

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

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14 **Abstract**

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

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

34

35 **1. Introduction**

36 Life history of temperate fish species is regulated by seasonal changes in climate, daylength,
37 and food supplies. These species show a marked seasonality particularly with regards to
38 reproductive cycle and spawning (Baggerman, 1980). In temperate fish, the synchronicity of
39 the reproductive cycle is important because it ensures that offspring are produced when
40 environmental conditions and food supply are the most suitable for their survival and
41 development (Sumpter, 1990; Teletchea and Fontaine, 2014). Both environmental cues and
42 intrinsic factors are involved in the control of the reproductive cycle. Rosa and Bryant (2003)
43 divided them into determining factors, responsible for the synchronicity of reproductive cycle,
44 and modulating factors that can influence the progress and the quality of reproduction. From
45 all these factors, photoperiod and temperature are commonly accepted as the two
46 environmental cues that provide a timing message (Bromage et al., 2001; Wang et al., 2010).
47 Studies showed that photoperiod provides an accurate, reliable, and reproducible signal that
48 determines the onset of the reproductive cycle in many temperate species (Migaud et al.,
49 2010). Photoperiodic manipulations alone can inhibit, advance, or delay the induction of the
50 reproductive cycle, depending on the species and the time of application (Migaud et al.,
51 2010). Applying a constant long photoperiod can inhibit the onset of the reproductive cycle in
52 many species, such as the European seabass, *Dicentrarchus labrax*, (Bayarri et al., 2004),
53 European perch, *Perca fluviatilis*, (Migaud et al., 2004a), yellow perch, *Perca flavescens*,
54 (Shewmon et al., 2007), and turbot, *Scophthalmus maximus*, (Imsland et al., 1997). However,
55 in other species like three-spined stickleback, *Gasterosteus aculeatus*, and catla, *Gibelion*
56 *catla*, constant long photoperiod seems to have a stimulating effect on the gametogenesis
57 (Borg and Ekström, 1981; Bornestaf et al., 1997; Dey et al., 2005). It can advance the ovarian
58 development like in stone moroko, *Pseudorasbora parva*, (Zhu et al., 2014), and even allow
59 obtaining two annual spawns in chub, *Squalius cephalus*, (Poncin et al., 1987).

60 Cyprinids have colonized a wide variety of freshwater biotopes and are the most widely
61 farmed fish in the world. It is accepted that constant conditions of both temperature and
62 photoperiod do not generally allow the induction of their reproductive cycle (Kestemont,
63 1990; Scott, 1979). However, studies have shown or suggested that a decrease in temperature
64 or photoperiod is required to induce the gonad recrudescence in some species, such as
65 gudgeon, *Gobio gobio*, (Kestemont, 1990) and common carp, *Cyprinus carpio*, (Davies et al.,
66 1986; Davies and Hanyu, 1986).

67 The roach, *Rutilus rutilus*, is a cyprinid fish that is ubiquitous in European freshwater
68 ecosystems. This species has numerous interests for recreational fishing, pond polyculture
69 (restocking market), and ecotoxicology as sentinel species for endocrine disruption (Tyler and
70 Jobling, 2008). Based on observations in natural conditions, several authors suggest the
71 cueing role of temperature, however, there is no knowledge regarding the role of photoperiod
72 or temperature on the onset of the reproductive cycle in this species. For example, Tarkan
73 (2006) suggests that temperature drives the reproductive cycle in this species. Escaffre and
74 Billard (1976) also strongly suggest the action of temperature, but with a potential implication
75 of photoperiod in the entrainment of reproductive cycle. Moreover, according to Gillet and
76 Quéting (2006), roach belongs to a group of cyprinids that initiate gonad development during
77 the autumn, under the effect of decreasing temperature and photoperiod together. The
78 application of a long constant photoperiod during the onset of the reproductive cycle is known
79 as inhibitory in some fish species, belonging to temperate early spring spawners and
80 stimulatory in other cyprinids. As roach is an early spring spawning cyprinid (Teletchea et al.,
81 2009), the aim of this work is to study the effect of such a photoperiod on the induction of the
82 reproductive cycle in order to improve knowledge about its potential cueing role in this
83 species.

