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,

which would be consistent with the brief temporal requirements for long days and T3 in the synchronization of the ovine circannual program.

Material & Methods

Ethics statement

All experimental procedures were performed in accordance with international (directive 2010/63/UE) and national legislation (décret n° 2013–118) governing the ethical use of animals in research (authorization n° E37–175-2 and n°A38 801). All procedures used in this work were evaluated by a local ethics committee (Comité d'Ethique en Expérimentation Animale Val de Loire; n°2012-10-5).

Experimental animals & procedures

Experiments were conducted in 30 adult Ile-de-France ewes (3–5 years old; weight 60–80 kg) kept under normal husbandry conditions at the research station of the Institut National de la Recherche Agronomique (Nouzilly, Unité Expérimentale PAO n°1297 (EU0028)). The natural photoperiod at the latitude of Nouzilly, France (47°N) ranges from ~16:8 (16h of light, 8h of night) at the summer solstice to ~8:16 at the winter solstice. All ewes were ovariectomized (OVX) and estradiol-implanted (E2; 1cm silastic implant) between Sept 2nd and Sept 6th (2012).

All surgeries were performed after sodium thiopental anesthesia (Nesdonal®, 1g/80kg), under constant isoflurane administration (Vetflurane®) and all efforts were made to minimize suffering. Following surgery, animals received an injection of antibiotics (oxytetracycline, Terramycine LA®, 1ml/10kg) and an injection of a non-steroidal anti-inflammatory drug (Finadyne®, flumixin megumine, 2ml/50kgs). Animals were followed daily throughout the experiment.

Following surgery, all ewes were initially kept outdoors (open barns) before being brought indoors in a light-tight building on Nov 19th (**Figure 1A**). The natural photoperiod at this time of year in Nouzilly is ~9h15min. Six ewes were randomly assigned to one of each photoperiodic treatment : Simulated Natural Photoperiod (PP nat), 11:13 (i.e. 11h of light per day), 12:12, 13:11 and 14:10 (**Figure 1A**). For each treatment, ewes were housed by groups of 3 in two separate light-tight pens. Ewes of the PP nat group were submitted to a photoperiodic treatment that mimics outdoor conditions through weekly stepwise modifications of daily light exposure. All other ewes were exposed to longer photoperiods by delaying the time of lights off (**Figure 1B**). Ewes were submitted to these photoperiodic treatments for ~2 months (**Figure 1A**).

Blood samples were collected twice weekly by jugular venipuncture in heparinized tubes from Sept 17th (2012) through to Jan 10th (2013). After centrifugation, serum was collected and frozen until assayed for hormones. At the end of the photoperiodic treatments, all animals were euthanized by decapitation under deep barbiturate anesthesia (Nesdonal®, 5mL). To minimize issues linked to potential time-of-day effects on gene expression all ewes were killed in the early morning between ZT2 and ZT4 (with ZT0 being the time of lights on; **Figure 1B**) on January 13th (n=15) and January 14th (n=14). Hypothalamic blocks were sampled and immediately frozen in isopentane kept on dry ice, before being stored at -80°C until sectioning, as described previously[3]. One ewe of the 12:12 group died in December and, at the end of the experiment, no E2 implant could be recovered from one ewe of the PP nat group and another ewe from the 13:11 group. Data from these 3 ewes were removed from the analysis of hormonal profiles.

Hormonal profiles

Plasma levels of LH, FSH and PRL were assayed by RIA. All samples from one experiment were included in a single assay and every sample was measured in duplicate. LH: the assay standard

was 1051-CY-LH (equivalent to 0.31 NIH-LH-S1). Intra- and inter- assay coefficients of variation averaged 9% and 15%, respectively with an assay sensitivity of 0.1 ng/mL. FSH: levels were measured using reagents supplied by Tucker Endocrine Research Institute (Atlanta, GA, USA). Intra- and inter- assay coefficients of variation averaged 8% and 9%, respectively with an assay sensitivity of 0.1 ng/mL relative to the standard (Tuenere oFSHstd. 1 equiv to 1.0 NIH-FSH-S1). The cross reactivity with ovine LH was 0.03%. PRL: Intra- and inter- assay coefficients of variation averaged 7% and 12%, respectively with an assay sensitivity of 2.5 ng/ml.

