis et al., 2014; Luchiari and

Pirhonen, 2008). Species-dependent preferences are a reflection of the photic environment the populations have evolved in (Migaud et al., 2007). Luchiari et al. (2009) showed an improved specific growth rate and feed efficiency under red light, only considering high light intensity. In parallel, Luchiari et al. (2006) described a preference for low light intensities. Among the three stress indicators tested in the present study, only brain serotonergic activity showed plausible correlation with husbandry performances, indicating that high stress level is one of the main causes of low husbandry performances in pikeperch culture. By elsewhere, brain serotonergic activity maybe considered as a more reliable stress indicator in percid fish than plasma cortisol. It has been suggested that a low degree of responsiveness to stressors in cultured fish may result in better performances (Pottinger and Pickering, 1997). The converse may also be true considering the greater advantage of fish with a high degree of stress responsiveness, through a faster acclimation to environmental and social changes (Pottinger and Carrick, 1999). While the latter authors have described no correlation between cortisol response and growth performances in rainbow trout, better growth was obtained in low cortisol responders (Fevolden et al., 2002). And, as well as plasma cortisol level, it was reported that brain serotonergic, noradrenergic and dopaminergic activities are influenced by dominant-subordinate relationships in several fish species, including the rainbow trout (Overli et al., 1999). Social dominance was not followed in the present experiment but could have influenced the lack of relationship between growth and cortisol level since it has been shown in other fish species that socially defeated animals exhibit increased glucocorticoid secretion, sustained sympathetic activation and other physiological stress responses (Winberg and Lepage, 1998; Overli et al., 1999).

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

Concerning immune status, humoral immune activities were slightly impacted by some tested factors during the 2-month period. The effects of low oxygen saturation on complement activity on day 36 were no longer observed on day 63, suggesting only a short-term effect. This decrease in the impact of low oxygen saturation may be account for the increase in ACH50 values between D36 and D63. On the other hand, the effect of low oxygen level on humoral immunity may be explained by the fact that pikeperch requires high oxygen saturation and recommendations are to maintain the oxygen level above 50% saturation to sustain growth and biofilter performance (Dalsgaard et al., 2013). In contrast to what was observed for growth rate, long photoperiod combined with high light intensity, which was characterized as a stressful factor, led to an increase in the immune expression level of both tested genes. Long photoperiod increased the exposure time to stressful light conditions that may have resulted in a short-term immune stimulation. However, since no single effect of photoperiod on stress and immune status was detected, the impact of such factor should be investigated in further experiments. The other factors tested in the present experiment were not revealed as directive variables for stress or immune status.

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

5. Conclusion

This experiment was based on a factorial fractional design study which allowed simultaneously to study the effects of 8 common husbandry practices in two modalities on pikeperch growth and welfare. Several husbandry practices, including light intensity, feed type and temperature were revealed to be directive factors for husbandry and stress parameters in pikeperch aquaculture while immune status was mainly influenced by photoperiod and oxygen saturation level. Best husbandry performances were thus obtained with sinking feed and low light intensity, and no obvious relation was established between stress status and growth parameters. Further investigations focusing on light characteristics, temperature and feed type are needed to improve management strategies of pikeperch culture, and knowledge on the interrelations between stress and immunity in percid fish.

Table 1. Selected modalities for the 8 environmental factors.

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

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

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

Table 3. Combinations (c1–c16) of the factors following a 2(8-4) reduced factorial design and results. Final weight heterogeneity (CV); Light spectrum: W = White, R = Red; Feed: S = Sinking, F = mid-Floating; Grading: Y = with manipulationsmimicking grading, N = without grading. Global score: note of interest for each combination, based on husbandry output variables. The grey lines correspond to the five best combinations according to the global score of interest.

