Discontinuity in the molecular neuroendocrine response to increasing daylengths in Ile-de-France ewes : is transient *Dio2* induction a key feature of circannual timing ?

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Abstract

In mammals, melatonin is responsible for synchronisation of seasonal cycles to the solar year. Melatonin is secreted by the pineal gland with a profile reflecting the duration of the night and acts via the pituitary *pars tuberalis* (PT), which in turn modulates hypothalamic thyroid hormone status through seasonal changes in the production of locally-acting thyrotropin (TSH). Recently we demonstrated that in the Soay sheep, photoperiodic induction of *Tshb* expression and consequent downstream hypothalamic changes occur over a narrow range of photoperiods between 12- and 14-h in duration. In the present study, we sought to extend our molecular characterization of this pathway, based on transcriptomic analysis of photoperiodic changes in the pituitary and hypothalamus of ovariectomized, estradiol-implanted Ile-de-France ewes. We demonstrate that photoperiodic treatments applied before the winter solstice elicit two distinctive modes of accelerated reproductive switch off compared to ewes held on a simulated natural photoperiod,

with shut-down occurring markedly faster on photoperiods of 13-h or more than on photoperiods of 12-h and less. This pattern of response was reflected in gene expression profiles of photoperiodically sensitive markers, both in the PT (*Tshb*, *Fam150b*, *Vmo1, Ezh2* and *Suv39H2*) and in tanycytes (*Tmem252* and *Dct*). Unexpectedly, the expression of *Dio2* in tanycytes did not show any noticeable increase in expression with lengthening photoperiods. Finally, expression of *Kiss1*, the key activator of GnRH release, was proportionately decreased by lengthening photoperiods, in a pattern that correlated strongly with gonadotropin suppression. These data show that stepwise increases in photoperiod lead to graded molecular responses at the level of the PT, a progressive suppression of *Kiss1* in the hypothalamic arcuate nucleus and LH/FSH release by the pituitary, in spite of seemingly unchanged *Dio2* expression in tanycytes. We hypothesize that this apparent discontinuity in the seasonal neuroendocrine response illustrates the transient nature of the thyroid hormone-mediated response to long days in the control of circannual timing.

Abbreviations

ISH: *in situ* hybridization; LP: long photoperiod; MBH: medio-basal hypothalamus; OVX: ovariectomized; PD: *pars distalis* of the pituitary; PT: *pars tuberalis* of the pituitary; SP: short photoperiod; TH: thyroid hormone; T3: triiodothyronine; TSH: thyrotropin; ZT: zeitgeber time.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Introduction

Seasonal breeding is a common adaptive feature of most mammals living at temperate latitudes, ensuring that birth takes place at the most favourable time of year in terms of environmental resources. Photoperiod is the main synchronizer of seasonal functions with additional factors such as food availability, stress and social interactions acting as modulators[1]. In mammals, melatonin is the endocrine messenger of photoperiod and coordinates seasonal switches in endocrinology and metabolism. To achieve this, melatonin targets a peculiar population of thyrotrophs within the *pars tuberalis* (PT) of the pituitary, leading to seasonal expression of *Tshb* and hundreds of genes expressed in the PT and medio-basal hypothalamus (MBH)[2-4]. PT-specific TSH appears to be the crux of photoperiod-dependent seasonal timing as it connects melatonin input with local hypothalamic T3 output through induction of deiodinase 2 (*Dio2*) in tanycytes lining the infra-lateral walls of the adjacent third ventricle[5-8]. While the basics of this TSH-DIO2-T3 axis have been well characterized in birds and mammals, cellular and molecular mechanisms that link T3 to seasonal GnRH output, and hence to control of the pituitary response and gonadal axis, remain unclear. However, a role for the KISS1 neuronal population of the arcuate nucleus seems inescapable[9-11].

Our understanding of how photoperiod controls this molecular neuroendocrine axis remains limited. We lack a comprehensive view of the impact of increasing daylengths on the expression of seasonal markers within the PT, tanycytes and hypothalamus. Indeed, the majority of studies in quail, hamsters and sheep have focused on comparisons between a long and a short photoperiod (typically 16h vs 8h). However, switches in physiology are triggered by intermediate photoperiods, as exemplified by experiments that defined the length of the critical photoperiod (CP) in quail and hamsters[12]. The CP is the minimal duration of daylight exposure (as determined by stepwise increases or decreases) that prompts the opposite reproductive state; i.e. activation in sexually

inactive animals and sexual arrest in reproductively active animals. In quail and hamsters the CP lies somewhere between 12.5h and 13h[13-16]. We recently determined that the CP is comprised between 11.75h and 12.5h in male Soay sheep[17]. While this study demonstrated a strong correlation between the molecular response at the PT-tanycytes level and the endocrine output (FSH and Testosterone), it also revealed that the endocrine response to TSH was logarithmic rather than linear, such that a very small increase in PT-derived TSH could elicit a large increase in *Dio2* expression. Interestingly, a recent study on maternal programming in hamsters also revealed non-linearity in the photoperiodic response of TSH-*Dio2*[18]. We proposed that such non-linearity along the molecular neuroendocrine axis of the MBH underpins the long-term effects of photoperiod on intrinsic seasonal programs, i.e. circannual timers[4,17,18].

Here, we used an approach similar to that developed for Soay lambs (CP protocol) in ovariectomized, estradiol-implanted (OVX+E2) ewes of the Ile-de-France breed. The OVX+E2 model normalizes the level of circulating E2, which uncovers the well-documented central seasonal shift in the negative feedback action of E2 on gonadotropin secretion[19]. In this model, serum levels of the gonadotropins LH and FSH provide a reliable index of the state of the GnRH pulse generator[3,19]. Our findings are consistent with the CP value defined in Soay rams as well as with the existence of non-linearity in the gonadotropic response to long days, such that a small increase in *Tshb* correlates with a large decrease in LH and FSH levels. Our findings also revealed a strong linear correlation between *Kiss1* expression in the arcuate nucleus and plasma LH/FSH levels, consistent with a major role for these neurons in the control of seasonal breeding. Finally, we observed uncoupling between expression levels of *Tshb* and *Dio2*: levels of *Dio2* were similar across the five groups at the end of the two months of photoperiodic treatments. We speculate that this discontinuity in the molecular TSH/DIO2/KISS1 pathway reflects transient *Dio2* induction,

98 which would be consistent with the brief temporal requirements for long days and T3 in the

99 synchronization of the ovine circannual program.

100

Material & Methods

Ethics statement

103 All experimental procedures were performed in accordance with international (directive

104 2010/63/UE) and national legislation (décret n° 2013–118) governing the ethical use of animals in

105 research (authorization n° E37–175-2 and n°A38 801). All procedures used in this work were

106 evaluated by a local ethics committee (Comité d'Ethique en Expérimentation Animale Val de

107 Loire; n°2012-10-5).

108

Experimental animals & procedures

110 Experiments were conducted in 30 adult Ile-de-France ewes (3–5 years old; weight 60–80 kg) kept

111 under normal husbandry conditions at the research station of the Institut National de la Recherche

112 Agronomique (Nouzilly, Unité Expérimentale PAO n°1297 (EU0028)). The natural photoperiod

113 at the latitude of Nouzilly, France (47°N) ranges from ~16:8 (16h of light, 8h of night) at the

114 summer solstice to ~8:16 at the winter solstice. All ewes were ovariectomized (OVX) and

115 estradiol-implanted (E2; 1cm silastic implant) between Sept 2nd and Sept 6th (2012).

116

117 All surgeries were performed after sodium thiopental anesthesia (Nesdonal®, 1g/80kg), under

118 constant isoflurane administration (Vetflurane®) and all efforts were made to minimize suffering.