In situ hybridization (ISH)

ISH was perfomed as described previously using validated ovine riboprobes[3,20,21]. A complete list of the riboprobes used in this study is provided in **Supplementary Table 1**.

Hypothalamic blocks for *in situ* hybridisation were cut into 20μm sections using a cryostat (CryoStar NX70, ThermoScientific) and thaw-mounted onto SuperFrost Plus slides (ThermoScientific). All radioactive cRNA riboprobes were prepared by plasmid linearisation and *in vitro* transcription (Riboprobe System, Promega) including ^{35}S-UTP (Perkin-Elmer). The probe was purified with Illustra Probe Quant G50 micro-columns (Fisher) and counted with a liquid scintillation counter (Tri-Carb 2900TR, Packard). Slides were post-fixed at 4°C for 20 min in 4% PFA, 0.1 M PB, rinsed with 0.1 M PB (2 X 5min), acetylated with 3.75% v/v of acetic anhydride in 0.1 TEA, 0.05 N NaOH (10min) and finally rinsed with 0.1 M PB (2 X 5min). Slides were then dehydrated through graded ethanol solutions (50%, 70%, 95% and 100%; 3min each) and dried under vacuum for 60 min. Sections were hybridized overnight at 58ºC with 10^6 cpm of probe per slide in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1 X Denhardt's solution, 300 mM NaCl, 10 mM Tris, 10 mM DTT, 1 mM EDTA, 500 μg/ml tRNA). Sections were then rinsed in 4 X SSC (3 X 5 min) and subjected to RNase-A digestion (20 μg/ml) in a buffer containing 500 mM NaCl, 1 mM Tris, 1 mM EDTA for 30 min at 37°C. Stringency washes

in SSC (with 1mM DTT) were performed to remove non-specific probe hybridisation: 2 X SSC (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 min). Slides were then dehydrated through graded ethanol solutions (50%, 70%, 95% and 100%; 3min each), dried under vacuum for 60 min and exposed for 1 to 3 weeks (depending on the target mRNA) to an autoradiographic film (BioMax MR, Kodak). Films were scanned on a transmittance image scanner (Amersham, UK) along with a calibrated optical density (OD) transmission step wedge (Stouffer, USA). Calibrated Integrated OD measurements of gene expression were performed using ImageJ software.

Data analysis

Data were analysed using GraphPad Prism 6.0 and are reported as mean ± sem. For hormonal profiles, RIA data were analyzed by Repeated Measures (RM) 2-way ANOVA. One-way ANOVA was also used to perform analyses limited to the two weeks before the photoperiodic transfer and the last two weeks of the experiment. ISH data were analyzed by 1-way ANOVA using treatment as a variable. The post-hoc Tukey test was used for multiple comparisons. $p<0.05$ was considered significant. Using individual values, linear regression analysis was performed to evaluate correlations between *Tshb*, *Dio2* or *Kiss1* mRNA levels and mean LH/FSH levels over the last two weeks of the experiment.

Results

Hormonal profiles

RIA was used to assess plasma levels for LH (**Figure 2A-C**), FSH (**Figure 2D-F**) and PRL (**Figure 2G-I**). For LH, RM two-way ANOVA revealed a strong time*group interaction ($P<0.0001$), which reflected the impact of the photoperiodic treatments, as no difference between groups were observed prior to these. Indeed, further analysis revealed no differences between groups over the

2-wks period before the photoperiodic transfer (**Figure 2B** ; one-way ANOVA ; P=0.25), while levels differed significantly over the last 2-wks period (**Figure 2C** ; one-way ANOVA ; P<0.0001). For FSH, RM 2-way ANOVA revealed a strong time*group interaction (P<0.0001), which mostly reflected the impact of the photoperiodic treatments. Indeed, further analysis revealed differences between groups over the 2-wks period before the photoperiodic transfer (**Figure 2E**) and the last 2-wks period of the experiment (**Figure 2F** ; one-way ANOVA ; P<0.0001 for both comparisons). The difference before the photoperiodic transfer is accounted for by slightly lower levels in the PP nat group compared to the 4 other groups. Linear regression analysis showed that levels of LH and FSH during last 2-wks period of the experiment were correlated (R^2=0.4679 ; P<0.0001).

