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Rapidly alternating photoperiods disrupt central and peripheral rhythmicity and decrease plasma glucose, but do not affect glucose tolerance or insulin secretion in sheep

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2 plasma glucose, but do not affect glucose tolerance or insulin secretion in sheep.

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1 • What is the central question of this study? Disrupting circadian rhythms in rodents

2 perturbs glucose metabolism and increases adiposity. In this study we asked whether

3 circadian rhythm disruption, induced by exposure of sheep to rapidly alternating

4 photoperiods (RAP), also disrupts metabolic homeostasis in a large diurnal animal

5 model.

6 • What is the main finding and its importance? RAP exposure disrupted central

7 (melatonin and core body temperature) and peripheral (skeletal muscle clock gene

8 expression) rhythmicity. This led to reduced nocturnal plasma glucose concentrations,

9 but did not affect glucose tolerance and glucose stimulated insulin secretion. These

10 results suggest that RAP-induced circadian rhythm disruption has minimal effect on

11 glucose homeostasis in the sheep.

12

13

1   **ABSTRACT**

2   Disrupting circadian rhythms in rodents perturbs glucose metabolism and increases adiposity.

3   To determine if these effects occur in a large diurnal animal we assessed the impact of

4   circadian rhythm disruption upon metabolic function in sheep. Adult ewes (n=7) underwent 3

5   weeks of a control 12L:12D photoperiod, followed by 4 weeks of rapidly alternating

6   photoperiods (RAP) whereby the time of light exposure was reversed twice each week.

7   Measures of central (melatonin secretion and core body temperature) and peripheral

8   rhythmicity (clock and metabolic gene expression in skeletal muscle) were obtained over 24

9   hours under both conditions. Metabolic homeostasis was assessed by glucose tolerance tests

10   and 24 hour glucose and insulin profiles. Melatonin and core body temperature rhythms re-

11   synchronised within 2 days of the last photoperiod shift. High amplitude *Bmal1, Clock,*

12   *Nr1d1, Cry2* and *Per3* mRNA rhythms were apparent in skeletal muscle, which were phase

13   advanced by up to 3.5 hours at 2 days after the last phase shift, whereas *Per1* expression was

14   down-regulated at this time. *Pparα, Pgc1α* and *Nampt* mRNA were constitutively expressed

15   under both conditions. Nocturnal glucose concentrations were reduced following chronic

16   phase shifts (ZT0 -5.5%, ZT12 -2.9%, ZT16 -5.7%), whereas plasma insulin, glucose

17   tolerance, and glucose stimulated insulin secretion were not altered. These results

18   demonstrate that clock gene expression within ovine skeletal muscle oscillates over 24 hours

19   and responds to changing photoperiods. However, metabolic genes which link circadian and

20   metabolic clocks in rodents were arrhythmic in sheep. Differences may be due to the

21   ruminant versus monogastric digestive organisation in each species. Together these results

22   demonstrate that despite disruptions to central and peripheral rhythmicity following rapidly

23   alternating photoperiod exposure, there was minimal impact on glucose homeostasis in the

24   sheep.

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4 **INTRODUCTION**

5 Shift work exposure is associated with increased risks of heart disease, diabetes and obesity

6 (Morris *et al.*, 2012; Figueiro & White, 2013). Given the high incidence of shift work (~16%

7 of the Australian working population; (Beers, 2000; Australian Bureau of Statistics, 2012)

8 this represents a significant health burden for the community. While determining causality is

9 inherently difficult, there is a growing consensus that shift work itself initiates the

10 development of chronic disease, independent of confounding factors such as socioeconomic

11 group or smoking, and that the risk increases with the number of years of exposure (Karlsson

12 *et al.*, 2005; Pan *et al.*, 2011; Wang *et al.*, 2012).

13

14 It has been proposed that circadian disruption is a key causal factor in the development of

15 metabolic disease in shift workers. Shift work interferes with normal patterns of

16 sleep/activity, feeding/fasting, and light/dark exposure (Watanabe *et al.*, 2004; Lowden *et al.*,

17 2010; Grundy *et al.*, 2011; McPherson *et al.*, 2011; Ferguson *et al.*, 2012). In humans,

18 circadian disruption in controlled environments induces adverse metabolic responses

19 including increased postprandial circulating glucose, triglyceride and insulin concentrations

20 (Hampton *et al.*, 1996; Al-Naimi *et al.*, 2004; Scheer *et al.*, 2009). Similarly, in rodents,

21 circadian disruption induced by gene mutation/deletion (Turek *et al.*, 2005; Kennaway *et al.*,

22 2013), suprachiasmatic nucleus ablation (Coomans *et al.*, 2013), or exposure to altered

23 lighting/food access (Arble *et al.*, 2009; Salgado-Delgado *et al.*, 2010) increases adiposity

24 and perturbs glucose metabolism.

