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Does constant photoperiod inhibit the onset of the reproductive cycle in northern pike (*Esox lucius*) males?

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Keywords

Pike males, constant photoperiod, onset of reproductive cycle.

Abstract

Abstract For most temperate and arctic fish species, photoperiod is the key abiotic factor determining the onset of the reproductive cycle, and, for many species, constant long photoperiod seems to have an inhibitory effect. Yet, there is no knowledge about the effect of photoperiod on the onset of the reproductive cycle in northern pike males. The aim of this work was to investigate the potential inhibitory effect of constant long photoperiod on pike males. Batches of fish were reared in triplicate under natural simulated photoperiod (NSP) or constant long photoperiod (CP; 16L:8D) from 14 October 2013 to 14 March 2014. Fish were sampled three times (at the beginning of the photoperiod decrease, at the beginning of the temperature decrease and at the end of the temperature decrease). Morphological parameters, sexual steroid levels and spermatogenic stages were investigated. Our results showed that CP partially inhibited gonadal development (gonado-somatic index or GSI, %; 2.5% under NSP and 1.2% under CP at day 152), spermatic development (96.2% of spermatozoa under NSP and 69.3% under CP at day 152) and the plasmatic levels of testosterone (7.20 ng/mL under NSP and 2.37 ng/mL under CP at day 152). In conclusion, photoperiod maybe one of the determinant factors that control the onset of the reproductive cycle in pike males, but temperature and endogenous rhythms also need to be investigated.

1. Introduction

The northern pike, *Esox lucius* (L., 1758), is a keystone piscivorous species in cool-water habitats that can influence species composition, abundance and distribution of many species (Balik et al. 2006; Hubenova et al. 2007; Craig 2008). It is one of the few predatory fish species that are most preferred by anglers throughout Europe, and it is intensively fished because of its flesh quality (Billard 1983; FAO 2014). In 2014, the global capture and aquaculture productions were about 24,055 and 728 tons, respectively (FAO 2016). According to Watson (2008), pikes typically supply niche markets that are relatively stable in their requirements. These markets are traditional and well-established in the fish-producing country. Yet, pike suffers from irregular supply and a decline in wild catches (from 45,000 tons in the 1950s and

1960s to 24,055 tons in 2014; Watson 2008; FAO 2016). As this species is reared in an extensive manner in mixed farming ponds, the intensive production of pike could offer development opportunities to small and medium-sized companies as it is currently the case for percids (Kestemont et al. 2015). However, there is little knowledge about the control and management of the reproductive cycle of this species in captivity, which is a vital step to produce high quality and quantity of larvae.

In pike, males are generally sexually mature about 2–3 years after hatching at a length range of 27–45 cm (Billard 1996). In the wild, the entire process of spermatogenesis lasts about 2 months for this species (Billard 1996). Testes are in sexual

arrest in July–August, containing mostly type A spermatogonia, and the main spermatogenesis activity starts in August (Billard 1996). All spermatogenic stages can be observed in September–October, type A and B spermatogonia, spermatocytes and spermatids, and, in November–December, testicular lobules are filled with spermatozoa. Spermatozoa can also appear in small quantities in August, and their number increases in October (Lofts and Marshall 1957). They remain in the lobules until spawning in spring. A maximum gonadosomatic index (GSI) of 2–3% is reached between August and September (Medford and Mackay 1978). When spermiogenesis is completed (November–December), and testes are filled with spermatozoa, a decrease in GSI is observed with values between 1.5 and 2% commonly reported before spawning.

This change is probably due to the removal of cytoplasm from spermatids (Billard 1983). As spermiation occurs in February–May depending on the latitude, the pike belongs to the early spring spawner group (Teletchea et al. 2009). After the spawning season, all spermatozoa are eliminated before the next cycle starts. The residual spermatozoa are resorbed in

the testis itself where they are phagocytized by Sertoli cells and by some macrophages identified in the lobular lumen; only type A spermatogonia are permanent (Billard 1986, 1996).