84 **2. Materials and methods**

85 This work is based on a morpho-anatomic (gonado-somatic index GSI, visceral-somatic index
86 VSI and hepato-somatic index HSI, oocyte diameter OD), histological (developmental stage
87 of gametogenesis) and physiological (estradiol E2, testosterone T, 11-ketotestosterone 11-KT
88 and Alkali-labile phosphate levels ALP) analysis of the broodstock sampled at three times
89 during and after the induction of their reproductive cycle which can be driven by photoperiod,
90 temperature or both of them. In females, the proliferation and the growth of the oocytes (GSI,
91 OD and the developmental stages) are known to be driven by the estradiol (produced by the
92 conversion of testosterone) and can be anterior or concomitant with the endocytosis of the
93 lipids and vitellus vesicles (Miura et al., 2007). The 11-Ketotestosterone (produced by the
94 conversion of testosterone) can also be involved in those stages (Rohr et al., 2001). The
95 vitellogenesis occurs right after with the incorporation of the vitellogenin (ALP levels)
96 produced by the liver (HSI) under the estrogenic control. In males, E2 at very low levels and
97 11-KT are implied in the renewal and the proliferation of the spermatogonia respectively
98 (Knapp and Carlisle, 2011) which affect the GSI and the developmental stages in testis.
99 Broodstock can use a part of their visceral reserves during these phases (VSI).

100 **2.1. Fish**

101 Roach (origin: pond, GAEC piscicole du Saulnois, Gelucourt, Moselle, France, reared under
102 natural conditions, mean weight 61 ± 8 g) were received on March, 14th 2012 (water
103 temperature at arrival 8°C). Fish were kept in an outdoor water-recirculating system, located
104 at the Domaine de la Bouzule, Laneuvelotte, Meurthe et Moselle, France (Migaud et al.,
105 2004b). They were placed into two 3m³ polyester tanks, partially covered for acclimation to
106 Recirculating aquaculture systems (RAS) conditions. During acclimation, roach were fed a
107 commercial feed (Le Gouessant Ombrine N°4). On June 21st 2012, 270 fish were transferred
108 to our laboratory facilities (University of Lorraine, France) in indoor water-recirculating

109 systems, under controlled environmental conditions. They were placed in six polyester tanks
110 (500L) at a density of 45 fish per tank. At the beginning of the experiment (Day1), fish
111 showed low gonado-somatic index GSI (1.4 ± 0.3 % in females and 0.7 ± 0.5 % in males) and
112 were in sexual rest period. Tanks were totally covered and light was provided by fluorescent
113 bulbs (Osram daylight, 150-300 lx at the water surface). Three tanks were maintained under
114 constant photoperiod 16L:8D (CP) which is known to be inhibitory and represents the longest
115 daylength to which those species are exposed under this latitude. Three other tanks were
116 maintained under natural simulated photoperiod (NSP) based on the natural variations
117 observed in Nancy, France (Figure 1). The temperature followed the natural decrease
118 occurring in Nancy (48.42N, 06.12E), as described in a previous works (Figure 1, Ben
119 Ammar et al., 2017, 2015). Experiment was performed according to the European (directive
120 2010/63/EU) and French legislation (law No 2010-788 of 12 July 2010 on national
121 commitment to the environment) for fish welfare during animal experimentation.

122 **2.2. Organ and blood sampling**

123 Fish were sampled three times: June 28th before the start of the photoperiod decrease in NSP
124 tanks (Day1), September 13th as temperature started decreasing (Day78) and November 21st
125 (Day149) corresponding to the gonadal maturation (GSI increase and appearance of
126 secondary oocytes in females, Geraudie et al. 2010a; Figure 1). At each sampling date, eight
127 fish were randomly caught in each tank to get, if possible, three females and three males (no
128 sexual dimorphism). Fish were anesthetized by immersion into a 2-phenoxyethanol solution
129 for 2–3 min (0.4 mL/L, Sigma). Blood was removed from the caudal vein using a syringe,
130 stored on ice in heparinised microtubes, and finally centrifuged at 2860 g at 15°C for 25 min
131 (centrifuge Jouan C-412). Aliquots of plasma were stored at -20 °C until sexual steroids
132 ELISA assays. After blood sampling, fish were euthanized by overdose of 2-phenoxyethanol
133 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 μm 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.

179 **2.4. Steroids and vitellogenin assays**

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

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

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

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

201

202

203 2.5. Statistical analysis

204 All statistical analyses were performed using the free software R version 3.3.1 (R
205 Development Core Team, 2005). For each modality, analyses were run in duplicate on eight
206 individual sampled at each time from each tank (three tanks per modality). For all the
207 dependant variables, homogeneity of variances was previously tested using Levene test
208 (leveneTest, package 'car', Fox et al. 2014). For each sex, data were analysed by a linear
209 mixed model (lmer, package 'lme4', Bates et al. 2014) with the photoperiod type and the
210 sampling time as fixed effects and the tank as random effect:
211 $\text{model} = \text{lmer}(Y \sim \text{photoperiod_type} * \text{sampling_time} + (1 | \text{Tank}))$ with Y : dependant variable. For
212 model validation, residuals were tested for homogeneity and normality using residuals vs
213 fitted values and sample vs theoretical quantiles (Q-Q) plots, respectively (plotresid, package
214 'RVAideMemoire', Hervé 2015). If necessary, data were log-transformed, arcsin root square
215 transformed (only for data expressed in percentage), or Box-Cox transformed. When the
216 model was validated, an anova table for various statistical models was performed to calculate
217 F-tests (Anova, package 'car', Fox et al. 2014) followed by a Least-squares means (predicted
218 marginal means) comparisons as a post-hoc test (lsmeans, package 'lsmeans', Lenth, 2016).
219 When data, even transformed, did not met the assumptions for linear mixed model
220 (homogeneity of variance and normality of residuals), we used the aligned rank
221 transformation for nonparametric factorial analysis (aligned.rank.transform, package 'ART',
222 Villacorta, 2015) from Higgins and Tashtoush (1994) formula, followed by a pairwise
223 comparison using Tukey and Kramer test with Tukey-Dist approximation for independent
224 samples (posthoc.kruskal.nemenyi.test, package 'PMCMR', Pohlert, 2016). The level of
225 significance used in all tests was $p < 0.05$.