For PRL, RM two-way ANOVA revealed a a strong time*group interaction (P=0.0004), which may reflect the inter-individual and inter-group variabilities before the photoperiodic treatments and slightly divergent trajectories after transfer. However, PRL levels did not differ bewteen groups before the photoperiodic transfer (**Figure 2H** ; one-way ANOVA ; P=0.53), while a trend towards higher levels with increasing photoperiods was noticed for the last 2-wks period of the experiment (**Figure 2I** ; one-way ANOVA ; P=0.053).

Gene expression

We used semi-quantitative ISH to assess the impact of the photoperiodic transfer on the mRNA expression levels of multiple seasonal markers within the MBH (**Figure 3**). Data were analysed by one-way ANOVA (**Figure 3A**). Photoperiod affected the expression of all PT markers except *Chga* [$F_{4,24}$ = 0.32 ; P=0.86] : *Tshb* [$F_{4,24}$ = 25.94 ; P<0.0001], *Fam150b* [$F_{4,24}$ = 22.04 ; P<0.0001], *Vmo1* [$F_{4,24}$ = 14.37 ; P<0.0001], *Ezh2* [$F_{4,24}$ = 14.37 ; P<0.0001], *Suv39H2* [$F_{4,24}$ = 6.61 ; P=0.001] and *Eya3* [$F_{4,24}$ = 6.29 ; P<0.005]. Post-hoc analysis revealed that expression

levels were not significantly different between the PP nat, 11:13 and 12:12 groups for any of these PT-expressed genes. Compared to PP nat, expression of *Tshb*, *Fam150b* and *Eya3* were significantly increased under 13:11. Expression of all markers was significantly increased by the 14:10 photoperiod compared to PP nat. Within markers of tanycytes, photoperiod significantly affected mRNA expression of *Tmem252* [$F_{4,23} = 7.55$; P=0.0005] and *Dct* [$F_{4,24} = 6.11$; P<0.005] but not *Dio2* [$F_{4,24} = 1.74$; P=0.17]. Similar to the PT markers, no significant differences were found between PP nat, 11:13 and 12:12 groups, while longer photoperiods of 13:11 and 14:10 increased expression of *Tmem252* and *Dct*, compared to PP nat. No detectable expression of *Dio3* was observed (not shown). Finally, the photoperiodic transfer affected expression of the hypothalamic marker *Kiss1* [$F_{4,24} = 6.06$; P<0.005], with expression significantly reduced under 13:11 and 14:10 compared to PP nat. Representative images of autoradiograms for all these markers at their peak of expression are provided in **Figure 3B**.

Linear regression analyses

Data for LH and FSH (using individual means for the last two weeks of treatment; data from Figure 2C and Figure 2F) are shown in **Figure 4A and 4B**, respectively. Levels of *Tshb* and *Kiss1* mRNA were negatively and positively correlated with LH (R^2=0.20, P=0.018 and R^2=0.66, P<0.0001) and FSH levels (R^2=0.29, P=0.004 and R^2=0.53, P<0.0001), respectively . Levels of *Dio2* mRNA were 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 LH/FSH, data could be better fitted with a hyperbolic curve, which is shown instead of the linear regression line.

Discussion

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

statistically significant changes in the expression of molecular seasonal markers. Current evidence in mouse[27] and sheep[2,26,28] points to a pivotal role for EYA3 in the photoperiodic response of the melatonin-responsive PT. In sheep, we proposed that the response to LP is triggered by the EYA3/SIX1 heterodimer, which acts as a co-activator of DNA-bound TEF at the *Tshb* promoter[1,7,26]. The LP increase in *Eya3* expression is crux to the system but the overall *Tshb* transcriptional output rests on proper phase relationship between *Eya3* and *Tef*, which are both circadian clock-controlled genes[26]. *Eya3* expression is timed to occur ~12h after dark onset (between 8h and 16h, tighter sampling schedule missing) and is potently inhibited by melatonin through a mechanism that remains unknown (as is the mechanism for *Eya3* induction by LP). This model predicts that longer photoperiods are more conducive to *Eya3* increase, due to melatonin being absent in the morning, and also predicts that the rise in *Tshb* is necessarily preceded by rising *Eya3*. Our data are consistent with this since *Eya3* has risen by 75% under 11:13 and by 250% under 12:12, when compared to PP nat ($P<0.05$ by t-test for both pairwise comparisons). Comparatively, *Tshb* does not display any statistically significant changes in expression. However, animals were killed in the early day (ZT2-4) ; a time which fits the expected *Eya3* peak under longer photoperiods but also corresponds to a marked trough in *Tshb* expression[26]. This might account for the apparent lack of difference observed for *Tshb* expression between PP nat, 11:13 and 12:12 photoperiods.