25

1   Whilst studies in rodents have provided some insight into the possible mechanisms by which

2   the circadian timing system regulates metabolism, it can be technically difficult to conduct

3   many of the necessary interventions and assessments in small animals. For example, it is not

4   possible to monitor central and peripheral rhythmicity within an individual rodent over time

5   in response to changing conditions. Sheep represent an alternative animal model in which to

6   interrogate the mechanisms whereby circadian disruption impacts upon physiological

7   processes. Sheep, like humans, are active and consume the majority of their food during the

8   day (Penning *et al.*, 1991; Piccione *et al.*, 2005), in contrast to the nocturnal activity patterns

9   of mice and rats (Kennaway, 1994; Kennaway *et al.*, 2003). Sheep also share similar rhythms

10   of core body temperature (Mendel & Raghavan, 1964; Piccione *et al.*, 2013) and hormonal

11   secretion to humans, including the production of melatonin during the rest phase, even during

12   constant darkness (Kennaway *et al.*, 1982; Earl *et al.*, 1990; Piccione *et al.*, 2005). Rhythmic

13   melatonin production in sheep is dependent upon post-transcriptional modulation of

14   arylalkylamine *N*-acetyltransferase, the rate-limiting enzyme critical for melatonin synthesis

15   (Klein *et al.*, 1997; Stehle *et al.*, 2001), whereas in rats melatonin synthesis is primarily

16   dependent upon rhythmic accumulation of the mRNA encoding this enzyme (Borjigin *et al.*,

17   1995). Cellular rhythmicity is apparent in peripheral and central tissues, with sheep

18   expressing the full suite of clock genes including *Bmal1, Clock, Per1, Per2, Cry1* and *Cry2*

19   mRNA in a circadian manner in liver, suprachiasmatic nucleus, pars tuberalis and cell lines

20   transfected with ovine clock components (Lincoln *et al.*, 2002; Andersson *et al.*, 2005;

21   Dardente *et al.*, 2009). Although similarities are evident in circadian rhythms and regulation,

22   sheep differ from humans and rodents in relation to their alimentary organisation. Being

23   ruminants, sheep metabolise cellulose to volatile fatty acids which are absorbed from the

24   rumen and provide the primary source of energy for the animal (Bergman, 1990).

25   Consequently, sheep display lower overall basal glucose levels, reduced post-prandial

1 nutrient fluctuations and lower insulin sensitivity (Elmahdi *et al.*, 1997; Kaske *et al.*, 2001).

2 Thus while ruminants show 24-hour rhythms of plasma metabolites such as glucose (Piccione

3 *et al.*, 2005), the relationship between circadian rhythms and metabolic regulation are likely

4 to differ between humans and sheep.

5

6 In this study we therefore determined the impact of rhythm disruption on circadian and

7 metabolic outcomes in sheep, for the first time in this species. Sheep were exposed to control

8 lighting conditions (12 hours of light:12 hours of dark) for 3 weeks followed by 4 weeks of

9 rapidly alternating photoperiods (RAP) with reversal of the photoperiod for 3 days each

10 week. We measured plasma melatonin, glucose and insulin concentrations, food

11 consumption, core body temperature and glucose tolerance during each condition. We also

12 collected sequential muscle biopsies from individual sheep over a 24 hour period to

13 quantitate peripheral tissue expression of clock genes and metabolic genes believed to link

14 circadian and metabolic rhythmicity.

15

16 **METHODS**

17 *Ethical approval*

18 All experiments were approved by the University of Adelaide Animal Ethics Committee and

19 were conducted in accordance with the *Australian Code of Practice for the Care and Use of*

20 *Animals for Scientific Purposes* (National Health and Medical Research Council, 2013).

21

22 *Experimental design*

23 Six year old Border Leicester x Merino female ewes (n=7) were transported from the field

24 (autumn, sunrise 0711h, sunset 1714h) into a light proof, temperature regulated room where

25 they were held in individual floor pens, and kept on a 12L:12D photoperiod (lights on

1   1000h). Ewes had *ad libitum* access to lucerne chaff and water, supplemented with 500 g

2   Rumevite pellets (Ridley AgriProducts, Melbourne, Australia) and 200 g lucerne hay daily.

3   Feed bins were filled with 1 kg of fresh chaff within 1 hour of lights on (1000h-1100h) and

4   refilled again in the afternoon (~1600h). Pellets and lucerne hay were given once only in the

5   morning 1 hour after lights on. All ewes were maintained on this control photoperiod for 3

6   weeks, followed by 4 weeks of exposure to rapidly alternating photoperiods (RAP, Figure 1).

7   The RAP protocol involved altering the timing of light exposure and feeding, such that the

8   photoperiod was reversed between Thursday and Monday of each week and animals were fed

9   chaff, pellets and hay within 1 hour of lights on (2200h-2300h), with further hay provided

10   prior to lights off (~0900h) when exposed to the reversed photoperiod. Daily food

11   consumption was recorded during week 2 (control) and week 7 (RAP) of the experiment by

12   weighing lucerne chaff and pellets offered each day and amounts remaining in the feed bin at

13   the beginning of each light period. Sheep were weighed on arrival in the facility, at the end of

14   week 3 (control) and at the end of week 9 (RAP). At the completion of the experiment, sheep

15   were killed by overdose of Lethabarb (Lyypards, Melbourne, Australia).

