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### **► To cite this version:**

Imen Ben Ammar, Sylvain Milla, Yannick Ledore, Fabrice Teletchea, Pascal Fontaine. Constant long photoperiod inhibits the onset of the reproductive cycle in roach females and males. *Fish Physiology and Biochemistry*, 2020, 46 (1), pp.89-102. <10.1007/s10695-019-00698-3>. <hal-02930917>

**HAL Id: hal-02930917**

**<https://hal.science/hal-02930917v1>**

Submitted on 4 Sep 2020

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**Constant long photoperiod inhibits the onset of the reproductive cycle in roach females  
and males**

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## Abstract

Photoperiod and temperature are commonly accepted as the determinant factors for the control of the reproductive cycle in freshwater fishes. However, this determining effect is dependent on fish species. While applying a constant long photoperiod has an inhibitory effect in some species, the same photoperiodic manipulation has a stimulating effect in others. In cyprinids, a decrease in temperature or photoperiod can induce the gonad recrudescence. However, in roach *Rutilus rutilus* an early spring spawner cyprinid, there is little knowledge about the cueing role of each environmental factor. The aim of this work was to study the effect of a constant long photoperiod on the gametogenesis in roach. Fish were kept under either naturally simulated photoperiod or artificial constant long photoperiod and sampled at three times: at the beginning of photoperiod decrease, at the beginning of temperature decrease, and at the end of temperature decrease. Morphological parameters (gonado-somatic, hepato-somatic and viscera-somatic indexes), plasma sexual steroids, and proportion of gametogenesis stages were estimated at each sampling time. The results showed that a constant, long photoperiod exerted inhibitory effects on gametogenesis advancement in both females and males that could stem from decrease of sex steroid production. Roach displayed a similar response to photoperiodic manipulations to other early spring spawners like percids, such as European perch, yellow perch and pikeperch. These results clearly showed the cueing role of the photoperiod in the induction of the reproductive cycle in roach.

**Key words:** Photoperiod manipulation, Inhibition, onset of reproductive cycle, Roach.

## 1. Introduction

Life history of temperate fish species is regulated by seasonal changes in climate, daylength, and food supplies. These species show a marked seasonality particularly with regards to reproductive cycle and spawning (Baggerman, 1980). In temperate fish, the synchronicity of the reproductive cycle is important because it ensures that offspring are produced when environmental conditions and food supply are the most suitable for their survival and development (Sumpter, 1990; Teletchea and Fontaine, 2014). Both environmental cues and intrinsic factors are involved in the control of the reproductive cycle. Rosa and Bryant (2003) divided them into determining factors, responsible for the synchronicity of reproductive cycle, and modulating factors that can influence the progress and the quality of reproduction. From all these factors, photoperiod and temperature are commonly accepted as the two environmental cues that provide a timing message (Bromage et al., 2001; Wang et al., 2010). Studies showed that photoperiod provides an accurate, reliable, and reproducible signal that determines the onset of the reproductive cycle in many temperate species (Migaud et al., 2010). Photoperiodic manipulations alone can inhibit, advance, or delay the induction of the reproductive cycle, depending on the species and the time of application (Migaud et al., 2010). Applying a constant long photoperiod can inhibit the onset of the reproductive cycle in many species, such as the European seabass, *Dicentrarchus labrax*, (Bayarri et al., 2004), European perch, *Perca fluviatilis*, (Migaud et al., 2004a), yellow perch, *Perca flavescens*, (Shewmon et al., 2007), and turbot, *Scophthalmus maximus*, (Immsland et al., 1997). However, in other species like three-spined stickleback, *Gasterosteus aculeatus*, and catla, *Gibelion catla*, constant long photoperiod seems to have a stimulating effect on the gametogenesis (Borg and Ekström, 1981; Bornestaf et al., 1997; Dey et al., 2005). It can advance the ovarian development like in stone moroko, *Pseudorasbora parva*, (Zhu et al., 2014), and even allow obtaining two annual spawns in chub, *Squalius cephalus*, (Poncin et al., 1987).

Cyprinids have colonized a wide variety of freshwater biotopes and are the most widely farmed fish in the world. It is accepted that constant conditions of both temperature and photoperiod do not generally allow the induction of their reproductive cycle (Kestemont, 1990; Scott, 1979). However, studies have shown or suggested that a decrease in temperature or photoperiod is required to induce the gonad recrudescence in some species, such as gudgeon, *Gobio gobio*, (Kestemont, 1990) and common carp, *Cyprinus carpio*, (Davies et al., 1986; Davies and Hanyu, 1986).

The roach, *Rutilus rutilus*, is a cyprinid fish that is ubiquitous in European freshwater ecosystems. This species has numerous interests for recreational fishing, pond polyculture (restocking market), and ecotoxicology as sentinel species for endocrine disruption (Tyler and Jobling, 2008). Based on observations in natural conditions, several authors suggest the cueing role of temperature, however, there is no knowledge regarding the role of photoperiod or temperature on the onset of the reproductive cycle in this species. For example, Tarkan (2006) suggests that temperature drives the reproductive cycle in this species. Escaffre and Billard (1976) also strongly suggest the action of temperature, but with a potential implication of photoperiod in the entrainment of reproductive cycle. Moreover, according to Gillet and Qu  tin (2006), roach belongs to a group of cyprinids that initiate gonad development during the autumn, under the effect of decreasing temperature and photoperiod together. The application of a long constant photoperiod during the onset of the reproductive cycle is known as inhibitory in some fish species, belonging to temperate early spring spawners and stimulatory in other cyprinids. As roach is an early spring spawning cyprinid (Teletchea et al., 2009), the aim of this work is to study the effect of such a photoperiod on the induction of the reproductive cycle in order to improve knowledge about its potential cueing role in this species.

## **2. Materials and methods**

This work is based on a morpho-anatomic (gonado-somatic index GSI, visceral-somatic index VSI and hepato-somatic index HSI, oocyte diameter OD), histological (developmental stage of gametogenesis) and physiological (estradiol E2, testosterone T, 11-ketotestosterone 11-KT and Alkali-labile phosphate levels ALP) analysis of the broodstock sampled at three times during and after the induction of their reproductive cycle which can be driven by photoperiod, temperature or both of them. In females, the proliferation and the growth of the oocytes (GSI, OD and the developmental stages) are known to be driven by the estradiol (produced by the conversion of testosterone) and can be anterior or concomitant with the endocytosis of the lipids and vitellus vesicles (Miura et al., 2007). The 11-Ketotestosterone (produced by the conversion of testosterone) can also be involved in those stages (Rohr et al., 2001). The vitellogenesis occurs right after with the incorporation of the vitellogenin (ALP levels) produced by the liver (HSI) under the estrogenic control. In males, E2 at very low levels and 11-KT are implied in the renewal and the proliferation of the spermatogonia respectively (Knapp and Carlisle, 2011) which affect the GSI and the developmental stages in testis. Broodstock can use a part of their visceral reserves during these phases (VSI).

