Endogenous circannual rhythm in luteinizing hormone secretion: insight from signal analysis coupled with mathematical modelling

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In sheep, as in many vertebrates, the seasonal pattern of reproduction is timed by the annual photoperiodic cycle, characterized by seasonal changes in the day length. The photoperiodic information is translated into a circadian profile of melatonin secretion. After multiple neuronal relays (within the hypothalamus), melatonin affects gonadotrophin-releasing hormone (GnRH) secretion, which in turn controls ovarian cyclicity. The pattern of GnRH secretion is mirrored by that of luteinizing hormone (LH) secretion, whose plasmatic level can be easily measured. We addressed the question of whether there exists an endogenous circannual rhythm in a tropical sheep (Blackbelly) population that exhibits clear seasonal ovarian activity when ewes are subject to temperate latitudes. We based our analysis on LH time series collected in the course of 3 years from ewes subject to a constant photoperiodic regime. Owing to intra- and interanimal variability and unequal sampling times, the existence of an endogenous rhythm is not straightforward. We have used time–frequency signal processing methods, and especially the smooth pseudo-Wigner–Ville distribution, to extract possible hidden rhythms from the data. To further investigate the low-frequency (LF) and high-frequency (HF) components of the signals, we have designed a simple mathematical model of the LH plasmatic level accounting for the effect of experimental sampling times. The model enables us to (i) confirm the existence of an endogenous circannual rhythm as detected by the LF signal component, (ii) investigate the action mechanism of the photoperiod on the pulsatile pattern of LH secretion (control of the interpulse interval), and (iii) conclude that the HF component is mainly due to the experimental sampling protocol.

Keywords: chronobiology; endogenous reproductive rhythm; time–frequency analysis; mathematical modelling

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1. Introduction

In temperate regions, sheep exhibit annual cycles of breeding activity (Karsch et al. 1989; Chemineau et al. 2007), timed by the photoperiodic cycle. The photoperiodic information is translated into circadian profiles of melatonin secretion. Melatonin affects gonadotrophin-releasing hormone (GnRH) secretion, which in turn controls ovarian cyclicity. Melatonin does not act directly on GnRH neurons, rather its action involves a network of neurons that relays the information from the target cells in the pre-mammillary hypothalamus to the GnRH neurons in the pre-optic area (Malpaux et al. 2001). The pattern of GnRH secretion is mirrored by that of luteinizing hormone (LH) secretion, whose plasmatic level can be easily measured. It has been generally assumed that the photoperiod entrains a circannual endogenous rhythm of sexual activity to a period of 1 year (Malpaux et al. 1989).

Sheep native to tropical regions often exhibit limited seasonality of their reproductive activity or are completely aseasonal (Mahieu et al. 2004; Valencia et al. 2006). Surprisingly, Blackbelly sheep transported as embryos in a temperate latitude displayed seasonal variations in reproduction in sharp contrast to the lack of seasonality displayed by their congener on their island of origin (Martinique; Chemineau et al. 2004), and it remains unknown whether such seasonal changes reflect an endogenous rhythm.

The aim of this study was to gather evidence for the existence of an endogenous circannual reproductive rhythm in Blackbelly ewes from the study of long-term variations in LH plasmatic levels. To get rid of the entrainment by the photoperiodic cycle, the ewes were exposed to a fixed day length corresponding to permanent short days.

The raw LH time series are characterized by a low signal-to-noise ratio (figure 1). As extracting a circannual trend from the raw data amounts to detecting time-varying hidden rhythms, we chose to use a time–frequency method, the smooth pseudo-Wigner–Ville distribution (SPWVD). This method provides meaningful time-varying spectral parameters and can discriminate efficiently between the low-frequency (LF) component and the high-frequency (HF) component in the LH signals. The SPWVD has enabled us to reveal a clear endogenous rhythm from the LF component (and to compare its properties with those of a purely periodic annual rhythm). It has also revealed that the HF component represents a well-located and quantifiable activity different from a white noise.

To further investigate the LF and HF components of the signals, we have designed a simple mathematical model of the LH plasmatic level, accounting for the main mechanisms underlying LH secretion and the effect of experimental sampling times. The comparative SPWVD analysis of the model outputs with the experimental data has enabled us to (i) confirm the existence of an endogenous circannual rhythm as detected by the LF signal component, (ii) investigate the action mechanism of the photoperiod on the pulsatile pattern of LH secretion (control of the interpulse interval), and (iii) conclude that the HF component is mainly due to the experimental sampling protocol.
Figure 1. Changes in LH plasmatic levels. Five ewes, ovariectomized and treated with a constant-release implant of oestradiol, were exposed to short days (8L:16D) for 3 years. Blood samples were retrieved twice a week during the 3 years of the experiment; each series consists of 312 experimental points. The LH level (ng ml$^{-1}$) is shown on a logarithmic scale. All ewes exhibit a low signal-to-noise ratio, owing to a strong HF activity, in the range of the sampling frequency of the measure, which complicates the research for a hidden endogenous circannual rhythm.