226

227 **3. Results**

228 **3.1. Morpho-anatomic indexes**

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

234 3.1.1. Gonad development

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

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

251 cortical alveoli stage respectively, in those reared under CP (Figure 3.1). At day 149, oocytes
252 reached early vitellogenesis stage (S4, 10.5%) and late vitellogenesis stage (S5, 35.2%) in
253 females reared under NSP while the ovaries of those reared under CP showed atresia (S6,
254 22%), in addition to a majority of oogonia (S1, 68.8%). The oocyte diameter (Figure 3.2)
255 showed a strong increase in females reared under NSP between day 1 and day 149 ($p < 0.001$),
256 and a slight increase in those reared under CP between day 1 and day 78 ($p = 0.031$). At day 78
257 and day 149, the oocyte diameter was significantly higher in females reared under NSP
258 relatively to those reared under CP ($p = 0.003$ and $p = 0.045$, respectively). Females from the
259 latter treatment also showed a large inter-individual variability in the oocyte diameter at day
260 149 ($442.3 \pm 187 \mu\text{m}$, $\text{CV} = 42.2\%$).

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

264 3.1.2. Plasma steroids

265 • Plasma estradiol

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

272 • Plasma androgens

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

275 depending on the interaction between photoperiod and sampling time in both females
276 ($p < 0.001$, Figure 5.1) and males ($p < 0.001$, Figure 5.2). These levels increased significantly
277 between day 1 and day 149 in females reared under CP ($p = 0.002$) while in those reared under
278 NSP, 11KT levels increased significantly between day 1 and day 78 ($p = 0.03$) and remained
279 stable between day 78 and day 149. At day 78 and day 149, 11KT levels were significantly
280 higher in females reared under NSP compared to those reared under CP ($p = 0.001$ and $p = 0.035$
281 respectively). In males, 11-KT levels increased significantly between day 1 and day 78 in
282 both group ($p < 0.001$ in NSP group and $p = 0.003$ in CP group) and remained stable between
283 day 78 and day 149 (Figure 5.2). At days 78 and 149, males reared under NSP showed
284 significantly higher 11-KT values than those reared under CP ($p < 0.001$ for day 78 and
285 $p = 0.017$ for day 149).

286 3.1.3. Plasma vitellogenin

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

291

292

293 **4. Discussion**

294 **4.1. Effect of photoperiod on females**

295 Gonad development, oogenesis development stage, and oocyte diameter, varied throughout
296 the experiment for females depending on the photoperiod. This development showed an
297 important increase of GSI under photoperiod decrease only (3 times higher than at day 1),
298 increase of oocyte diameter (2.5 times higher than at day 1) and advanced oogenesis
299 developmental stages (appearance of early vitellogenesis at day 78). The decrease of
300 photoperiod by more than three hours between day 1 and day 78 allowed the induction of the
301 reproductive cycle in females. Our results are in concordance with the observations made by
302 Geraudie et al. (2010a, b) that found GSI close to 7% in November in wild populations of
303 roach in the region of Haute Normandie (France), and strengthen the observed similarities in
304 the gonad development rhythms between roach and Eurasian perch caught in the wild
305 (Lukšienė and Svedang 1997). In fact, this response is similar to results obtained in percids
306 like Eurasian perch (Migaud et al., 2006), yellow perch *Perca flavescens* (Shewmon et al.,
307 2007), and pikeperch *Sander lucioperca* (Ben Ammar et al., 2015), in which the decreasing
308 photoperiod triggers the onset of the reproductive cycle. Our results also showed a partial
309 inhibition of the induction of the reproductive cycle by a long constant photoperiod. In fact, in
310 females reared under CP, the sudden temperature decrease between day 78 and day 149
311 induced the reproductive cycle, with a significant increase of GSI and oocyte diameter, and
312 the appearance in the ovaries of oocytes at cortical alveoli stage. Thus, it seems that, even
313 under constant long photoperiod, oocytes maintain a real potential of growth and oogenetic
314 development. Therefore, we have little knowledge of the oogenesis developmental stage from
315 which we can consider that the reproductive cycle is well induced and can lead to the progress
316 of vitellogenesis, final oocyte maturation, and spawning. In the study of Milla et al. (2009),
317 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.,

342 2007; Rohr et al., 2001). The presence of alveoli cortical oocytes in the females reared under
343 CP seems to be linked to the slight increase of 11-KT observed at day 149.