### **2.1. Fish**

Roach (origin: pond, GAEC piscicole du Saulnois, Gelucourt, Moselle, France, reared under natural conditions, mean weight  $61 \pm 8$  g) were received on March, 14<sup>th</sup> 2012 (water temperature at arrival 8°C). Fish were kept in an outdoor water-recirculating system, located at the Domaine de la Bouzule, Laneuvelotte, Meurthe et Moselle, France (Migaud et al., 2004b). They were placed into two 3m<sup>3</sup> polyester tanks, partially covered for acclimation to Recirculating aquaculture systems (RAS) conditions. During acclimation, roach were fed a commercial feed (Le Gouessant Ombrine N°4). On June 21<sup>st</sup> 2012, 270 fish were transferred to our laboratory facilities (University of Lorraine, France) in indoor water-recirculating

systems, under controlled environmental conditions. They were placed in six polyester tanks (500L) at a density of 45 fish per tank. At the beginning of the experiment (Day1), fish showed low gonado-somatic index GSI ( $1.4 \pm 0.3$  % in females and  $0.7 \pm 0.5$  % in males) and were in sexual rest period. Tanks were totally covered and light was provided by fluorescent bulbs (Osram daylight, 150-300 lx at the water surface). Three tanks were maintained under constant photoperiod 16L:8D (CP) which is known to be inhibitory and represents the longest daylength to which those species are exposed under this latitude. Three other tanks were maintained under natural simulated photoperiod (NSP) based on the natural variations observed in Nancy, France (Figure 1). The temperature followed the natural decrease occurring in Nancy (48.42N, 06.12E), as described in a previous works (Figure 1, Ben Ammar et al., 2017, 2015). Experiment was performed according to the European (directive 2010/63/EU) and French legislation (law No 2010-788 of 12 July 2010 on national commitment to the environment) for fish welfare during animal experimentation.

## **2.2. Organ and blood sampling**

Fish were sampled three times: June 28<sup>th</sup> before the start of the photoperiod decrease in NSP tanks (Day1), September 13<sup>th</sup> as temperature started decreasing (Day78) and November 21<sup>st</sup> (Day149) corresponding to the gonadal maturation (GSI increase and appearance of secondary oocytes in females, Geraudie et al. 2010a; Figure 1). At each sampling date, eight fish were randomly caught in each tank to get, if possible, three females and three males (no sexual dimorphism). Fish were anesthetized 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 heparinised microtubes, and finally centrifuged at 2860 g at 15°C for 25 min (centrifuge Jouan C-412). Aliquots of plasma were stored at –20 °C until sexual steroids ELISA assays. After blood sampling, fish were euthanized by overdose of 2-phenoxyethanol solution (4 mL/L). Fish were measured (cm), weighed (g), and dissected for gonad, viscera

134 and liver weight (g) determination in order to calculate the gonado-somatic ( $GSI =$   
135  $\frac{100 \times \text{Gonad weight}}{\text{Body weight}}$ , %), the visceral-somatic ( $VSI = \frac{100 \times \text{Visera weight}}{\text{Body weight}}$ , %) and the hepato-  
136 somatic indexes ( $HSI = \frac{100 \times \text{Liver weight}}{\text{Body weight}}$ , %), respectively.

### 137 **2.3. Gonadal histology**

138 Samples of both ovaries and testis were stored in a Bouin-Holland solution for one week,  
139 washed once with water, twice with 70% ethanol and stored in absolute ethanol (Ben Ammar  
140 et al., 2015). Then, fragments of gonads were cut into 5 mm thick slices, dehydrated with  
141 OTTIX solution (DIAPATH SpA, Italy/ MM France), and embedded in a paraffin substitute  
142 dubbed Diawax (DIAPATH SpA, Italy / MM France) (adapted from Langeron, 1913).

143 For females, sections of 6  $\mu\text{m}$  were cut from the Diawax block and stained with a trichrome:  
144 haematoxylin Gill III (VWR), 0.5% phloxine B (VWR) and 0.5% light green (Sigma),  
145 according to Rinchard and Kestemont (1996). The stage of oocyte development was  
146 determined according to Le Menn et al. (2007). Six stages were considered:

- 147       ▪ Primary oocyte (S1): oocytes with vacuole free cytoplasm
- 148       ▪ Early cortical stage (S2): Appearance of yolk vesicles, occupying 2 or 3 rings  
149       in the cytoplasm periphery.
- 150       ▪ Late cortical stage (S3): Oocytes are full of yolk vesicles. Follicular and  
151       cellular layers are differentiated.
- 152       ▪ Early vitellogenesis (S4): Appearance of yolk globules in the centre of oocytes.
- 153       ▪ Later vitellogenesis (S5): Oocytes accumulate yolk globules and yolk vesicles  
154       at the periphery of the cytoplasm.



155                   ▪ Atretic oocyte (S6): Degenerated cells without nuclear, the cytoplasm is filled  
156                   with yolk granules giving a red-orange-coloured oocyte (Geraudie et al.,  
157                   2010a)

158 As roach showed synchronous gonad development (Rinchard and Kestemont, 1996), the  
159 oocyte diameter was measured on 30 oocytes representing the most frequently observed stage  
160 by using an optical microscope (Nikon eclipse Ni) equipped with an ocular camera (Nikon  
161 DS-Fi2, 5MP) and the software provided with NIS-Elements®. Percentages of the different  
162 developmental stages were calculated by counting the number of oocytes in each development  
163 stage, multiplied by 100 and divided on the total number of oocytes.

164 For males, sections of 4 µm were cut from the Diawax block and stained with a trichrome:  
165 Regaud iron haematoxylin (solution from crystalline haematoxylin Merck), 1% Ponceau-  
166 Fushine of Masson (Sigma) and 0.1% light green (Sigma). Testis development was monitored  
167 according to the presence of different sperm cell types (Turner, 1919):

- 168                   ▪ Type A spermatogonia (SpgA): the largest cells of the germ line with a voluminous,  
169                   irregular nucleus surrounded by voluminous cytoplasm.
- 170                   ▪ Type B spermatogonia (SpgB): similar to type A spermatogonia but smaller.
- 171                   ▪ Spermatocytes (SPC): smaller than spermatogonia and identified by densely staining  
172                   chromatin.
- 173                   ▪ Spermatids (SPD): larger than spermatozoa with a regularly spherical, dense nucleus.
- 174                   ▪ Spermatozoa (SPZ): recognized by their kidney-shaped cell with a light-coloured hair-  
175                   like tail.