Various environmental factors play a role in the regulation of spermatogenesis and spermiation in fish. Spermatogenesis depends on long-term seasonal variations in day-length and temperature (Billard 1986). In many temperate species, photoperiod is the main environmental factor that drives and regulates the onset of the reproductive cycle (Bromage et al. 2001; Migaud et al. 2010; Wang et al. 2010). In many species, applying a constant long photoperiod at the beginning of the reproductive cycle leads to the inhibition of both gonad development and sexual steroid synthesis, particularly for European seabass *Dicentrarchus labrax* (Bayarri et al. 2004), turbot *Scophthalmus maximus* (Imstrand et al. 1997) and some early spring spawners such as Eurasian perch (Abdulfatah et al. 2011) and yellow perch *Perca flavescens* (Shewmon et al. 2007). As the pike belongs to this group (Teletchea et al. 2009), the aim of this work was to evaluate the potential inhibitory effect of constant long photoperiod on the onset of the reproductive cycle of males.

2. Material and Methods

2.1. Fish

Pike eggs were obtained from the artificial reproduction of a female (4.5 kg) and two males, which was carried out at the Domaine de Lindre (Dieuze, Moselle, France) on 19 March 2012. Larvae were reared from hatching (28 March 2012) under constant photoperiod 12L:12D and ambient temperature conditions at the experimental facilities of Lorraine University (UR AFPA, Nancy Aquarium-Museum, Lorraine, France). On 28 June 2012, fingerlings were transferred to 1700 L tanks and reared under constant photoperiod 16L:8D and temperature (21 °C ± 1 °C) conditions. On 10 September 2013, pike adults (mean total length = 34.86 ± 3.39 cm; mean weight = 288 ± 87 g; age = 19 months) were transferred into a water-recirculating system under controlled environmental conditions. They were placed in six polyester tanks (500 L) at a density of 33–35 fish per tank. Tanks were completely covered, and light was provided by fluorescent lamps (Osram Lumilux Daylight, L18W/860, 200 lx at water surface). Three tanks were maintained under constant photoperiod 16L:8D (hereafter CP), and three were maintained under natural simulated photoperiod (NSP) based on the natural variations observed in Nancy, France (Fig. 1). The temperature followed the natural decrease recorded in Nancy (48.42N, 06.12E) during a previous experiment (Fig. 1; see also Ben Ammar et al. 2014). Thus, the experiment started on 14 October 2013 and lasted 152 days. During the experiment, pike were fed, once a day, at a rate of 1% of the body weight with a commercial feed (Le Gouessant Ombrine No 7, 47% proteins, 13% lipids, 10% ashes and 1.45% crude fibres).

2.2. Organ and blood sampling

Fish were sampled three times: on 14 October 2013 before photothermal induction, on 16 December 2013 when temperatures started to decrease and on 14 March 2014 corresponding to the end of the chilling period as previously

recorded (Ben Ammar et al. 2014) (Fig. 1). At each sampling time, four males were taken from each tank. They were anaesthetized by immersion into a 2-phenoxyethanol solution for 2–3 min (0.4 mL/L, Sigma). Blood was removed from the caudal vein using a syringe, stored on ice in heparinized microtubes and finally centrifuged at 4000 rpm for 25 min (Centrifuge Jouan C-412). Aliquots of plasma were stored at –80 °C until sexual steroid ELISA assays. After blood sampling, fish were euthanized by overdose of 2-phenoxyethanol solution (4 mL/L). Fish were measured (cm) and weighed (g) to calculate the condition factor K1 and dissected for gonad, viscera and liver weight (g) determination in order to calculate the condition factor K2, the gonadosomatic index (GSI), the visceral-somatic index (VSI) and the hepato-somatic index (HSI), respectively, with the following formulas:

$$K1 = \frac{100 \times \text{Body weight}}{\text{Length}^3}, (\%)$$

$$K2 = \frac{100 \times (\text{Body weight} - \text{Gonad weight})}{\text{Length}^3}, (\%)$$

$$GSI = \frac{100 \times \text{Gonad weight}}{\text{Body weight}}, (\%)$$

$$VSI = \frac{100 \times \text{Viscera weight}}{\text{Body weight}}, (\%)$$

$$HSI = \frac{100 \times \text{Liver weight}}{\text{Body weight}}, (\%).$$

2.3. Gonad histology

Samples of the testis were stored in a Bouin-Holland solution for 1 week, washed once with water, twice with 70% ethanol and stored in absolute ethanol (Abdulfatah et al. 2011). Then, fragments of gonads were cut into 5-mm-thick slices, dehydrated with OTTIX solution (DIAPATH SpA, Italy/MM France) and embedded in a paraffin substitute-dubbed Diawax (DIAPATH SpA, Italy/MM France) (adapted from Langeron 1942; Ben Ammar et al. 2014).