16

17   *Blood and tissue collection*

18   Venous blood and muscle biopsies were sampled across a 24 h cycle before phase shifting

19   (week 3, control photoperiod), and after 4 weeks of phase shifting (week 8, RAP). To track

20   the response of central circadian rhythms to a phase shift, blood sampling was extended to a

21   60 h period following RAP, with the muscle biopsies collected in the last 24 h of this period

22   (Figure 1). A catheter (BD angiocaths, 16G, Becton Dickinson, Franklin Lakes, NJ) was

23   inserted into the left jugular vein of each sheep two days before the control photoperiod

24   sampling period, and maintained by daily flushing with 500 U/ml heparinised saline (Pfizer,

25   New York City, NY). Blood (5 ml) was collected into lithium heparin tubes at 1000h, 1400h,

1   1800h, 2200h, 0200h, 0600h and 1000h and briefly placed in ice, centrifuged and plasma

2   harvested and stored at -20ºC for later analysis. The timing of the collections correspond to

3   ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT24 respectively (ZT = zeitgeber time where ZT0

4   is the time of lights on). In week 8, catheters were inserted at ~ZT2 into the right jugular vein

5   of each sheep as above, and blood collections commenced at ZT12 the same night and

6   continued for 60 hours, from 12 until 72 hours after the last photoperiod shift (Figure 1B).

7   Skeletal muscle was sampled at ZT0, ZT6, ZT12 and ZT18 on the day of blood sampling

8   during control conditions, and in the final 24 hours of blood sampling under RAP conditions.

9   Under local anaesthesia (1% Lignocaine without epinephrine; Lyypards, Melbourne,

10   Australia) and using a percutaneous skeletal muscle biopsy needle (5.0 mm diameter) with

11   aspiration, biopsies were collected alternately from the left and right *semimembranosus*

12   muscle, snap frozen in liquid nitrogen and stored at -80ºC until processing.

13

14   *Glucose tolerance tests*

15   Glucose tolerance and glucose-stimulated insulin secretion were measured during an

16   intravenous glucose tolerance test (IVGTT, Gatford *et al.*, 2004) under control and RAP

17   conditions, two days after collection of muscle biopsies. In brief, sheep were weighed

18   following an overnight fast, then a bolus of glucose (0.25 g glucose/kg body weight) was

19   injected 1-2 hours after lights on. Blood (2 ml) was collected before and until 3.5 h after

20   administration of the glucose bolus, and plasma collected and stored at -20 ºC for subsequent

21   glucose and insulin analysis. Due to catheter failure in one sheep, glucose tolerance tests were

22   only performed on 6 sheep under control conditions.

23

24   *Measurement of core body temperature*

1   Temperature data loggers were inserted into the peritoneal cavity of each sheep under general

2   anaesthesia and asepsis. Anaesthesia was induced by intravenous injection of thiopentone

3   sodium (1.5 g/kg body weight in sterile water, Troy Laboratories, Glendenning, NSW,

4   Australia), and maintained by inhalation of 1.5 - 2.5% isoflurane in oxygen. Antibiotics (1 g

5   cephalazolin in 3 mL sterile water i.m., Hospira Australia, Mulgrave, VIC, Australia) were

6   administered after induction of anaesthesia, and ketoprofen analgesia (300 mg i.m., Troy

7   Laboratories) was administered at extubation and the day after surgery. Three Thermochron

8   iButtons® DS129H (Maxim Integrated, San Jose, CA) were programmed to sequentially

9   record core body temperature every 10 minutes for 40 days and inserted aseptically into the

10   peritoneal cavity of each ewe. The iButtons were recovered at post-mortem and data

11   retrieved. A 3 point moving average was calculated for each sheep for the 3 days prior to the

12   first photoperiod shift and thereafter for the remainder of the experiment. Upon recovery, all

13   iButtons were found lodged within fat deposits of the peritoneal cavity.

14

15   *Metabolite and hormonal analyses*

16   Plasma melatonin was analysed in duplicate using a solid phase extraction RIA kit according

17   to the manufacturer's protocol (Buhlmann Laboratories, Schonenbuch, Switzerland)

18   (Voultsios *et al.*, 1997). The intra-assay coefficients of variation (CV) for the melatonin assay

19   were 5.9% and 17.4%, and inter-assay CV were 6.7% and 14.4% for QC samples containing

20   2.0 and 22.0 pg/ml melatonin respectively (n = 4 assays). Sensitivity of the assay was 1

21   pg/ml. Insulin was analysed by double antibody RIA (HI-11K, Merck Millipore, Darmstadt,

22   Germany). The intra-assay CV for the insulin assay were 14.6% and 5.3%, and inter-assay

23   CV were 19.4% and 9.5% for QC samples containing 11.7 and 53.1 µU/ml insulin

24   respectively, and assay sensitivity was 3.13 µU/ml (n = 6 assays). Glucose was analysed

1 colourimetrically on a Hitachi 912 Automatic Analyzer using kits obtained from Roche

2 Diagnostics (Mannheim, Germany).

3

4 *Gene expression analyses*

5 Gene expression in muscle biopsies was measured by Real Time RT-PCR using primers

6 specific for the clock genes *Bmal1, Clock, Per1, Per2, Per3, Cry1, Cry2, Dec1, Nr1d1 (Rev-*

7 *erbα), Nr1d2 (Rev-erbβ), Nr1f1 (RORα)* and *Nr1f3 (RORγ),* and the metabolic genes

8 peroxisome proliferator-activated receptor alpha (*Pparα*), peroxisome proliferator-activated

9 gamma co-activator 1 alpha *(Pgc1α)* and nicotinamide phosphoribosyltransferase *(Nampt).*

10 Sheep mRNA primers were designed for each gene of interest within NCBI nucleotide (Table

11 1). In brief, muscle biopsies (50-100 mg) were homogenised in 1 ml of TriReagent (Sigma

12 Aldrich, St. Louis, MO) using a PowerLyzer 24 (Mo Bio Laboratories, Carlsbad, CA), and

13 RNA extracted according to the manufacturer's protocol. Residual contaminating DNA from

14 all samples was removed using Ambion DNA*free*™ kits (Life Technologies, Carlsbad, CA).