176 Percentages of the different developmental stages were calculated by counting the area of  
177 cysts from each spermatogenic stage, multiplied by 100, and divided on the total area in three  
178 images taken from the periphery, the middle, and the lumen of the testis.

## 2.4. Steroids and vitellogenin assays

Estradiol (E<sub>2</sub>) was assayed on 50 µL of plasma using the DIAsource E2-ELISA kit (DIAsource, KAP0621). When necessary, dilutions from 1/20 to 1/70 of the plasma samples were performed. Sensitivity was 5 pg/mL, CV intra-assay varied between 3.1% and 2.6% and CV inter-assays varied between 4.7% and 2.4% for low and high levels, respectively.

Testosterone (T) was assayed on 25 µL of plasma using the DIAsource Testosterone ELISA Kit (DIAsource, KAPD1559). When necessary, a 1/2 dilution of the plasma samples was performed. Sensitivity was 0.083 ng/mL, CV intra-assay varied between 1.5% and 9.5% and CV inter-assays varied between 7.6% and 8.7% for low and high levels, respectively.

The androgen 11-ketotestosterone (11KT) was assayed on 50 µL of plasma diluted 1/50 in ELISA buffer using the Cayman 11-keto Testosterone EIA Kit (Cayman, 582751). Sensitivity was 1.3 pg/mL, CV intra-assay varied between 2.6% and 8.4% and CV inter-assays varied between 9.4% and 12.1% for low and high levels, respectively.

Vitellogenin was indirectly estimated with Alkali-labile phosphate levels (ALP, µg/ml). ALP levels were assayed on 80 µL of plasma. The procedure consists of precipitation of high molecular weight proteins in the presence of trichloroacetic acid (20%), followed by three pellet washing sessions using pure ethanol; a solution of chloroform, diethyl ether and pure ethanol (1:2:2), and finally diethyl ether. The pellet was dried for 20 minutes, then dissolved in NaOH solution (2N), heated to 100°C for 15 minutes, and finally phosphate release was neutralized by adding cold HCl solution (2N). Phosphates were assayed by Stanton method (Stanton, 1968) and ALP levels were calculated from a standard curve of phosphorus that ranged between 0.2 and 6 µg/ml (adapted from Gagné and Blaise, 2000).

## 2.5. Statistical analysis

All statistical analyses were performed using the free software R version 3.3.1 (R Development Core Team, 2005). For each modality, analyses were run in duplicate on eight individual sampled at each time from each tank (three tanks per modality). For all the dependant variables, homogeneity of variances was previously tested using Levene test (leveneTest, package 'car', Fox et al. 2014). For each sex, data were analysed by a linear mixed model (lmer, package 'lme4', Bates et al. 2014) with the photoperiod type and the sampling time as fixed effects and the tank as random effect:

model=lmer( $Y \sim \text{photoperiod\_type} * \text{sampling\_time} + (1|\text{Tank})$ ) with  $Y$ : dependant variable. For model validation, residuals were tested for homogeneity and normality using residuals *vs* fitted values and sample *vs* theoretical quantiles (Q-Q) plots, respectively (plotresid, package 'RVAideMemoire', Hervé 2015). If necessary, data were log-transformed, arcsin root square transformed (only for data expressed in percentage), or Box-Cox transformed. When the model was validated, an anova table for various statistical models was performed to calculate F-tests (Anova, package 'car', Fox et al. 2014) followed by a Least-squares means (predicted marginal means) comparisons as a post-hoc test (lsmeans, package 'lsmeans', Lenth, 2016). When data, even transformed, did not met the assumptions for linear mixed model (homogeneity of variance and normality of residuals), we used the aligned rank transformation for nonparametric factorial analysis (aligned.rank.transform, package 'ART', Villacorta, 2015) from Higgins and Tashtoush (1994) formula, followed by a pairwise comparison using Tukey and Kramer test with Tukey-Dist approximation for independent samples (posthoc.kruskal.nemenyi.test, package 'PMCMR', Pohlert, 2016). The level of significance used in all tests was  $p < 0.05$ .

### 3. Results

#### 3.1. Morpho-anatomic indexes

In both sexes, hepato-somatic index (HSI) varied with sampling time, showing similar values between day 1 and 78 and increasing significantly between day 78 and day 149 ( $p < 0.001$ ) (Table 1). Visceral-somatic index (VSI) varied significantly depending on the interaction between sampling time and photoperiod ( $p \leq 0.001$ ). It was significantly higher in females and males reared under constant photoperiod (CP) at day 149 ( $p < 0.05$ ).

##### 3.1.1. Gonad development

In both sexes, gonado-somatic index (GSI) varied depending on the interaction between photoperiod and sampling time (Figure 2). At day 1, there was no significant difference between fish reared under natural simulated photoperiod (NSP) and those reared under CP. In females, GSI increased significantly between day 1 and day 78 (Figure 2.1) in the group reared under NSP ( $p = 0.025$ ) and between day 78 and day 149 for those reared under CP ( $p = 0.022$ ). Moreover, GSI was significantly higher in females reared under NSP compared to those reared under CP at both day 78 ( $p < 0.001$ ) and day 149 ( $p < 0.001$ ). In males (Figure 2.2), GSI increased significantly between day 1 and day 78 in roach reared under NSP only ( $p < 0.001$ ), but did not show any significant difference over this period relating to photoperiod. GSI increased between day 78 and day 149 in all males ( $p < 0.001$  in both cases), and displayed significantly higher values in males reared under NSP compared to those reared under CP ( $p < 0.001$ ).

Females displayed oocytes at the primary growth stage (S1, Figure 3.1), with a low oocyte diameter ( $189.4 \pm 13.4 \mu\text{m}$ ) at day 1 (Figure 3.2). At day 78, oocytes at the late cortical alveoli stage (S3, 23.8%) and early vitellogenesis (S4, 14.5%) were observed in females reared under NSP, whereas only 7.8% and 1.4% of oocytes attained early (S2) and late (S3)

cortical alveoli stage respectively, in those reared under CP (Figure 3.1). At day 149, oocytes reached early vitellogenesis stage (S4, 10.5%) and late vitellogenesis stage (S5, 35.2%) in females reared under NSP while the ovaries of those reared under CP showed atresia (S6, 22%), in addition to a majority of oogonia (S1, 68.8%). The oocyte diameter (Figure 3.2) showed a strong increase in females reared under NSP between day 1 and day 149 ( $p < 0.001$ ), and a slight increase in those reared under CP between day 1 and day 78 ( $p = 0.031$ ). At day 78 and day 149, the oocyte diameter was significantly higher in females reared under NSP relatively to those reared under CP ( $p = 0.003$  and  $p = 0.045$ , respectively). Females from the latter treatment also showed a large inter-individual variability in the oocyte diameter at day 149 ( $442.3 \pm 187 \mu\text{m}$ ,  $\text{CV} = 42.2\%$ ).