2. Material and methods

(a) Animals and experimental design

The study was carried out with five Blackbelly ewes exposed for 3 years, from January 2002 to January 2005, to a constant short-day length (SD) of an 8 h light/16 h dark regime (8L:16D). Prior to this experiment, they had previously

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been exposed for three months to a constant long-day length (LD) of a 16L:8D regime (October–December 2001). In January 2002, the ewes were ovariectomized and received a constant-release oestradiol implant (OVX+E₂). The subcutaneous Silastic implant contains a 30 mm column of oestradiol-17β to maintain a serum concentration of 3–5 pg ml⁻¹, which is intermediate between the concentrations observed in the luteal and follicular phase of the ovarian cycle. They were kept in the light-controlled room of an experimental breeding station near Tours (France, 47° N). The artificial photoperiodic regime was tuned by means of electric timers, whereas temperature was not controlled. The reproductive endocrine state was monitored by regular measurements of the LH plasmatic level. Blood samples were collected from the jugular vein, twice a week, early in the morning and stored at −20°C. The sensitivity of the plasma LH assay was 0.2 ng ml⁻¹.

(b) Data analysis: signal processing

To follow instantaneous changes in spectral activities, we selected as the time–frequency method the SPWVD, which is based on the Wigner–Ville distribution (Cohen 1989; Pola et al. 1996). The Wigner–Ville distribution is always real-valued, with a very good time and frequency resolution preserving time and frequency shifts. This distribution satisfies the marginal properties: the energy spectral density and the instantaneous power can be obtained as marginal distributions of the Wigner–Ville distribution. As a main drawback, interference between components of different frequencies may occur, leading to oscillatory cross-terms (Martin & Flandrin 1985; Monti et al. 2002). To overcome this problem, the SPWVD is computed as a time average of the Wigner–Ville distribution

\[
\text{SPW}(t, \nu) = \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} g_M(s - t) h_N^2(\tau) x\left(s + \frac{\tau}{2}\right) \\
\times x^*(s - \frac{\tau}{2}) e^{-i2\pi\nu\tau} d\tau ds,
\]

where \( t \) represents the time (h), \( \nu \) the frequency (h⁻¹), \( N \) and \( M \) are, respectively, the size of the \( h_N \) and \( g_M \) windows, the \( h_N \) window weights the input time series \( x(t) \) and the \( g_M \) window averages the instantaneous spectrum. In the SPWVD, a trade-off is required between the time–frequency resolution and the removal of the cross terms: smoothing in time by \( g_M \) and/or in frequency by \( h_N \) reduces the time resolution and/or the frequency resolution (Pola et al. 1996).

Even if the marginal properties are no longer strictly valid and the time resolution reduced (depending on the duration of \( g_M \)), the SPWVD can be used to estimate both the instantaneous power (IPow) and frequency (IF) of the signal from the first two order moments of the distribution

\[
\text{IPow}(t) = \int_{-\infty}^{+\infty} \text{SPW}(t, \nu) d\nu \\
\text{IF}(t) = \frac{\int_{-\infty}^{+\infty} \nu \text{SPW}(t, \nu) d\nu}{\int_{-\infty}^{+\infty} \text{SPW}(t, \nu) d\nu}.
\]

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Figure 2. Definition of the parameters characterizing the LF component of the LH signal resulting from the time–frequency analysis based on the SPWVD. xmax (day), vmax (ng ml\(^{-1}\)): occurrence and value of the maximal LF amplitude of a phase; xmin (day), vmin (ng ml\(^{-1}\)): occurrence and value of the minimal LF amplitude of a phase; max–min amplitude (ng ml\(^{-1}\)): difference between the maximal and minimal LF values of a phase; cycle period (day): duration between two xmax.

The instantaneous power (IPow) can be converted into the instantaneous amplitude as follows:

\[ \text{IAmp}(t) = \alpha \sqrt{\text{IPow}(t)}, \]

where \(\alpha\) is a constant depending on the filter properties.