344 **4.2. Effect of photoperiod on males**

345 The GSI variation in males reared under NSP showed an increase about 1.9 fold when
346 photoperiod decreased alone (between day 1 and day 78), which was followed by a stronger
347 increase (3 fold) when both photoperiod and temperature decreased concomitantly. The
348 increase of GSI is mainly due to the increase of spermatogonia number during successive
349 rounds of mitotic duplication (Schulz and Nóbrega, 2011). Conversely to what we observed in
350 females, spermatogenic development was interrupted between day 78 and day 149, and male roach
351 testis did not exhibit cells in more advanced stage than spermatocytes in the same proportion.
352 However, roach males reared under CP exhibit only spermatogonia A and B in their testis. It
353 seems that this spermatogenesis process does not display the same advancement, compared to
354 other early spring spawners like Eurasian perch (Sulistyo et al., 2000), pikeperch, (Ben
355 Ammar et al., 2015) and yellow perch (Shewmon et al., 2007). This delay observed in roach
356 males reared under NSP suggests that this species may present other regulatory mechanisms
357 or different sensitivity to the environmental factors. For example, in pike males, another early
358 spring spawner, applying a constant long photoperiod seems to slow spermatogenesis (Ben
359 Ammar et al. 2017). Some authors have reported an interruption of spermatogenesis in the
360 beginning of winter, with resumption in February, in temperate waters (Escaffre and Billard,
361 1976). The most differentiated stages appear in March (spermatid) and in April
362 (spermatozoa), with males spermiating between May and July (Leman Lake, France, Escaffre
363 and Billard, 1976). These observations made in the wild are close to our results, although
364 caution should be taken when comparing wild fish and RAS reared ones. The interruption of
365 spermatogenic development observed in roach can be explained by the characteristics of the
366 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.

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

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625 **Legends**

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

629 **Fig. 2** Effect of photoperiod treatment (NSP: Natural simulated photoperiod, CP: Constant
630 photoperiod) in day 1, day 78 and day 149 on gonado-somatic index (%) in females (1) and males (2)
631 of roach *Rutilus rutilus*. The horizontal line in the box represents the median; the bottom and the top of
632 the box are the first and third quartiles. Whiskers show values within 1.5 times of the interquartile
633 range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B)
634 indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c)
635 indicate significant differences among the sampling times ($p < 0.05$). Females: N=8, 11 and 11 for
636 natural simulated photoperiod NSP and 8, 9 and 12 for constant photoperiod CP at day 1, 78 and 149
637 respectively. Males: N=8, 11 and 11 for NSP and 8, 9 and 12 for CP at day 1, 78 and 149 respectively.

638 **Fig. 3** Effect of photoperiod treatment (NSP: Natural simulated photoperiod, CP: Constant
639 photoperiod) on oogenesis development stages observed in ovaries (1) on oocyte diameter (μm) and
640 spermatogenesis development stages (3) of roach *Rutilus rutilus* females and males. (1) S1: Primary
641 growth, S2: early cortical alveoli stage, S3: late cortical alveoli stage, S4: early vitellogenesis, S5:
642 vitellogenesis and S6: atresia. (2) SPGA: spermatogonia A, SPGB: spermatogonia B, SPC:
643 spermatocyte, SPD: spermatid and SPZ: spermatozoa. (3) The horizontal line in the box represents the
644 median; the bottom and the top of the box are the first and third quartiles. Whiskers show values
645 within 1.5 times of the interquartile range from the boxes and outliers are plotted as little white circle.
646 Different capital letters (A, B) indicate significant differences between the photoperiod treatment and,
647 lower case letters (a, b, c) indicate significant differences among the sampling times ($p < 0.05$). N=3 per
648 tank for both sexes in all modalities of photoperiod and sampling time.