15 RNA was reverse transcribed using a Superscipt III reverse transcription kit (Life

16 Technologies) and random hexamer primers (Geneworks, Adelaide, Australia). Amplification

17 of cDNA was performed on a GeneAmp 7500 Sequence Detection System (Life

18 Technologies, Carlsbad, CA). The expression of each gene within each sample was

19 normalised against the housekeeper *Rplpo1* (Muhlhausler *et al.*, 2007) and expressed relative

20 to a calibrator using the ΔΔCt method. For each gene, the calibrator was designated as the

21 time point of maximal expression under control conditions and was set as 1 (Varcoe &

22 Kennaway, 2008).

23

24 *Data analysis*

1   Statistical analyses were conducted using SPSS v.20. Circulating melatonin, glucose and

2   insulin concentrations, and gene expression data were analysed using two-factor repeated

3   measures ANOVA with *post-hoc* Bonferroni tests to compare conditions (control cf. RAP) at

4   individual time points, during the 24-hour collection periods in both groups, i.e. comparisons

5   between 24-hour measures made during control conditions and at 48-72 hours following the

6   last phase shift during RAP. Data within each condition, including longer sampling periods

7   where appropriate, was also analysed for effects of time using one-way repeated measures

8   ANOVA with *post-hoc* Bonferroni tests. Additionally, Circwave analyses (developed by R.A

9   Hut, http://hutlab.nl, (Oster *et al.*, 2006) were performed for gene expression measures during

10   control and RAP conditions to determine whether gene expression data fit to a sine curve

11   with a 24 hour period, and the times of peak and trough gene expression for each

12   experimental condition. Data are presented as mean ± SEM unless otherwise stated. $P < 0.05$

13   was accepted as significant.

14

15

1   **RESULTS**

2   *Body weight and food consumption*

3   Sheep gained 9.7 ± 1.5 kg from the time of arrival in the facility to completion of the

4   experiment and were 6.8 ± 0.8 kg heavier at the time of data collection following RAP

5   exposure (week 8) than during control data collection (week 3, $P < 0.001$, Table 2). However,

6   the rate of weight gain did not differ between control and RAP conditions ($P > 0.05$). Chaff

7   consumption was not different between conditions ($P > 0.05$, Table 2). Absolute daily sheep

8   pellet consumption was higher when the ewes were exposed to RAP than control ($P < 0.05$,

9   Table 2), but did not differ between conditions when expressed relative to body weight ($P >$

10   0.05).

11

12   *Circulating melatonin, glucose and insulin*

13   Plasma melatonin concentrations (Figure 2A) of sheep maintained on a control photoperiod

14   changed with time ($P < 0.001$), with high nocturnal concentrations evident at ZT16 (120 ± 36

15   pg/ml) and ZT20 (117 ± 25 pg/ml), which declined to 24 ± 4 pg/ml at the time of lights on

16   (ZT0). Following exposure to 4 weeks of RAP, plasma melatonin concentration also changed

17   with time ($P < 0.001$) with peak secretion still occuring during the dark phase at ZT16-20.

18   During the first night of collections (12-24 hours after the last shift), however, melatonin

19   secretion following RAP was reduced by ~40% compared to secretion at the same zeitgeber

20   time on the last night of collections, 60-72 hours after the last shift. Plasma melatonin in the

21   first 24 h after the final phase shift peaked briefly at   70 ± 26 pg/ml at ZT16 ($P > 0.05$

22   compared to the final night of collections), then decreasing to 19 ± 7 pg/ml at ZT20 ($P <$

23   0.01) and 7.5 ± 0.6 pg/ml at ZT0 ($P<0.05$). The following night, 36-48 hours after the last

24   shift, the pattern of secretion had returned to that observed under control conditions, with

1 high levels at night and low levels during the day (P > 0.05 compared to final night of

2 collections).

3

4 Plasma glucose concentrations (Figure 2B) of sheep maintained on the control photoperiod

5 changed with time (P < 0.05), with peak concentrations of 4.0 ± 0.03 mM occuring at ZT16

6 and a nadir of 3.6 ± 0.1 mM at ZT4. Plasma glucose following RAP exposure also changed

7 with time (P < 0.05) with peak levels occurring during the day rather than at night as seen

8 during control conditions. Effects of condition varied with sample time (condition X time

9 interaction, P < 0.05), such that plasma glucose was lower under RAP than control conditions

10 at 48 h (ZT0), 60 h (ZT12) and 64 h (ZT16) after the final phase shift (each P < 0.05).

11 Condition did not alter plasma insulin (P > 0.05, Figure 2C), which did not change with time

12 under the control conditions (P > 0.05). Under RAP conditions, plasma insulin changed with

13 time during the 72 h after the final phase change (P < 0.05), such that plasma insulin

14 concentrations were highest during the day, and lowest at the time of lights on.