119 Following surgery, animals received an injection of antibiotics (oxytetracycline, Terramycine

120 LA®, 1ml/10kg) and an injection of a non-steroidal anti-inflammatory drug (Finadyne®, flumixin

121 megumine, 2ml/50kgs). Animals were followed daily throughout the experiment.

122

123   Following surgery, all ewes were initially kept outdoors (open barns) before being brought indoors

124   in a light-tight building on Nov 19th (**Figure 1A**). The natural photoperiod at this time of year in

125   Nouzilly is ~9h15min. Six ewes were randomly assigned to one of each photoperiodic treatment :

126   Simulated Natural Photoperiod (PP nat), 11:13 (i.e. 11h of light per day), 12:12, 13:11 and 14:10

127   (**Figure 1A**). For each treatment, ewes were housed by groups of 3 in two separate light-tight pens.

128   Ewes of the PP nat group were submitted to a photoperiodic treatment that mimics outdoor

129   conditions through weekly stepwise modifications of daily light exposure. All other ewes were

130   exposed to longer photoperiods by delaying the time of lights off (**Figure 1B**). Ewes were

131   submitted to these photoperiodic treatments for ~2 months (**Figure 1A**).

132

133   Blood samples were collected twice weekly by jugular venipuncture in heparinized tubes from

134   Sept 17th (2012) through to Jan 10th (2013). After centrifugation, serum was collected and frozen

135   until assayed for hormones. At the end of the photoperiodic treatments, all animals were

136   euthanized by decapitation under deep barbiturate anesthesia (Nesdonal®, 5mL). To minimize

137   issues linked to potential time-of-day effects on gene expression all ewes were killed in the early

138   morning between ZT2 and ZT4 (with ZT0 being the time of lights on; **Figure 1B**) on January 13th

139   (n=15) and January 14th (n=14). Hypothalamic blocks were sampled and immediately frozen in

140   isopentane kept on dry ice, before being stored at -80°C until sectioning, as described previously[3].

141   One ewe of the 12:12 group died in December and, at the end of the experiment, no E2 implant

142   could be recovered from one ewe of the PP nat group and another ewe from the 13:11 group. Data

143   from these 3 ewes were removed from the analysis of hormonal profiles.

144

145   **Hormonal profiles**

146   Plasma levels of LH, FSH and PRL were assayed by RIA. All samples from one experiment were

147   included in a single assay and every sample was measured in duplicate. <u>LH</u>: the assay standard

148 was 1051-CY-LH (equivalent to 0.31 NIH-LH-S1). Intra- and inter- assay coefficients of variation

149 averaged 9% and 15%, respectively with an assay sensitivity of 0.1 ng/mL. <u>FSH:</u> levels were

150 measured using reagents supplied by Tucker Endocrine Research Institute (Atlanta, GA, USA).

151 Intra- and inter- assay coefficients of variation averaged 8% and 9%, respectively with an assay

152 sensitivity of 0.1 ng/mL relative to the standard (Tuenere oFSHstd. 1 equiv to 1.0 NIH-FSH-S1).

153 The cross reactivity with ovine LH was 0.03%. <u>PRL</u>: Intra- and inter- assay coefficients of

154 variation averaged 7% and 12%, respectively with an assay sensitivity of 2.5 ng/ml.

155

156 ***In situ* hybridization (ISH)**

157 ISH was perfomed as described previously using validated ovine riboprobes[3,20,21]. A complete list

158 of the riboprobes used in this study is provided in **Supplementary Table 1**.

159 Hypothalamic blocks for *in situ* hybridisation were cut into 20μm sections using a cryostat

160 (CryoStar NX70, ThermoScientific) and thaw-mounted onto SuperFrost Plus slides

161 (ThermoScientific). All radioactive cRNA riboprobes were prepared by plasmid linearisation and

162 *in vitro* transcription (Riboprobe System, Promega) including ^{35}S-UTP (Perkin-Elmer). The probe

163 was purified with Illustra Probe Quant G50 micro-columns (Fisher) and counted with a liquid

164 scintillation counter (Tri-Carb 2900TR, Packard). Slides were post-fixed at 4°C for 20 min in 4%

165 PFA, 0.1 M PB, rinsed with 0.1 M PB (2 X 5min), acetylated with 3.75% v/v of acetic anhydride

166 in 0.1 TEA, 0.05 N NaOH (10min) and finally rinsed with 0.1 M PB (2 X 5min). Slides were then

167 dehydrated through graded ethanol solutions (50%, 70%, 95% and 100%; 3min each) and dried

168 under vacuum for 60 min. Sections were hybridized overnight at 58ºC with 10^6 cpm of probe per

169 slide in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1 X Denhardt's

170 solution, 300 mM NaCl, 10 mM Tris, 10 mM DTT, 1 mM EDTA, 500 μg/ml tRNA). Sections

171 were then rinsed in 4 X SSC (3 X 5 min) and subjected to RNase-A digestion (20 μg/ml) in a

172 buffer containing 500 mM NaCl, 1 mM Tris, 1 mM EDTA for 30 min at 37°C. Stringency washes

173   in SSC (with 1mM DTT) were performed to remove non-specific probe hybridisation: 2 X SSC

174   (2 X 5 min), 1 X SSC (10 min), 0.5 X SSC (10 min), 0.1 X SSC (30 min at 60°C), 0.1 X SSC (5

175   min). Slides were then dehydrated through graded ethanol solutions (50%, 70%, 95% and 100%;

176   3min each), dried under vacuum for 60 min and exposed for 1 to 3 weeks (depending on the target

177   mRNA) to an autoradiographic film (BioMax MR, Kodak). Films were scanned on a transmittance

178   image scanner (Amersham, UK) along with a calibrated optical density (OD) transmission step

179   wedge (Stouffer, USA). Calibrated Integrated OD measurements of gene expression were

180   performed using ImageJ software.

181

182   **Data analysis**

183   Data were analysed using GraphPad Prism 6.0 and are reported as mean ± sem. For hormonal

184   profiles, RIA data were analyzed by Repeated Measures (RM) 2-way ANOVA. One-way ANOVA

185   was also used to perform analyses limited to the two weeks before the photoperiodic transfer and

186   the last two weeks of the experiment. ISH data were analyzed by 1-way ANOVA using treatment

187   as a variable. The post-hoc Tukey test was used for multiple comparisons. $p<0.05$ was considered

188   significant. Using individual values, linear regression analysis was performed to evaluate

189   correlations between *Tshb*, *Dio2* or *Kiss1* mRNA levels and mean LH/FSH levels over the last two

190   weeks of the experiment.

191

192   **Results**

193   **Hormonal profiles**

194   RIA was used to assess plasma levels for LH (**Figure 2A-C**), FSH (**Figure 2D-F**) and PRL (**Figure**

195   **2G-I**). For LH, RM two-way ANOVA revealed a strong time*group interaction (P<0.0001), which

196   reflected the impact of the photoperiodic treatments, as no difference between groups were

197   observed prior to these. Indeed, further analysis revealed no differences between groups over the

198   2-wks period before the photoperiodic transfer (**Figure 2B** ; one-way ANOVA ; P=0.25), while

199   levels differed significantly over the last 2-wks period (**Figure 2C** ; one-way ANOVA ;

200   P<0.0001). For FSH, RM 2-way ANOVA revealed a strong time*group interaction (P<0.0001),

201   which mostly reflected the impact of the photoperiodic treatments. Indeed, further analysis

202   revealed differences between groups over the 2-wks period before the photoperiodic transfer

203   (**Figure 2E**) and the last 2-wks period of the experiment (**Figure 2F** ; one-way ANOVA ;

204   P<0.0001 for both comparisons). The difference before the photoperiodic transfer is accounted for

205   by slightly lower levels in the PP nat group compared to the 4 other groups. Linear regression

206   analysis showed that levels of LH and FSH during last 2-wks period of the experiment were

207   correlated (R^2=0.4679 ; P<0.0001).