The time–frequency analysis was performed within the Scilab–Scicos academic environment.\(^1\) Owing to the interference between the spectra, the use of SPWVD needs some pre-processing of the LH time series. The data were resampled by interpolation with a third-order spline function to get equally spaced time series with a daily sampling time. Two mono-frequency components were extracted after filtering. The LF component corresponds to long-term fluctuations in the LH level (alternation between ovarian cyclic activity and inactivity), while the HF component covers the range of the experimental sampling frequency. Accordingly, the LF activity was upper-bounded at five months with a low-pass finite impulse response filter (FIR). The five-month bound was chosen to encompass relatively short-term changes in LH levels. The HF activity was bounded between one and a half and three weeks (around the experimental sampling frequency) with a band-pass FIR.

After SPWVD processing, several parameters were extracted from the resulting time series for LF amplitude (as illustrated in figure 2) to describe phases and cycle periods of LH secretion. An activity phase encompasses the period where plasmatic LH levels remain high, resulting from the release of LH at HF in the sexual season. An activity phase is defined as a time interval \([t_1, t_2]\) such that

(i) the LH level is over 0.2 ng ml\(^{-1}\) during this whole interval,
(ii) the LF component derivative at time \(t_1\) becomes greater than a threshold \((7 \times 10^{-3} \text{ ng ml}^{-1} \text{ d}^{-1})\), and

\(^1\text{http://www.scilab.org/; http://www.scicos.org/}\)

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(iii) the upper bound $t_2$ is the lowest time greater than $t_1$ at which the signal derivative is negative, increasing and becomes greater than $-7 \times 10^{-3}$ ng ml$^{-1}$ d$^{-1}$.

A cycle period corresponds to the sequence of one activity phase and the subsequent inactivity phase,

(i) $\text{xmax (day)}$ and $\text{vmax (ng ml}^{-1})$ stand for the occurrence and value of the maximal amplitude of a phase, respectively; hence the time separating two subsequent values of $\text{xmax}$ is analogous to a cycle period,

(ii) $\text{xmin (day)}$ and $\text{vmin (ng ml}^{-1})$ stand for the occurrence and value of the minimal amplitude of a phase, respectively,

(iii) max–min amplitude (ng ml$^{-1}$) stands for the difference between the maximal and minimal values of a phase,

(iv) mean amplitude (ng ml$^{-1}$ d$^{-1}$) stands for the sum of instantaneous amplitudes normalized by the number of days between $\text{xmin1 and xmax1}$, and

(v) phase duration (day) begins at the midpoint joining $\text{xmin1 and xmax}$, and ends up at the midpoint joining $\text{xmax}$ and $\text{xmin2}$ of a phase.

These parameters are useful in assessing the existence of a circannual trend in the LH series and comparing the LH series between the five ewes of the study. In particular, the synchronization is estimated by the standard error of the mean (s.e.m.) of the time occurrences of the minimal and maximal amplitude values in each phase. Average parameter values can also be used to compare the Blackbelly strain with other strains originating from temperate regions, such as the Suffolk strain. Statistical comparisons have been performed as a one-way repeated-measure analysis of variance. All results are expressed as mean and s.e.m.

(c) Mathematical modelling

To assess the results of the time–frequency analysis and improve our interpretation of the frequency-filtered components, we have built a simple mathematical model of the plasmatic LH level. It is based on a representative function of the pulsatile LH secretion by the pituitary gland coupled with a term accounting for dissemination into the blood. We have introduced the influence of the photoperiodic regime on LH release frequency at the pituitary level. Indeed, in OVX+$E_2$ ewes, LH pulse frequency varies considerably throughout the year from a minimum of one pulse every 24 h during the rest season to a maximum of one pulse every 20 or 30 min during the reproductively active season (Karsch et al. 1984; Malpaux et al. 2001). This seasonal effect is mediated by photoperiod and can be reproduced by alternating exposures to short and long days. Thus, the interspike interval function represents in a compact way both the direct effect of melatonin on the LH-secreting gonadotroph cells (modulation of the GnRH-induced LH release after a GnRH pulse; Skinner & Robinson 1997) and the indirect, upstream effect on GnRH pulse frequency. To compare the photoperiod-entrained circannual rhythm with the free-running rhythm, we have tested the effect of both sinusoidal (1 year period) and damped sinusoidal (1 year fundamental period) function of time for the changes in the interspike interval along the years. Synthetic (model output) LH series comparable to
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Figure 3. Design of the LH secretion rate in the case of constant interspike interval. Each panel represents the graph of the function LH(t) (defined in equation (2.4)) as the function controlling the interspike interval is held constant to either (a) 36 or (b) 2.

the experimental series have been obtained after mimicking the process of experimental sampling and are subject to the same SPWVD analysis as the experimental data.

