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ABSTRACT

This study aimed to assess the effect of constant darkness applied to fish during controlled breeding on reproductive traits in domesticated females of Eurasian perch. Based on the assumption that keeping fish in constant darkness during the reproduction operation may reduce stress, suspected to be responsible for variable spawning effectiveness in this species. Two conditions were assessed (16 h light per day [group **16L**] and constant darkness [group **0L**], two tank replicates per condition). The reproductive protocol involved a 7-day-long adaptation period for group 0L where photoperiod was reduced by 2.3 h a day down to constant darkness. After the adaptation period, two hormone injections (salmon gonadoliberin analogue) were applied to both groups: priming (10 μg/kg) and resolving (25 μg/kg) with a 7-day interval between them. During the study, morphometric indices were recorded and blood, brain, and pituitary samples were collected to assess stress markers and determine hypothalamic-pituitary-gonadal axis functioning via measuring blood plasma hormones, as well as gonadoliberin and gonadotropins (luteinising hormone [**LH**] and follicle-stimulating hormone [**FSH**]) transcript abundance (n = 7 for each group at each sampling point). In addition, kinetics of the final oocyte maturation (**FOM**) process, ovulation rate, and egg quality of each group was monitored (n = 12 for each group). The results indicated that there were no differences in terms of morphometry, FOM kinetics, and most stress indices between groups throughout the experiment, except haematocrit, which increased immediately following the acclimation period in fish kept in darkness. Constant darkness negatively affected plasma levels of 17α,20β-dihydroxy-4-pregnen-3-one (**DHP**) and LH transcript expression at the time of the second hormone injection. This indicated that exposure to constant darkness negatively affected priming of the hormonal dose applied, resulted in the disruption of ovulation, and reduced ovulation rates (50%) for group 0L, as compared to 16L (91%). The findings of this study clearly indicate that constant darkness may have significant deleterious effects on reproductive traits throughout out-of-season induced, hormonally supported, controlled reproduction. Therefore, we advise against the use of constant darkness when managing broodstock reproduction in domesticated Eurasian perch.

Implications

In this study, we tested the literature-data-based hypothesis that constant darkness may improve the reproductive traits of domesticated *Perca fluviatilis* females by diming the stress reaction coming from frequent handling. The results have revealed that constant darkness did not affect the stress response but rather reproductive hormones level, leading to lowered reproductive performance (i.e. lack of ovulation and overmaturation phenomena). The findings of this study provide new and valuable knowledge related to the physiological reactions of hormonally stimulated fish to modified lighting conditions, which has been largely ignored until now in commercially-relevant fish species.

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Introduction

Controlling reproduction in domesticated broodstock is a major factor that affects the profitability of commercial fish reared exclusively in recirculating aquaculture systems (**RASs**) (Fontaine and Teletchea, 2019), such as Eurasian perch (*Perca fluviatilis*). Despite the fact that commercial production of Eurasian perch has already been established in several countries, expansion of its production will require further optimisation of production technology with selective breeding programmes considered as highest priority (Fontaine and Teletchea, 2019; Policar et al., 2019). The success of selection is highly dependent on reproductive control and performing crossbreeding (through *in vitro* fertilisation) using specimens exhibiting desired traits. Therefore, a great deal of attention has been paid to the development of controlled reproductive protocols in this species (Żarski et al., 2019).

Recently, a novel, highly efficient hormonal treatment protocol was developed for Eurasian perch (Żarski et al., 2019). It allowed considerable synchronisation of ovulation, contributing to minimisation of cannibalism during the early life stages and facilitation of hatchery functioning (Król et al., 2019). However, despite the great degree of progress that has been achieved regarding the controlled reproduction of Eurasian perch, variable egg quality remains a serious concern (Policar et al., 2019).

Photoperiod (Bromage et al., 2001) and temperature (Wang et al., 2010) are two of the best known environmental factors that affect gonadogenesis in captively grown fish. However, when it comes to hormonally induced spawning in percids, primarily the effect of temperature on final oocyte maturation (**FOM**) and spawning has been studied in detail (Żarski et al., 2013). The effect of lighting conditions on these processes has been largely ignored. However, it is well known that the endocrine system in fish is controlled by lighting conditions via neurohormones, such as light-specific monoamines, e.g. melatonin (Bromage et al., 2001) or serotonin (Baekelandt et al., 2020), which have been reported to affect the FOM process and regulate ovulation (Maitra et al., 2013; Prasad et al., 2015). One of the factors suspected to be responsible for problems with spawning quality in Eurasian perch is stress (Castets et al., 2012). Especially, that controlled reproduction involves highly stressful multiple handling of the same fish to assess spawning readiness (Żarski et al., 2017b). That is why special attention should be given to the modulation of lighting conditions, which were reported to have a strong modulatory effect on stress and immune responses in cultured percids (Baekelandt et al., 2019 and 2020). Therefore, there is a need for more detailed investigations on the effect of lighting conditions during hormonally stimulated reproduction in Eurasian perch, especially, when the modulation of lighting conditions is reconsidered as a tool for the reduction of stress during controlled reproduction operations.

During controlled, hormonally supported reproduction in Eurasian perch, the use of a photoperiod between 12 and 16 h of light is typically applied (Żarski et al., 2015 and 2019). It is commonly known that light is a strong modulator of endocrine system in fish, with constant darkness usually having negative effects on the functioning of endocrine axis (Cowan et al., 2017). However, in controlled hormone-supported reproduction of other predatory freshwater fish species, such as European catfish (*Silurus glanis*), constant darkness has been applied, aiming to reduce stress during hormonally induced out-of-season reproduction (Ulikowski, 2004) by reducing the number of visual stimuli during the reproductive operation. The effect of constant darkness, to reduce stress load, was also tested in pikeperch (*Sander lucioperca*) where reduced glucose levels, a chronic stress marker, in fish exposed to handling stress was recorded (Pourhosein Sarameh et al., 2013). In another study by the same group, constant darkness was reported not to affect the overall reproductive traits significantly, when compared to photoperiod of 12**L**:12**D** (**L**ight:**D**ark phases expressed in hours per day) or 24L:0D (Pourhosein Sarameh et al., 2012). However, in that case, fish were left to spawn spontaneously on the nests, which limited the overall stress load significantly (lack of frequent handling and manipulation) and it is not clear whether constant darkness would have positive or negative effect during highly stressful, typical controlled reproduction operations (which involves hormonal treatment, frequent manipulation and hand stripping of the gametes; Żarski et al., 2017b and 2019). Additionally, in the study on pikeperch, Pourhosein Sarameh et al. (2012) did not apply hormonal stimulation. It should be mentioned that Khoo (1980) reported the lack of significant influence of constant darkness on hypothalamic-pituitary-gonadal axis in hormonally treated goldfish (*Carassius auratus*), indicating that photoperiod does not play a modulatory role on reproduction-related endocrine system following application of exogenous hormones. Considering that in pikeperch, various stress markers varied between the light and dark phase in juveniles reared at 12L:12D photoperiod (Baekelandt et al., 2019), we have decided to study the application of constant darkness as potentially beneficial practice in controlled reproduction in RAS-grown domesticated Eurasian perch during induced spawning. The potential benefits could be associated with a reduced stress load during the frequent ripeness checks that fish are usually exposed to, without negative darkness-related effect on functioning of endocrine system.