Males reared under NSP showed testis with some spermatocytes (5.8% and 5%) at days 78 and 149 respectively, while those reared under CP showed only Type A and B spermatogonia (Figure 3.3).

### 3.1.2. Plasma steroids

- Plasma estradiol

In females, plasma levels of E2 varied significantly depending on photoperiod ( $p < 0.001$ , Figure 4.1), and sampling time ( $p < 0.001$ , Figure 4.2) independently. Plasma levels of E2 were significantly higher in females reared under NSP than in those reared under CP ( $p = 0.002$ ) throughout the time. At day 149, these levels were significantly higher than at day 78 regardless of the photoperiod ( $p = 0.002$ ) but were not significantly different from those observed at day 1 ( $p = 0.105$ ).

- Plasma androgens

The plasma testosterone levels varied greatly between individuals ( $\text{CV} > 65\%$ ) with no significant effect of photoperiod or sampling time. Plasma levels of 11KT varied significantly

depending on the interaction between photoperiod and sampling time in both females (p<0.001, Figure 5.1) and males (p<0.001, Figure 5.2). These levels increased significantly between day 1 and day 149 in females reared under CP (p=0.002) while in those reared under NSP, 11KT levels increased significantly between day 1 and day 78 (p=0.03) and remained stable between day 78 and day 149. At day 78 and day 149, 11KT levels were significantly higher in females reared under NSP compared to those reared under CP (p=0.001 and p=0.035 respectively). In males, 11-KT levels increased significantly between day 1 and day 78 in both group (p<0.001 in NSP group and p=0.003 in CP group) and remained stable between day 78 and day 149 (Figure 5.2). At days 78 and 149, males reared under NSP showed significantly higher 11-KT values than those reared under CP (p<0.001 for day 78 and p=0.017 for day 149).

### 3.1.3. Plasma vitellogenin

Plasma ALP levels measured in females varied significantly depending on photoperiod, and sampling time (Figure 6). These levels were significantly higher in females reared under NSP compared to those reared under CP (p<0.001). Additionally, plasma ALP levels increased significantly only between day 78 and day 149 (p<0.001).

## 4. Discussion

### 4.1. Effect of photoperiod on females

Gonad development, oogenesis development stage, and oocyte diameter, varied throughout the experiment for females depending on the photoperiod. This development showed an important increase of GSI under photoperiod decrease only (3 times higher than at day 1), increase of oocyte diameter (2.5 times higher than at day 1) and advanced oogenesis developmental stages (appearance of early vitellogenesis at day 78). The decrease of photoperiod by more than three hours between day 1 and day 78 allowed the induction of the reproductive cycle in females. Our results are in concordance with the observations made by Geraudie et al. (2010a, b) that found GSI close to 7% in November in wild populations of roach in the region of Haute Normandie (France), and strengthen the observed similarities in the gonad development rhythms between roach and Eurasian perch caught in the wild (Lukšienė and Svedang 1997). In fact, this response is similar to results obtained in percids like Eurasian perch (Migaud et al., 2006), yellow perch *Perca flavescens* (Shewmon et al., 2007), and pikeperch *Sander lucioperca* (Ben Ammar et al., 2015), in which the decreasing photoperiod triggers the onset of the reproductive cycle. Our results also showed a partial inhibition of the induction of the reproductive cycle by a long constant photoperiod. In fact, in females reared under CP, the sudden temperature decrease between day 78 and day 149 induced the reproductive cycle, with a significant increase of GSI and oocyte diameter, and the appearance in the ovaries of oocytes at cortical alveoli stage. Thus, it seems that, even under constant long photoperiod, oocytes maintain a real potential of growth and oogenetic development. Therefore, we have little knowledge of the oogenesis developmental stage from which we can consider that the reproductive cycle is well induced and can lead to the progress of vitellogenesis, final oocyte maturation, and spawning. In the study of Milla et al. (2009), the vitellogenesis stage seems to be the critical stage that cannot be reached if the photoperiod

318 and temperature applied to Eurasian perch broodstock are constant. The same phenomenon  
319 seems to exist in roach females when only temperature decreases. In fact, under this  
320 photoperiod, the most advanced stage reached was alveoli cortical, with high variability in  
321 oocyte diameter between individuals (CV=42.2). Moreover, we observed 25% of atresia at  
322 day 149 only in roach female's ovaries reared under CP, suggesting that constant long  
323 photoperiod displays also deleterious effects on oogenesis. All these results allow us to  
324 conclude that, even though constant long photoperiod seems to be permissive when associated  
325 to a temperature decrease, this photoperiod partly inhibits the reproductive cycle, and may  
326 lead to oocyte regression before the spawning period.

327 The estradiol-controlled production of vitellogenin seems to be a conserved mechanism in  
328 teleosts (Atkinson and Yoshioka, 2007). The increase of plasma E2 and ALP levels in females  
329 reared under NSP is due to the hepatic vitellogenin synthesis and its incorporation in the  
330 oocytes under the regulation of E2 (Babin et al., 2007; Lubzens et al., 2010). This vitellogenin  
331 synthesis increases the liver weight, which may explain the more elevated HSI observed in  
332 NSP females, as shown in other species like walleye *Sander vitreus* (Henderson et al., 1996)  
333 and pikeperch (Ben Ammar et al., 2015).

334 Plasmatic levels of 11-ketotestosterone increased at the alveoli cortical stage (day 78) and  
335 remained higher in females reared under NSP, compared to those reared under CP. A slight  
336 but significant increase was also observed at day 149 in females reared under CP, when  
337 ovaries showed oocytes at the alveoli cortical stage. Similar to pikeperch, our results suggest a  
338 potential role of this non-aromatizable androgen at the alveoli cortical stage for female roach  
339 reared under the natural photoperiod (Ben Ammar et al., 2015). Other studies suggest that 11-  
340 KT may be involved in the transition to the alveoli cortical stage, and the increase of the  
341 oocyte diameter in short-finned eel *Anguilla australis* (Divers et al., 2010; Lokman et al.,



2007; Rohr et al., 2001). The presence of alveoli cortical oocytes in the females reared under CP seems to be linked to the slight increase of 11-KT observed at day 149.