	Variables tested							Variables studied																			
Combination of the factors	Light intensity (lux)	Density (kg·m^{-3})	Light spectra	Photoperiod (h)	Temperature (°C)	Feed	Grading	Oxygen saturation (%)	Final individual weight (g)	Mortality Rate (%)	CV (%)	Specific growth rate (%d-1)	Plasma Cortisol (D36, ng·mL^{-1})	Plasma Cortisol (D63, ng·mL^{-1})	Plasma Glucose (D36, µg·mL^{-1})	Plasma Glucose (D63, µg·mL^{-1})	Serotonergic activity (D36)	Serotonergic activity (D63)	ACH50 (D36)	ACH50 (D63)	Lysozyme activity (D36, U)	Lysozyme activity (D63, U)	Relative C3 gene expression to ef1+βactin	Relative *lysozyme* gene expression to ef1+βactin (D63)	Relative C3 gene expression to ef1+βactin (D63)	Relative *lysozyme* gene expression to ef1+βactin	Global score of interest	Rank of the global score
c1	10	30	W	24	21	S	Y	90	168	4	37	0.9	84	31	457	318	0.84	0.61	98	202	17	19	0.011	0.018	0.00019	0.079	3.9	2
c2	100	15	R	10	26	F	N	60	146	3	50	0.7	17	13	378	427	0.71	0.77	145	142	16	19	0.027	0.016	0.00034	0.027	1.3	6
c3	100	15	W	24	21	S	N	60	172	13	40	1.0	20	15	355	371	0.94	0.74	162	215	16	18	0.014	0.045	0.00014	0.114	3.1	5
c4	100	30	R	10	21	S	N	90	143	31	29	0.7	19	14	264	340	0.79	0.72	153	216	17	19	0.027	0.024	0.00014	0.042	0.4	8
c5	10	15	R	10	21	S	Y	60	131	7	53	0.5	15	14	424	379	0.96	0.81	157	181	16	19	0.0016	0.029	0.00026	0.025	−0.5	10
c6	10	15	W	10	21	F	N	90	88	7	67	0	16	14	342	365	0.53	0.82	76	264	15	15	0.004	0.061	0.00050	0.090	−5.4	16
c7	100	15	R	24	21	F	Y	90	113	24	61	0.3	17	13	273	343	0.69	0.78	140	210	14	19	0.021	0.024	0.00083	0.065	−4.0	15
c8	10	15	W	24	26	F	Y	60	146	10	53	0.7	60	13	322	359	0.60	0.72	125	244	16	21	0.004	0.022	0.00049	0.030	0.4	7
c9	100	15	W	10	26	S	Y	90	158	13	39	1.1	26	13	371	411	0.87	0.75	132	225	17	18	0.015	0.055	0.00248	0.088	3.1	4
c10	100	30	W	10	21	F	Y	60	122	41	52	0.7	17	14	339	317	1.06	0.76	173	261	17	20	0.008	0.028	0.00005	0.011	−3.0	13
c11	100	30	W	24	26	F	N	90	148	18	61	0.8	89	29	328	405	0.81	0.72	101	257	15	20	0.012	0.045	0.00100	0.015	−0.6	11
c12	10	30	R	10	26	F	Y	90	114	24	57	0.3	23	13	304	325	1.19	0.68	96	250	11	17	0.006	0.061	0.00027	0.014	−3.6	14
c13	100	30	R	24	26	S	Y	60	151	32	37	0.8	47	15	466	412	0.71	0.77	100	233	18	20	0.019	0.067	0.00059	0.021	0.3	9
c14	10	30	R	24	21	F	N	60	117	4	72	0.4	17	13	271	337	0.94	0.75	132	298	15	16	0.012	0.033	0.00132	0.019	−2.8	12
c15	10	30	W	10	26	S	N	60	167	3	36	0.9	27	15	420	373	0.67	0.73	124	249	16	16	0.027	0.049	0.00059	0.017	4.0	1
c16	10	15	R	24	26	S	N	90	169	7	40	0.9	18	15	375	369	0.89	0.64	67	267	14	18	0.003	0.019	0.00012	0.010	3.4	3
Mean									140	15	49	0.7	32	16	356	366	0.82	0.73	124	232	16	18	0.014	0.037	0.00058	0.042		
SD									24	12	13	0.3	24	6	63	35	0.17	0.06	31	38	2	2	0.008	0.017	0.00062	0.034		

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

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

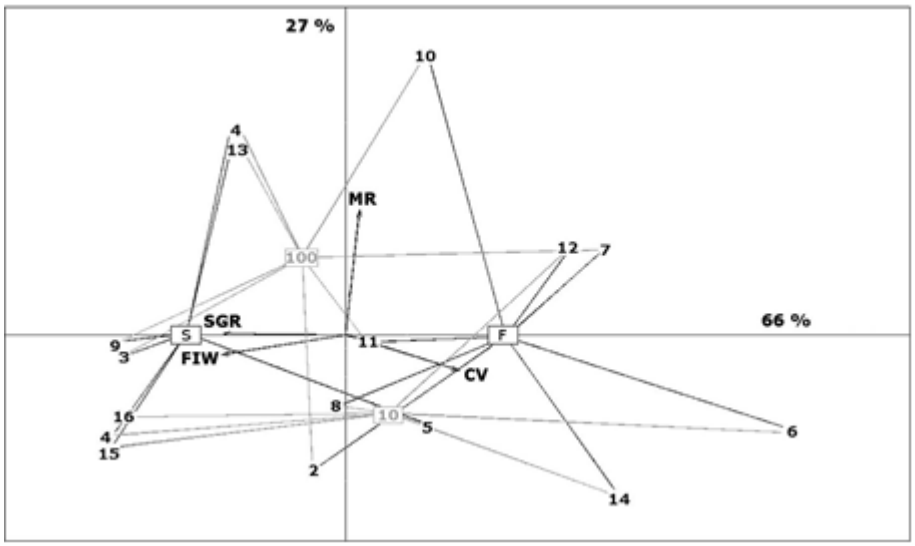

Figure 1. Projections of husbandry outputs and combinations (1–16) on the two first axis of the principal component analysis. The plans 1 and 2 of the PCA explain 93% of the projected inertia. Axis 1 (inertia 66%) represents Specific growth rate (SGR), Final individual weight (FIW), final weight heterogeneity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