208

209   For PRL, RM two-way ANOVA revealed a a strong time*group interaction (P=0.0004), which

210   may reflect the inter-individual and inter-group variabilities before the photoperiodic treatments

211   and slightly divergent trajectories after transfer. However, PRL levels did not differ bewteen

212   groups before the photoperiodic transfer (**Figure 2H** ; one-way ANOVA ; P=0.53), while a trend

213   towards higher levels with increasing photoperiods was noticed for the last 2-wks period of the

214   experiment (**Figure 2I** ; one-way ANOVA ; P=0.053).

215

216   **Gene expression**

217   We used semi-quantitative ISH to assess the impact of the photoperiodic transfer on the mRNA

218   expression levels of multiple seasonal markers within the MBH (**Figure 3**). Data were analysed

219   by one-way ANOVA (**Figure 3A**). Photoperiod affected the expression of all PT markers except

220   *Chga* [$F_{4,24}$ = 0.32 ; P=0.86] : *Tshb* [$F_{4,24}$ = 25.94 ; P<0.0001], *Fam150b* [$F_{4,24}$ = 22.04 ;

221   P<0.0001], *Vmo1* [$F_{4,24}$ = 14.37 ; P<0.0001], *Ezh2* [$F_{4,24}$ = 14.37 ; P<0.0001], *Suv39H2* [$F_{4,24}$ =

222   6.61 ; P=0.001] and *Eya3* [$F_{4,24}$ = 6.29 ; P<0.005]. Post-hoc analysis revealed that expression

223   levels were not significantly different between the PP nat, 11:13 and 12:12 groups for any of these

224   PT-expressed genes. Compared to PP nat, expression of *Tshb*, *Fam150b* and *Eya3* were

225   significantly increased under 13:11. Expression of all markers was significantly increased by the

226   14:10 photoperiod compared to PP nat. Within markers of tanycytes, photoperiod significantly

227   affected mRNA expression of *Tmem252* [$F_{4,23} = 7.55$; P=0.0005] and *Dct* [$F_{4,24} = 6.11$; P<0.005]

228   but not *Dio2* [$F_{4,24} = 1.74$; P=0.17]. Similar to the PT markers, no significant differences were

229   found between PP nat, 11:13 and 12:12 groups, while longer photoperiods of 13:11 and 14:10

230   increased expression of *Tmem252* and *Dct*, compared to PP nat. No detectable expression of *Dio3*

231   was observed (not shown). Finally, the photoperiodic transfer affected expression of the

232   hypothalamic marker *Kiss1* [$F_{4,24} = 6.06$; P<0.005], with expression significantly reduced under

233   13:11 and 14:10 compared to PP nat. Representative images of autoradiograms for all these

234   markers at their peak of expression are provided in **Figure 3B**.

235

236   **Linear regression analyses**

237   Data for LH and FSH (using individual means for the last two weeks of treatment; data from Figure

238   2C and Figure 2F) are shown in **Figure 4A and 4B**, respectively. Levels of *Tshb* and *Kiss1* mRNA

239   were negatively and positively correlated with LH (R^2=0.20, P=0.018 and R^2=0.66, P<0.0001) and

240   FSH levels (R^2=0.29, P=0.004 and R^2=0.53, P<0.0001), respectively . Levels of *Dio2* mRNA were

241   not correlated with either LH (R^2=0.04, P=0.34) or FSH levels (R^2=0.1; P=0.11). For *Tshb* and

242   LH/FSH, data could be better fitted with a hyperbolic curve, which is shown instead of the linear

243   regression line.

244

245   **Discussion**

246   There is a strong intrinsic component to the seasonally timed changes in reproductive status in

247   sheep, which under constant long photoperiods manifests itself as a circannual rhythm of ~10

248   months[22,23]. By employing OVX+E2 ewes of the Ile-de-France breed, this cycle of activation and

249   quiescence can be clearly seen in changes in gonadotropin secretion, and hence the 3 trajectories

250   of reproductive shutdown observed in the present study can be interpreted against the circannual

251   framework. These endocrine changes mirror changes in expression level of key molecular markers

252   in the MBH. However, our data also point to a discontinuity along the neuroendocrine

253   gonadotropic axis, since *Dio2* levels in tanycytes - unlike *Tshb* in the PT and *Kiss1* in the

254   hypothalamic parenchyma - were not correlated to the differential photoperiodic LH/FSH output

255   (**Figure 4**).

256

257   Our molecular analysis supports the key role of the PT as a reliable decoder of the photoperiodic

258   message carried out by melatonin. Beyond *Tshb*, we and others recently identifed hundreds of

259   PT-expressed genes displaying strong T3-independent seasonal changes in expression[2,3]. The

260   expression profiles reported in this study are also consistent with the acute LP-responsiveness

261   of most of these markers (*Tshb*, *Fam150b*, *Vmo1*, *Ezh2* and *Eya3* ; see[3]). We note that

262   expression of *Chga*, a marker for SP in the PT[2,3], was not diminished upon exposure to increased

263   daylengths, consistent with its lack of responsiveness to acute LP exposure[3]. This finding is

264   compatible with a model in which most photoperiodic markers in the PT are induced/repressed

265   at different daylengths and/or located in pathways downstream of a core of "1st order LP-

266   responsive genes", in a parallel with the organization of the molecular circadian clock, with a

267   handful of core clock genes and a myriad of (tissue-specific) clock-controlled genes[24,25].

268

269   Our data are consistent with a role for PT-expressed *Eya3*/*Tshb* in the photoperiodic read-

270   out[7,26], and with the implication of *Kiss1*-expressing neurons of the arcuate nucleus in the

271   seasonal control of GnRH and gonadotropins[10,11]. These data further indicate that a photoperiod

272   as short as 11:13 is sufficient to elicit a marked response in both LH and FSH, without

273 statistically significant changes in the expression of molecular seasonal markers. Current

274 evidence in mouse[27] and sheep[2,26,28] points to a pivotal role for EYA3 in the photoperiodic

275 response of the melatonin-responsive PT. In sheep, we proposed that the response to LP is

276 triggered by the EYA3/SIX1 heterodimer, which acts as a co-activator of DNA-bound TEF at

277 the *Tshb* promoter[1,7,26]. The LP increase in *Eya3* expression is crux to the system but the overall

278 *Tshb* transcriptional output rests on proper phase relationship between *Eya3* and *Tef*, which are

279 both circadian clock-controlled genes[26]. *Eya3* expression is timed to occur ~12h after dark onset

280 (between 8h and 16h, tighter sampling schedule missing) and is potently inhibited by melatonin

281 through a mechanism that remains unknown (as is the mechanism for *Eya3* induction by LP).

282 This model predicts that longer photoperiods are more conducive to *Eya3* increase, due to

283 melatonin being absent in the morning, and also predicts that the rise in *Tshb* is necessarily

284 preceded by rising *Eya3*. Our data are consistent with this since *Eya3* has risen by 75% under

285 11:13 and by 250% under 12:12, when compared to PP nat ($P<0.05$ by t-test for both pairwise

286 comparisons). Comparatively, *Tshb* does not display any statistically significant changes in

287 expression. However, animals were killed in the early day (ZT2-4) ; a time which fits the

288 expected *Eya3* peak under longer photoperiods but also corresponds to a marked trough in *Tshb*

289 expression[26]. This might account for the apparent lack of difference observed for *Tshb*

290 expression between PP nat, 11:13 and 12:12 photoperiods.