Ovulation in finfishes is preceded by temporal induction of specific hormones that regulate the hypothalamic-pituitary-gonadal axis (**HPG** axis) (Fontaine et al., 2003; Żarski et al., 2015). In teleosts, this involves a surge of luteinising hormone (**LH**), which affects production of 17α-hydroxyprogesterone (Nagahama and Yamashita, 2008). This is further converted to either estradiol (**E2**), which is mainly produced during oocyte growth, or the maturation inducing steroid, which is produced during FOM (Nagahama and Yamashita, 2008). In percids, 17α, 20β-dihydroxy-4-pregnen-3-one (**DHP**) is a putative maturation inducing steroid that is also responsible for promoting the ovulation process (Yamashita et al., 1992) and has been recognised as a main maturation inducing steroid in other fish (Żarski et al., 2015). Therefore, while considering the effect of evaluating different external factors, such as lighting conditions, on spawning performance, analysis of various indices that are indicators of HPG axis function should be evaluated to reveal mechanisms that affect potential reproductive disorders. This includes measuring expression levels of gonadotropin-releasing-hormone (**GnRH**) genes in the brain, gonadotropin levels in the pituitary, and levels of sex steroids in blood plasma (Khendek et al., 2017 and 2018). When considering the association of the stress response in this process, various stress and immune response markers need to be analysed due to their interdependent nature in fish (for details see Tort, 2011), including elevated cortisol level which was already reported to have negative effect on reproductive effectiveness in percids (Falahatkar and Poursaeid, 2013; Pourhosein Sarameh et al., 2013).

The aim of this study was to evaluate whether constant darkness affects reproductive performance in hormonally treated, domesticated Eurasian perch when compared to a typical light regime (16 h light per day) applied during hormonal treatment. Throughout the experiment, we aimed to assess functioning of the HPG axis and determine levels of various stress markers to determine which processes were affected by eliminating the photoperiod information throughout induced spawning.

Material and methods

Preparation of broodstock

Fish (F3 generation reared in captivity; age 2+; 59 females and 41 males; average weight, 204 ± 36 g) were obtained from a commercial fish farm (SARL Asialor, Pierrevillers, France). The experiment was carried out using an Aquaculture Experimental Platform (registration number for animal experimentation C54-547-18) belonging to the Unit Research Animal And Functionality Of Animal Products laboratory located at the Faculty of Sciences of the University of Lorraine (Nancy, France). Fish were transferred to the facility and placed in 2000 L tanks operated using a RAS (total volume 3000 L). The system was supplied with tap water with c. a. 5% of water exchange a day. Fish were fed once a day until apparent satiation with a commercial feed (47% protein, 13% fat, 1.9% cellulose, 11% ash, 14% starch, 1.5% phosphorus) throughout the study (feeding rates ranged between 0.2 and 1.2%, depending on the water temperature). Water parameters (mean ± SD) were measured once a week: dissolved oxygen (11.2 ± 1.1 mg/L), ammonia (NH_4^+; 0.01 ± 0.02 mg/L), and nitrite (NO_2^-; 0.03 ± 0.02 mg/L). To obtain a pH greater than 7.4, sodium bicarbonate ($NaHCO_3$) was used. Next, the fish, which had not previously spawned, were exposed to a 300-day-long photo-thermal programme that facilitated the induction of gonadal development, as described by Fontaine et al. (2015). After that period, the experiment was initiated when the temperature following the wintering period reached 10 °C (Fig. 1A).

Experimental design

At the beginning of the experiment, fish were sorted according to sex. Sex was confirmed each time with catheterisation (Supplementary material S1), and seven randomly chosen females were sacrificed for initial sampling [S1] (as described in section *Sampling procedure*). Next, the remaining females were randomly assigned to one of the four batches. Each batch consisted of 13 females and was placed in a separate RAS. Each RAS was independent and located in a different room (hereinafter referred to as an *ecotron*), within which biological filtration, UV sterilisation, and aeration were provided. In each *ecotron,* temperature and photoperiod were independently adjustable and automatically controlled. Two *ecotrons* constituted an experimental group 16L (16L:8D) and two others were used for experimental group 0L (constant darkness). After