Sections of 4µm were cut from the Diawax block and stained with trichrome: Regaud's iron haematoxylin (solution from Merck's crystalline haematoxylin), 1% Masson's Ponceau-Fuchsin (Sigma) and 0.1% light green (Sigma). Testis development was evaluated according to the presence of different sperm cell types (Turner 1919) on three sections per male (Fig. 2):

Type A spermatogonia (SpgA): the largest cells of the germ line with a voluminous, irregular nucleus surrounded by voluminous cytoplasm.

Type B spermatogonia (SpgB): similar to type A spermatogonia but smaller.

Spermatocytes (SPC): smaller than spermatogonia and identified by densely staining chromatin.

Spermatids (SPD): larger than spermatozoa with a regularly spherical, dense nucleus.

Spermatozoa (SPZ): recognized by their kidney-shaped cell with alight-colored hair-like tail.

2.4. Steroid assays

Testosterone (T, ng/mL) was assayed on 25 µL of plasma using the DIAsource Testosterone ELISA kit (DIAsource, KAPD1559). When necessary, a 1/2 dilution of the samples was performed. Sensitivity was 0.083 ng/mL, CV intra-assays were 1.5, 7.9 and 9.5%, and CV inter-assays were 7.6, 13.9 and 8.7% for 0.2, 2 and 16 ng/mL, respectively.

The androgen 11-keto testosterone (11-KT, ng/mL) was assayed on 50 µL of plasma diluted 1/50 in ELISA buffer using the Cayman 11-KT EIA kit (Cayman, 582751). Sensitivity was 1.3 pg/mL, CV intra-assays were 2.6, 1.9 and 8.4%, and CV inter-assays were 9.4, 9.6 and 12.1% for 0.78, 6.25 and 100 pg/mL, respectively.

17β-estradiol (E2, ng/mL) was assayed on 50 µL of plasma using the DIAsource E2-ELISA kit (DIAsource, KAP0621). When necessary, a 1/20 to 1/70 dilution of the samples was performed. Sensitivity was 5 pg/mL, CV intra-assays were 3, 2.3 and 2.6%, and CV inter-assays were 4.7, 4.1 and 2.4% for 37, 118 and 880 pg/mL, respectively.

2.5. Statistical analysis

Data are expressed as mean ± SEM with individual fish as the experimental unit. Normality and homogeneity of variances were tested using Shapiro-Wilk and Levene tests, and data were either log-transformed or arcsin root square-transformed (for data expressed in percentage) when needed. When data respected the assumptions of normality, a two-way analysis of variance (ANOVA) with two independent factors (photoperiod and time) was performed followed by Bonferroni's post-hoc tests to identify significant differences. The level of significance used in all tests was $p < 0.05$. When data were not normally distributed, a Kruskal-Wallis test was performed to test the photoperiod or time effect, followed by the nonparametric Mann Whitney test. We tested the distribution of the gonadal stages by a chi-squared test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 20.0) program (Chicago, IL, USA).

3. Results

3.1. Morpho-anatomic indexes

In all groups of males, condition factors K1 and K2 increased significantly from day 1 to day 64 ($p < 0.01$) and remained stable from day 64 to day 152 (Table 1). HSI increased significantly between day 1 and day 64 under both photoperiod conditions ($p < 0.01$). While it remained stable between day 64 and day 152 in males reared under CP, it decreased over the same time period for those reared under NSP ($p < 0.05$). But at day 152, there was no significant difference in HSI between the two photoperiod treatments. VSI increased significantly between day 1 and day 64 with much higher values in males reared under CP ($p < 0.01$). Then, VSI decreased drastically with both treatments between day 64 and day 152 ($p < 0.05$) to attain initial values, but it was significantly higher in males reared under NSP at the end of the experiment ($p < 0.01$).

3.2. Gonadal development

Before application of the photoperiodic treatments (day 1), GSIs were at the lowest level ($0.53\% \pm 0.66\%$) for both photothermal regimes (Fig. 3a). GSI increased between day 1 and day 64 to reach 3.85% only in males reared under NSP ($p < 0.001$) and remained stable in those reared under CP. In males reared under NSP, GSI decreased significantly between day 64 and day 152 to reach 2.5% ($p < 0.01$) while it increased to reach 1.18% for those reared under CP ($p < 0.05$). Finally, at day 152, GSI showed a significantly higher value in males reared under NSP compared to the other group ($p < 0.001$).