291

292 We recently used a similar methodology to investigate the impact of quantitative increases in

293 photoperiod on the neuroendocrine response in intact Soay ram lambs[17]. In that study, we also

294 found induction of *Eya3* at a shorter photoperiod (11.75h) compared to *Tshb* (12.5h), while the

295 pituitary and gonadal responses (FSH and Testosterone, respectively) were more clear-cut and

296 pointed to the existence of a CP between 11.75h and 12.5h. It is difficult to compare the two

297 studies with regards to the physiological output since we used intact ram lambs in the former

study and OVX+E2 ewes in the current study. However, in this OVX+E2 model, it has been established that LH values above and below ~1ng/ml do correspond to active and anestrus states of intact ewes of both Suffolk[29,30] and Ile-de-France breeds[3,31,32]. Considering this, ewes of the 13:11 and 14:10 groups, but not of the 11:13 and 12:12 groups, would have been in – or very close to – a state of anestrus. Furthermore, LH levels are not significantly different between the groups exposed to daylengths of 13h and 14h while *Tshb* levels are more than doubled under 14:10 compared to 13:11. This seems to indicate that *Tshb* levels attained with exposure to a photoperiod equal to – and likely shorter than – 13h are sufficient to trigger maximal response of the hypothalamic-pituitary axis. Therefore, we conclude that the critical photoperiod in Ile-de-France ewes probably lies between 12h and 13h, similar to the situation in Soay ram lambs[17].

In comparing the outcomes of these independent studies, it is also important to stress that the duration of photoperiodic treatments differed between the two protocols: Soay rams were exposed for 4 weeks, while ewes of the current study were exposed for ~8 weeks. This duration has to be taken into account when interpreting the physiological status at the end of the experiment. Considering the trajectory for LH (and FSH) in ewes of the 11:13 and 12:12 groups, it seems likely that longer exposure to these photoperiods would eventually have led to LH values <1ng/ml (signing an anestrus-like state ; see above). The impact of photoperiodic history also has to be considered as the same photoperiod triggers opposite responses of the GnRH pulse generator and LH output, according to the initial photoperiod to which ewes were exposed[30].

In contrast to LH and FSH, plasma levels of PRL did not change significantly throughout exposure to graded photoperiods, even though a trend towards higher levels was seen, most obvious for the 14:10 group. We also note a much larger inter-individual variability, with

multiple peaks, before than after the transfer to the light-tight building. This most likely reflects the well-characterized susceptibility of PRL secretion to various unpredictable stressors such as noise or human activity and temperature fluctuations[33-35], which are common in open barns but very limited in our light-tight building. PRL displays a seasonal pattern of secretion, with higher levels during spring and summer[5,33,35-38] and rapid increase or decrease upon acute exposure to longer or shorter daylengths, respectively[26,28,39,40]. Photoperiodic history also affects the long-day response of PRL secretion in ewes[41]. In sheep, gonadotropic (LH/FSH) and lactotropic axes (PRL) display opposite responses to daylength, which are driven by two distinct neuroendocrine axes[12,42,43]: the gonadotropic axis uses the retrograde TSH/DIO2/T3 axis while the lactotropic axis relies on anterograde signaling from the PT to the *pars distalis*, independently of T3 (in both rams and ewes[3,12,44,45]), through one or several endocrine factors (known as tuberalin(s)) whose identity remains unclear[4]. Our findings that a 14h-daylength does not significantly increase PRL is in line with early findings in Siberian hamster[46], which showed that both axes have distinct CP, the CP for PRL being longer than that for LH/FSH.

Overall, the concept of CP is of theoretical value but must be used with caution as the CP value differs according to the species and latitude of natural habitat, molecular or neuroendocrine output considered, duration of exposure and moment in the seasonal (circannual) cycle at which exposure occurs (i.e. photoperiod history ; see[47,48]). CP might also differ slightly between sexes since rams typically display an advance of their breeding season compared to ewes (~1 month), which ensures that all ewes get pregnant - hence deliver lambs - within a very narrow time span at the end of winter[49]. However, our protocols with different breeds (Soay vs Ile-de-France), intact ram lambs vs OVX+E2 adult ewes, different duration of photoperiodic treatments and a temporal resolution of 1h in the current study are not adapted to rigorously test for small differences in CP value between sexes.

348

349 Perhaps, the most striking difference with the Soay lamb study[17] is the disconnection between

350 *Tshb* and *Dio2* : *Tshb* expression steadily increases with stepwise increases in photoperiod

351 while *Dio2* does not. This is very surprising considering our prior finding that *Dio2* (amongst

352 other genes, see below) is acutely induced – 4-fold increase as assessed by RT-qPCR – by

353 exposure to a LP of 15.5h[3]. However, here again, the length of the photoperiodic treatment, 3.5

354 weeks, was much shorter than the 8 weeks treatment of the current study. In addition, the ISH

355 methodology used here is less sensitive than RT-qPCR, which might be a problem considering

356 the modest (1.5-2-fold) seasonal amplitude of the *Dio2* rhythm in tanycytes of Ile-de-France

357 ewes[3], compared to Soay rams[50]. Nevertheless, considering the decrease in *Kiss1* expression

358 and the concurrent decreases in LH/FSH, we infer that photoperiodic treatments were efficient.

359 Since the DIO2-triggered hypothalamic local increase in T3 at the beginning of spring is crucial

360 to the progression of the ovine seasonal cycle towards reproductive arrest[12,44,51-53], we conclude

361 that *Dio2* induction occurred, but was not sustained throughout the 8 weeks. We therefore

362 hypothesize that the disconnection between *Tshb* and *Dio2* is due to a transient impact of TSH

363 upon *Dio2* and T3 production, which is nevertheless enough to entrain the circannual clock and

364 trigger the response of the hypothalamo-pituitary axis. We can exclude that this disconnection

365 reflects a general uncoupling between PT and tanycytes since two other tanycyte-specific

366 markers, *Tmem252*[3] and *Dct*[21], showed increased expression levels with increasing

367 photoperiods, in a pattern resembling that of PT-expressed genes. Interestingly, using

368 thyroidectomized OVX+E2 ewes we demonstrated that *Tmem252* induction by LP is genuinely

369 dependent upon T3[3]. The profile of *Tmem252* observed in this study would therefore indicate

370 that T3 levels remain high under LP in spite of constant *Dio2* expression. Overall, these

371 observations point to a *Dio2*-specific mechanism of negative feed-back, which rapidly follows

372 induction by LP.

374 Indeed, under natural conditions, Siberian hamster display sharp transient peaks of *Dio2* and

375 *Dio3* expression during the year[54]. There is also strong evidence in sheep that a transient

376 exposure to LP – 45-60 long days – is sufficient to synchronize the circannual cycle[12,55,56]. This

377 is in line with the idea that T3, even though it exerts a key organizing role, is required only

378 during a limited window of time in sheep and hamsters[12,45,57,58]. Then, after being acutely

379 induced, *Dio2* expression would return to lower levels, in spite of increased PT-derived TSH.

380 The mechanisms for such an uncoupling are unknown but a few hypotheses can be made. The

381 induction of *Dio2* by TSH depends on the cAMP pathway ; *Dio2* is a CRE-dependent gene[53,58].