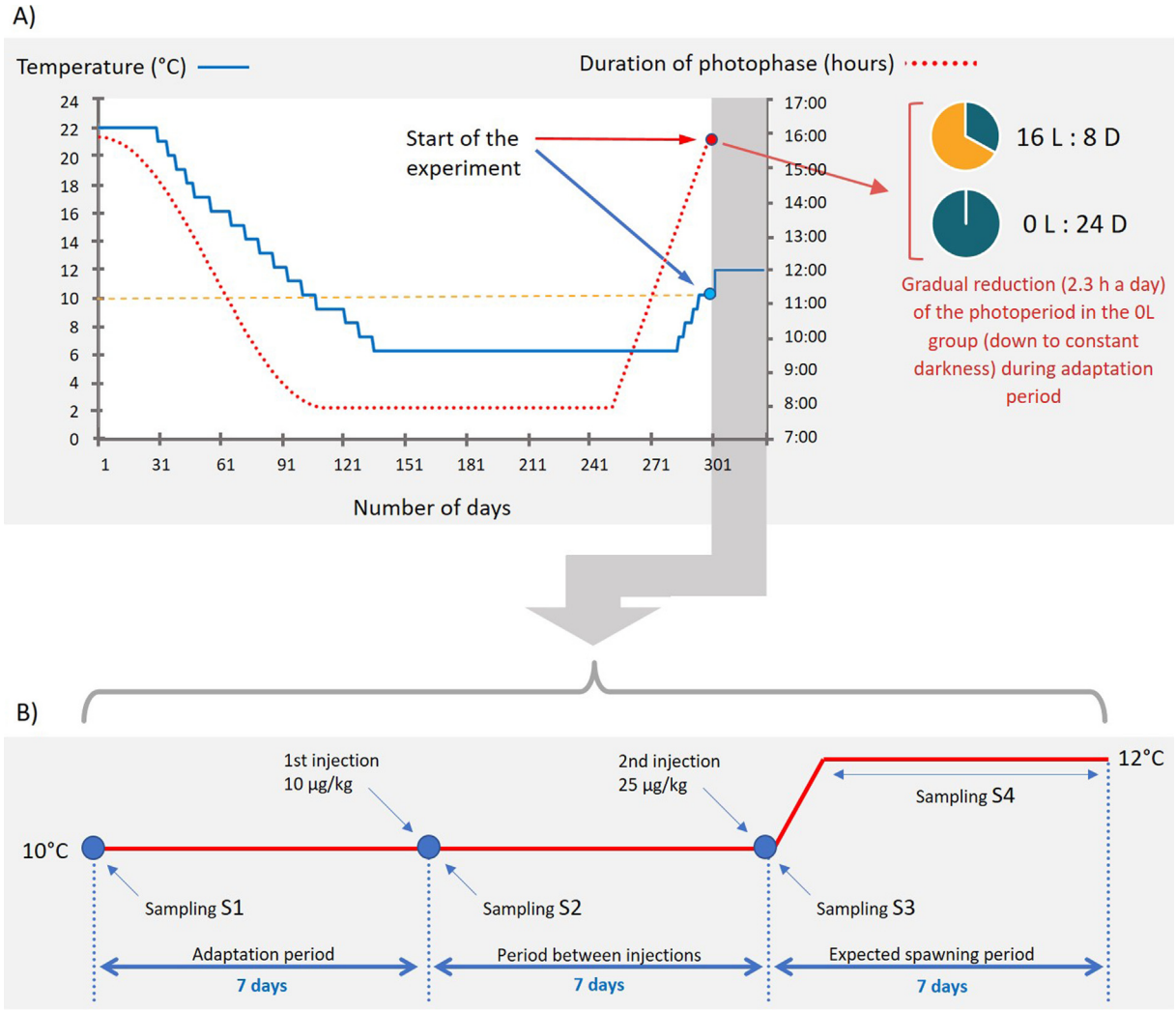

Fig. 1. A scheme summarising the overall experimental approach is shown. (A) The photo-thermal programme applied before initiation of the experiment (during which the fish in both groups were kept together and exposed for 301 days to the same photo-thermal conditions). (B) The thermal regime, experimental protocol (including hormonal treatment protocol) and sampling strategy used. 16L:8D refers to a group exposed to 16 h of light and 8 h of darkness per day. 0L:24D refers to group exposed to constant darkness.

fish were distributed in their respective *ecotrons,* a 7-day-long adaptation period was initiated for 0L group during which the photoperiod was gradually changed by 2.3 h a day. This rapid adaptation period was designed in order to keep the balance between the need for following the photo-thermal programme during initial phases of FOM and to not extend the process by prolonged acclimation. Such an approach was considered commercially relevant and feasible, and therefore included in the existing reproductive protocols. At the end of this period, either a 16 or 0 h light photophase was reached (Fig. 1A and B). At the end of the photoperiod adjustments, when the 0L group had been in constant darkness for 24 h, seven fish from each group were used for sampling [S2], and the remaining fish received a priming salmon GnRHa (amino acid sequence: Pyr-His-Trp-Ser-Tyr-D-Arg-Trp-Leu-Pro-NHEt; Bachem, Switzerland) hormonal injection (at a dose: 10 μg/kg) (Fig. 1B). After another seven days, seven more females from each group were sampled [S3] and the remaining females received a resolving hormonal injection (25 μg/kg). Such hormonal treatment procedure and dosage were recommended by Żarski et al. (2019). After the resolving injection, temperature was gradually raised to 12 °C and then remained constant until the end of the experiment (Fig. 1B). Afterwards, each female was assessed every 6 h for ovulation by applying gentle abdominal pressure. Each female was removed from the experiment when one of the following three criteria were met: (i) eggs were stripped, (ii) egg atresia (lack of typical egg-ribbon structure, semi-liquid mass with individual oocytes stripped – typical symptoms of egg atresia, followed by overripening) was observed, or (iii) lack of ovulation was determined for at least 7 days following the resolving injection. When ovulation was determined, eggs were collected and subjected to fertilisation (as described in section *Fertilisation and egg quality evaluation*). The female was then euthanised and subjected to the fourth round of sampling [S4]. Females that began resorption or did not spawn for 7 days following the resolving injection were not sampled throughout S4. Thermal conditions and the hormonal injection protocol used were in accordance with those described in a previous report (Żarski et al., 2019) and were visualised in Fig. 1B, along with the experimental scheme and sampling strategy.

During the sampling events, doors of each *ecotron* were opened and within less than a minute, fish were taken out with a net and placed into the anaesthetic bath. During that period, ambient light from the corridor reflected into the *ecotron* illuminated the tank and water surface. Therefore, during the sampling, fish were exposed to dim light for a very short period. During the hormonal injection and ovulation control, similar procedure was applied, though it lasted longer (20 min for hormonal injection and 5–10 min during ovulation control). Tissue sampling and morphometric analysis were always performed between 8:00 and 10:30, after which the remaining fish were injected with the hormonal preparations (between 10:30 and 12:00). Additionally, to minimise the potential bias linked to the sampling time, the samplings of each replicate from each group were done in a randomised order for each sampling day.

Males were kept in a separate, fifth *ecotron* and were exposed to the same photo-thermal programme as females of group 16L. The males were also hormonally stimulated when females received priming injections of 100 μg/kg of salmon GnRH analogue, as recommended by Żarski et al. (2017a).

Sampling procedure

Before fish were handled and sampling procedures were performed, females were anaesthetised via immersion in a 150 mg/L MS-222 solution. Blood samples were taken from the caudal vein of fish using a heparinised syringe. Blood samples from each fish taken at S1, S2, and S3 were performed within a maximum of five min from opening the doors of the *ecotron*, as required for the evaluation of cortisol levels within blood plasma (Milla et al., 2010). Next, fish were euthanised via overexposure to MS-222 solution (300 mg/L; Topic Popovic et al., 2012) after wet weight was recorded. Afterwards, the gonad and liver were dissected, and their weights were determined. Simultaneously, the brain and pituitary were removed from each sampled fish, placed in a cryotube, and snap-frozen in liquid nitrogen for subsequent RNA extraction procedures. Determination of the GnRH in the whole brain was reported to be an appropriate method allowing investigation of transcription regulation of GnRH along the reproductive cycle in perch (Khendek et al., 2017). Additionally, the maturation stage of oocytes was determined based on the 6-stage FOM classification system, as described previously (Żarski et al., 2011) (for details see: Supplementary material S1). During sampling period S4 (Fig. 1B), eggs were stripped before their weights were determined. Next, remaining parameters (except gonad weight) were assessed, and sampling of the tissues was performed using a method similar to that described for previous rounds of sampling (S1-S3).