At day 1, the testis showed all developmental stages with 39.3% of spermatogonia, 35.1% of spermatocytes, 12.7% of spermatids and 12.8% of spermatozoa (Fig. 3b). At day 152,

the testis showed a significantly larger proportion ($p < 0.05$) of advanced stages in males reared under NSP (96.2% of spermatozoa) than in those reared under CP (69.3% of spermatozoa). For all the other stages, no significant difference related to time and photoperiod was shown.

3.3. Plasma steroids

In males reared under NSP, plasma testosterone levels increased significantly between day 1 and day 64 ($p < 0.001$) and remained stable from day 64 to day 152 (8.34 ng/mL and 7.20 ng/mL, respectively) (Fig. 4a). There was no significant variation in these levels in males reared under CP. Plasma

testosterone levels were significantly higher in males reared under NSP than in males reared under CP at day 64 and day 152 ($p < 0.05$). Plasma 11-KT levels increased between day 1 and day 64 ($p < 0.05$) and remained stable between day 64 and day 152 (Fig. 4b) in both groups. For all sampling times, no significant difference was detected in 11-KT concentration between pike exposed to CP conditions compared to those exposed to NSP.

Plasma E2 levels showed a significant increase between day 64 and day 152 only for males reared under NSP (Fig. 5) ($p < 0.001$). At day 152, plasma E2 levels were significantly lower in males reared under CP ($p < 0.05$) than in the other group.

4. Discussion

Rearing pike males under constant photothermal conditions allows us to induce earlier puberty by an adequate photothermal regime than that reported under natural conditions (2–3 years, 27–45 cm in length, depending on populations, Billard 1996). Our pike males were sexually mature at 19 months old and 35 cm in length. As previously observed in Eurasian perch (Ben Ammar et al. 2012), it seems that puberty in pike depends more on size than on age and that our rearing conditions allow early sexual maturation.

The GSI profile under NSP showed a sharp increase with decreasing photoperiod followed by a moderate decrease with the decreasing temperature. Under CP, there was no GSI increase before the temperature decrease. The profile observed under NSP is close to the observations described under natural conditions. Indeed, in the wild, the maximum GSI in pike males is reached between August and September (approximately 2 months after the beginning of photoperiod decrease in temperate regions) and then it decreases in November–December when testes are filled with spermatozoa (Billard 1983). Because spermatozoa are smaller cells than spermatocytes and spermatogonia, the increase observed in their abundance rates in testes under NSP may explain the concomitant GSI decrease observed between day 64 and day 152. However, at the beginning of the experiment, even though pike males were maintained under CP (16L:8D) and high temperature (21 °C) conditions, we observed all the cellular types in testes, even the most advanced ones. Some observations in the wild suggest that spermatogenesis in pike is asynchronous (testes contain cohorts of cells at different developmental stages) and occurs over a very short time period (2 months, Billard 1983). The gonadal development was more advanced under NSP than under CP even though we observed a notable rate of spermatozoa (40%) at the end of the experiment under the second condition. Altogether, we suggest that part of the spermatogenesis process is driven independently of photothermal conditions but its full completion requires variations in photoperiod.

The condition factors remained stable between day 64 and day 152 in all groups. We observed a loss of appetite, probably due to the temperature as described by Diana (1979), which can inhibit and even depress male growth. HSI remained stable between day 64 and day 152 in males reared under CP and decreased in those reared under NSP. This result is in accordance with those observed under natural conditions

(Medford and Mackay 1978; Diana and Mackay 1979). In fact, during spermatogenetic activity, the testis showed caloric gain of the same magnitude as the loss observed in the liver. As pike males showed a loss of appetite, the energy for testicular growth, observed in males reared under NSP, may have come from endogenous stores in the liver.