382 Considering that tanycytes are a hub for a host of signals[4,59,60], it seems plausible that other

383 GPCR-dependent signaling pathways, coupled to either Gαs or Gαi, also impinge on *Dio2*

384 expression. Furthermore, *Dio2* expression is enhanced in both hypothyroid rats[61] and ewes[3],

385 which indicates that T3 normally exerts a brake on *Dio2* expression in tanycytes. Such an

386 autocrine short feedback loop is predicted to keep *Dio2* levels in check during LP exposure,

387 and might be responsible for the apparent disconnection. Also, T3 strongly impacts local

388 metabolism[62] and *Dio2* is itself sensitive to metabolic cues[63-66], which provides yet another

389 potential short feedback loop. Other unrelated cues, such as LPS injection[67,68] and hypoxia[69]

390 also impact either *Dio2* expression or DIO2 stability. In conclusion, multiple signals – not

391 limited to PT-derived TSH – converge onto tanycytes and overall *Dio2* expression reflects

392 integration of all these inputs. We conclude that our experimental design, which examined

393 expression of genes after ~2 months of photoperiodic exposure, reveals the transient nature of

394 the LP impact upon the circannual clock.

395

396 Our prior work in Soay lambs[17], Ile-de-France ewes[3] and in Siberian hamsters[18] has provided

397 hints that a very modest increase in PT-derived TSH at the end of winter might be enough to

398   yield a large increase in *Dio2* expression. We ascribed this logarithmic response to sensitization

399   of the TSHR pathway within tanycytes, a proposal which remains to be addressed rigorously.

400   Here, we report that very modest changes in *Tshb* expression trigger comparatively large

401   responses in LH/FSH (i.e. logarithmic response rather than linear), which are associated with a

402   rather linear and progressive decrease of *Kiss1* expression (**Figure 4**). Our data in OVX+E2

403   ewes are consistent with those in intact Soay ram lambs and point to a CP value comprised

404   between 12-13h. More importantly, our data provide strong evidence that non-linear responses

405   and discontinuity occur along the TSH/DIO2/T3/KISS1 axis. These features may be part of of

406   the circannual timing device and will have to be considered in the interpretation of future

407   studies.

408

Acknowledgements

414

415

Figure legends

417   **Figure 1**: Schematic of the experimental design. (A) Overview of the photoperiodic treatments.

418   OVX+E2 ewes were initially kept in open barns, exposed to the natural decrease in daylength,

419   before being brought indoors and exposed for ~2 months to either a simulated natural photoperiod

420   (PP nat) or to photoperiod of increasing duration: 11:13, 12:12 , 13:11 and 14:10. (B) Ewes were

421   exposed to constant photoperiods by delaying the time of lights off. All ewes were killed in the

422   early day (ZT2-4).

423

Figure 2: Hormonal profiling of the response to the five photoperiodic treatments. (A) Mean LH levels in blood plasma of ewes sampled bi-weekly throughout the experiment. (B) Mean LH for the two weeks (i.e. four time points) before the photoperiodic transfer. (C) Mean LH for the last two weeks of the experiment. Different letters indicate statistically different groups (P<0.05). (D-F) FSH profiles – legends identical to those for LH. (G-I) PRL profiles – legends identical to those for LH. (n=6 for groups 11:13 and 14:10 , n=5 for groups PPnat, 12:12 and 13:11; see M&M).

Figure 3: Impact of photoperiodic treatments on select photoperiod-responsive genes in the MBH as assessed by ISH. (A) Bar charts showing normalized expression for PT markers (*Tshb* through to *Chga*), tanycytic markers (*Dio2*, *Tmem252* and *Dct*) and the hypothalamic marker *Kiss1*. Different letters indicate statistically different groups (P<0.05). (B) Images representative of high ISH signal for each gene as revealed by autoradiography. (n=6 for all groups, except PPnat n=5; see M&M).

Figure 4: Discontinuity in the molecular neuroendocrine pathway of seasonality revealed by linear regression analysis. Individual means for LH and FSH correspond to the last two weeks of treatment (i.e. same data as in Figure2) (A) Linear regression analysis reveals statistically significant correlation between *Tshb* / *Kiss1* and LH, but not *Dio2* and LH. (B) Linear regression analysis reveals statistically significant correlation between *Tshb* / *Kiss1* and FSH but not *Dio2* and FSH. P values for linear regressions are provided on top of each panel. Note that the relationships between *Tshb* and LH/FSH could be better fitted by a non-linear hyperbolic function, which is shown, instead of the linear regression line. The dashed grey line arbitrarily set a x=1 is meant to help visualization of the extent of the distribution. (n=6 for groups 11:13 and 14:10 , n=5 for groups PPnat, 12:12 and 13:11; see M&M)

18

References

1. Dardente H, Lomet D, Robert V, Decourt C, Beltramo M, Pellicer-Rubio MT. Seasonal breeding in mammals: From basic science to applications and back. *Theriogenology*. 2016;1:324-332.

2. Wood SH, Christian HC, Miedzinska K, Saer BR, Johnson M, Paton B, Yu L, McNeilly J, Davis JR, McNeilly AS, Burt DW, Loudon AS. Binary Switching of Calendar Cells in the Pituitary Defines the Phase of the Circannual Cycle in Mammals. *Curr Biol*. 2015;20:2651-2662.

3. Lomet D, Cognie J, Chesneau D, Dubois E, Hazlerigg D, Dardente H. The impact of thyroid hormone in seasonal breeding has a restricted transcriptional signature. *Cell Mol Life Sci*. 2018;5:905-919.

4. Dardente H, Wood S, Ebling F, Saenz de Miera C. An integrative view of mammalian seasonal neuroendocrinology. *J Neuroendocrinol*. 2019;5:e12729.

5. Wood S, Loudon A. Clocks for all seasons: unwinding the roles and mechanisms of circadian and interval timers in the hypothalamus and pituitary. *J Endocrinol*. 2014;2:R39-59.

6. Shinomiya A, Shimmura T, Nishiwaki-Ohkawa T, Yoshimura T. Regulation of seasonal reproduction by hypothalamic activation of thyroid hormone. *Front Endocrinol (Lausanne)*. 2014;5:12.

7. Dardente H, Hazlerigg DG, Ebling FJ. Thyroid hormone and seasonal rhythmicity. *Front Endocrinol (Lausanne)*. 2014;5:19.

8. Helfer G, Barrett P, Morgan PJ. A unifying hypothesis for control of body weight and reproduction in seasonally breeding mammals. *J Neuroendocrinol*. 2019;3:e12680.

9. Smith JT. The role of kisspeptin and gonadotropin inhibitory hormone in the seasonal regulation of reproduction in sheep. *Domest Anim Endocrinol*. 2012;2:75-84.

10. Beltramo M, Dardente H, Cayla X, Caraty A. Cellular mechanisms and integrative timing of neuroendocrine control of GnRH secretion by kisspeptin. *Mol Cell Endocrinol*. 2014;1:387-399.

11. Simonneaux V. A Kiss to drive rhythms in reproduction. *Eur J Neurosci*. 2018;doi: 10.1111/ejn.14287.

12. Dardente H. Melatonin-dependent timing of seasonal reproduction by the pars tuberalis: pivotal roles for long daylengths and thyroid hormones. *J Neuroendocrinol*. 2012;2:249-266.

13. Gaston S, Menaker M. Photoperiodic control of hamster testis. *Science*. 1967;3803:925-928.

14. Follett BK, Maung SL. Rate of testicular maturation, in relation to gonadotrophin and testosterone levels, in quail exposed to various artificial photoperiods and to natural daylengths. *J Endocrinol*. 1978;2:267-280.

15. Elliott JA. Circadian rhythms and photoperiodic time measurement in mammals. *Fed Proc*. 1976;12:2339-2346.

16. Hoffmann K. The critical photoperiod in the Djungarian hamster Phodopus sungorus. *Springer-Verlag Berlin, Heidelberg, Vertebrate Circadian Systems, Eds J.Aschoff, S.Daan and G.Groos*. 1982;297-304.