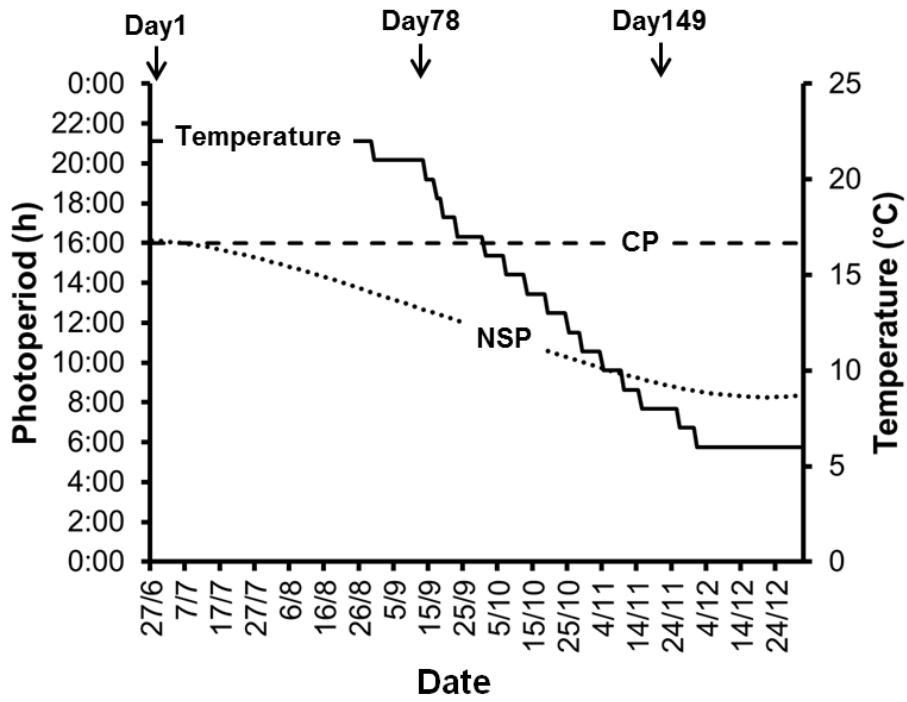
649 **Fig. 4** Effect of photoperiod treatment (1, NSP: Natural simulated photoperiod, CP: Constant
650 photoperiod) and sampling time (2) on plasma estradiol levels E2 (ng/mL) in females of roach *Rutilus*
651 *rutilus*. The horizontal line in the box represents the median; the bottom and the top of the box are the

652 first and third quartiles. Whiskers show values within 1.5 times of the interquartile range from the
653 boxes and outliers are plotted as little white circle. Different capital letters (A, B) indicate significant
654 differences between the photoperiod treatment and, lower case letters (a, b, c) indicate significant
655 differences among the sampling times ($p < 0.05$). (1): N=30 for NSP treatment and 29 for CP treatment.
656 (2): N=16, 20 and 23 at day 1, 78 and 149 respectively.

657 **Fig. 5** Effect of photoperiod treatment (NSP: Natural simulated photoperiod, CP: Constant
658 photoperiod) on plasma 11-ketotestosterone levels 11-KT (ng/mL) in females (1) and males (2) of
659 roach *Rutilus rutilus*. The horizontal line in the box represents the median; the bottom and the top of
660 the box are the first and third quartiles. Whiskers show values within 1.5 times of the interquartile
661 range from the boxes and outliers are plotted as little white circle. Different capital letters (A, B)
662 indicate significant differences between the photoperiod treatment and, lower case letters (a, b, c)
663 indicate significant differences among the sampling times ($p < 0.05$). Females: N=8, 11 and 11 for
664 natural simulated photoperiod NSP and 8, 9 and 12 for constant photoperiod CP at day 1, 78 and 149
665 respectively. Males: N=8, 11 and 11 for NSP and 8, 9 and 12 for CP at day 1, 78 and 149 respectively.

666 **Fig. 6** Effect of photoperiod treatment (1, NSP: Natural simulated photoperiod, CP: Constant
667 photoperiod) and sampling time (2) on plasma Alkaline Labile Phosphate levels ALP ($\mu\text{g/mL}$) in
668 females of roach *Rutilus rutilus*. The horizontal line in the box represents the median; the bottom and
669 the top of the box are the first and third quartiles. Whiskers show values within 1.5 times of the
670 interquartile range from the boxes and outliers are plotted as little white circle. Different capital letters
671 (A, B) indicate significant differences between the photoperiod treatment and, lower case letters (a, b,
672 c) indicate significant differences among the sampling times ($p < 0.05$). (1): N=30 for NSP treatment
673 and 29 for CP treatment. (2): N=16, 20 and 23 at day 1, 78 and 149 respectively.