The most important androgen (11-KT) in many temperate species was not modified by photoperiod conditions, unlike testosterone. Plasma testosterone levels increased considerably under NSP but not under CP. These results suggest that the photoperiod effect on the first stages of the reproductive cycle is mediated through the production of this androgen. Plasma E2 levels showed a significant increase with decreasing photoperiod. Some studies highlighted the presence of E2 in blood serum at low concentrations (Miura et al. 1999; Amer et al. 2001; Chaves-Pozo et al. 2007), increasing temporarily at the beginning of reproduction (Gomez et al. 1999). These findings and our results suggest that oestrogens have a role in spermatogenesis. Schulz et al. (2010) also proposed that oestrogens exhibited regulatory effects on the expression of some genes involved in the regulation of steroidogenesis and especially linked to spermatogenesis. Low doses had stimulatory effects on spermatogonial stem cell renewal (Miura et al. 1999), while high doses had an inhibitory effect (Song and Gutzeit 2003; Chaves-Pozo et al. 2007).

Constant long photoperiod affects the onset of the reproductive cycle in pike males and photoperiod seems to be one of the factors that determine the initiation of spermatogenesis in this species. As we did not evaluate the temperature effect in this study, we cannot assess whether photoperiod is the main factor in the induction of pike spermatogenesis. CP delayed the GSI increase (GSI increased only between day 64 and day 152) and the gonadal development was slower compared to that observed under NSP. However, CP did not totally inhibit the GSI increase and gonad development as observed in other temperate species such as Eurasian perch (Abdulfatah et al. 2011), yellow perch (Shewmon et al. 2007) and European seabass (18L:6D; Bayarri et al. 2004). Consequently, we hypothesise that temperature might be another strong driver of the environmental control of spermatogenesis in male pikes.

The progress of spermatogenesis under CP can be explained either by the coexistence of several factors potentially involved

in the regulation of the onset of the reproductive cycle or by the duration of the applied CP (Migaud et al. 2010). On the one hand, the presence of the most advanced stages at day 1 independently of the applied photoperiod can be explained by the role of endogenous clocks and free-running melatonin rhythms at the onset of the reproductive cycle. In fact, the model of neuro-endocrine control of reproduction in temperate teleosts suggests that the photic information, perceived by photosensitive neurones, initiates the reproductive cascade (Masuda et al. 2003). However, endogenous clocks exist in this system and can interact with the reproductive cascade at different levels (brain, pineal gland, retina, pituitary gland and gonads). Melatonin is synthesised in response to photic information perceived by the retina or pineal gland (Migaud et al. 2007) and can interact with the reproductive cascade at

presence of spermatozoa at day 1 and for the observed gonadal development, GSI increase and plasma 11-KT increase in males reared under CP.

On the other hand, the applied daylength 16L:8D could not be long enough to have a real inhibitory effect on the onset of the reproductive cycle in pike males. For example, in European seabass, applying a constant long photoperiod 18L:6D is enough to totally inhibit the onset of the reproductive cycle (Bayarri et al. 2004), while applying 15L:9D for 4 years did not block the reproductive cycle but delayed it (Prat et al. 1999). Pike is an early-spring spawner like Eurasian and yellow perch (Teletchea et al. 2009), but the photoperiod threshold that allows inhibition of spermatogenesis may be different and testing other daylength durations could allow determining this threshold.

many key steps (Sébert et al. 2008). Circadian rhythms of melatonin are regulated by environmental factors but can also be autonomous and controlled by endogenous clocks (Falcón 1999; Fukada and Okano 2002; Holzberg and Albrecht 2003; Falcón et al. 1994, 2007). In pike, two studies highlighted the existence of autonomous rhythms of melatonin under total darkness (Falcón et al. 1989, 1994). These rhythms may be heritable and responsible for the initiation of the reproductive cascade. As a consequence, they may be responsible for the

In conclusion, applying a constant long photoperiod 16L:8D slows down spermatogenesis. This is illustrated by lower changes in GSI, spermatogenetic development, and testosterone and estradiol levels. The photoperiod may be one of the determinant factors of the onset of the reproductive cycle in pike males. However, we cannot rule out the involvement of other external factors, such as temperature, and the potential important role of endogenous rhythms.