We recently used a similar methodology to investigate the impact of quantitative increases in photoperiod on the neuroendocrine response in intact Soay ram lambs[17]. In that study, we also found induction of *Eya3* at a shorter photoperiod (11.75h) compared to *Tshb* (12.5h), while the pituitary and gonadal responses (FSH and Testosterone, respectively) were more clear-cut and pointed to the existence of a CP between 11.75h and 12.5h. It is difficult to compare the two 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.

Perhaps, the most striking difference with the Soay lamb study[17] is the disconnection between *Tshb* and *Dio2* : *Tshb* expression steadily increases with stepwise increases in photoperiod while *Dio2* does not. This is very surprising considering our prior finding that *Dio2* (amongst other genes, see below) is acutely induced – 4-fold increase as assessed by RT-qPCR – by exposure to a LP of 15.5h[3]. However, here again, the length of the photoperiodic treatment, 3.5 weeks, was much shorter than the 8 weeks treatment of the current study. In addition, the ISH methodology used here is less sensitive than RT-qPCR, which might be a problem considering the modest (1.5-2-fold) seasonal amplitude of the *Dio2* rhythm in tanycytes of Ile-de-France ewes[3], compared to Soay rams[50]. Nevertheless, considering the decrease in *Kiss1* expression and the concurrent decreases in LH/FSH, we infer that photoperiodic treatments were efficient. Since the DIO2-triggered hypothalamic local increase in T3 at the beginning of spring is crucial to the progression of the ovine seasonal cycle towards reproductive arrest[12,44,51-53], we conclude that *Dio2* induction occurred, but was not sustained throughout the 8 weeks. We therefore hypothesize that the disconnection between *Tshb* and *Dio2* is due to a transient impact of TSH upon *Dio2* and T3 production, which is nevertheless enough to entrain the circannual clock and trigger the response of the hypothalamo-pituitary axis. We can exclude that this disconnection reflects a general uncoupling between PT and tanycytes since two other tanycyte-specific markers, *Tmem252*[3] and *Dct*[21], showed increased expression levels with increasing photoperiods, in a pattern resembling that of PT-expressed genes. Interestingly, using thyroidectomized OVX+E2 ewes we demonstrated that *Tmem252* induction by LP is genuinely dependent upon T3[3]. The profile of *Tmem252* observed in this study would therefore indicate that T3 levels remain high under LP in spite of constant *Dio2* expression. Overall, these observations point to a *Dio2*-specific mechanism of negative feed-back, which rapidly follows induction by LP.

Indeed, under natural conditions, Siberian hamster display sharp transient peaks of *Dio2* and *Dio3* expression during the year[54]. There is also strong evidence in sheep that a transient exposure to LP – 45-60 long days – is sufficient to synchronize the circannual cycle[12,55,56]. This is in line with the idea that T3, even though it exerts a key organizing role, is required only during a limited window of time in sheep and hamsters[12,45,57,58]. Then, after being acutely induced, *Dio2* expression would return to lower levels, in spite of increased PT-derived TSH. The mechanisms for such an uncoupling are unknown but a few hypotheses can be made. The induction of *Dio2* by TSH depends on the cAMP pathway ; *Dio2* is a CRE-dependent gene[53,58]. Considering that tanycytes are a hub for a host of signals[4,59,60], it seems plausible that other GPCR-dependent signaling pathways, coupled to either Gαs or Gαi, also impinge on *Dio2* expression. Furthermore, *Dio2* expression is enhanced in both hypothyroid rats[61] and ewes[3], which indicates that T3 normally exerts a brake on *Dio2* expression in tanycytes. Such an autocrine short feedback loop is predicted to keep *Dio2* levels in check during LP exposure, and might be responsible for the apparent disconnection. Also, T3 strongly impacts local metabolism[62] and *Dio2* is itself sensitive to metabolic cues[63-66], which provides yet another potential short feedback loop. Other unrelated cues, such as LPS injection[67,68] and hypoxia[69] also impact either *Dio2* expression or DIO2 stability. In conclusion, multiple signals – not limited to PT-derived TSH – converge onto tanycytes and overall *Dio2* expression reflects integration of all these inputs. We conclude that our experimental design, which examined expression of genes after ~2 months of photoperiodic exposure, reveals the transient nature of the LP impact upon the circannual clock.