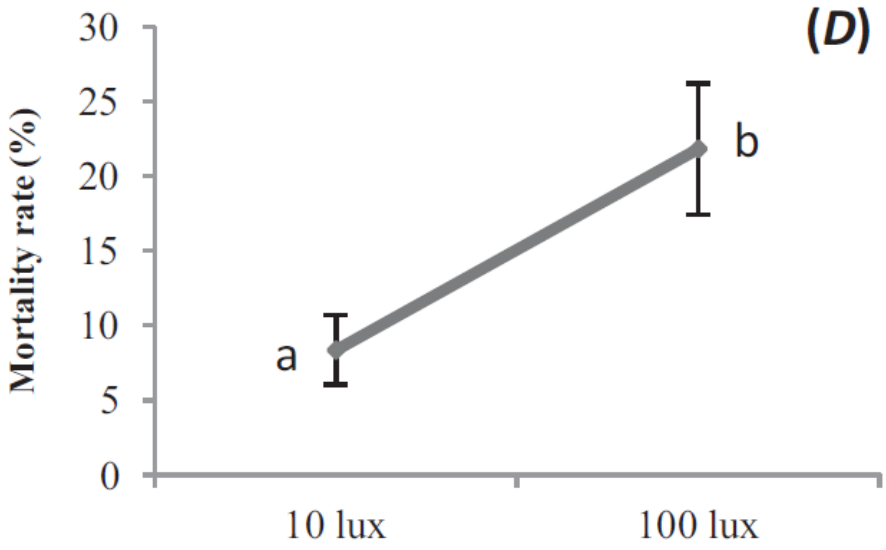

Figure 2. Effects of tested factors on (A) specific growth rate, (B) final individual weight, (C) final weight heterogeneity, and (D) mortality rate. Day 63. Data are presented as mean ± SEM (n = 8). Lowercase letters indicate a significant difference atp < 0.05.