Table 1. Effect of the photoperiod treatment on condition factors (K1 and K2), hepato-somatic index (HSI) and viscero-somatic index (VSI) in pike males (mean ± SEM)

Photoperiod treatment		Time (days)		
		1	64	152
K1	NSP	0.66 ± 0.01 (A)	0.76 ± 0.02 (B)	0.72 ± 0.01 (B)
	CP	0.66 ± 0.01 (A)	0.75 ± 0.02 (B)	0.74 ± 0.01 (B)
K2	NSP	0.66 ± 0.01 (A)	0.73 ± 0.02 (B)	0.70 ± 0.01 (B)
	CP	0.66 ± 0.01 (A)	0.75 ± 0.02 (B)	0.73 ± 0.01 (B)
HSI	NSP	1.59 ± 0.07 (A)	1.94 ± 0.13 (C)	1.66 ± 0.12 (AB)
	CP	1.59 ± 0.07 (A)	2.44 ± 0.15 (C)	2.28 ± 0.19 (BC)
VSI	NSP	4.85 ± 0.17 (a)	5.53 ± 0.23 (*b)	4.71 ± 0.24 (*a)
	CP	4.85 ± 0.17 (a)	6.60 ± 0.37 (*b)	4.06 ± 0.25 (*a)

Different major letters indicate significant differences due to the interaction, minor letters indicate significant differences due to the time and asterisks indicate significant differences due to the photoperiodic treatment alone ($p < 0.05$). N = 17, 12 and 15 for constant photoperiod (CP) treatment and 18, 12 and 15 for natural simulated photoperiod (NSP) treatment at days 1, 64 and 152, respectively

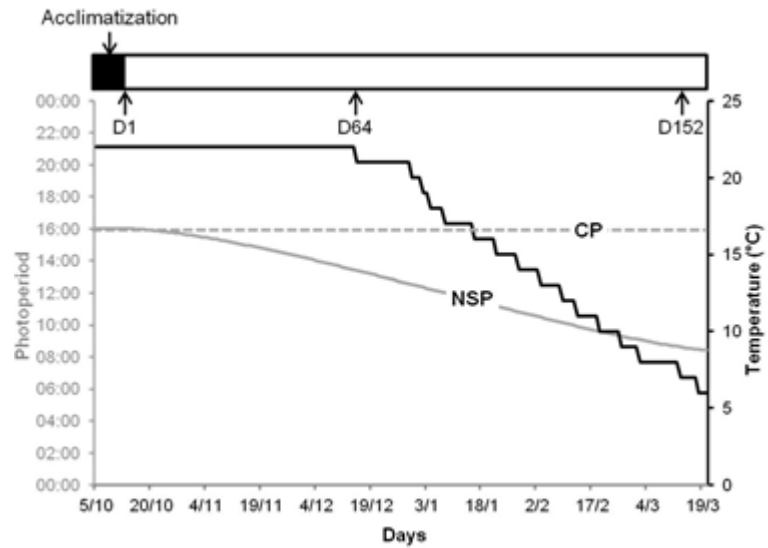


Figure 1.

Photothermal kinetics applied with natural simulated photoperiod (NSP, grey continuous line), constant photoperiod (CP, grey discontinuous line) and temperature (°C, black continuous line) values over the entire time course of the experiment. Sampling times are also indicated (arrows)