Our prior work in Soay lambs[17], Ile-de-France ewes[3] and in Siberian hamsters[18] has provided hints that a very modest increase in PT-derived TSH at the end of winter might be enough to

yield a large increase in *Dio2* expression. We ascribed this logarithmic response to sensitization of the TSHR pathway within tanycytes, a proposal which remains to be addressed rigorously. Here, we report that very modest changes in *Tshb* expression trigger comparatively large responses in LH/FSH (i.e. logarithmic response rather than linear), which are associated with a rather linear and progressive decrease of *Kiss1* expression (**Figure 4**). Our data in OVX+E2 ewes are consistent with those in intact Soay ram lambs and point to a CP value comprised between 12-13h. More importantly, our data provide strong evidence that non-linear responses and discontinuity occur along the TSH/DIO2/T3/KISS1 axis. These features may be part of of the circannual timing device and will have to be considered in the interpretation of future studies.

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Figure legends

Figure 1: Schematic of the experimental design. (A) Overview of the photoperiodic treatments. OVX+E2 ewes were initially kept in open barns, exposed to the natural decrease in daylength, before being brought indoors and exposed for ~2 months to either a simulated natural photoperiod (PP nat) or to photoperiod of increasing duration: 11:13, 12:12 , 13:11 and 14:10. (B) Ewes were exposed to constant photoperiods by delaying the time of lights off. All ewes were killed in the early day (ZT2-4).

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)

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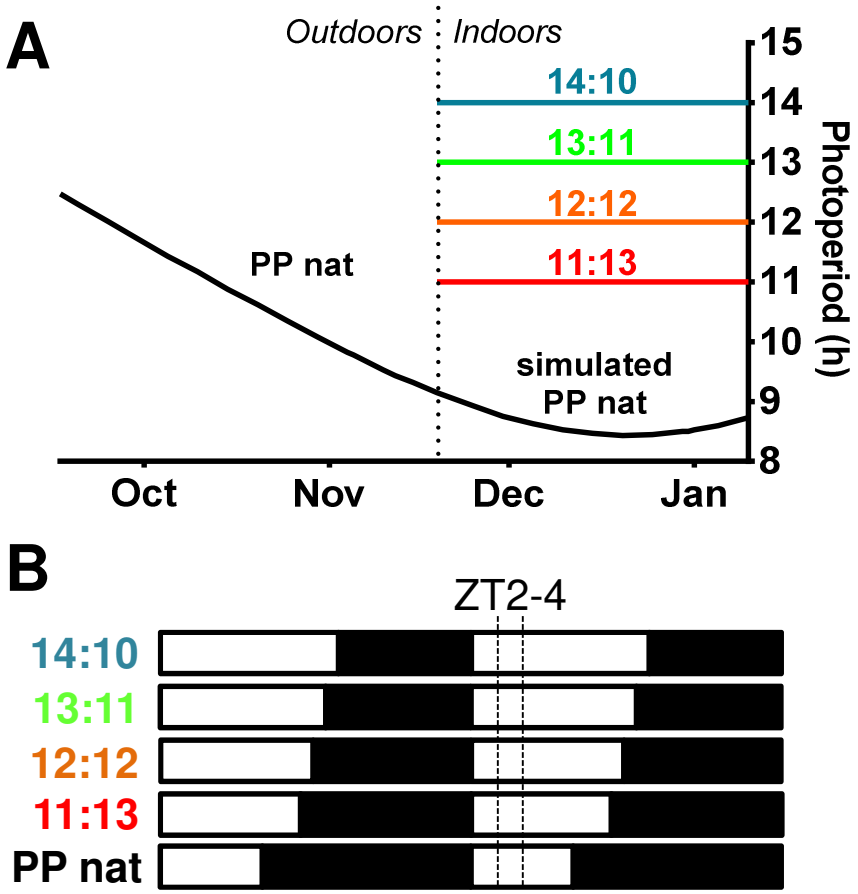
A
Outdoors
Indoors
14:10
13:11
12:12
11:13
PP nat
simulated
PP nat
15
14
13
12
11
10
9
8
Photoperiod (h)
Oct
Nov
Dec
Jan
B
ZT2-4
14:10
13:11
12:12
11:13
PP nat