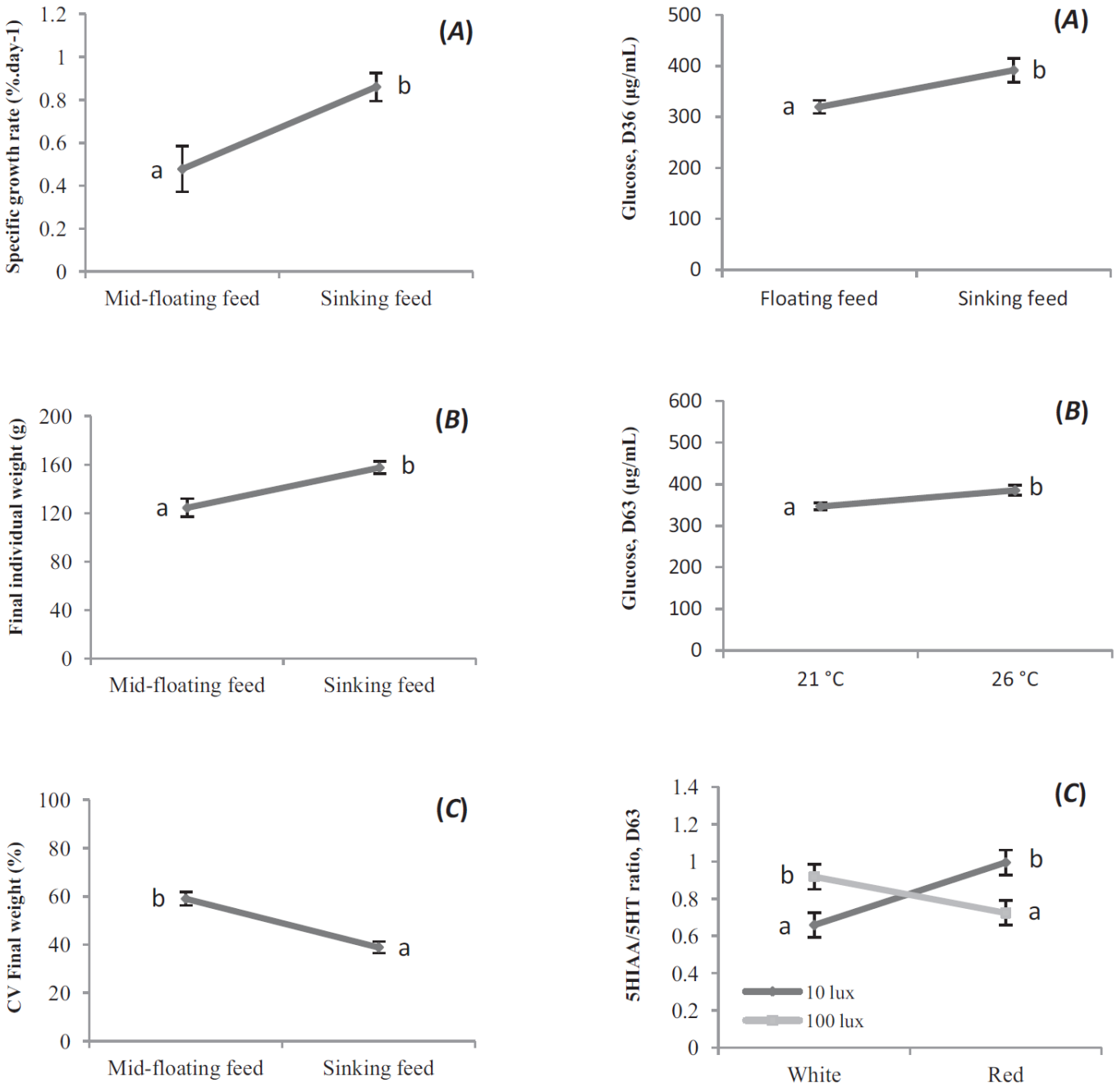

Figure 3. Effects of tested factors on (A) plasma glucose D36, (B) plasma glucose D63, and (C) serotonergic activity in brain. Data are presented as mean ± SEM (n = 8 (A); 8 (B); 4(C)). Lowercase letters indicate a significant difference at $p < 0.05$.

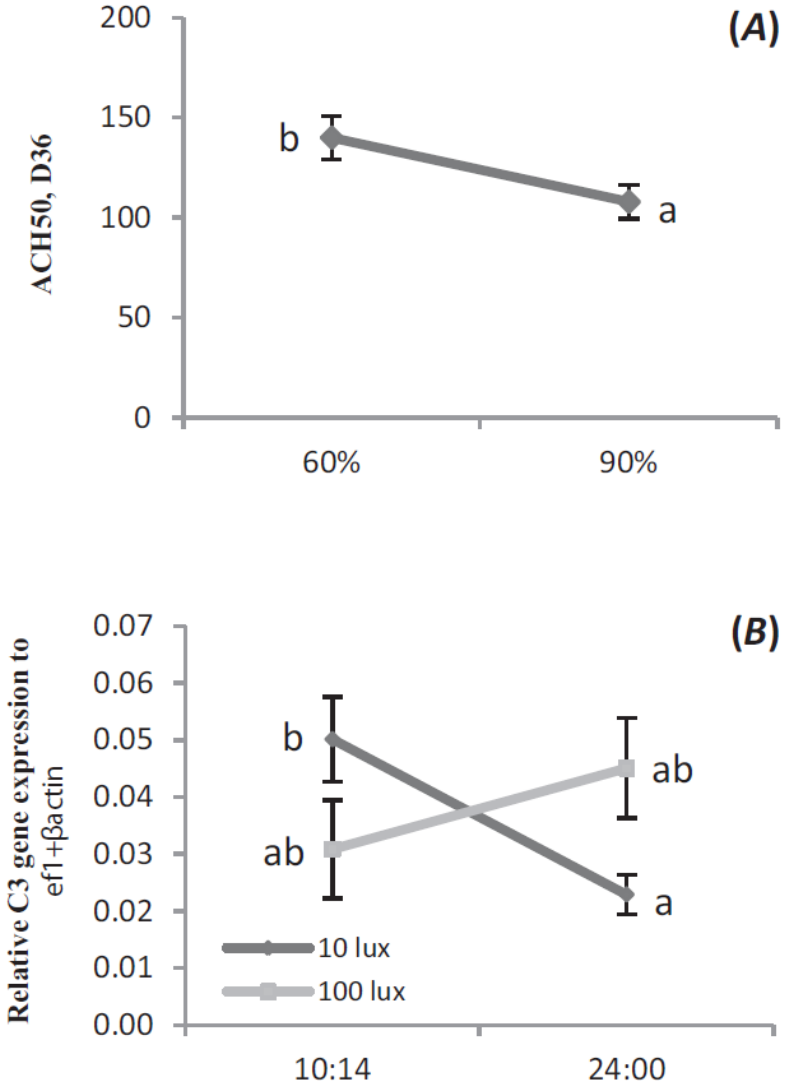

Figure 4. Effects of tested factors on (A) plasma complement activity, and (B) relative C3 gene expression. Data are presented as mean ± SEM (n = 8 (A); 4 (B)). Lowercase letters indicate a significant difference at $p < 0.05$.

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