15

16 *Clock and metabolic gene expression in skeletal muscle*

17 Expression of many, but not all of the clock genes was rhythmic in skeletal muscle under

18 control conditions (Figure 3). Expression of *Bmal1*, *Clock*, *Per3, Nr1d1* and *Cry2* mRNA

19 changed with time (each P < 0.001) and fitted a sine curve with a 24 hour period (each P <

20 0.05). Under control conditions, peak expression for *Bmal1* and *Clock* mRNA occurred

21 during the early dark phase (ZT17.5 and ZT17.2 respectively), whereas for *Nr1d1* mRNA it

22 was around dawn (ZT22.2) and *Per3* and *Cry2* mRNA expression peaked during the day

23 (ZT5.8 and ZT8.3 respectively). While the expression of *Per1* and *Per2* mRNA also changed

24 with time (each P < 0.001), the data did not fit a 24 hour sine curve (each P > 0.05). Peak

25 expression of *Per1* and *Per2* mRNA under control conditions occurred around the time of

1   lights on. Expression of *Cry1*, *Dec1, Nr1d2*, *Nr1f1* and *Nr1f3* mRNA did not change with

2   time (each P > 0.05).

3

4   Four weeks of RAP exposure altered patterns of clock gene expression in skeletal muscle.

5   Effects of condition varied with sample time (condition X time interaction) for the expression

6   of *Bmal1* (P < 0.05) and *Nr1d1* mRNA (P < 0.05), with RAP exposure phase advancing the

7   peak expression of these genes by 2.5 and 2 hours respectively. Condition also altered *Per1*

8   (P < 0.05) and *Nr1d2* mRNA (P < 0.05) expression such that RAP decreased *Per1* mRNA

9   expression at ZT0 and ZT6, and of *Nr1d2* mRNA at ZT12 compared to control conditions.

10 Expression of *Clock, Per2*, *Dec1*, *Cry1*, *Cry2, Nr1f1* and *Nr1f3* mRNA was similar under

11 control conditions and after RAP (each P > 0.05).

12

13 Gene expression of *Pgc1α, Pparα* and *Nampt* (Figure 3) did not change with time (each P >

14 0.05) or in response to RAP exposure (each P > 0.05).

15

16 *Glucose tolerance*

17 Fasting plasma glucose (Figure 4A) was higher under control than RAP conditions (Control =

18 4.3 ± 0.3 mM; RAP = 3.8 ± 0.3 mM; P < 0.05). Fasting insulin concentrations did not differ

19 with condition, however (Control = 15.5 ± 2.2 µU/ml; RAP = 14.1 ± 2.0 µU/ml; P > 0.05).

20 Plasma glucose concentrations throughout the IVGTT (Figure 4A) were lower following

21 RAP exposure than under control conditions (P < 0.05). Glucose tolerance did not differ

22 between conditions (Glucose area under the curve: Control = 616 ± 8 mmol.min.l^{-1}; RAP =

23 596 ± 54 mmol.min.l^{-1}, P < 0.05). Effect of condition on plasma insulin concentrations

24 throughout the IVGTT (Figure 4B) varied with sample time (condition X time interaction, P

25 <0.01), such that plasma insulin concentrations at 50 minutes after glucose administration

1 were higher in control than RAP sheep ($P < 0.05$). Condition did not affect absolute insulin

2 secretion (insulin area under the curve) overall (Control = 3180 ± 430 µU.min.ml^{-1}; RAP =

3 2947 ± 275 µU.min.ml^{-1}), during the 1st phase (Control = 410 ± 116 µU.min.ml^{-1}; RAP = 491

4 ± 155 µU.min.ml^{-1}) or during the 2nd phase of secretion (Control = 2586 ± 406 µU.min.ml^{-1};

5 RAP = 2002 ± 341 µU.min.ml^{-1}).

6

7 *Core body temperature*

8 Under control conditions, the ewes maintained a bimodal pattern of core body temperature

9 (shaded profile in Figure 5) whereby the temperature rose to peak values during the light

10 period, before briefly dropping at the time of lights off. Core body temperature then gradually

11 increased during the night, before decreasing to minimum levels at dawn. During RAP, the

12 daily pattern of temperature changes also shifted to remain aligned with the timing of the new

13 light cycle. This pattern of temperature response to phase shifting remained similar from the

14 first week of RAP (Figure 5A) to the last week of RAP (Figure 5B). Upon reversal to the

15 original photoperiod each Monday morning, the sheep very quickly adjusted such that the

16 core body temperature pattern became virtually indistinguishable from that measured during

17 control conditions within 24 hours of the photoperiod shift.

18

19

1 **DISCUSSION**

2 The main purpose of this study was to evaluate the impact of rapidly alternating photoperiods

3 on central and peripheral rhythmicity, and to determine how this impacts upon a range of

4 metabolic outcomes. We found that under a control 12L:12D photoperiod, adult ewes express

5 stable central and peripheral rhythms including the nocturnal secretion of melatonin, regular

6 changes in core body temperature and, for the first time, also rhythmic clock gene expression

7 in skeletal muscle. These variables were each affected by RAP exposure, indicative of an

8 attempt by the ewes to adjust to the changing photoperiod. While there were some metabolic

9 changes observed following RAP exposure, in particular lower plasma glucose levels at

10 night, there was no effect of treatment upon food consumption, glucose tolerance, glucose

11 stimulated insulin secretion, or metabolic gene expression in skeletal muscle.