#### **4.2. Effect of photoperiod on males**

The GSI variation in males reared under NSP showed an increase about 1.9 fold when photoperiod decreased alone (between day 1 and day 78), which was followed by a stronger increase (3 fold) when both photoperiod and temperature decreased concomitantly. The increase of GSI is mainly due to the increase of spermatogonia number during successive rounds of mitotic duplication (Schulz and Nóbrega, 2011). Conversely to what we observed in females, spermatogenic development was interrupted between day 78 and day 149, and male roach testis did not exhibit cells in more advanced stage than spermatocytes in the same proportion. However, roach males reared under CP exhibit only spermatogonia A and B in their testis. It seems that this spermatogenesis process does not display the same advancement, compared to other early spring spawners like Eurasian perch (Sulistyo et al., 2000), pikeperch, (Ben Ammar et al., 2015) and yellow perch (Shewmon et al., 2007). This delay observed in roach males reared under NSP suggests that this species may present other regulatory mechanisms or different sensitivity to the environmental factors. For example, in pike males, another early spring spawner, applying a constant long photoperiod seems to slow spermatogenesis (Ben Ammar et al. 2017). Some authors have reported an interruption of spermatogenesis in the beginning of winter, with resumption in February, in temperate waters (Escaffre and Billard, 1976). The most differentiated stages appear in March (spermatid) and in April (spermatozoa), with males spermiating between May and July (Leman Lake, France, Escaffre and Billard, 1976). These observations made in the wild are close to our results, although caution should be taken when comparing wild fish and RAS reared ones. The interruption of spermatogenic development observed in roach can be explained by the characteristics of the spermatogenic cycle. In other early spring spawners like Eurasian perch (Sulistyo et al.,

2000), pike *Esox lucius* (Cakić and Lenhardt, 2002), yellow perch (Shewmon et al., 2007) and  
pikeperch (Ben Ammar et al., 2015), the spermatogenesis and spermiogenesis occurs rapidly  
and the spermiating males show testis full of spermatozoa about 150 days after the beginning  
of photoperiod decrease. In roach, it seems that the spermatogenesis phase would last longer,  
with a shorter spermiogenesis phase, and a rapid elimination of spermatozoa after their  
formation. The time-course of this spermatogenic cycle may be related to other abiotic  
factors, like temperature, as the spermatogenetic changes require some temperature threshold  
(Koskimies-Soininen and Nyberg, 1991; Lofts et al., 1968). In Lake Chub, *Coesius plumbeus*  
(cyprinid), high temperatures promote proliferation of spermatogonia and hasten or terminate  
spermiation, while low temperatures are essential to the formation of the primary  
spermatocytes (Koskimies-Soininen and Nyberg, 1991). In roach, it may be possible that the  
return to warm temperature is necessary to hasten the meiotic divisions and engage the  
spermiogenesis and spermiation in testis as observed in wild (Geraudie et al., 2010a).

In fish, 11-KT is considered as the major androgen regulating spermatogenesis (Mylonas et  
al., 2010). In the present study, constant long photoperiod negatively affected its synthesis,  
and 11-KT presented higher levels in males reared under NSP at the end of the experiment.

Androgens mediate and regulate the whole process, or at least some steps, of fish  
spermatogenesis (Billard et al., 1982; Borg, 1994; Fostier et al., 1983). Previous studies in  
Japanese eel *Anguilla japonica*, goldfish *Carassius auratus*, and Japanese huchen *Hucho*  
*perryi* support the involvement of 11-KT in this process from spermatogonial renewal to  
spermiogenesis (Amer et al., 2001; Miura and Miura, 2011; Miura et al., 1991; Schulz et al.,  
2010). In Eurasian perch (Sulistyo et al., 2000), walleye (Malison et al., 1994), and yellow  
perch (Dabrowski, 1996), previous studies found that 11-KT remained at high levels until the  
spawning season. It seems that, like in those species, 11-KT may be involved in all  
spermatogenetic changes in roach.

Constant long photoperiod had an inhibitory effect especially on the most integrative parameters like GSI in both females and males, oocyte diameter and oogenesis process in females. It also affected the most conserved mechanism like synthesis of E2 and the regulation of vitellogenin synthesis under E2 control in females and the regulation of the spermatogenesis by 11-KT in males. In contrast, the photoperiod decrease allowed the induction of the reproductive cycle in both females and males. However, the partial inhibition of GSI observed in females, or the interruption of the spermatogenic development observed in males can be explained by the potential role of other abiotic factors, like temperature, or the existence of different regulation mechanisms.

## **Acknowledgments**

We would like to thank all members of the AFPA research unit for their active collaboration in this study. We would also like to thank Julien Ruelle and Maryline Harroué from UMR 1092 AgroParisTech INRA Laboratory of study of forest and wood resources "LERFoB", technical platform xylosciences for the provision of the necessary equipment to realize the histological analyses (Technical Platform of functional ecogenomics, INRA Champenoux, France). We are thankful to Daniel Van Vlaender (Facultés Universitaires Notre-Dame de la Paix FUNDP, Belgium) for his help with histological protocols, Jos Fey (Faculty of Kinesiology and Rehabilitation Science, University of Leuven), Marie Fernandez (University of Saint Etienne, France) for their advices about statistics with R software, and Mark Holmes (University of Namur, Belgium) for the proofreading.

## **Funding**

This study was partly funded by the Tunisian Ministry of Higher Education and Research.

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## Legends

**Fig. 1** Graphical representations of the applied natural photoperiod (NSP, grey continuous line), constant photoperiod (CP, grey discontinuous line) and temperature (°C, black continuous line) values during the whole time-course of the experiment. Sampling times (arrows) are also indicated.

**Fig. 2** Effect of photoperiod treatment (NSP: Natural simulated photoperiod, CP: Constant photoperiod) in day 1, day 78 and day 149 on gonado-somatic index (%) in females (1) and males (2) of roach *Rutilus rutilus*. 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 of the interquartile range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant differences among the sampling times ( $p < 0.05$ ). Females: N=8, 11 and 11 for natural simulated photoperiod NSP and 8, 9 and 12 for constant photoperiod CP at day 1, 78 and 149 respectively. Males: N=8, 11 and 11 for NSP and 8, 9 and 12 for CP at day 1, 78 and 149 respectively.

**Fig. 3** Effect of photoperiod treatment (NSP: Natural simulated photoperiod, CP: Constant photoperiod) on oogenesis development stages observed in ovaries (1) on oocyte diameter ( $\mu\text{m}$ ) and spermatogenesis development stages (3) of roach *Rutilus rutilus* females and males. (1) S1: Primary growth, S2: early cortical alveoli stage, S3: late cortical alveoli stage, S4: early vitellogenesis, S5: vitellogenesis and S6: atresia. (2) SPGA: spermatogonia A, SPGB: spermatogonia B, SPC: spermatocyte, SPD: spermatid and SPZ: spermatozoa. (3) 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 of the interquartile range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant differences among the sampling times ( $p < 0.05$ ). N=3 per tank for both sexes in all modalities of photoperiod and sampling time.