(i) Luteinizing hormone secretion by the pituitary gland

The pituitary gland releases LH as successive spikes. We chose to represent each spike of LH release by an instantaneous impulse followed by a fast exponential decrease. The interspike interval is controlled by a time-varying function (accounting for the photoperiod action) $P_{\text{spike}}(t)$. Hence, the instantaneous release of LH by the pituitary gland is given by

$$LH(t) = A_{\text{spike}} \exp \left[ -k_{\text{hl}} \left( t - \left\lfloor \frac{t}{P_{\text{spike}}(t)} \right\rfloor P_{\text{spike}}(t) \right) \right], \quad (2.4)$$

where $\lfloor x \rfloor$ refers to the greatest integer smaller than $x$ (integer part). Figure 3 displays the graph of the LH(t) function when $P_{\text{spike}}(t)$ is constant and equals 36 (figure 3a) or 2 (figure 3b). We will give the precise expressions for $P_{\text{spike}}(t)$ in the next subsection. The $A_{\text{spike}}$ parameter represents the spike amplitude. The $k_{\text{hl}}$ stiffness coefficient is directly linked to the spike half-life, $\tau_{\text{hl}}$, by the following relation:

$$k_{\text{hl}} = \frac{\ln 2}{\tau_{\text{hl}}}.$$  

The parameter values are chosen so as to fit, at best, the shape of the LH pulse in the ewe. We first based our choice on the few experiments that have investigated the LH release by the pituitary gland in the ewe from regular sampling in portal...
hypophyseal blood (Pincus et al. 1998; Keenan et al. 2004). We also used the results from deconvolution methods applied to time series of LH sampled in jugular blood to get indications about the amplitude and the half-life of the LH spike (Veldhuis et al. 1987; Veldhuis & Johnson 1990; De Nicolao et al. 1999). Accordingly, we chose the following parameter values for the model outputs: $A_{\text{spike}} = 150\,\text{ng}$ and $k_{\text{hl}} = 2$, to obtain a spike half-life $\tau_{\text{hl}}$ around 20 min.

(ii) Control of the interspike interval

The $P_{\text{spike}}$ function represents, in a compact way, the effect of light on LH secretion at the pituitary level and reproduces the alternation between high LH release frequency (i.e. short interspike interval) and low LH release frequency (i.e. long interspike interval). We will use two different expressions of $P_{\text{spike}}$ to mimic either the natural temperate photoperiod or the constant SD light regime used in the experimental protocol.

Information on the daylight duration is encoded as melatonin secretion by the pineal gland and conveyed to GnRH neurons through complex neuronal circuits. GnRH secretion in turn controls the frequency of LH secretion. In ewes, LH pulse frequency increases as the daylight duration gets shorter, which sets sexual activity on. In contrast, LH pulse frequency decreases as the daylight duration gets longer, leading to sexual inactivity. Approximating the annual variations in daylight by a sinusoidal function, we can introduce the impact of the light regime in temperate regions as sustained oscillations of the interspike interval

$$P_{\text{spike}}(t) = \frac{\Delta P}{2} \left( \sin \left( \frac{2\pi t}{P_{\text{photo}}} \right) + 1 \right) + P_{\text{min spike}}$$

with

$$\Delta P = P_{\text{max spike}} - P_{\text{min spike}}.$$

$P_{\text{max spike}}$ corresponds to the maximal interspike interval during the inactivity phase in the ewe (36 h), $P_{\text{min spike}}$ corresponds to the minimal interspike interval in the activity phase (1.5 h) and the photoperiod $P_{\text{photo}}$ is 1 year (8760 h).

In the experimental constant SD light regime, we may expect that the oscillations in $P_{\text{spike}}$ progressively weaken as there is no more entrainment. This can be tested using a damped sinusoid for $P_{\text{spike}}$, for which the annual increase in the interspike interval is less and less pronounced with time

$$P_{\text{spike}}(t) = \begin{cases} 
\frac{\Delta P}{2} (\sin(\omega t) + 1) + P_{\text{min spike}} & \text{if } t < P_{\text{photo}}, \\
\frac{\Delta P}{2} \left( \sin \left( \sqrt{1 - \zeta^2} \frac{2\pi t}{P_{\text{photo}}} \right) + 1 \right) K e^{-\zeta \omega (t - P_{\text{photo}})} + P_{\text{min spike}} & \text{if } t \geq P_{\text{photo}},
\end{cases}$$

where $\omega = \frac{2\pi}{P_{\text{photo}}}$. For $t$ in $[0, P_{\text{photo}}]$, the expression of $P_{\text{spike}}$ is the same as before. For $t > P_{\text{photo}}$, the sinusoid is damped with coefficient $\zeta$. After several tests, the more appropriate
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Figure 4. Design of the time-varying function controlling the interspike interval ($P_{\text{spike}}(t)$).