Fertilisation and egg quality evaluation

After eggs were hand-stripped and placed into a dry, plastic container, their weights were determined. Next, milt from male perch was collected by direct stripping into a dry syringe, as recommended for the controlled reproduction of fish whenever sperm is used within a short period of time (Król et al., 2018). Collected gametes were subjected to a fertilisation procedure, described previously (Żarski et al., 2012). Briefly, eggs were first activated in hatchery water and after 30 s, sperm (1 ml mixture of sperm obtained from three males per 100 g of eggs) was added to promote fertilisation. After gentle mixing for five min, eggs were placed in an individual incubator at 14 °C. The incubators were vertical small "raceways" (width × length × depth: 12 × 39 × 7.5 cm) with a nylon net bottom (mesh size 0.2 mm), individually fed with fresh, well oxygenated water.

After three days of incubation (72 h postfertilisation), two subsamples were removed from the egg-ribbon (150–250 eggs each) and the number of eggs, as well as viable embryos present in each sample, was determined via direct counting using a stereoscopic microscope. Next, each subsample was placed in a separate incubator, where it was incubated until hatching. During incubation, 80% oxygen saturation and a temperature of 14 °C were maintained. Freshly hatched larvae were collected and transferred to 30% ethanol (Gagliano et al., 2006) and larvae from each subsample were collected individually. Hatched larvae were counted to determine hatching rates. Each larva was evaluated using a stereoscope for the presence of body deformities, as described by Alix et al. (2017), to determine the larval deformity rate. In summary, these procedures allowed researchers to determine the fertilisation rate 72 h postfertilisation, hatching rate, and deformity rate of hatched larvae (for details see: Supplementary material S1).

Analysis of blood parameters

Blood samples were divided into three aliquots. The first was used to determine haematocrit (for details see Supplementary material S1); the second was used to determine leukocyte populations (leukogram; for details see Supplementary material S1); and the third was centrifuged (15 min at 6 700*g*) to obtain blood plasma needed to determine biochemical characteristics and hormone levels. Blood plasma was aliquoted into smaller portions, immediately snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent use.

Biochemical parameters

Blood plasma samples were analysed for various stress-related indices with an automatic chemistry analyser (Catalyst Dx Chemistry Analyzer; Idexx Labs; USA) using dedicated test slides (custom panel). Biochemical tests for glucose (catalogue test number 98-11076), total protein (cat. test number 98-11085), albumin (cat. test number 98-11065), alanine aminotransferase (cat. test number 98-11067), calcium ions (cat. test number 98-11086) and triglycerides (cat. test number 98-11086) were performed. Each sample was thawed only once at room temperature and fibrin clots were removed by a needle, if present. All indices listed above were assessed at the same time to eliminate the need to perform multiple freeze/thaw cycles. Prior testing, quality control panel (QC Clip Panel™, cat. number 98-13700) was run using VetTrol™ (standardised, lyophilised bovine serum, in a special formulation, designed to control the operation of the Catalyst Dx analyzer) control fluid (cat. number 98-11379). Standard curves for each test lot were updated automatically online by manufacturer service.

Determination of hormonal level

Evaluation of plasma hormone levels was performed using commercially available ELISA kits. For this study, estradiol (582251-96, Cayman Chemicals, Ann Arbor, MI, USA; Hermelink et al., 2013), cortisol (KAPDB270, DIAsource, Louvain-la-Neuve, Belgium; as described by Khendek et al., 2018) and DHP (MBS2602842, MyBiosource, San Diego, CA, USA; as described by Roche et al., 2018) kits were purchased. For specific information on each kit, please see also Supplementary file S1. All analytical procedures, as well as calculations of the final concentrations, were performed according to the manufacturer's protocols. Optical density values were obtained using a Multiskan FC Microplate Photometer (Thermo Scientific).

Analysis of expression of luteinising hormone, follicle-stimulating hormone and gonadoliberine

Total RNA was extracted from each brain and pituitary using TRIzol reagent (Cat. No. 15596026, Life Technologies Corporation, Carlsbad, CA, USA) following the protocol provided by the manufacturer. The concentration and purity of RNA were assessed using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) instrument. RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA), and all the samples had RIN (RNA integrity number) values higher than 8.0. Next, the samples were treated with TURBO DNase (Cat. No. AM2238, Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) to remove genomic DNA contamination. Complementary DNA was generated using the MAXIMA First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). Real-time quantitative PCR was performed using a LightCycler 480 II (Roche, Bazylea, Switzerland) and a DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific Inc). Enzyme activation and denaturation were performed by incubating samples for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min (Żarski et al., 2017c). Analysis was preceded by earlier verification of the reaction efficiency by analysing C_T values of each primer pair using serial 10-fold dilution of template cDNA. During the verification of reaction efficiency, appropriate negative control samples were included (RNA, RNA mixed with all chemicals used for reverse transcription but without reverse transcriptase [RT-] and deionised, diethylpyrocarbonate water used for dilution of the samples and reagents). After amplification of the template, melting curve analysis was performed for each sample to verify amplification specificity. Each pair of primers exhibited efficiency values between 85 and 105%.

Primers targeting genes encoding LH (*βlh*), FSH (*βfsh*), GnRH 3 (*gnrh*), and β-actin (*β-actin*; used as a housekeeping gene) for Eurasian perch were used following Khendek et al. (2017) (Table 1). From among three main forms of GnRH usually recorded in fishes, we have analysed GnRH 3 which was reported earlier to be the form involved in functioning of the HPG axis in Eurasian perch (Khendek et al., 2017) and in a range of other fish species (Chen and Fernald, 2008). Real-time quantitative PCR reactions to assess the expression of each gene within each sample were performed in duplicate. Expression values were determined using the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001) and further presented as relative expression to *β-actin*. The coefficient of variation (CV = SD/mean * 100) of C_T values for reference gene (*β-actin*) for all samples analysed was below 1.9% (mean C_T = 20.5) in the brain and below 3.4% in the pituitary (mean C_T = 15.58).