**Fig. 4** Effect of photoperiod treatment (1, NSP: Natural simulated photoperiod, CP: Constant photoperiod) and sampling time (2) on plasma estradiol levels E2 (ng/mL) in females of roach *Rutilus rutilus*. 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 of the interquartile range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant differences among the sampling times ( $p < 0.05$ ). (1): N=30 for NSP treatment and 29 for CP treatment. (2): N=16, 20 and 23 at day 1, 78 and 149 respectively.

**Fig. 5** Effect of photoperiod treatment (NSP: Natural simulated photoperiod, CP: Constant photoperiod) on plasma 11-ketotestosterone levels 11-KT (ng/mL) in females (1) and males (2) of roach *Rutilus rutilus*. 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 of the interquartile range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant differences among the sampling times ( $p < 0.05$ ). Females: N=8, 11 and 11 for natural simulated photoperiod NSP and 8, 9 and 12 for constant photoperiod CP at day 1, 78 and 149 respectively. Males: N=8, 11 and 11 for NSP and 8, 9 and 12 for CP at day 1, 78 and 149 respectively.

**Fig. 6** Effect of photoperiod treatment (1, NSP: Natural simulated photoperiod, CP: Constant photoperiod) and sampling time (2) on plasma Alkaline Labile Phosphate levels ALP ( $\mu\text{g/mL}$ ) in females of roach *Rutilus rutilus*. 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 of the interquartile range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant differences among the sampling times ( $p < 0.05$ ). (1): N=30 for NSP treatment and 29 for CP treatment. (2): N=16, 20 and 23 at day 1, 78 and 149 respectively.

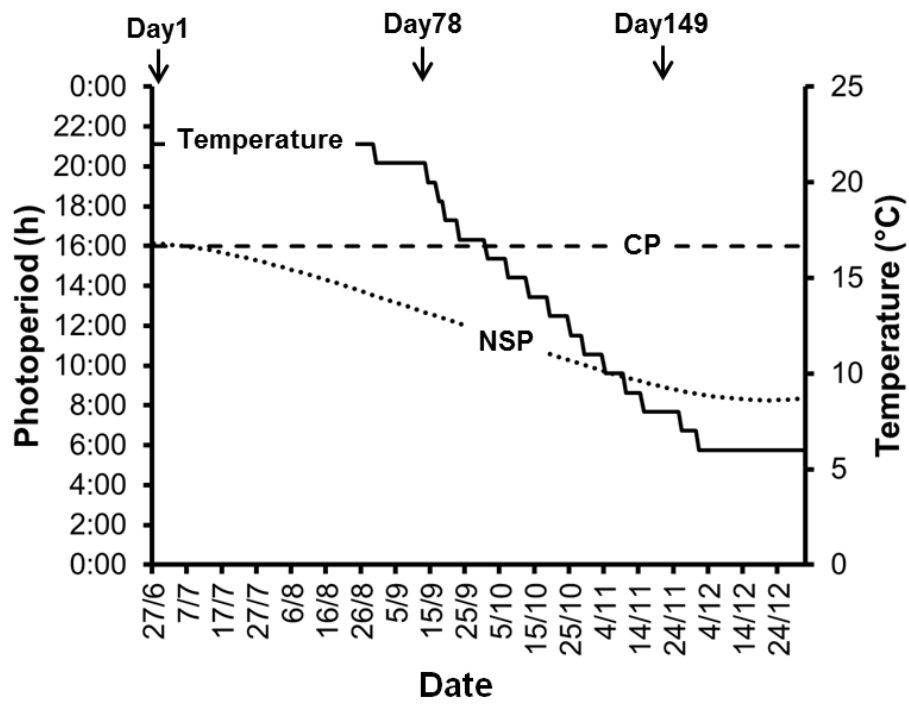


Figure 1 Ben Ammar et al.

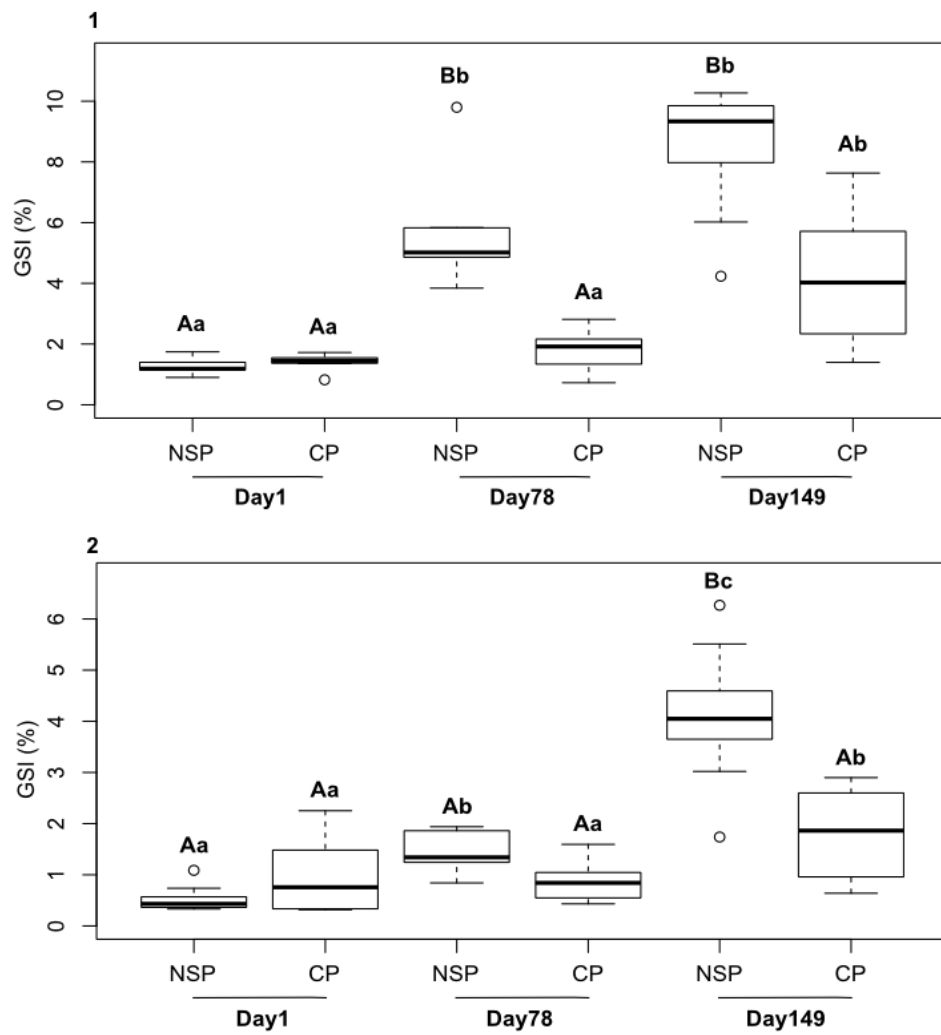


Figure 2. Ben Ammar et al.