Figure 1

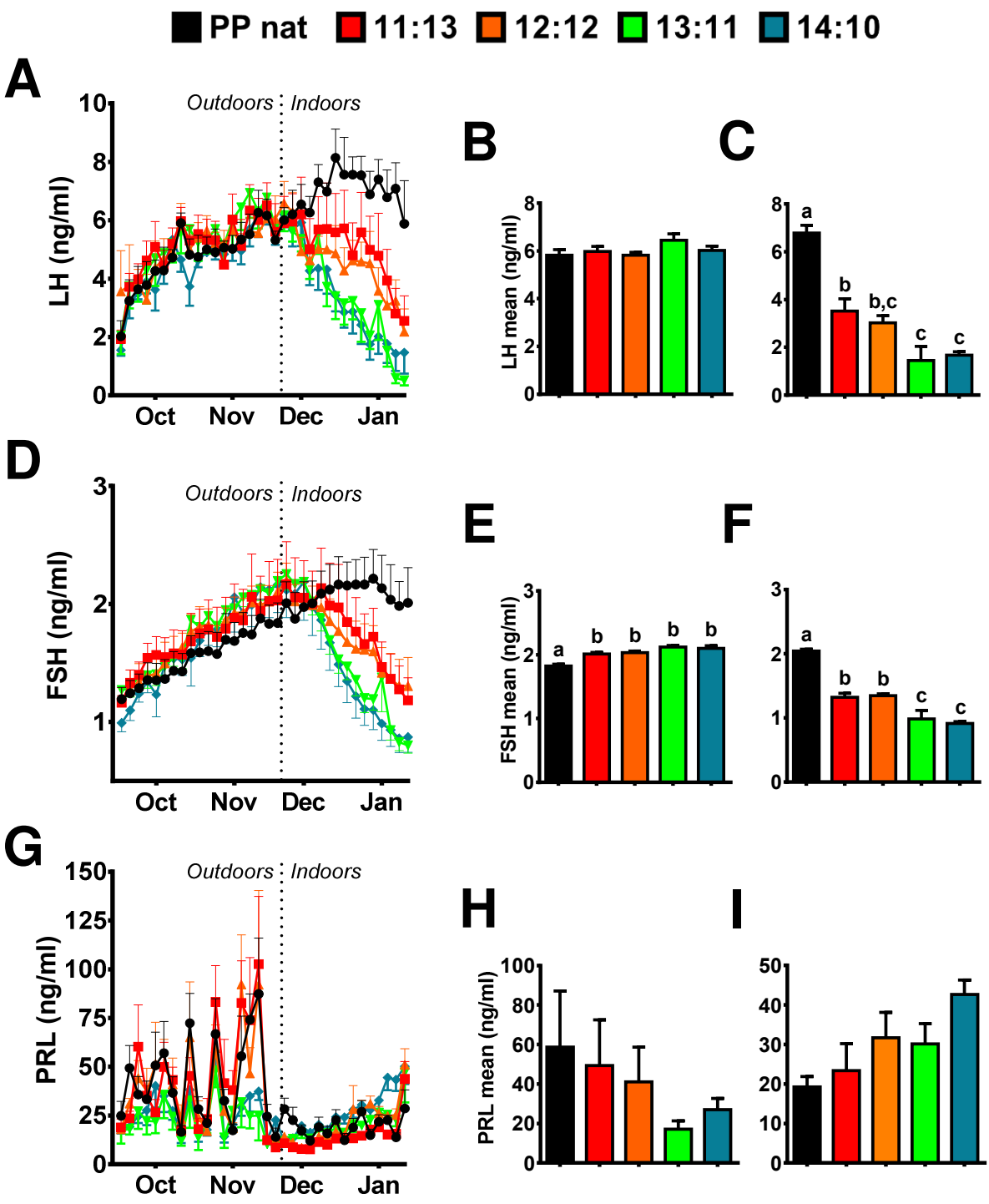
PP nat
11:13
12:12
13:11
14:10
A
Outdoors
Indoors
LH (ng/ml)
Oct
Nov
Dec
Jan
B
LH mean (ng/ml)
C
a
b
b,c
c
c
D
FSH (ng/ml)
E
FSH mean (ng/ml)
F
G
PRL (ng/ml)
H
PRL mean (ng/ml)
I