(a) The interspike interval function as defined in equation (2.5): a sinusoid of 1 year period. (b) The interspike interval function as defined in equation (2.6): a sinusoid of 1 year period up to day 365, followed by a damped sinusoid of 1 year fundamental period, the damping coefficient $\zeta$ was set to 0.14. The vertical segment marks day 365.

value to reproduce the experimental behaviour appeared to be $\zeta = 0.14$. For sake of continuity of $P_{\text{spike}}$, $K$ is given by

$$K = \frac{1}{\left(\sin\left(2\sqrt{1-\zeta^2}\pi\right) + 1\right)}.$$  

Figure 4 illustrates the changes in $P_{\text{spike}}(t)$ with the sinusoidal $P_{\text{spike}}$ function defined by equation (2.5) (figure 4a) and the damped sinusoidal $P_{\text{spike}}$ function defined by equation (2.6) (figure 4b) with the following parameter values that we fix from now on:

$$P_{\text{max}}^{\text{spike}} = 36 \text{ h}, \quad P_{\text{min}}^{\text{spike}} = 1.5 \text{ h}, \quad \Delta P = 34.5 \text{ h} \quad \text{and} \quad P_{\text{photo}} = 8760 \text{ h}. \quad (2.7)$$

(iii) Plasmatic luteinizing hormone level and synthetic sampling process

The LH blood level changes according to

$$\frac{dLH_p}{dt}(t) = LH(t) - \alpha LH_p(t), \quad (2.8)$$

where the LH spiking release $LH(t)$ is given by equation (2.4). Parameter $\alpha$ represents the LH clearance rate from the blood. To be consistent with the 1 h half-life of LH pulses in the jugular blood, we have fixed $\alpha = 6$.

Then, we extracted (unequally spaced) time series from the fine-step numerical integration of equation (2.8), with a sampling frequency of twice a week (comparable to the experimental protocol). The sampling times (expressed in hours) are defined by

$$t_i = 84t + a(i).$$

The random numbers $a(i) \in [-0.5, 0.5]$ are generated through a Mersenne Twister algorithm (Matsumoto & Nishimura 1998) to take into account the inherent variability of the sampling times.
Figure 5. Time changes in the LH plasmatic secretion and the corresponding LF and HF components during the 3 years of the experiment in one ewe. (a) The raw LH time series (ng ml\(^{-1}\)) in one ewe, ovariectomized and treated with a constant-release implant of oestradiol, and exposed to short days (8L:16D) for 3 years. (b,c) The LF and HF components extracted from the raw series by time–frequency analysis.

Once we had simulated the model outputs, we produced synthetic time series that can be subject to the same SPWVD analysis as the experimental data. We took advantage of our full control of the frequency components to gain insight into the interpretation of the LF and HF components of the experimental time series. In particular, we were able to study the HF component and separate the variability owing to the experimental protocol.

3. Results

(a) Signal analysis

(i) Low-frequency band: luteinizing hormone secretion phases

Figure 5 shows the time changes in the LH plasmatic level during the 3 years of the experiment in one ewe. Figure 5a represents the raw LH time series. Figure 5b represents the LF amplitude time series, after being extracted from the raw series by the SPWVD analysis.

The LF amplitude captures the basal LH plasmatic level correctly and its profile does appear to be compatible with an endogenous circannual rhythm. Four phases have been evidenced in each ewe over the 3 years, the last phase
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Figure 6. Time changes in the amplitude of the LF and HF components of the LH plasmatic time series for the five ewes. (a) The LF component of the LH time series. It reveals three consecutive phases followed by the beginning of a fourth one, which is compatible with an endogenous circannual rhythm. In each ewe, the average basal level within each phase progressively increases. The patterns are comparable between the ewes. (b) The HF component of the LH time series. There is an increasing HF activity along the years, especially from the second year on.

Table 1. Parameters characterizing each phase of the LF component of the LH signal resulting from the time–frequency analysis. The parameters are defined in both figure 2 and §2b.