12

13 In the current study, RAP exposure affected the normal pattern of melatonin secretion, such

14 that circulating concentrations during the first night after resumption of the normal

15 photoperiod were lower than that under control conditions. This response has been observed

16 in rats, where pineal N-acetyltransferase levels (Illnerova & Vanecek, 1987) and urinary 6-

17 sulphatoxymelatonin excretion (Kennaway & Rowe, 2000) were significantly reduced during

18 the first night following a 12 hour extension of the photoperiod. In our study the timing and

19 amplitude of nocturnal secretion had returned to normal by the second night, suggesting

20 central rhythmicity had re-aligned to the new photoperiod within 48 hours of the last

21 photoperiod shift. Core body temperature rhythm following RAP similarly re-aligned with

22 the new photoperiod within this time. Previous studies have assessed the time taken for rat

23 melatonin rhythms to adjust to a change in phase of the photoperiod. When rats were exposed

24 to a phase delay of the photoperiod through extension of the dark period by 12 hours, it took

25 up to a week for melatonin secretion to completely entrain (Liu & Borjigin, 2005). However,

1   further analysis of the data reveals that in fact the onset of melatonin secretion immediately

2   phase locked with the new photoperiod, whereas the timing of melatonin offset gradually

3   realigned over subsequent days. In our previous studies we also found that following

4   exposure of pregnant rats to a similar protocol of rapidly alternating photoperiods, melatonin

5   secretion on the night following return to the original photoperiod was very similar to that of

6   control rats maintained on an unchanging photoperiod, with high secretion at night (Varcoe *et*

7   *al.*, 2013). The two protocols did however differ slightly, such that the last photoperiod

8   reversal occurred through an extension to the dark phase in the rat model, whereas here in

9   sheep the light phase was extended. Given that we did not assess melatonin secretion during

10 the time of photoperiod reversal, we cannot determine the degree of adjustment required to

11 re-align to the original photoperiod. Nevertheless, together the previous studies in rats and

12 our work here in sheep demonstrate that melatonin secretion adjusts relatively quickly to

13 large phase shifts of the photoperiod.

14

15 In contrast to the centrally-regulated rhythms of melatonin and core body temperature,

16 muscle cellular rhythmicity in these sheep had not adjusted to the current photoperiod 2 days

17 after the last photoperiod shift. At this time the rhythm of expression of *Bmal1, Clock, Nr1d1*

18 and *Cry2* mRNA in skeletal muscle was phase advanced by 2 - 3.5 hours following RAP

19 exposure compared to the control photoperiod. Together these results suggest that in sheep,

20 central rhythmicity adjusts to a phase shift of the photoperiod faster than peripheral rhythms,

21 consistent with previous reports in rats (Yamazaki *et al.*, 2000).

22

23 At the basis of cellular rhythmicity lies a transcription-translation feedback loop whereby a

24 suite of clock genes regulate their own transcription over a period of 24 hours. Briefly,

25 CLOCK and BMAL1 heterodimers drive transcription of *Period*, *Cryptochrome* and *Nr1d1/2*

1   genes. PER and CRY proteins feedback to repress their own transcription, whereas NR1D1/2

2   together with NR1F1/2/3 regulate the expression of *Bmal1* (Boden *et al.*, 2013). Rhythmic

3   clock gene expression has previously been demonstrated in the ovine SCN, pars tuberalis and

4   liver (Lincoln *et al.*, 2002; Andersson *et al.*, 2005), but to our knowledge this is the first time

5   that clock gene expression in sequential *ex vivo* muscle biopsies has been reported in sheep.

6   A similar approach using repeated biopsies of the gluteal muscle has been used to investigate

7   clock gene expression in the horse (Martin *et al.*, 2010), whereas studies in mice have

8   collected muscle tissues from different animals at each time point (Zambon *et al.*, 2003). We

9   observed high amplitude rhythms of *Bmal1, Clock, Per3, Cry2* and *Nr1d1* mRNA in ovine

10   skeletal muscle, with peak *Bmal1* expression in antiphase to *Per3* and *Cry2* mRNA

11   expression. Expression of *Per1* and *Per2* mRNA and to a lesser extent, *Cry1* and *Dec1*

12   mRNA also changed across 24 hours in the control sheep, although the amplitude changes

13   were small and/or could not be fitted to a sine curve. *Bmal1, Nr1d1, Per1* and *Per2* mRNA

14   also change with time in horse skeletal muscle with a similar phase of expression to that

15   observed here, but patterns of *Clock* and *Cry2* mRNA differ between these species, being

16   constitutively expressed in horses (Martin *et al.*, 2010). We observed an additional species

17   difference in patterns of *Nr1d2* mRNA expression, which did not change over 24 hours in the

18   sheep in the present study, in contrast to high amplitude oscillations in *Nr1d2* mRNA

19   expression in horse and mouse skeletal muscle (Guillaumond *et al.*, 2005; Martin *et al.*,

20   2010). The lack of rhythmic expression of *Nr1f1* and *Nr1f3* mRNA expression in skeletal

21   muscle of sheep in the present study is consistent with that in horse and mice (Guillaumond *et*

22   *al.*, 2005; Mongrain *et al.*, 2008; Martin *et al.*, 2010). Together, the gene expression profiles

23   observed here are consistent with a transcription-translation feedback loop driving cellular

24   rhythmicity within ovine skeletal muscle.