Data analyses and statistics

During the study, an animal (and, when relevant, the tissues collected from it) was considered as an experimental unit. All data have been presented as means ± SD. Data (except for maturation stages, gonadosomatic index, hepatosomatic index, ovulation rate, and latency time) were found to be normally distributed (as revealed by a Shapiro-Wilk test) and they were further assessed using a two-way ANOVA (using a Sidak correction) with 95% confidence interval. Sampling (S1–S4) time and lighting conditions were the two factors tested. Statistical differences between lighting condition comparisons at each sampling point and intra-group comparison between sampling periods were observed. The differences between groups at each sampling point with respect to maturation stage, gonadosomatic as well as hepatosomatic indices and latency time, were assessed using the Mann–Whitney test (with a 95% confidence interval), whereas the differences between groups and between the sampling points were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test (at a 95% confidence interval). Additionally, ovulation rate comparisons between the groups were analysed using the chi-square test. Data analysis and visualisation were performed using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, CA, USA).

Table 1
Gene primer sequences used for real-time quantitative PCR analysis (following Khendek et al., 2017) used in analysis of expression level of genes (in pituitary and brain) during the Eurasian perch (*Perca fluviatilis*) hormonally induced reproduction of fish kept at standard photoperiod (16 h of light:8 h of darkness per day) or at constant darkness.

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size
βlh	5′-CAGCTCATCAACCAGACGGTGTC-3′	5′-TGACAGGGTCCTTGGTGATGCA-3′	165 bp
βfsh	5′-CCAAAGAACATCAGCATCCCTGTGG-3′	5′-GACCAGATCCTCGTGGTAGCAC-3′	181 bp
gnrh	5′-CAGCACTGGTCATAYGGACTGAG-3′	5′-AGGVGATTCCTCDGCACAACCC-3′	135 bp
β-actin	5′-ACCTTCTACAACGAGCTGAGAGTT-3′	5′-AGTGGTACGACCAGAGGCATAC-3′	183 bp

Abbreviations of genes: *Blh* – gene encoding luteinising hormone; *Bfsh* – gene encoding follicle-stimulating hormone; *gnrh* – gene encoding gonadoliberine; *β-actin* – gene encoding beta-actin.

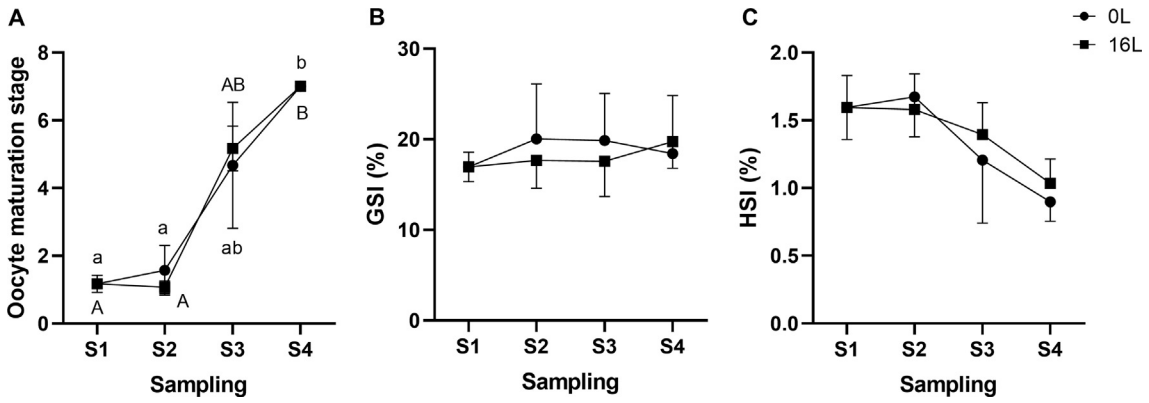

Fig. 2. Oocyte maturation stages, hepatosomatic (HSI), and gonadosomatic (GSI) indices (mean ± SD) recorded in female Eurasian perch throughout the experimental period, in which fish were either exposed to constant darkness (group 0L) or a typically used photoperiod (16 h light:8 h dark; group 16L). For an explanation of the sampling strategy, please refer to Fig. 1. Data of both groups at each sampling point were not significantly different. The absence of a letter indicates a lack of statistically significant differences. Statistically significant differences ($P < 0.05$) among the same group but between different sampling points were indicated with either lowercase (for group 0L) or CAPITAL (for group 16L) letters.

Results

Kinetics of maturation, morphometric indices, and spawning effectiveness

All the fish at S1 reached FOM at stages 1–2. The lack of intergroup differences regarding the kinetics of maturation (Fig. 2A), as well as gonadosomatic index (Fig. 2B) and hepatosomatic index (Fig. 2C), at each sampling point were noted ($P > 0.05$). There were significant differences ($P < 0.05$) in terms of ovulation rate between groups, and higher values were determined when the 16L group was assessed (91%) compared to the 0L group (50%; 6 out of 12). When considering only ovulating females, fish from 16L group ovulated 52 h (±15) after receiving a resolving injection, which was significantly later ($P < 0.05$) than the latency time observed in the spawned females from the 0L group (20 h ± 9). Interestingly, the fish that did not ovulate were seen to resorb the eggs (liquid containing single oocytes/eggs, instead of a ribbon, could be collected from the female's genital pore). This applied to six females of the 0L group and one female of the 16L group. In both groups, eggs obtained were of comparable quality, since no differences in terms of fertilisation rate (57.3 ± 26.1 and 82.8 ± 20.7% in 0L and 16L, respectively), hatching rate (42.3 ± 33.3 and 74.0 ± 25.6% in 0L and 16L, respectively), and deformity rate (38.0 ± 18.7 and 20.5 ± 18.1% in 0L and 16L, respectively) were observed ($P > 0.05$).

Kinetics of gene expression and hormonal level

For both experimental groups, expression of genes encoding GnRH (Fig. 3A) and FSH (Fig. 3C) remained at similar ($P > 0.05$) levels throughout the study. Expression of *βlh*, however, exhibited different kinetics, which depended on the group. In group 16L, the initial hormone dose induced expression of *βlh* when the resolving injection (S3) was performed, and mRNA abundance decreased with ovulation (S4) (Fig. 3B). When fish were maintained in the dark, the initial injection did not affect the increased expression of *βlh* at sampling point S3, but the resolving injection did enhance expression of the gene (Fig. 3B). At sampling point S3, significant differences ($P < 0.05$) between expression levels of *βlh* of both groups were observed (Fig. 3B). In terms of plasma concentrations of DHP, the highest levels were observed in fish of group 16L at sampling point S3, which were higher ($P < 0.05$) than the mean value recorded for the 0L group at the same sampling point (Fig. 3D). In the latter group, DHP levels remained stable throughout the study. Furthermore, plasma concentrations of E2 did not change significantly throughout the study for either group with decreasing tendency along the experimental period (Fig. 3E).