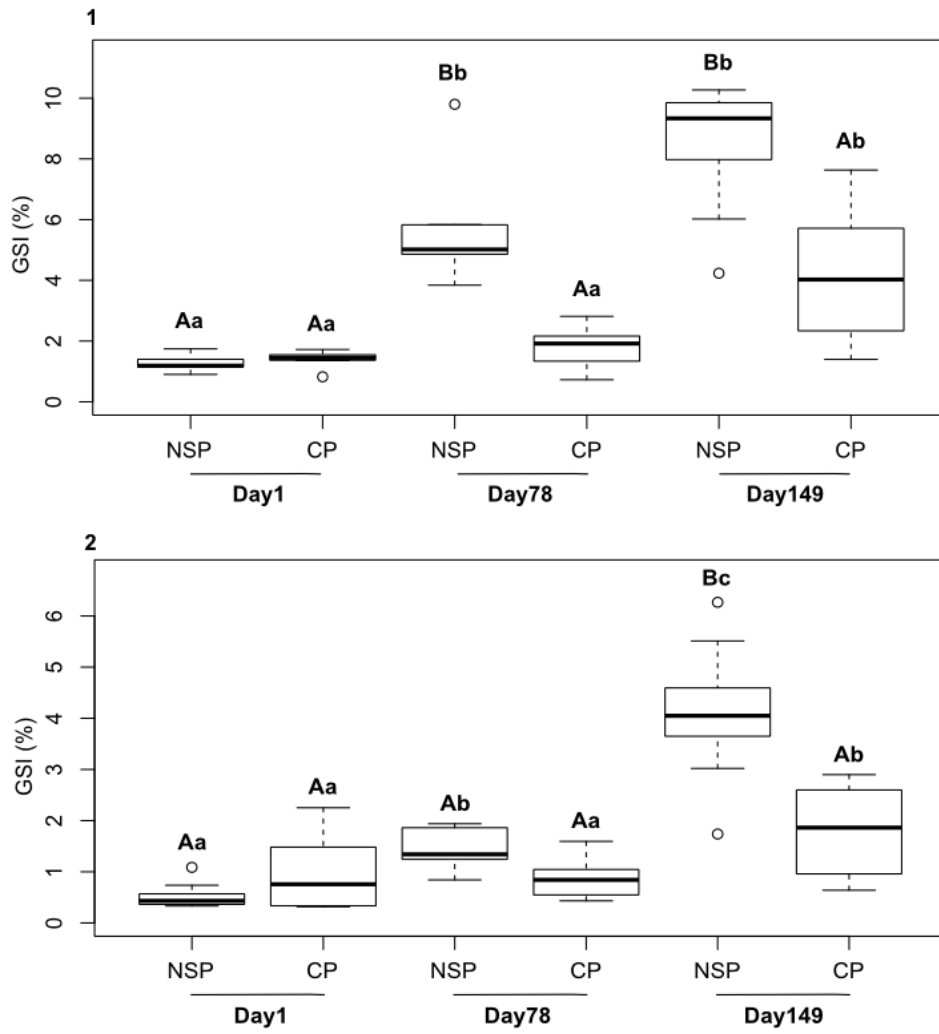
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676 Figure 1 Ben Ammar et al.

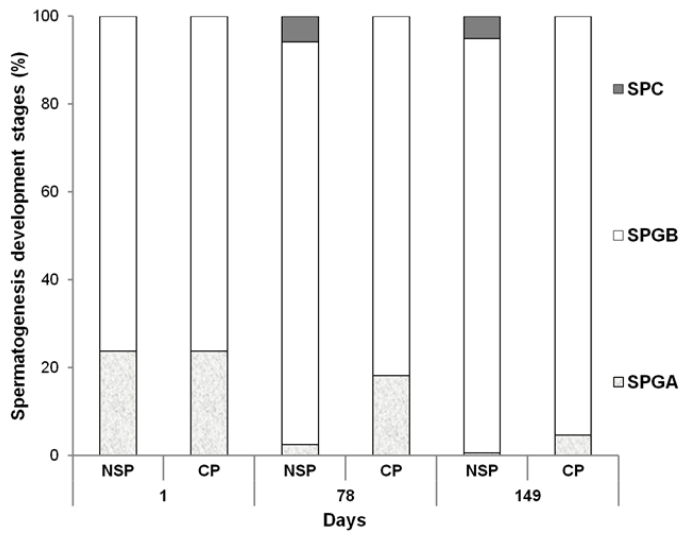
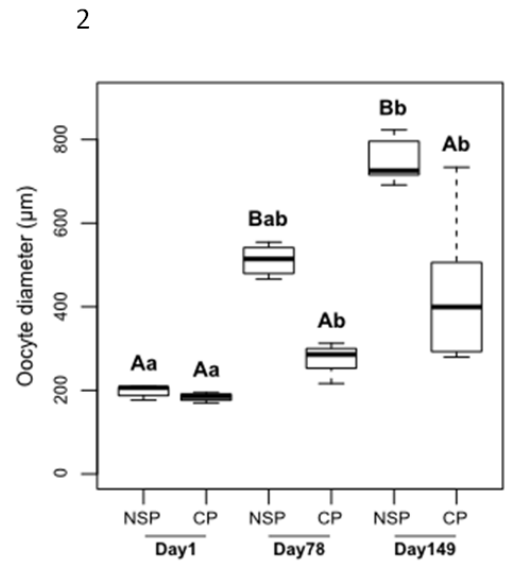
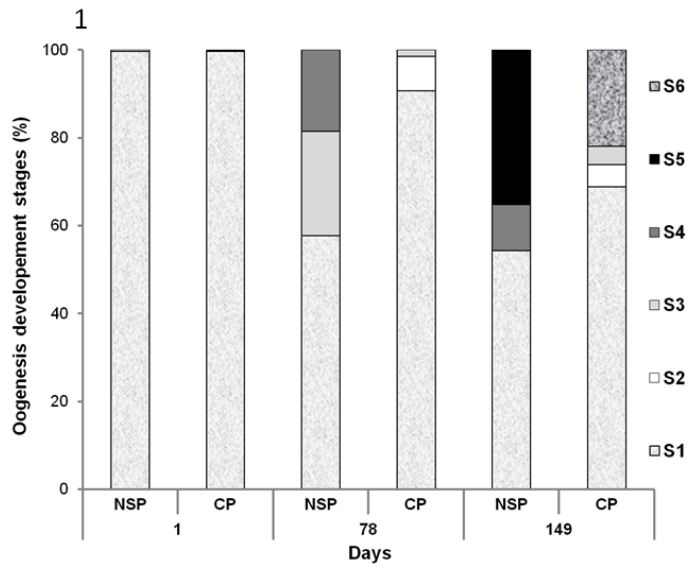
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679 Figure 2. Ben Ammar et al.