25

1   Due to obvious practical and ethical issues, *ex vivo* studies on rhythmic clock gene expression

2   in humans have been limited to studies on blood leukocytes (Boivin *et al.*, 2003), hair

3   follicles (Akashi *et al.*, 2010), subcutaneous fat biopsies (Otway *et al.*, 2011; Gomez-Abellan

4   *et al.*, 2012) and oral mucosa (Polidarova *et al.*, 2013). Interestingly, the phase of expression

5   of the clock genes in humans is in antiphase to that observed in nocturnal species. In

6   particular, highest expression of *Bmal1* mRNA occurs at dusk, whereas the *Period* and

7   *Cryptochrome* genes peak around dawn in human adipose tissue (Otway *et al.*, 2011). The

8   similar profiles of rhythmic clock gene expression we observed here in sheep skeletal muscle

9   suggests that other diurnal species may share similar profiles, and further suggest that the

10   sheep will be a useful animal model for interrogating the effects of circadian rhythms and

11   their disruption in human physiology.

12

13   In contrast to the clock genes, expression of 3 metabolically important genes (*Ppara*, *Pgc1a*

14   and *Nampt*) did not change with time across a 24 h profile in ovine skeletal muscle in the

15   present study. This contradicts findings from mice and rats where these genes are

16   rhythmically expressed and are postulated to play an important role in linking the circadian

17   timing system and cellular metabolism. PPARα, a master transcriptional regulator of lipid

18   and carbohydrate metabolism (Lemberger *et al.*, 1994; Steineger *et al.*, 1994), exhibits a

19   circadian rhythm of gene expression in the liver of rats (Lemberger *et al.*, 1996) and mice

20   (Patel *et al.*, 2001). Mutation of CLOCK or BMAL1 globally or in cultured mouse fibroblasts

21   leads to arrhythmic *Pparα* mRNA expression (Oishi *et al.*, 2005; Canaple *et al.*, 2006),

22   suggesting the rhythmicity of *Pparα* mRNA expression is driven by cellular clocks.

23   Furthermore, the *Pparα* promoter contains multiple E-boxes upon which BMAL1:CLOCK

24   heterodimers bind and drive rhythmic transcription, independent of insulin or glucocorticoid

25   signalling (Oishi *et al.*, 2005). Interestingly, PPARα is in turn believed to affect cellular

1   rhythmicity through *Bmal1* (Canaple *et al.*, 2006) and *Nr1d1* (Gervois *et al.*, 1999)

2   regulation. Similarly, the expression of *Pgc1α* mRNA, an inducible transcriptional

3   coactivator that regulates cellular energy metabolism (Finck & Kelly, 2006), is rhythmic in

4   liver and skeletal muscle of mice (Liu *et al.*, 2007), and its stability and hence activity is

5   regulated by the circadian clock through CK1δ-dependent phosphorylation (Li *et al.*, 2011).

6   Like *Pparα*, *Pgc1α* also participates in consolidating circadian rhythms through its regulation

7   of *Bmal1* (Liu *et al.*, 2007). Finally, *Nampt* which is a rate-limiting enzyme in the NAD^+

8   biosynthetic pathway, is expressed rhythmically in liver, adipose and muscle of mice, leading

9   to parallel changes in NAD^+ (Nakahata *et al.*, 2009; Ramsey *et al.*, 2009; Um *et al.*, 2011).

10   *Nampt* transcription is driven by CLOCK:BMAL1 binding to E-boxes (Nakahata *et al.*, 2009;

11   Ramsey *et al.*, 2009), and rhythmicity of *Nampt* transcription, and subsequent NAD^+

12   oscillations are abolished in *Clock* mutant mice (Nakahata *et al.*, 2009; Ramsey *et al.*, 2009).

13   Interestingly, through NAD+ dependent SIRT1 regulation of CLOCK/BMAL1 binding,

14   NAMPT indirectly regulates the cellular clock, and hence its own transcription (Nakahata *et*

15   *al.*, 2008a).

16

17   Given the body of evidence supporting a role for each of these 3 genes in the cross-talk

18   between circadian and metabolic clocks, why did we find no evidence of rhythmicity within

19   the sheep skeletal muscle? The frequency of muscle biopsy collection (6 hourly) may have

20   been insufficient to detect transient changes in gene expression. An examination of the latest

21   version   of   the   sheep   genome   (Ovis   aries   Oar_v3.1,   2012;

22   http://www.livestockgenomics.csiro.au/sheep/) indicated that each of these genes contains at

23   least 1 canonical or non-canonical E-box within 2 kb of their transcriptional start site,

24   suggesting a potential mechanism for their circadian regulation. However, multiple E-boxes

25   (E-E elements) may be required for rhythmic CLOCK:BMAL1 driven transcription

1 (Nakahata *et al.*, 2008b). Alternatively, other incoming signals may override circadian