Kinetics of haematocrit and leukogram

The adaptation period (between samplings S1 and S2) induced haematocrit differences between groups, and values recorded for the 0L group were higher ($P < 0.01$) than those of the 16L group. In group 0L, a significant reduction in haematocrit values was observed ($i < 0.05$) between S2 and S4; the parameter remained stable throughout the study in fish of group 16L (Fig. 4A).

The percentage share of white blood cells did not differ between the groups, though some differences in kinetics were observed. Significantly decreased lymphocyte abundance was observed at the end, relative to the beginning of the study ($P < 0.05$; Fig. 4B). Neutrophils, however, exhibited the opposite trend, and values at the end of the study were higher than at the previous two sampling points ($P < 0.05$; Fig. 4C). The percentage share of monocytes remained constant throughout the study ($P > 0.05$; Fig. 4D).

Blood plasma cortisol levels and biochemical indices

Cortisol levels in both groups exhibited increasing tendency throughout the study, though statistical significance could only be detected in fish maintained in the dark. No difference between each group was noted in terms of plasma cortisol level ($P > 0.05$) (Fig. 5A). Glucose levels determined for both groups remained similar for the first two weeks of the experiment (between sample points S1 and S3). However, in blood sampled during egg collection, the average glucose levels were greater than two-fold higher in comparison to previous sampling points ($P < 0.05$) for both groups, and no intergroup differences in glucose levels were observed (Fig. 5B). In both groups, calcium ions, total protein, albumin, globulin, alanine aminotransferase, and triglycerides levels remained similar throughout the study ($P > 0.05$; Fig. 5C–H).

Discussion

The lighting conditions provided in our study did not affect the kinetics of FOM in Eurasian perch. However, constant darkness did affect the ovulation rate and promoted overmaturation (overripening – the oocytes/eggs undergo decomposition through the process called atresia; Miranda et al., 1999), which was observed in half of the fish acclimated to this condition prior to spawning. This contrasts with previous studies involving wild Eurasian perch, in

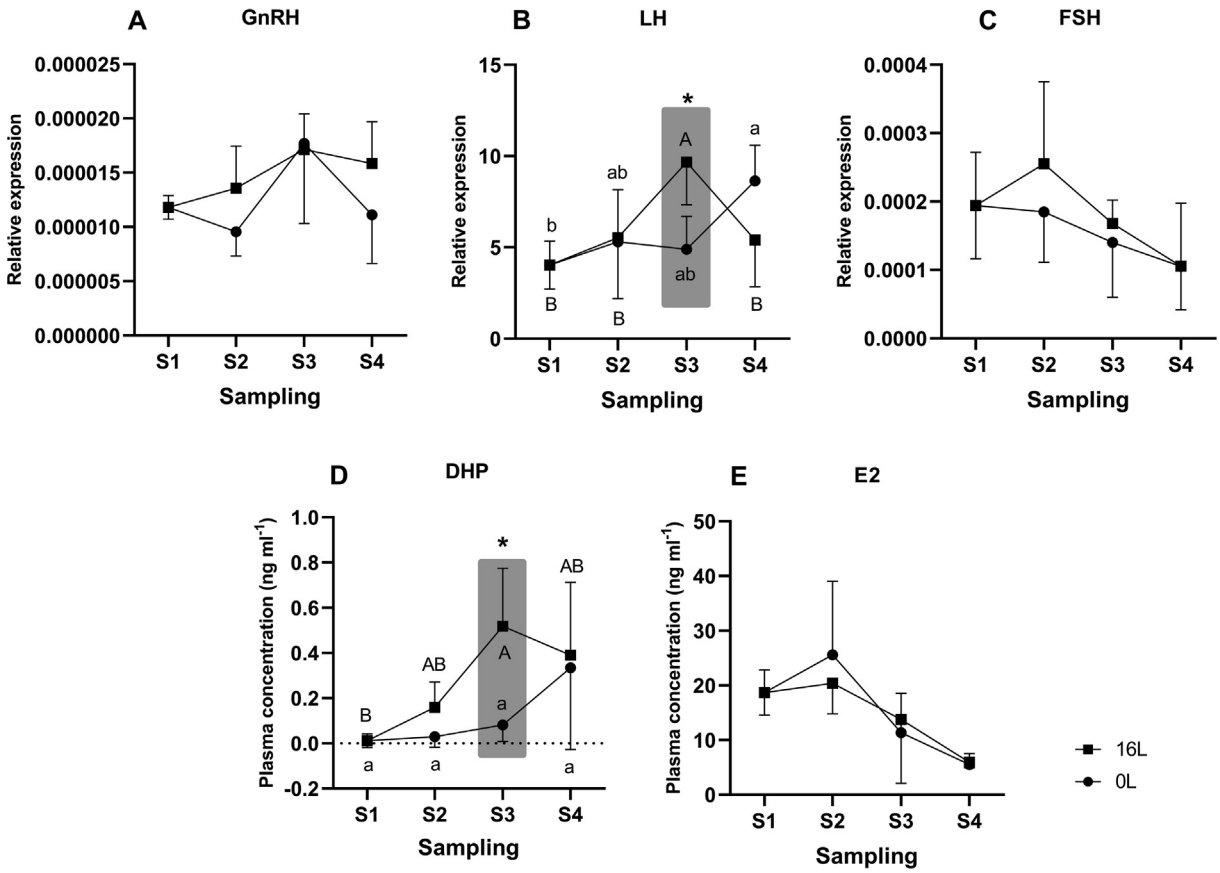

Fig. 3. Expression level of genes encoding gonadoliberin (GnRH), luteinising-hormone (LH), and follicle-stimulating-hormone (FSH), as well as blood plasma levels of DHP (17α, 20β-dihydroxy-4-pregnen-3-one) and estradiol (E2) (mean ± SD) in female Eurasian perch over the course of the experiment are shown. Fish were exposed to either constant darkness (group 0L) or a typical photoperiod (16 h light:8 h dark; group 16L). For an explanation of the sampling strategy, please refer to Fig. 1. Data determined for groups that are shadowed with a grey box and marked with an asterisk were determined to be different ($P < 0.05$). Statistically significant differences ($P < 0.05$) among the same group but between different sampling points were indicated with either lowercase (for group 0L) or CAPITAL (for group 16L) letters. The absence of a letter indicates a lack of statistically significant differences.

which ovulation has typically been achieved via hormonal treatment. In wild females, when eggs are ready to be spawned, they are usually spontaneously released in the tank, regardless of their quality or whether males are present (Żarski et al., 2011 and 2017b). That is why overmaturation has not been documented in wild specimens of this species so far (Żarski et al., 2017b). However, overmaturation has been reported in domesticated Eurasian perch exposed to photo-thermally induced spawning (Migaud et al., 2004). This suggests that domesticated fish are more susceptible to external factor-induced disruption of ovulation than wild fish. However, this phenomenon should be more closely studied before any conclusions are drawn. Nevertheless, the results of our study clearly suggest that exposure to constant darkness did not affect FOM but did affect the ovulation process.