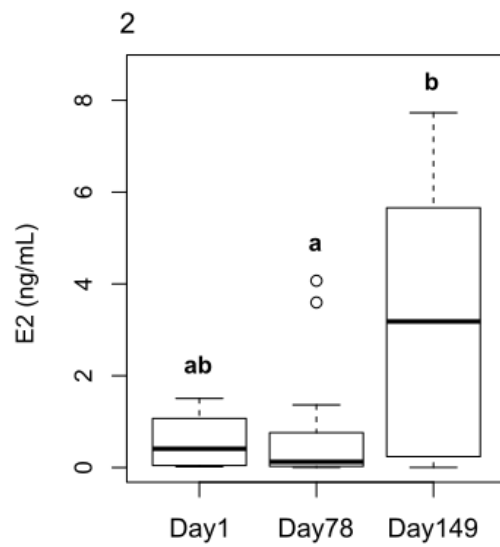
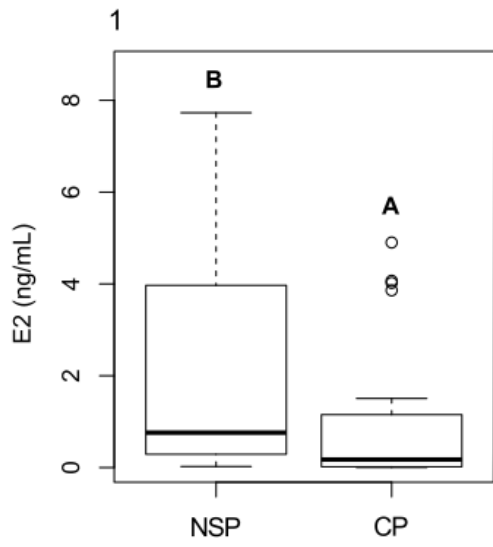
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682 Figure 3 Ben Ammar et al