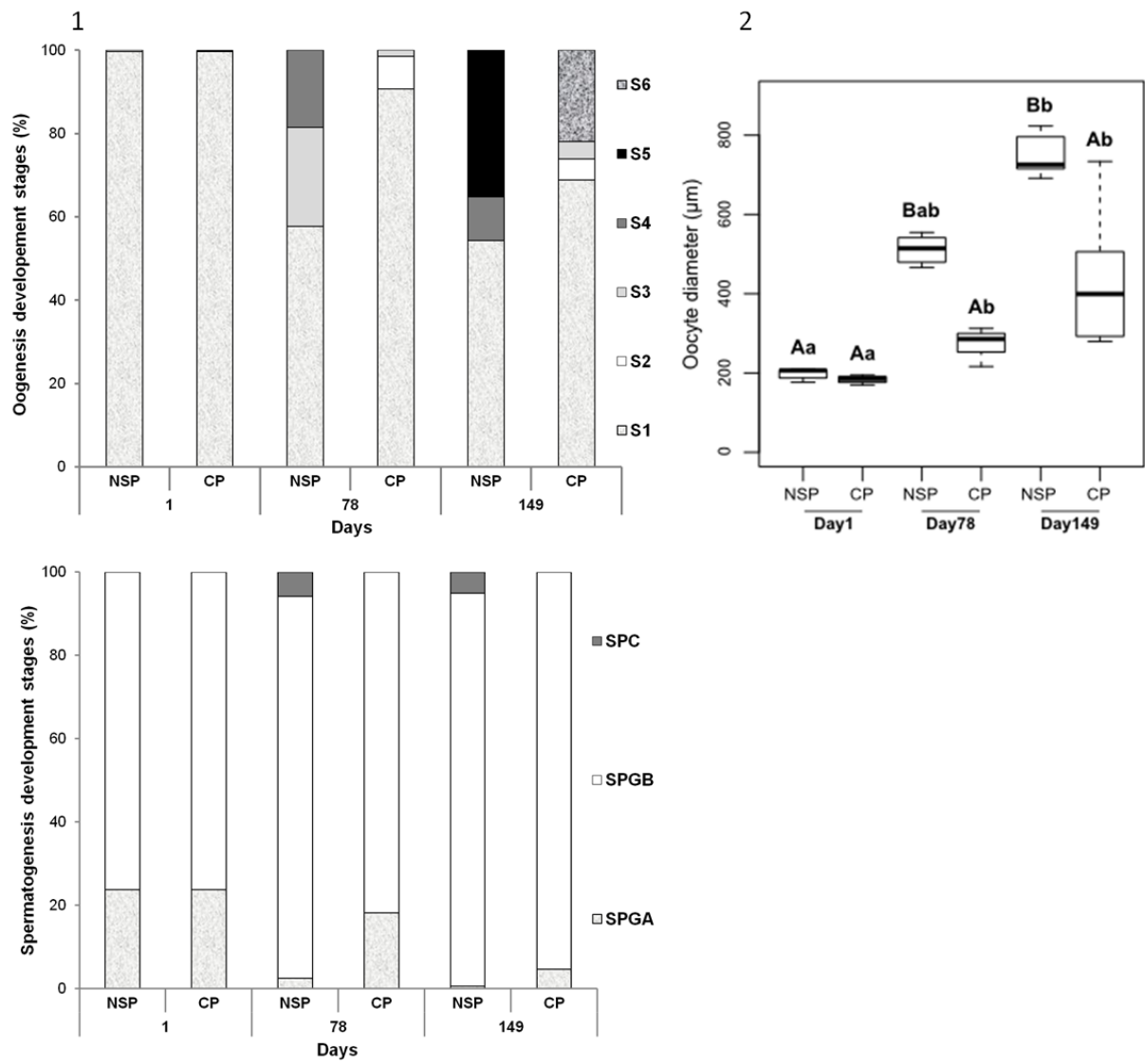


Figure 3 Ben Ammar et al

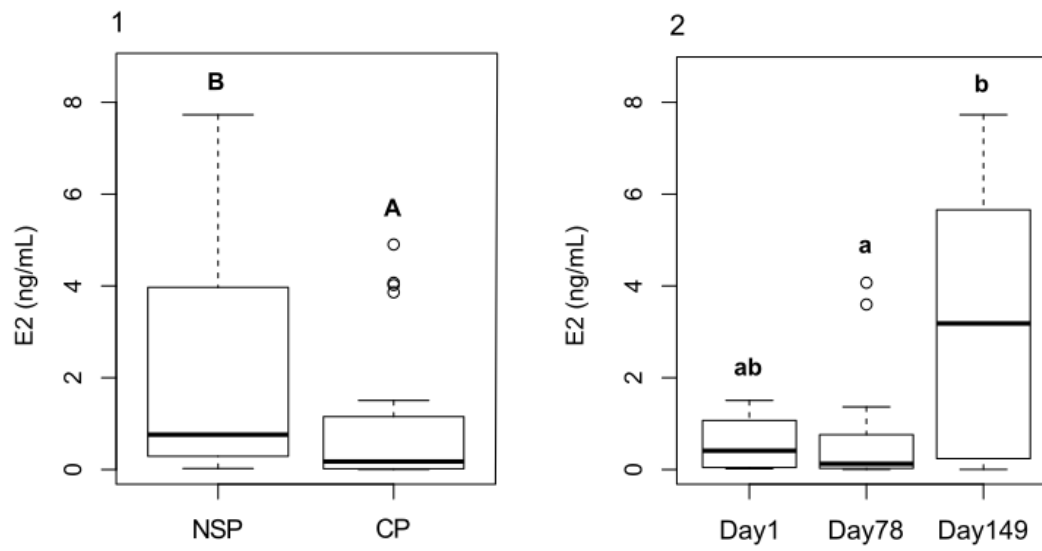
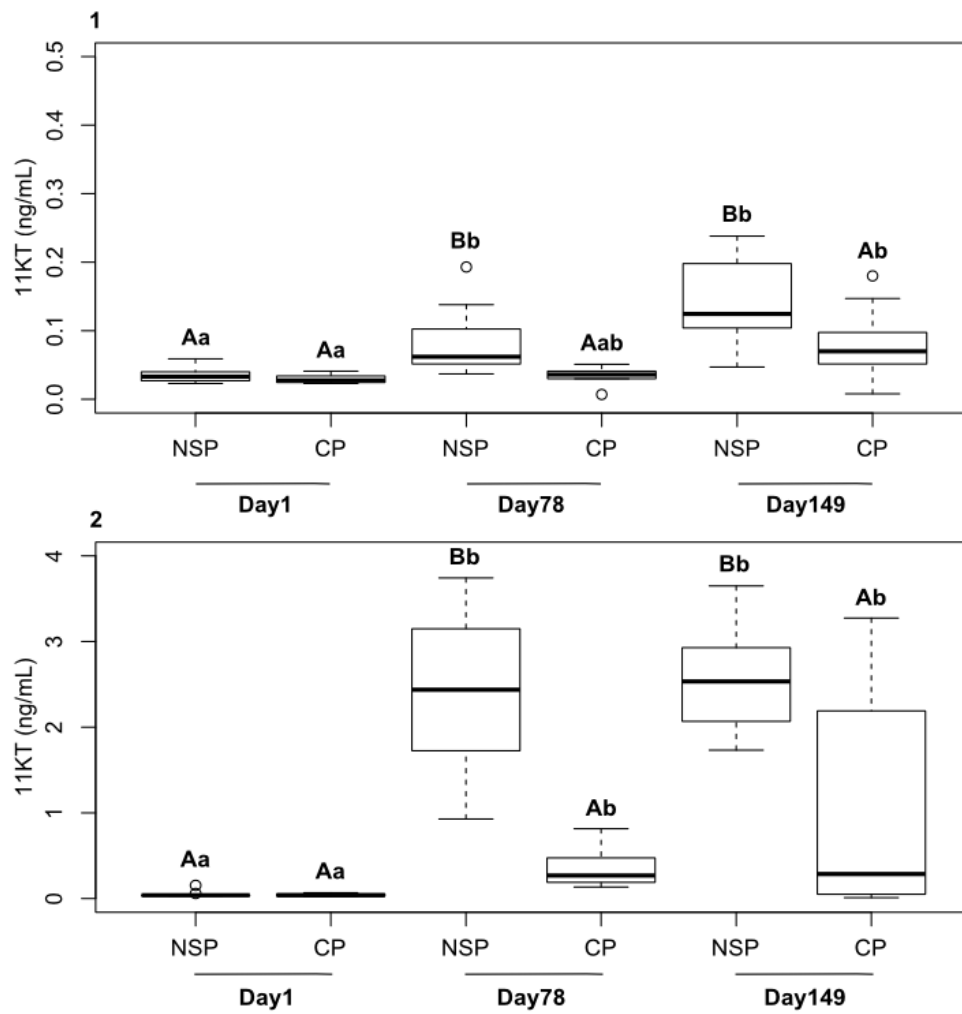