Ovulation in fish can be disrupted by stress (Shourbela et al., 2016). This connection between alteration of the reproductive performances and stress may be partly due to the close bidirectional interactions between the hypothalamus-pituitary-gonadal axis and the hypothalamus-gonad-interrenal axis. For example, cortisol elevation triggers changes in hypothalamic LH and depression of sex steroids (Milla et al., 2009). In our study, plasma levels of stress indices, including cortisol and glucose, exhibited similar kinetics with similar values in both groups throughout the study. This also applies to the remaining stress-related indices tested such as total protein, albumin, alanine aminotransferase, calcium ions and triglycerides. The only parameter measured that differed between groups was haematocrit (Fig. 4A), which was higher in group 0L after a 7-day-long adaptation to the targeted lighting conditions. Increased haematocrit levels have been recognised as a secondary stress response marker in fish (Barton and Iwama, 1991). Elevated haematocrit values were recorded in sea bream (*Sparus aurata*) (Fazio et al., 2015) as well as in Eurasian perch (Ekström et al., 2016) following exposure to stress. Therefore, these findings suggest that the adaptation period induced a stress response in groups having the photoperiod reduced, though not confirmed by any other stress marker investigated. After an adaptation period, haematocrit values of both groups were similar, which was likely due to fish adaptation to the targeted lighting conditions. It should be emphasised, however, that the overall condition of fish along the experimental period declined, which was well reflected via observed increment of cortisol level as well as neutrophilia and lymphopenia, which are also relevant indicators of stress in fish (Grzelak et al., 2017). However, similar kinetic trends were observed in both groups (extreme values were observed in sampling period S4; Figs. 4B, C, and 5A) and were likely caused by the frequent handling of fish during the control of ovulation and not modulated by lighting conditions. Thus, results indicate that constant darkness caused stress to fish only throughout the adaptation period (between samplings S1 and S2), and after this period, fish most likely had adequately adapted and no differences between groups were recorded. However, the attention should be paid to the fact that stressful adaptation period (between samplings S1 and S2) could be responsible for significant increment

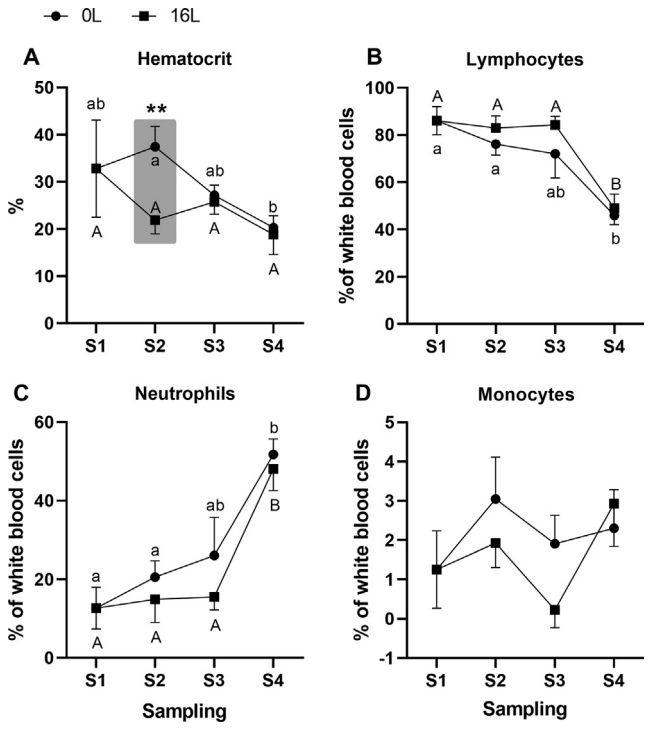

Fig. 4. Hematological indices (mean ± SD) were recorded for female Eurasian perch over the course of the experiment in which fish were exposed to either constant darkness (group 0L) or a typical photoperiod (16 h light:8 h dark; group 16L). For an explanation of the sampling strategy used, please refer to Fig. 1. Statistical differences between groups are indicated using shadowed grey boxes marked with double asterisks ($P < 0.01$). Statistically significant differences ($P < 0.05$) among the same group but between different sampling points were indicated with either lowercase (for group 0L) or CAPITAL (for group 16L) letters. The absence of a letter indicates a lack of statistically significant differences.

in plasma cortisol level in the 0L group, while not recorded in the 16L group. Nevertheless, the data suggest that differences in the reproductive performance of fish exposed to different lighting conditions likely stemmed from factors other than stress, such as for example the lack of appropriate melatonin rhythmicity. However, this should be addressed with appropriate research, in order to validate this hypothesis.

In the present study, the priming hormone dose significantly induced expression of *βlh* and production of DHP in fish exposed to 16 h light per day relative to fish maintained in the dark, in which the expression level of *βlh* and plasma concentration of DHP did not change postapplication of the priming dose of a hormone (Fig. 3B and D). This suggests that the effectiveness of low hormonal doses applied during the priming injection (10 μg/kg) were strongly reduced by altering the lighting conditions. As reported by Bayarri et al. (2004), levels of endogenous hormones, including LH and GnRH, in sea bass (*Dicentrarchus labrax*) were controlled by the circadian system, and a dark period often had suppressive effects on the observed hormone levels. In fact, the most drastic change in levels of sea bass hormones was recorded during the period in which fish were shifted from dark to light and light to dark periods. This suggests that changing lighting conditions have a huge effect on the level of reproductive hormones. Considering that circadian system of Eurasian perch is characteristic for diurnal chronotype, in which light has an inhibitory effect on the melatonin production (Bruning et al., 2015), the differences observed are likely associated also with disruption of the daily rhythm. The lack of a hormone response in female Eurasian perch maintained in the dark, following the application of a priming dose of hormone, suggests that even exogenous hormones may be insufficient for inducing LH and DHP production when the circadian