Figure 2

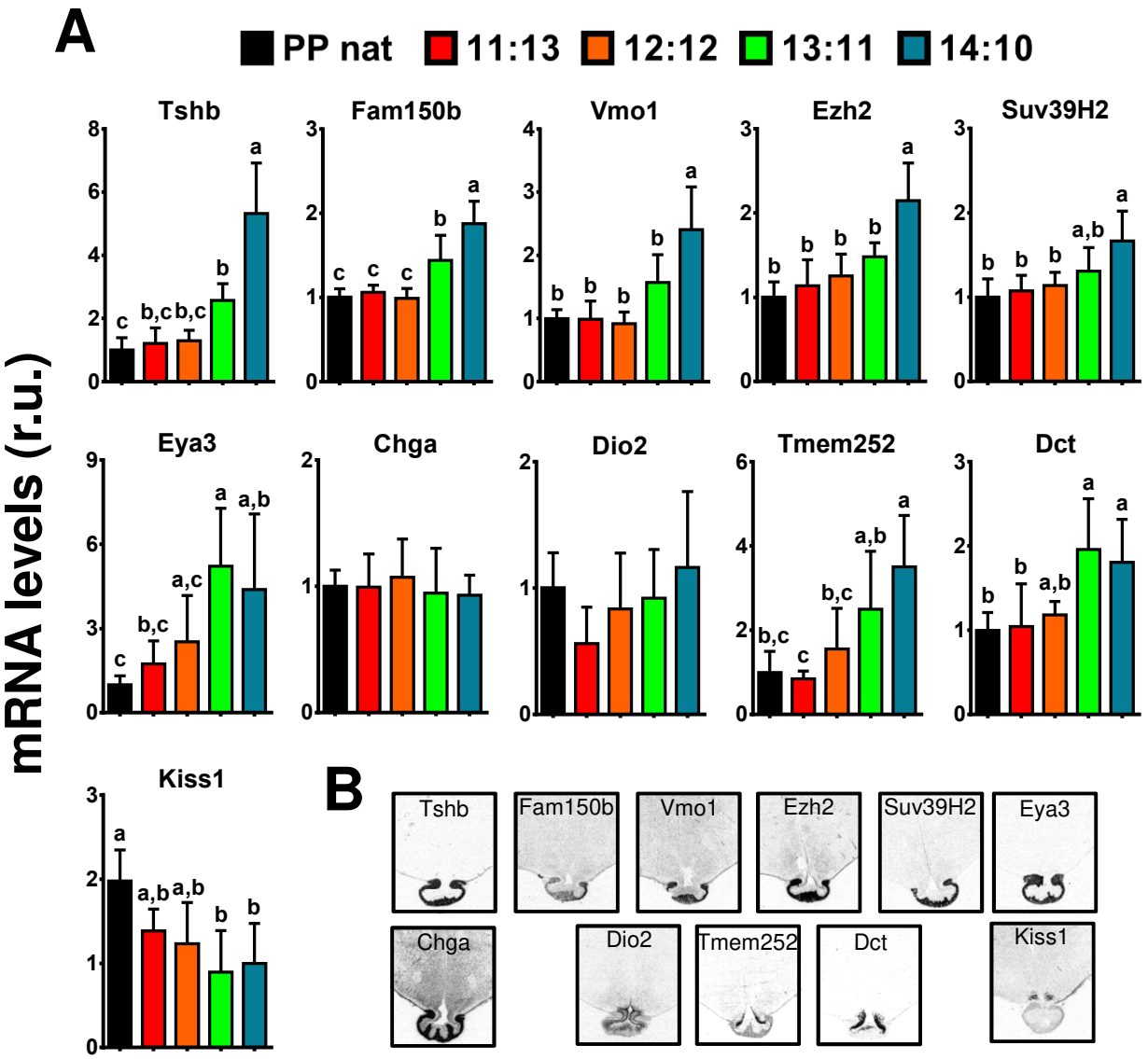
A
PP nat
11:13
12:12
13:11
14:10
mRNA levels (r.u.)
Tshb
Fam150b
Vmo1
Ezh2
Suv39H2
Eya3
Chga
Dio2
Tmem252
Dct
Kiss1
B
Tshb
Fam150b
Vmo1
Ezh2
Suv39H2
Eya3
Chga
Dio2
Tmem252
Dct
Kiss1