Figure 4 Ben Ammar et al



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688 Figure 5. Ben Ammar et al.



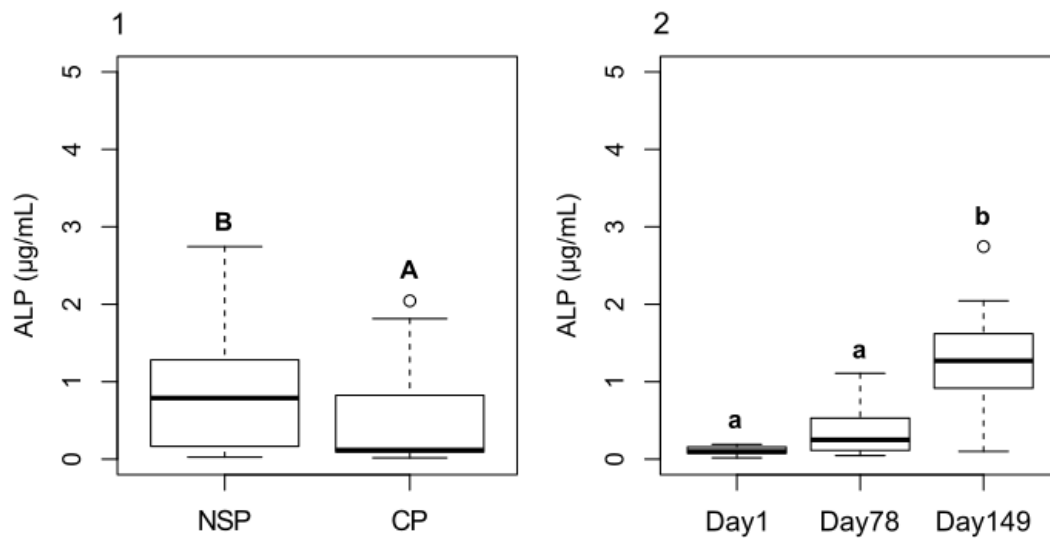


Figure 6. Ben Ammar et al.

**Table 1:** Effect of the photoperiod treatment on hepato-somatic index (HSI) and viscero-somatic index (VSI) in females and males of roach *Rutilus rutilus* (mean  $\pm$  SEM). Different capital letters (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant differences among the sampling times ( $p < 0.05$ ). Females: N=8, 11 and 11 for natural simulated photoperiod NSP and 8, 9 and 12 for constant photoperiod CP at day 1, 78 and 149 respectively. Males: N=8, 11 and 11 for NSP and 8, 9 and 12 for CP at day 1, 78 and 149 respectively.

		Females			Males		
		Time (days)			Time (days)		
		1	78	149	1	78	149
HSI	NSP	1.10 $\pm$ 0.04 <sup>a</sup>	1.01 $\pm$ 0.06 <sup>a</sup>	1.71 $\pm$ 0.11 <sup>b</sup>	1.14 $\pm$ 0.06 <sup>a</sup>	0.95 $\pm$ 0.07 <sup>a</sup>	1.59 $\pm$ 0.19 <sup>b</sup>
	CP	1.10 $\pm$ 0.04 <sup>a</sup>	0.99 $\pm$ 0.09 <sup>a</sup>	1.97 $\pm$ 0.12 <sup>b</sup>	1.14 $\pm$ 0.06 <sup>a</sup>	1.05 $\pm$ 0.08 <sup>a</sup>	2.00 $\pm$ 0.19 <sup>b</sup>
VSI	NSP	5.31 $\pm$ 0.21 <sup>Aa</sup>	5.77 $\pm$ 0.18 <sup>Aa</sup>	5.54 $\pm$ 0.27 <sup>Aa</sup>	5.52 $\pm$ 0.27 <sup>Aa</sup>	6.67 $\pm$ 0.41 <sup>Aa</sup>	6.68 $\pm$ 0.24 <sup>Aa</sup>
	CP	5.31 $\pm$ 0.21 <sup>Aa</sup>	6.12 $\pm$ 0.29 <sup>Aa</sup>	8.38 $\pm$ 0.34 <sup>Bb</sup>	5.52 $\pm$ 0.27 <sup>Aa</sup>	6.05 $\pm$ 0.67 <sup>Aa</sup>	8.86 $\pm$ 0.50 <sup>Bb</sup>