17. Hazlerigg D, Lomet D, Lincoln G, Dardente H. Neuroendocrine correlates of the critical day length response in the Soay sheep. *J Neuroendocrinol*. 2018;9:e12631.

18. Saenz de Miera C, Bothorel B, Jaeger C, Simonneaux V, Hazlerigg D. Maternal photoperiod programs hypothalamic thyroid status via the fetal pituitary gland. *Proc Natl Acad Sci USA*. 2017;31:8408-8413.

484   19. Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ, Robinson JE. Neuroendocrine basis of seasonal
485   reproduction. *Recent Prog Horm Res*. 1984;40:185-232.

486   20. Lomet D, Piegu B, Wood SH, Dardente H. Anti-angiogenic VEGFAxxxb transcripts are not expressed in the
487   medio-basal hypothalamus of the seasonal sheep. *PLoS One*. 2018;5:e0197123.

488   21. Dardente H, Lomet D. Photoperiod and thyroid hormone regulate expression of l-dopachrome tautomerase
489   (Dct), a melanocyte stem-cell marker, in tanycytes of the ovine hypothalamus. *J Neuroendocrinol*. 2018;9:e12640.

490   22. Lincoln GA, Clarke IJ, Hut RA, Hazlerigg DG. Characterizing a mammalian circannual pacemaker. *Science*.
491   2006;5807:1941-1944.

492   23. Lincoln G. A brief history of circannual time. *J Neuroendocrinol*. 2019;3:e12694.

493   24. Dardente H, Cermakian N. Molecular circadian rhythms in central and peripheral clocks in mammals.
494   *Chronobiol Int*. 2007;2:195-213.

495   25. Takahashi JS. Transcriptional architecture of the mammalian circadian clock. *Nat Rev Genet*. 2017;3:164-179.

496   26. Dardente H, Wyse CA, Birnie MJ, Dupre SM, Loudon AS, Lincoln GA, Hazlerigg DG. A molecular switch
497   for photoperiod responsiveness in mammals. *Curr Biol*. 2010;24:2193-2198.

498   27. Masumoto KH, Ukai-Tadenuma M, Kasukawa T, Nagano M, Uno KD, Tsujino K, Horikawa K, Shigeyoshi
499   Y, Ueda HR. Acute induction of Eya3 by late-night light stimulation triggers TSHbeta expression in
500   photoperiodism. *Curr Biol*. 2010;24:2199-2206.

501   28. Dupre SM, Miedzinska K, Duval CV, Yu L, Goodman RL, Lincoln GA, Davis JR, McNeilly AS, Burt DD,
502   Loudon AS. Identification of Eya3 and TAC1 as long-day signals in the sheep pituitary. *Curr Biol*. 2010;9:829-
503   835.

504   29. Robinson JE, Karsch FJ. Refractoriness to inductive day lengths terminates the breeding season of the Suffolk
505   ewe. *Biol Reprod*. 1984;4:656-663.

506   30. Robinson JE, Karsch FJ. Photoperiodic history and a changing melatonin pattern can determine the
507   neuroendocrine response of the ewe to daylength. *J Reprod Fertil*. 1987;1:159-165.

508   31. Thimonier J, Mauleon P. Variations saisonnières du comportement d'oestrus et des activités ovarienne et
509   hypophysaire chez les ovins. *Ann Biol anim Bioch Biophys*. 1969;9:233-250.

510   32. Chanvallon A, Sagot L, Pottier E, Debus N, Francois D, Fassier T, Scaramuzzi RJ, Fabre-Nys C. New insights
511   into the influence of breed and time of the year on the response of ewes to the 'ram effect'. *Animal*. 2011;10:1594-
512   1604.

513   33. Curlewis JD. Seasonal prolactin secretion and its role in seasonal reproduction: a review. *Reprod Fertil Dev*.
514   1992;1:1-23.

515   34. Lincoln GA, Clarke IJ. Photoperiodically-induced cycles in the secretion of prolactin in hypothalamo-pituitary
516   disconnected rams: evidence for translation of the melatonin signal in the pituitary gland. *J Neuroendocrinol*.
517   1994;3:251-260.

518   35. Grattan DR. 60 years of neuroendocrinology: The hypothalamo-prolactin axis. *J Endocrinol*. 2015;2:T101-22.

519   36. Ravault JP. Prolactin in the ram: seasonal variations in the concentration of blood plasma from birth until three
520   years old. *Acta Endocrinol (Copenh)*. 1976;4:720-725.

521 37. Lincoln GA. Correlation with changes in horns and pelage, but not reproduction, of seasonal cycles in the
522 secretion of prolactin in rams of wild, feral and domesticated breeds of sheep. *J Reprod Fertil*. 1990;1:285-296.

523 38. Dardente H. Does a melatonin-dependent circadian oscillator in the pars tuberalis drive prolactin seasonal
524 rhythmicity? *J Neuroendocrinol*. 2007;8:657-666.

525 39. Lincoln GA, McNeilly AS, Cameron CL. The effects of a sudden decrease or increase in daylength on prolactin
526 secretion in the ram. *J Reprod Fertil*. 1978;2:305-311.

527 40. Hazlerigg DG, Andersson H, Johnston JD, Lincoln G. Molecular characterization of the long-day response in
528 the Soay sheep, a seasonal mammal. *Curr Biol*. 2004;4:334-339.

529 41. Sweeney T, Kelly G, O'Callaghan D. Seasonal variation in long-day stimulation of prolactin secretion in ewes.
530 *Biol Reprod*. 1999;1:128-133.

531 42. Donham RS, Palacio E, Stetson MH. Dissociation of the reproductive and prolactin photoperiodic responses
532 in male golden hamsters. *Biol Reprod*. 1994;3:366-372.

533 43. Morgan PJ, Hazlerigg DG. Photoperiodic signalling through the melatonin receptor turns full circle. *J
534 Neuroendocrinol*. 2008;6:820-826.

535 44. Parkinson TJ, Follett BK. Effect of thyroidectomy upon seasonality in rams. *J Reprod Fertil*. 1994;1:51-58.

536 45. Billings HJ, Viguie C, Karsch FJ, Goodman RL, Connors JM, Anderson GM. Temporal requirements of
537 thyroid hormones for seasonal changes in LH secretion. *Endocrinology*. 2002;7:2618-2625.

538 46. Duncan MJ, Goldman BD, Di Pinto MN, Stetson MH. Testicular function and pelage color have different
539 critical daylengths in the Djungarian hamster, Phodopus sungorus sungorus. *Endocrinology*. 1985;1:424-430.

540 47. Saenz de Miera C. Maternal photoperiodic programming enlightens the internal regulation of thyroid-hormone
541 deiodinases in tanycytes. *J Neuroendocrinol*. 2019;1:e12679.

542 48. Goldman BD. Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of
543 photoperiodic time measurement. *J Biol Rhythms*. 2001;4:283-301.

544 49. Lincoln GA, Short RV. Seasonal breeding: nature's contraceptive. *Recent Prog Horm Res*. 1980;36:1-52.

545 50. Saenz de Miera C, Hanon EA, Dardente H, Birnie M, Simonneaux V, Lincoln GA, Hazlerigg DG. Circannual
546 variation in thyroid hormone deiodinases in a short-day breeder. *J Neuroendocrinol*. 2013;4:412-421.

547 51. Nicholls TJ, Follett BK, Goldsmith AR, Pearson H. Possible homologies between photorefractoriness in sheep
548 and birds: the effect of thyroidectomy on the length of the ewe's breeding season. *Reprod Nutr Dev*. 1988;2B:375-
549 385.