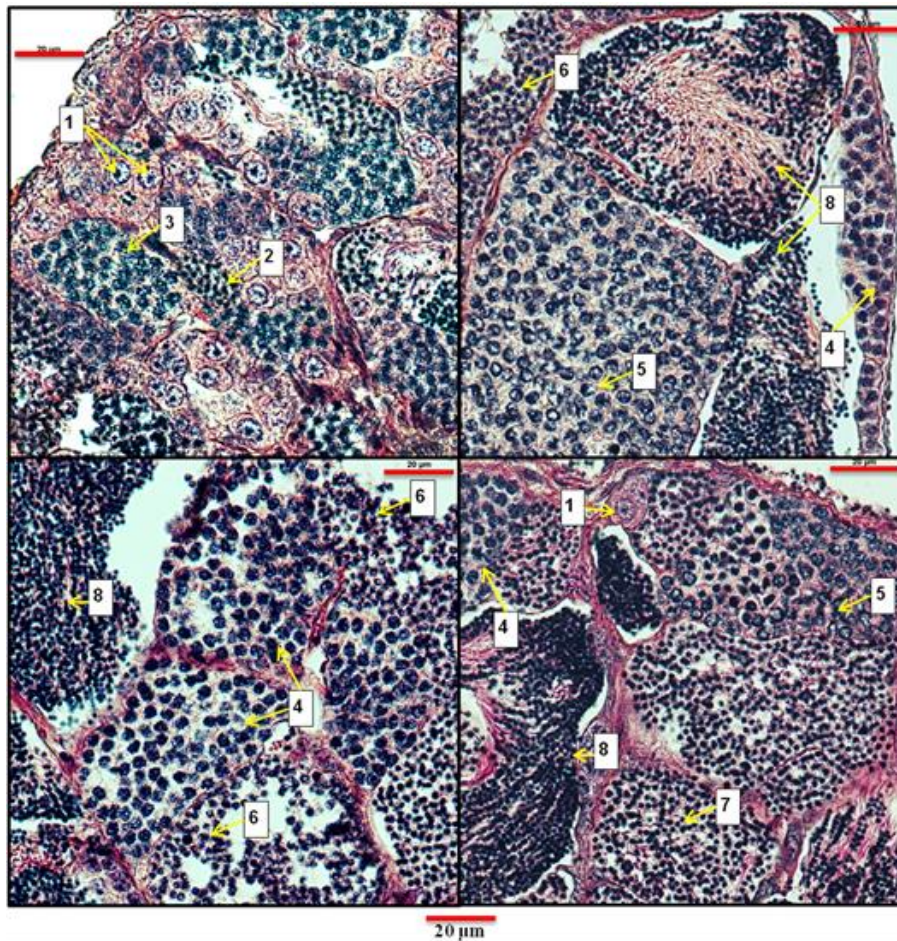


Figure 2.

Developmental stages of spermatogenesis observed in the testis of pike *Esox lucius* during the experiment (according to the description of Turner 1919). 1 (x600): type A spermatogonia; 2 (x600): type B spermatogonia; 3 (x600): spermatocytes at the pachytene stage; 4 (x600): spermatocytes at the diplotene/first meiotic stage; 5 (x600): spermatids E1; 6 (x600): spermatids E2; 7 (x600) spermatids E3; 8 (x600): spermatozoa. Scale (red bar): 20 µm

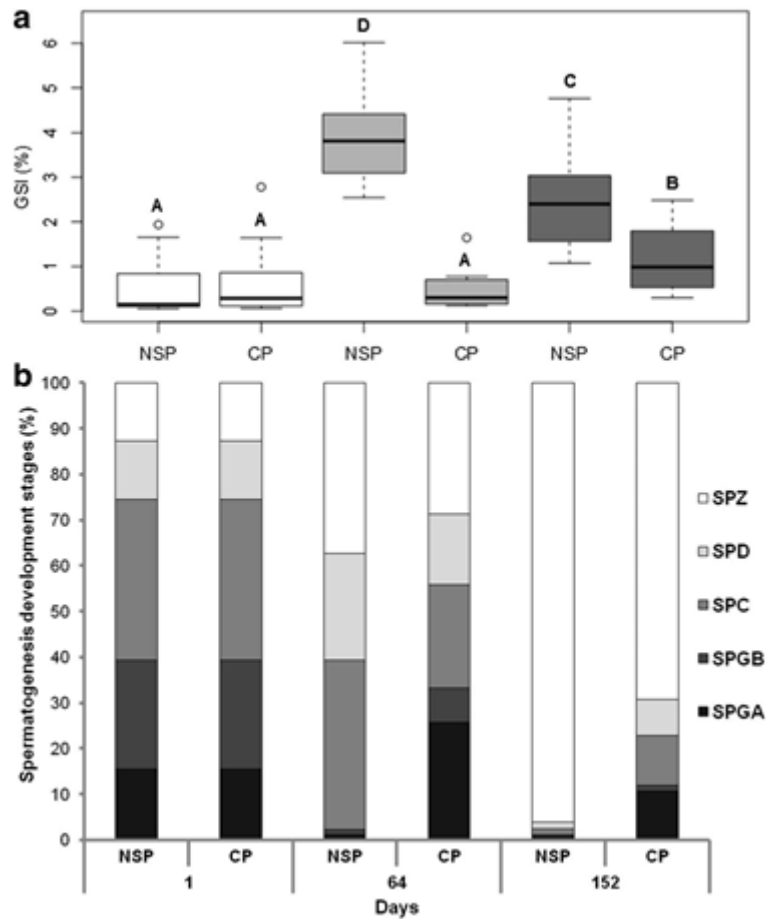


Figure 3.