Figure 3

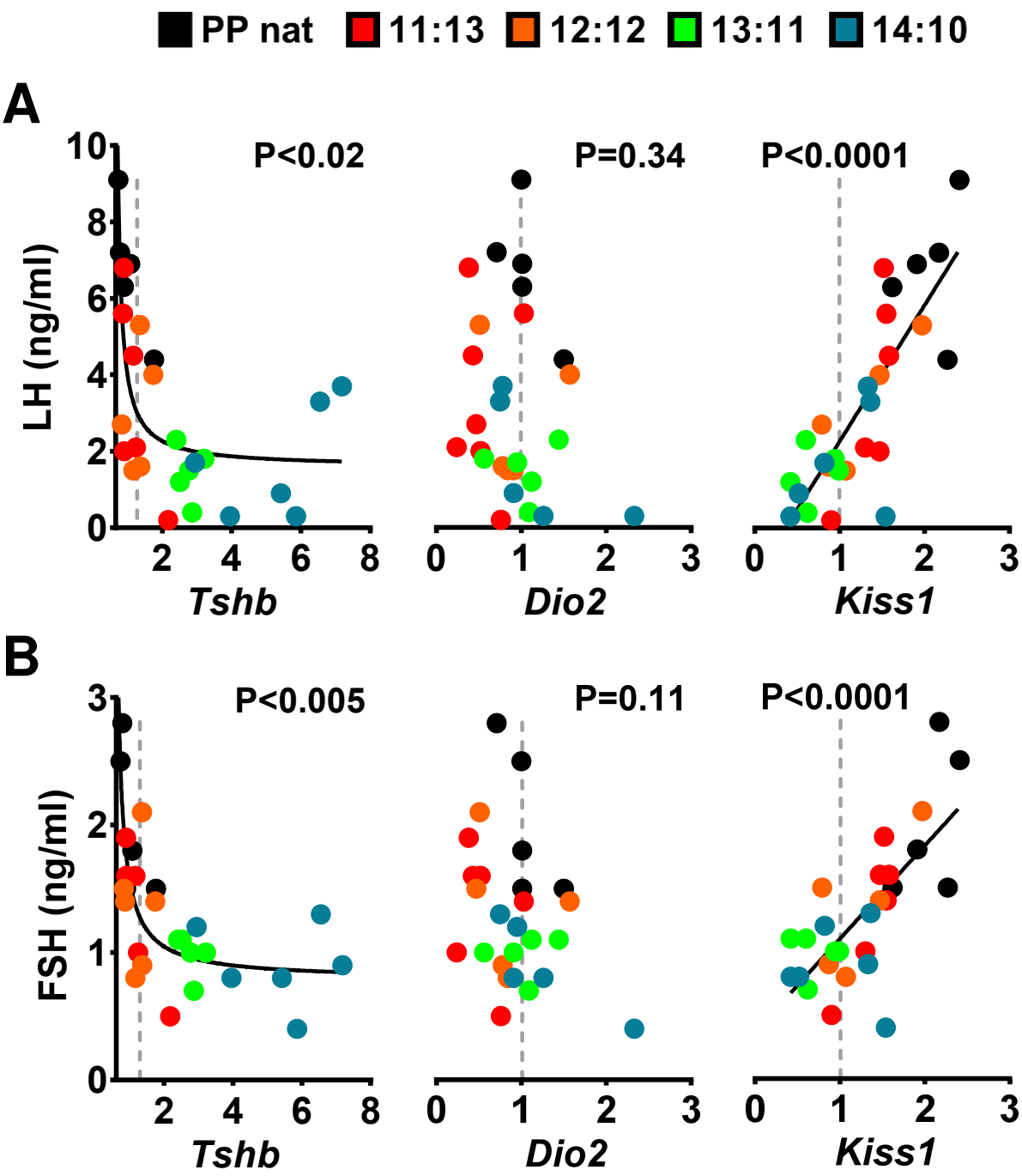
PP nat
11:13
12:12
13:11
14:10
A
LH (ng/ml)
P<0.02
P=0.34
P<0.0001
Tshb
Dio2
Kiss1
B
FSH (ng/ml)
P<0.005
P=0.11
P<0.0001
Tshb
Dio2
Kiss1

Figure 4