<table>
<thead>
<tr>
<th>LF parameters</th>
<th>phase 1</th>
<th>phase 2</th>
<th>phase 3</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>xmin, xmax synchro (day)</td>
<td>2; 4</td>
<td>8; 5</td>
<td>4; 10</td>
<td></td>
</tr>
<tr>
<td>min. amplitude (ng ml(^{-1}))</td>
<td>0.37 ± 0.06</td>
<td>0.42 ± 0.08</td>
<td>2.25 ± 0.52</td>
<td>0.003</td>
</tr>
<tr>
<td>max. amplitude (ng ml(^{-1}))</td>
<td>3.55 ± 0.24</td>
<td>4.30 ± 0.59</td>
<td>5.77 ± 0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>mean amplitude (ng ml(^{-1}) d(^{-1}))</td>
<td>1.96 ± 0.13</td>
<td>2.88 ± 0.36</td>
<td>3.95 ± 0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>max–min amplitude (ng ml(^{-1}))</td>
<td>3.18 ± 0.19</td>
<td>3.88 ± 0.51</td>
<td>3.52 ± 0.53</td>
<td>0.3</td>
</tr>
<tr>
<td>phase duration (day)</td>
<td>98 ± 3</td>
<td>116 ± 4</td>
<td>149 ± 5</td>
<td>0.001</td>
</tr>
</tbody>
</table>

being truncated. The LF parameters are summarized in table 1, as mean values (averaged over the five ewes) for each phase of the LH plasmatic level. These results show a significant change in the LH secretion in the course of the experiment: the minimal and maximal values, as well as the duration of the LH phases, progressively increase from phase 1 to 3, so that the phase mean amplitude gets greater.

Figures 6a and 7 illustrate the individual changes in the LF amplitude for each of the five ewes. Figure 6a illustrates the progressive increase in the basal level of LH. Figure 7 displays the characteristic times (beginning, end
and maximal value) of the activity phases: the synchronization between the ewes decreased, whereas the phase duration increased from the first to the last phase. The cycle periods were successively $334 \pm 4, 276 \pm 9, 294 \pm 22$, with $p \leq 0.07$. Hence, the times of maximal LF amplitude are around June for the first phase, around May for the second phase, around February for the third phase and spread around November for the last phase. On the other hand, the first activity phase begins in February, the second in December, the third in August and the fourth between June and September according to the ewe.

(ii) High-frequency band

Figure 5 shows the change in the LH plasmatic level during the 3 years of the experiment in one ewe. Figure 5a represents the raw LH time series, and figure 5c represents the HF component, after being extracted from the raw series by the SPWVD analysis. The level of HF amplitude increases during the experiment, especially from the second year on. The HF amplitude is not negligible at all compared with the LF one, which is consistent with the difficulty encountered in elucidating an LF rhythm from raw series, as shown in figure 1. It is worth noting that the HF and LF peaks do not occur simultaneously. This tendency is shared between the five ewes (figure 6b).
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Figure 8. LH model output and the corresponding LF and HF components in the case of a sinusoidal interspike interval function. (a) The sinusoidal interspike interval function defined by equation (2.5) to model the response to the natural temperate photoperiod. It is drawn from the beginning of the sampling process, marked by the vertical segment in figure 4. (b) A raw LH time series obtained after a simulated (twice per week) sampling process. The three inserts represent the pulsatile changes along days 42 and 43, day 500 and day 940 in the jugular LH level obtained from the high temporal resolution of the solution of equation (2.8). (c,d) The LF and HF components, extracted from the raw series by the time–frequency analysis based on the SPWVD. The increase in the LH release frequency (as the interspike interval decreases) implies the increase in the basal jugular LH level in the insert day 940.

(b) Mathematical modelling

(i) A sinusoidal evolution of the interspike interval models the influence of the natural temperate photoperiod

Figure 8b displays a typical output of the model with the sinusoidal function $P_{\text{spike}}$ defined in equation (2.5). When $P_{\text{spike}}$ takes its highest values, LH pulses are scarce, so that the sampling times occur most of the time when the
plasmatic LH level is negligible (under the detection threshold 0.2 ng ml$^{-1}$). However, it may happen that a sampling time occurs shortly after a spike, leading to isolated great sampled values such as those visible around day 1150. When the values of $P_{\text{spike}}$ are at their lowest, the LH pulses are much more frequent, so that the basal level remains at a relatively elevated level between two pulses, while the maximal values achieved after a spike increase. Moreover, the basal level of $LH(t)$ increases, leading necessarily to great sample values.

The LF component, extracted from the SPWVD analysis, distinguishes between the activity and inactivity phases (figure 8c). The LF maxima all have the same amplitude, which is a characteristic feature of the response to the natural temperate photoperiod.

The HF amplitude is not negligible but remains very weak compared with that of LF (figure 8d). Furthermore, there is no systematic coincidence between the changes in HF activity and LF activity. It is also worth noting that the HF component does not have an impact on the LF component dynamics in a significant way. For instance, despite the sequence of great spikes around day 1150 (during an inactivity phase), the LF activity pattern just undergoes a small variation (less than 8% of the maximal amplitude).