Effect of photoperiod treatment (NSP: natural simulated photoperiod, CP: constant photoperiod) at day 1 (white), day 64 (light grey) and day 152 (dark grey) on the gonado-matic index (a) and spermatogenesis developmental stages (b) in pike males. The horizontal line in the box represents the median; the bottom and the top of the box are the first and third quartiles. Whiskers show values within 1.5 times the interquartile range from the boxes and outliers are plotted as little white circle. Different letters indicate significant differences ($p < 0.05$), $N = 12$ for each sampling time and each photoperiod treatment

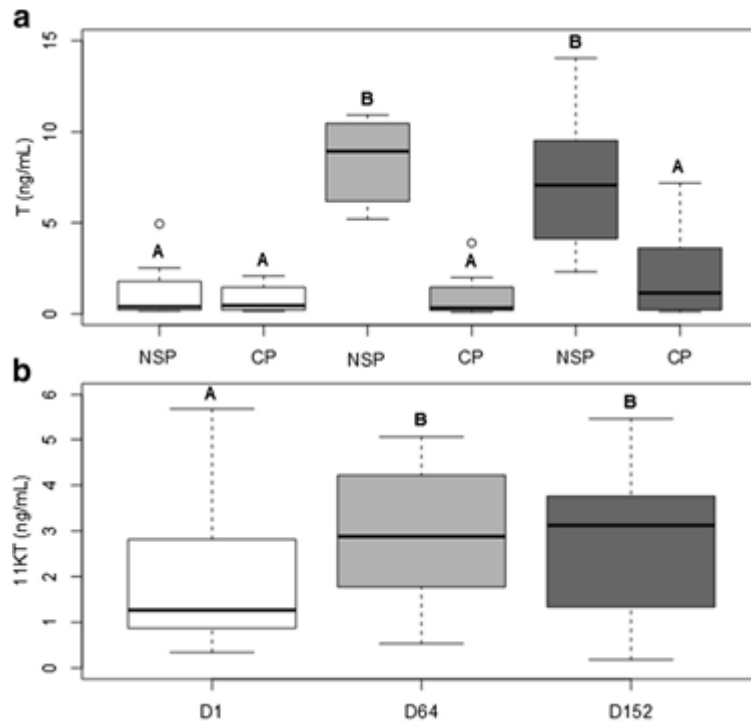


Figure 4.

Effect of photoperiod treatment (NSP: natural simulated photoperiod, CP: constant photoperiod) at day 1 (white), day 64 (light grey) and day 152 (dark grey) on plasma testosterone (a) and 11-keto testosterone (b) levels in pike males. The horizontal line in the box represents the median; the bottom and the top of the box are the first and third quartiles. Whiskers show values within 1.5 times the interquartile range from the boxes and outliers are plotted as little white circle. Different letters indicate significant differences ($p < 0.05$), $N = 12$ for each sampling time and each photoperiod treatment

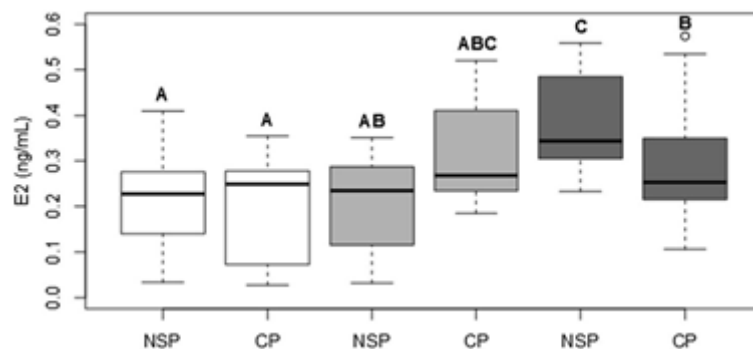


Figure 5.

Effect of photoperiod treatment (NSP: natural simulated photoperiod, CP: constant photoperiod) at day 1 (white), day 64 (light grey) and day 152 (dark grey) on plasma 17- β -estradiol levels in pike males. The horizontal line in the box represents the median; the bottom and the top of the box are the first and third quartiles. Whiskers show values within 1.5 times the interquartile range from the boxes and outliers are plotted as little white circle. Different letters indicate significant differences ($p < 0.05$), $N = 12$ for each sampling time and each photoperiod treatment

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