550 52. Moenter SM, Woodfill CJ, Karsch FJ. Role of the thyroid gland in seasonal reproduction: thyroidectomy blocks
551 seasonal suppression of reproductive neuroendocrine activity in ewes. *Endocrinology*. 1991;3:1337-1344.

552 53. Hanon EA, Lincoln GA, Fustin JM, Dardente H, Masson-Pevet M, Morgan PJ, Hazlerigg DG. Ancestral TSH
553 mechanism signals summer in a photoperiodic mammal. *Curr Biol*. 2008;15:1147-1152.

554 54. Petri I, Diedrich V, Wilson D, Fernandez-Calleja J, Herwig A, Steinlechner S, Barrett P. Orchestration of gene
555 expression across the seasons: Hypothalamic gene expression in natural photoperiod throughout the year in the
556 Siberian hamster. *Sci Rep*. 2016;6:29689.

557 55. Woodfill CJ, Wayne NL, Moenter SM, Karsch FJ. Photoperiodic synchronization of a circannual reproductive
558 rhythm in sheep: identification of season-specific time cues. *Biol Reprod*. 1994;4:965-976.

559 56. Sweeney T, Donovan A, Karsch FJ, Roche JF, O'Callaghan D. Influence of previous photoperiodic exposure
560 on the reproductive response to a specific photoperiod signal in ewes. *Biol Reprod*. 1997;4:916-920.

561 57. Barrett P, Ebling FJ, Schuhler S, Wilson D, Ross AW, Warner A, Jethwa P, Boelen A, Visser TJ, Ozanne DM,
562 Archer ZA, Mercer JG, Morgan PJ. Hypothalamic thyroid hormone catabolism acts as a gatekeeper for the seasonal
563 control of body weight and reproduction. *Endocrinology*. 2007;8:3608-3617.

564 58. Nakao N, Ono H, Yamamura T, Anraku T, Takagi T, Higashi K, Yasuo S, Katou Y, Kageyama S, Uno Y,
565 Kasukawa T, Iigo M, Sharp PJ, Iwasawa A, Suzuki Y, Sugano S, Niimi T, Mizutani M, Namikawa T, Ebihara S,
566 Ueda HR, Yoshimura T. Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature*.
567 2008;7185:317-322.

568 59. Prevot V, Dehouck B, Sharif A, Ciofi P, Giacobini P, Clasadonte J. The Versatile Tanycyte: A Hypothalamic
569 Integrator of Reproduction and Energy Metabolism. *Endocr Rev*. 2018;3:333-368.

570 60. Rodriguez E, Guerra M, Peruzzo B, Blazquez JL. Tanycytes: A rich morphological history to underpin future
571 molecular and functional investigations. *J Neuroendocrinol*. 2019;3:e12690.

572 61. Tu HM, Kim SW, Salvatore D, Bartha T, Legradi G, Larsen PR, Lechan RM. Regional distribution of type 2
573 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid
574 hormone. *Endocrinology*. 1997;8:3359-3368.

575 62. Kong WM, Martin NM, Smith KL, Gardiner JV, Connoley IP, Stephens DA, Dhillo WS, Ghatei MA, Small
576 CJ, Bloom SR. Triiodothyronine stimulates food intake via the hypothalamic ventromedial nucleus independent
577 of changes in energy expenditure. *Endocrinology*. 2004;11:5252-5258.

578 63. Diano S, Naftolin F, Goglia F, Horvath TL. Fasting-induced increase in type II iodothyronine deiodinase
579 activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. *Endocrinology*.
580 1998;6:2879-2884.

581 64. Coppola A, Hughes J, Esposito E, Schiavo L, Meli R, Diano S. Suppression of hypothalamic deiodinase type
582 II activity blunts TRH mRNA decline during fasting. *FEBS Lett*. 2005;21:4654-4658.

583 65. Coppola A, Liu ZW, Andrews ZB, Paradis E, Roy MC, Friedman JM, Ricquier D, Richard D, Horvath TL,
584 Gao XB, Diano S. A central thermogenic-like mechanism in feeding regulation: an interplay between arcuate
585 nucleus T3 and UCP2. *Cell Metab*. 2007;1:21-33.

586 66. Fekete C, Lechan RM. Central regulation of hypothalamic-pituitary-thyroid axis under physiological and
587 pathophysiological conditions. *Endocr Rev*. 2014;2:159-194.

588 67. Fekete C, Gereben B, Doleschall M, Harney JW, Dora JM, Bianco AC, Sarkar S, Liposits Z, Rand W, Emerson
589 C, Kacskovics I, Larsen PR, Lechan RM. Lipopolysaccharide induces type 2 iodothyronine deiodinase in the
590 mediobasal hypothalamus: implications for the nonthyroidal illness syndrome. *Endocrinology*. 2004;4:1649-1655.

591 68. Lamirand A, Ramauge M, Pierre M, Courtin F. Bacterial lipopolysaccharide induces type 2 deiodinase in
592 cultured rat astrocytes. *J Endocrinol*. 2011;2:183-192.