Finally, the four to six months activity phases are similar to the duration of sexual activity phases in seasonal ewes under natural photoperiod.

(ii) A damped sinusoidal evolution of the interspike interval models the LH sampled level under LD–SD light control

Figure 9b displays a typical output of the model with the damped sinusoid $P_{\text{spike}}$ function defined in equation (2.6). We can easily identify a first activity period similar to that obtained with a sinusoid function, which consists of a few spikes during the inactivity phase followed by an increase in the LH level and a return to a zero basal level. After the first year, the transitions between the activity and inactivity phases are less obvious as the signal becomes noisier.

The LF component extracted from the SPWVD analysis (figure 9c) displays three other phases, one each year. The maximal LF amplitude (occurring when $P_{\text{spike}}(t)$ is minimal) increases from one cycle to the next, while the basal level (corresponding to the local maxima of $P_{\text{spike}}(t)$) increases from one year to the next.

The HF activity (figure 9d) remains weak during the first year but increases significantly afterwards. As for the natural photoperiod, the HF variations are not related to activity or inactivity phases.

(iii) Comparison with experimental data

The SPWVD analysis on simulated time series with damped sinusoid $P_{\text{spike}}$ displays the same basic properties as the experimental data. In particular, there is a strong similarity between the LF components of the synthetic and experimental data. The fundamental pseudo-period of the damped sinusoid produces a circannual oscillation in the model outputs, which is detected as the LF activity by the SPWVD analysis.
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Figures 9. LH model output and the corresponding LF and HF components in the case of a damped sinusoidal interspike interval function. (a) The damped sinusoidal interspike interval function defined by equation (2.6) to model the response to the controlled SD photoperiod. It is drawn from the beginning of the sampling process, marked by the vertical segment in figure 4. (b) A raw LH time series obtained after a simulated (twice per week) sampling process. The four inserts represent the pulsatile changes along day 100, day 500, day 590 and day 760 in the jugular LH level obtained from the high temporal resolution of the solution of equation (2.8). (c,d) The LF and HF components, extracted from the raw series by the time–frequency analysis based on the SPWVD. The increase in the LH release frequency (as the interspike interval decreases) implies the increase in the basal jugular LH level as displayed by the insert day 590 compared with the insert day 500.

The secretion rate LH(t) generates HF, which drives the fast oscillations in the solutions of equation (2.8). For the chosen $P_{\text{spike}}(t)$ function and parameter values of equation (2.7), the frequency window corresponding to this activity remains between 1 per 36 h and 1 per 90 min. Thus, the HF component extracted by the SPWVD analysis results from this very HF activity distorted by the sampling process of twice per week frequency. Additionally, the HF components obtained from the experimental data and the model outputs display the same properties and seem to proceed from comparable underlying mechanisms.
Table 2. Comparison between Suffolk SD ewes (Karsch et al. 1989) and Blackbelly SD ewes (present study). For sake of comparability, the parameters given in table 1 are averaged (except for the synchronization criterion) over the five ewes and the consecutive phases, except the first one (as in Karsch et al. 1989). The synchronization is estimated by the s.e.m. of the time occurrences of the minimal and maximal amplitude values in each phase.

<table>
<thead>
<tr>
<th>LH parameters</th>
<th>Suffolk SD</th>
<th>Blackbelly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synchronization (s.e.m. day)</td>
<td>18</td>
<td>7</td>
</tr>
<tr>
<td>Phase duration (day)</td>
<td>126 ± 14</td>
<td>132 ± 4</td>
</tr>
<tr>
<td>Max. amplitude (ng ml(^{-1}))</td>
<td>5.2</td>
<td>5.03 ± 0.58</td>
</tr>
<tr>
<td>Min. amplitude (ng ml(^{-1}))</td>
<td>0.4</td>
<td>1.33 ± 0.30</td>
</tr>
<tr>
<td>Cycle duration (day)</td>
<td>336 ± 16</td>
<td>285 ± 15</td>
</tr>
</tbody>
</table>