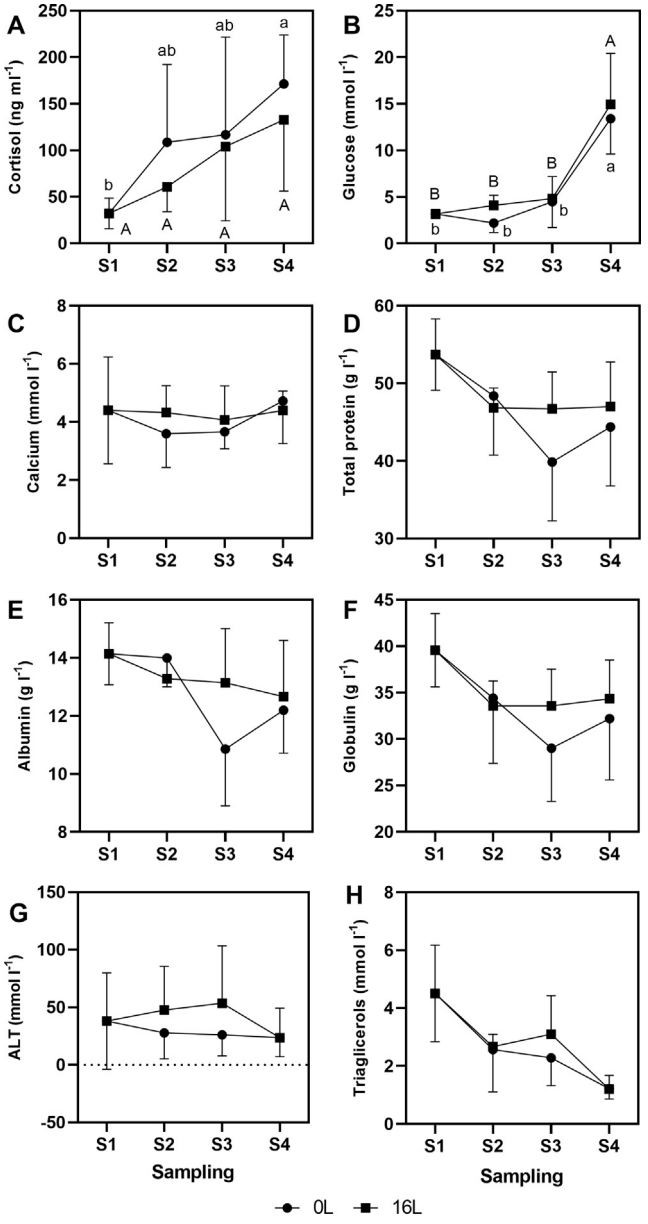

Fig. 5. Plasma cortisol levels and biochemical indices (mean ± SD) determined for female Eurasian perch throughout the course of the experiment, in which fish were exposed to either constant darkness (group 0L) or a typical photoperiod (16 h light/8 h dark; group 16L), are shown. For an explanation of the sampling strategy used, please refer to Fig. 1. Between group differences were not significantly significant ($P > 0.05$). Statistically significant differences ($P < 0.05$) among the same group but between different sampling points were indicated with either lowercase (for group 0L) or CAPITAL (for group 16L) letters. The absence of a letter indicates a lack of statistically significant difference. Abbreviations: ALT = alanine aminotransferase.

system is altered via the modification of photoperiod. Interestingly, an increased dose of hormone (25 μg/kg) applied at the resolving injection time point appeared to overcome the negative effects of exposure to constant darkness and adequately induced the production of both LH and DHP. However, this induction likely occurred at a time point that was too late to promote ovulation in half of the females assessed and eggs underwent atresia (overmaturation). Considering the atresia is driven by, among others, inadequate hormone levels (Nagahama, 1983; Miranda et al., 1999), the overmaturation phenomenon observed can be considered as a specific effect of the constant darkness applied in our study.

Overmaturation, or atresia, is a process that has not been well studied in fish so far. Despite the fact that it is widely observed

in fish undergoing induced spawning (Targońska et al., 2011), the processes driving pre-ovulatory oocytes to atresia are unknown. Migaud et al. (2002) suggested that in cultured Eurasian perch, there may be a lack of an ovulation-triggering factor and suggested that ovulation be promoted via hormonal treatment. The results of the present study suggest that atresia may occur even in hormonally treated fish. Moreover, the obtained results suggest that a few-day-delay in the elevation of DHP, associated with application of constant darkness in our study, may lead to atresia. This may be related to the lack of activation of the maturation promoting factor, which is triggered by elevated levels of DHP and is indispensable for promoting ovulation (Yamashita et al., 1992; Nagahama and Yamashita, 2008). However, altered ovulation may also be associated with impaired production of prostaglandins, which has also been reported to be important for triggering ovulation in teleosts (Takahashi et al., 2018). Moreover, in yellow perch (*Perca flavescens*), a closely related species to Eurasian perch, prostaglandins levels considerably increased in response to DHP before ovulation (Berndtson et al., 1989). Therefore, we speculated that fish kept in constant darkness could have DHP levels that were high enough to promote FOM but not high enough to promote ovulation. However, this hypothesis should be addressed by appropriate study before any conclusions can be drawn. Nevertheless, the results of the present study clearly indicate that lighting conditions have the potential to negatively affect the ovulation process during induced reproduction in domesticated Eurasian perch.

Data obtained in the present study clearly show that photoperiod management during induced spawning in Eurasian perch may be crucial for obtaining high ovulation rates. Indeed, from a practical point of view, the application of darkness during the peri-ovulatory period is not advised in domesticated stocks. It should be also emphasised that the study brings us novel information that is crucial for understanding the processes involved in ovulation during induced Eurasian perch spawning. Furthermore, these data shed a light on the importance of DHP in the activation of maturation promoting factor or other ovulation mediators, such as prostaglandines, in Eurasian perch, meaning the process is sensitive to environmental conditions, including lighting conditions. Widely observed problems regarding the variability of spawning effectiveness and overmaturation in domesticated stocks of this species may be associated with improper levels of DHP. This could be explained by the fact that the level of DHP may be modulated by external factors, including lighting conditions. Therefore, further studies aimed to improve reproductive protocols for this species should more closely address the effects of DHP on the ovulation process, including the kinetics of DHP accumulation and factors that affect its production.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100340.

Ethics approval

The study complies with international and national regulations for experiments on animals and is in accordance with the permission of local ethical committee No. 12482-2017120711508699.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request to the corresponding author (D. Żarski).

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Declaration of interest

None.

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