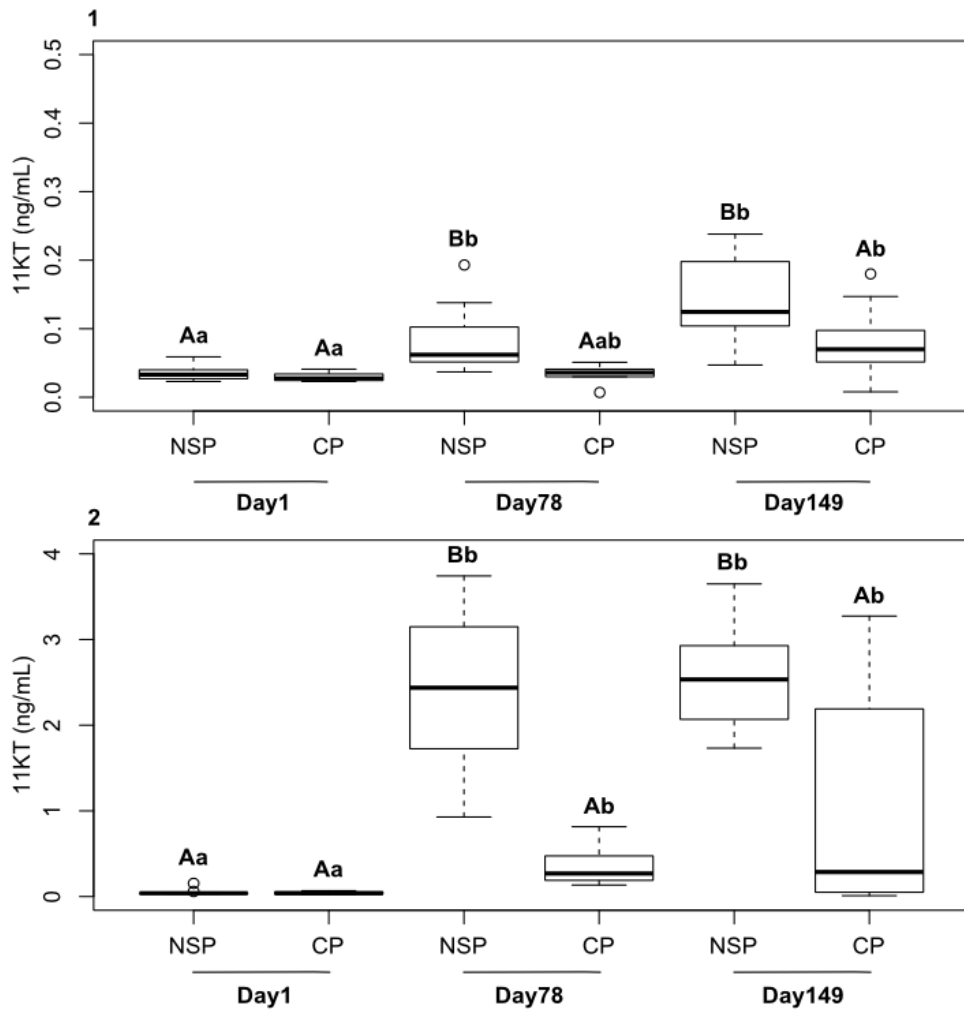
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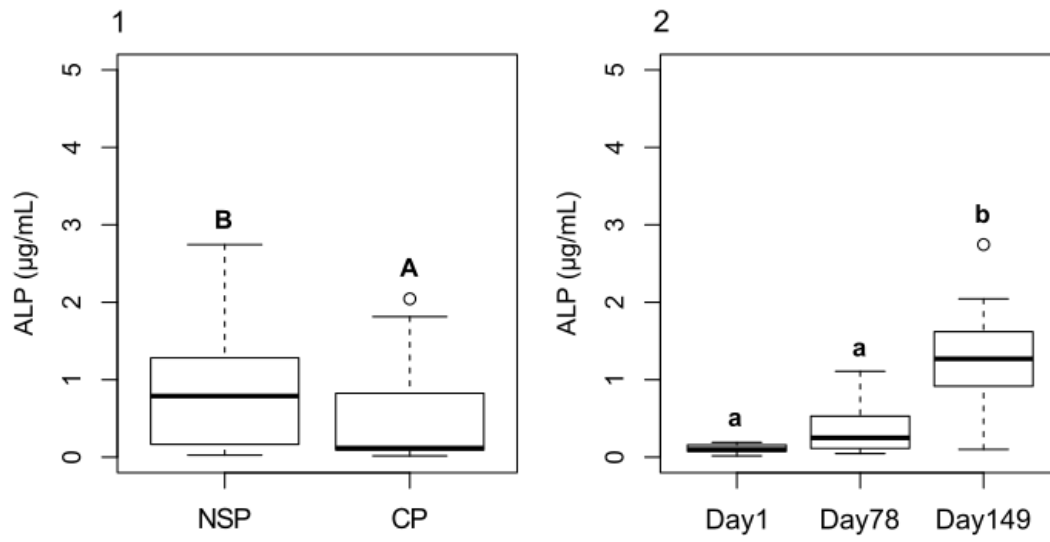
685 Figure 4 Ben Ammar et al

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688 Figure 5. Ben Ammar et al.



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690 Figure 6. Ben Ammar et al.

691 **Table 1:** Effect of the photoperiod treatment on hepato-somatic index (HSI) and
 692 viscerosomatic index (VSI) in females and males of roach *Rutilus rutilus* (mean \pm
 693 SEM). Different capital letters (A, B) indicate significant differences between the
 694 photoperiod treatment and, lower case letters (a, b, c) indicate significant differences
 695 among the sampling times ($p < 0.05$). Females: N=8, 11 and 11 for natural simulated
 696 photoperiod NSP and 8, 9 and 12 for constant photoperiod CP at day 1, 78 and 149
 697 respectively. Males: N=8, 11 and 11 for NSP and 8, 9 and 12 for CP at day 1, 78 and
 698 149 respectively.

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

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