4. Discussion

The present study gives convincing evidence for the existence of an endogenous circannual rhythm in Blackbelly ewes. To our knowledge, this is the first time that such a rhythm has been revealed in ewes originating from tropical regions. Up to now, it had only been studied in ewes originating from temperate regions. The SPWVD has been shown to be well suited both to extract this circannual hidden rhythm that was blurred by a very noisy environment and to follow the changes over the whole of the 3 years. As the SPWVD has not already been applied to neuroendocrine time series, we compared the parameter values given by the SPWVD with another method used by Karsch et al. on five Suffolk ewes, which were exposed to the same experimental design including an LD–SD regimen (Karsch et al. 1989). This protocol differed from ours on three points: (i) the Suffolk SD ewes originated from a temperate region (conversely tropical region for the Blackbelly), (ii) they were studied for 5 years (conversely 3 years for the Blackbelly), and (iii) the oestradiol implant was replaced every year (conversely not replaced in the Blackbelly; however, it was checked that the oestradiol plasma levels did not decrease throughout the experiment). In Karsch’s study, the first phase was eliminated because it was induced by the initial transfer from long to short days. To compare our results with those of Karsch, we have averaged the parameter values over all ewes and activity phases, except the first one (as Karsch et al. did) induced by the long-day regimen. Despite these differences, the results of the two studies are in quite good agreement, so that they reinforce each other (table 2). The maximal LH level is similar between the two groups. The Blackbelly ewes are characterized by a higher minimal LH amplitude, a longer LH phase duration and a shorter cycle duration. These results suggest that LH secretion is globally higher in the Blackbelly ewes than in Suffolk SD. Nevertheless, despite these small differences, our results confirm those obtained by Karsch et al.

When applied to the model outputs obtained from the sinusoidal interspike interval function (2.5), the SPWVD analysis correctly extracts the LF component, which is not affected by the HF component. Additionally, the variations in the LF component allow us to identify the circannual alternation from activity to inactivity phases. Hence, the analysis of the model outputs confirms those
obtained by the SPWVD analysis of the experimental data. More precisely, the synthetic and experimental data share the same basic features as far as the LF component is concerned: (i) the identification of four distinct phases, (ii) the increase in LF maximal amplitude, and (iii) the increase in the LF basal level. The damped sinusoid seems to reproduce quite well the action of light on LH secretion in the experimental context.

Therefore, we can propose some assumptions about the response to photoperiod from the construction of the function $P_{\text{spike}}$. It appears that, using a sinusoidal $P_{\text{spike}}$, we have obtained a typical LH level pattern comparable to what would be observed in a control ewe maintained under a natural photoperiod. We are thus prone to interpret the response to the natural photoperiod as sustained oscillations in the secretion frequency. In contrast, using a damped sinusoid $P_{\text{spike}}$ leads to the rhythmic priming fading progressively away, as a damped oscillator that is no more entrained. The sinusoidal part of the function represents the onset of the rhythmic process that may be due to the prior three-month exposure to an SD regime followed by a three-month LD regime. During the first year cycle, the physiological response to the experimental photoperiod seems to be comparable to the response to a temperate photoperiod. Then, during the 3 year exposure to SD, only the endogenous rhythm can produce oscillations in LH pulse frequency, and they fade away in the absence of a suitable photoperiodic entrainment. This opens the way to study the photoperiod influence on the physiological system from the viewpoint of oscillator-driving analysis. It is noticeable that the first phase of inactivity following the first activity phase appears to be longer than the following ones. This may reflect the transition between a first phase influenced by the residual effects of previous exposure to photoperiodic changes and the true expression of the endogenous oscillator. Similarly, in the circadian literature, a transient stage has been described in the course of the first cycle. In the model, the inactivity duration following the first phase is subject to the precise time when the photoperiodic driving cue owing to the LD–SD alternation is cut off. This time controls in turn the precise time when the interspike interval begins to decrease.

Furthermore, we can infer the origin of the HF component of the experimental data by exploring which mechanisms produce the HF component of the synthetic data. Indeed, by construction, the solution of equation (2.8) cannot display an oscillatory activity in the frequency window covered by the HF component. Nevertheless, with the damped sinusoid $P_{\text{spike}}$ function, the LH secretion function (defined in equation (2.4)) generates (very) high frequency spikes (1 per 36 h to 1 per 90 min), which drive the pulse frequency of the solutions of equation (2.8). Hence, the HF component results from the distortion of the (very) high frequency pulsatility by the sampling process of twice per week frequency. The increase in HF amplitude ensues from this phenomenon: as the local maxima of $P_{\text{spike}}$ are getting smaller and smaller, the probability of obtaining isolated spikes during expected inactivity phases becomes larger. We deduce that the experimental sampling on its own is sufficient to generate the HF components of increasing amplitude in the experimental LH time series. However, other factors may act as well: to distinguish between these artifactual features and an additional physiological mechanism, experimental sampling at a higher frequency should be undertaken. Further work will consist in using the model to try and find an optimal sampling frequency allowing us to capture the essential rhythmic behaviour while minimizing the irrelevant influence of the sampling process.

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2http://www-rocq.inria.fr/who/Frederique.Clement/regate.html

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