593 69. Lamirand A, Mercier G, Ramauge M, Pierre M, Courtin F. Hypoxia stabilizes type 2 deiodinase activity in rat
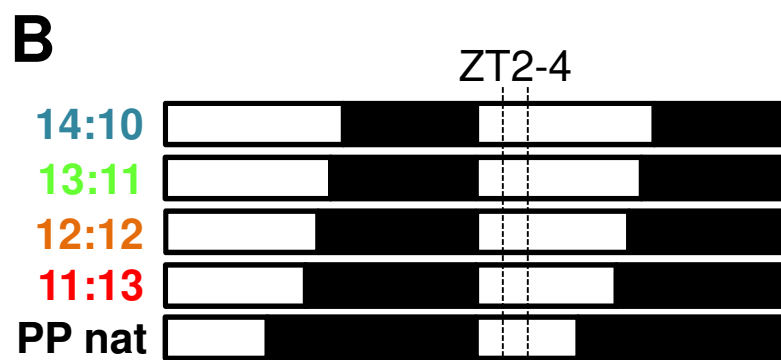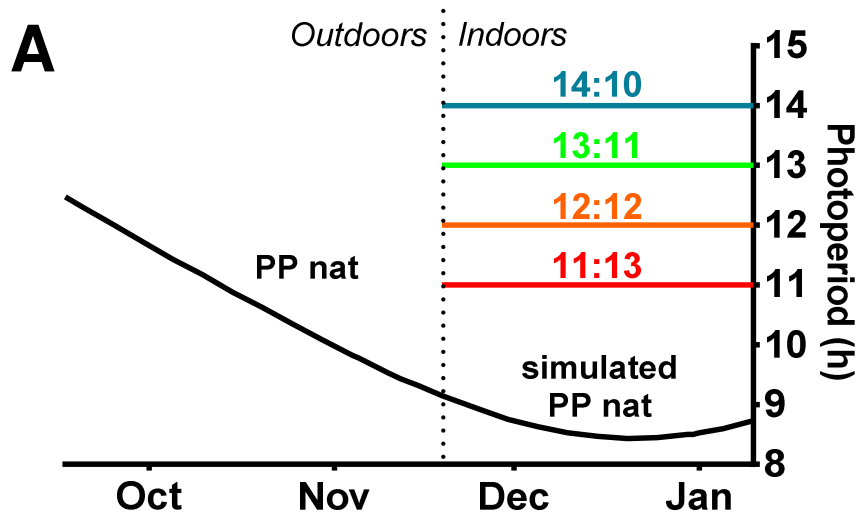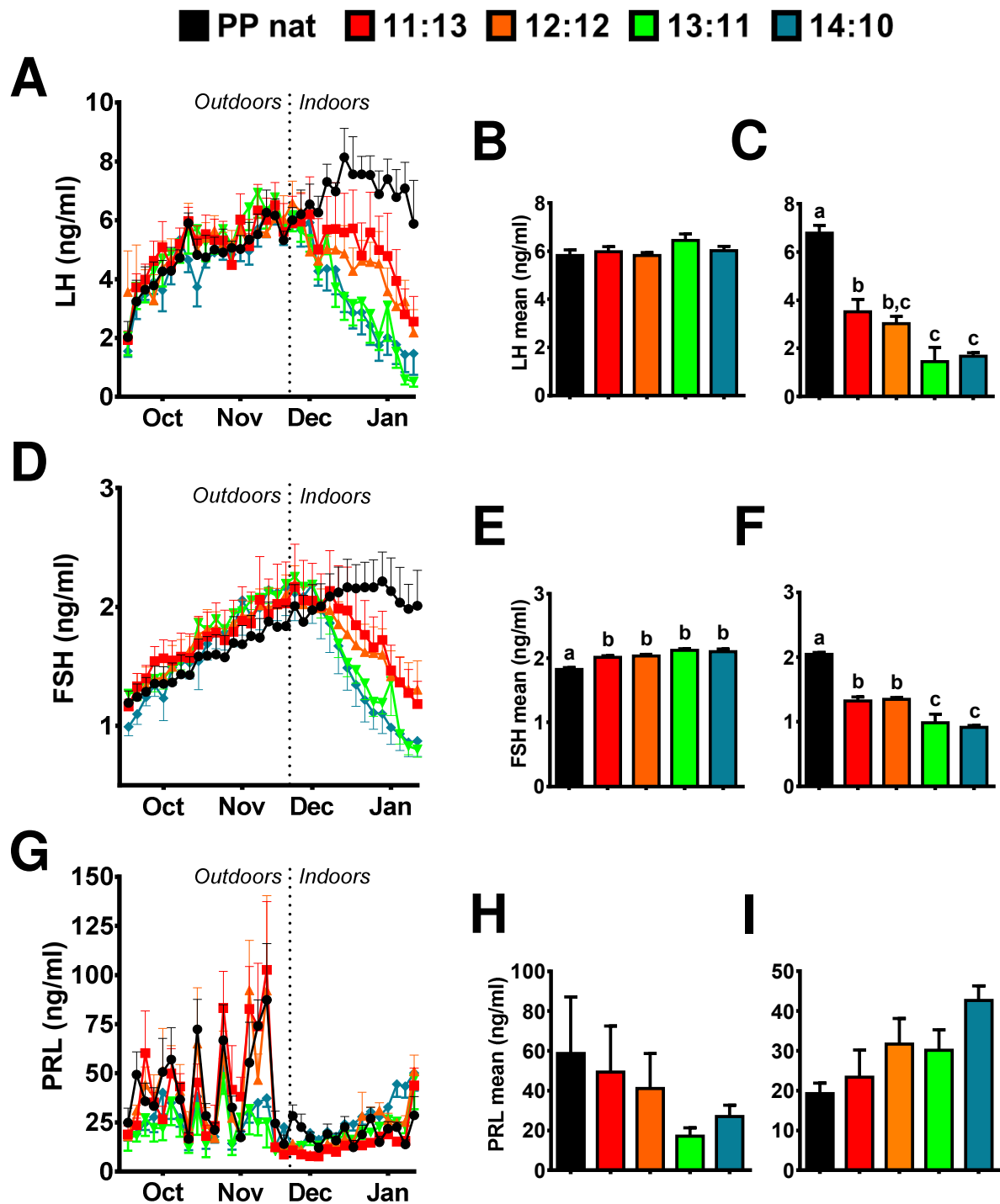594 astrocytes. *Endocrinology*. 2007;10:4745-4753.
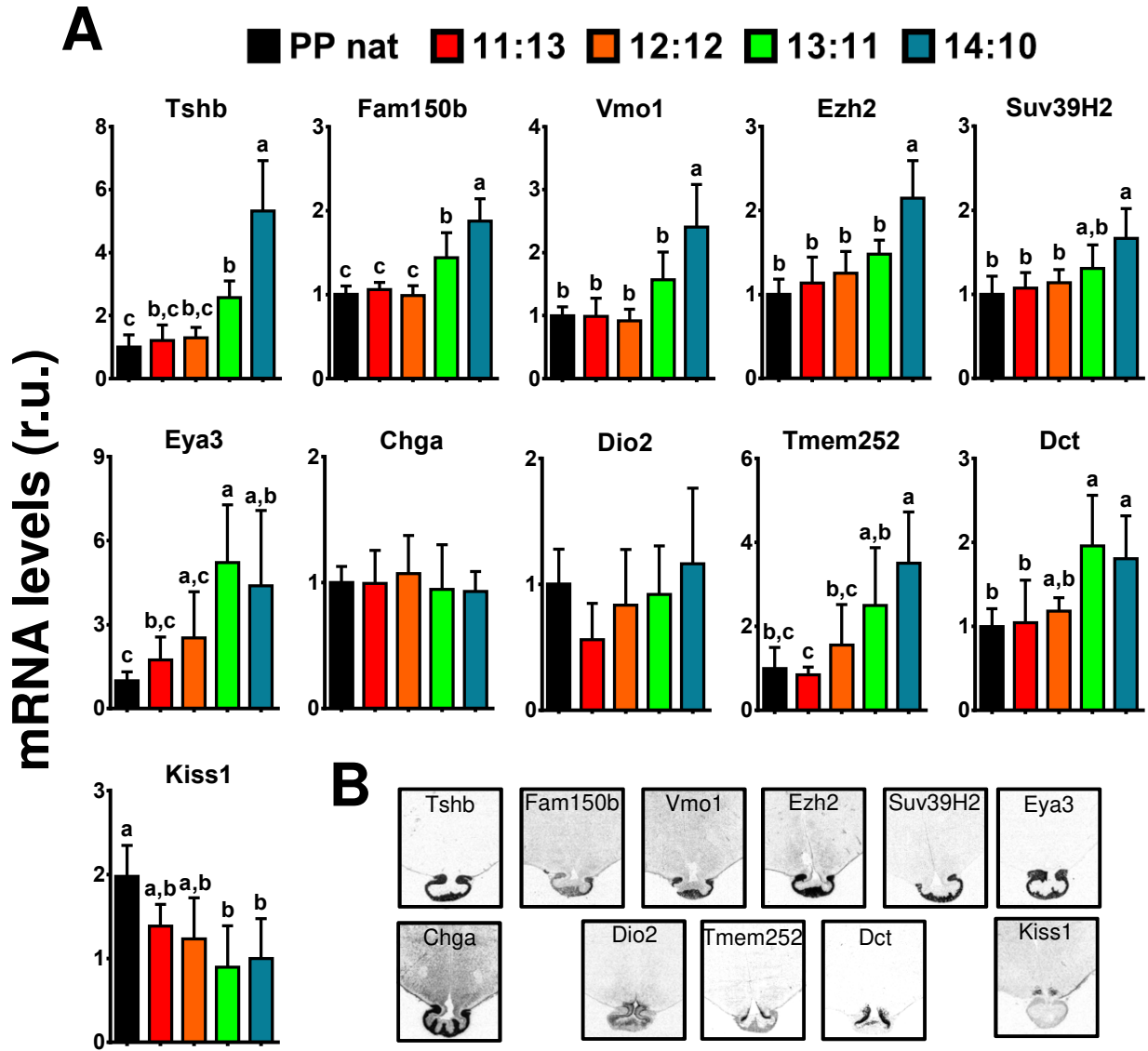
Figure 1

Figure 2

Figure 3

Figure 4