2 regulation of transcription of certain genes in ovine skeletal muscle. For example,

3 glucocorticoids regulate *Pparα* transcription, at least in liver (Lemberger *et al.*, 1996), but the

4 amplitude of the rhythm of cortisol secretion in sheep is low compared to other species

5 (Fulkerson & Tang, 1979; Simonetta *et al.*, 1991; Jaquiery *et al.*, 2013) and this may

6 underpin the lack of a 24 hour change in *Pparα* expression. Furthermore, since sheep are

7 ruminants with initial stages of digestion through bacterial fermentation, lower overall basal

8 glucose levels, reduced post-prandial nutrient fluctuations and lower insulin sensitivity

9 (Elmahdi *et al.*, 1997; Kaske *et al.*, 2001; Piccione *et al.*, 2005), daily fluctuations in signals

10 that regulate the expression of these genes within muscle, may differ between species.

11 Nevertheless, the lack of rhythmic metabolic gene expression in sheep skeletal muscle

12 observed here suggests that the relationship between cellular clocks and metabolic function

13 may vary between species, and further work is needed to assess this.

14

15 One of the aims of the current study was to investigate the impact of 4 weeks of phase

16 shifting of the photoperiod on glucose tolerance. Previous studies have shown that disrupting

17 the timing of sleep opportunities, activity and/or food consumption perturbs glucose

18 metabolism in rodents (Barclay *et al.*, 2012; Salgado-Delgado *et al.*, 2013). However, in the

19 current study glucose tolerance and glucose stimulated insulin secretion in the sheep were not

20 altered by RAP. Given that the GTT was conducted 4 days after the last photoperiod shift,

21 short term or transient effects may have been missed. Although glucose tolerance was not

22 impaired after RAP, the rhythm of plasma glucose was altered, with lower night-time

23 circulating glucose concentrations after RAP than in the control photoperiod condition.

24 Furthermore, this effect was maintained for 4 days, with decreased fasting glucose after RAP

25 still evident prior to the IVGTT, 4 days after the final phase-shift. A possible explanation for

1   reduced plasma glucose concentrations after RAP exposure could be altered timing of food

2   consumption. Exposure of pregnant rat dams to a similar RAP protocol led to intermittent

3   grazing rather than consolidated bouts of feeding during the dark period (Varcoe *et al.*, 2013).

4   While we know that the ewes in the current study did not alter their total food consumption

5   relative to body weight in response to RAP, we do not know at what time of day the food was

6   consumed. Changes to food consumption may also underlie the changing insulin profile

7   observed following RAP. Under control conditions, insulin levels were constant across 24

8   hours, whereas following RAP, insulin concentrations are elevated during the light period. It

9   would be interesting to continue the RAP exposure for longer to determine if these changes in

10   plasma glucose and insulin profiles were maintained and if they led to an altered metabolic

11   phenotype.

12

13   Due to the within-subject design of the current study we were able to compare measures of

14   circadian rhythmicity and glucose homeostasis in each sheep in response to the changing

15   conditions. While this approach reduced the variability and hence increased the power of the

16   analyses, the experimental design may have introduced a confounding variable. Between the

17   control and post intervention periods the sheep gained on average 6.8 kg or approximately

18   8% of their body weight, likely due to *ad libitum* access to food and limited capacity for

19   exercise (Sebert *et al.*, 2010). While the rate of weight gain was not different under the

20   control or RAP conditions, this may have affected glucose homeostasis. Future studies should

21   either adjust the amount of food offered daily to maintain a constant body weight or include a

22   second group of ewes maintained upon the control photoperiod throughout the study

23   duration.

24

1   Finally, the experimental design utilised here was aimed at causing a significant level of

2   circadian rhythm disruption. Obviously animal models do not recreate all of the human

3   aspects of shift work. Indeed, there is no such thing as a standard shift roster in use, with the

4   types of shifts, durations of work period, number of rest days, etc. varying significantly

5   across countries, industries and even workplaces. Furthermore, the individual behavioural

6   responses to identical shift schedules vary considerably (Gamble *et al.*, 2011). Nevertheless,

7   models of shift work can be designed to replicate the disruption to circadian rhythms that

8   occur in shift workers by manipulating the timing of light exposure, food consumption and

9   activity. Different approaches have been used previously in animals to test the effects of

10   circadian disruption on physiological systems with varying degrees of relevance to shift

11   work. In our experiment, sheep were exposed to a complete reversal of the photoperiod twice

12   each week, with 2 periods of 24 hours of light. There is no such thing as a "standard shift

13   roster", but 12 hour day/night shifts are widely used in many industries (Ferguson & Dawson,

14   2012). Furthermore, a recent study found that 25% of nurses engaged in shift work extend the

15   period of wakefulness (and hence light exposure) for 24 hours during each shift transition

16   (Gamble *et al.*, 2011). We therefore believe that this experiment is a necessary early step

17   towards a mechanistic understanding of the systemic, cellular and molecular changes

18   experienced by shift workers.

19

20   In conclusion, exposure of ewes to a rapidly alternating photoperiod for 4 weeks disrupted

21   central and peripheral rhythmicity, and plasma glucose, but not glucose tolerance or glucose

22   stimulated insulin secretion. Through repeat *semimembranous* muscle biopsies, we have

23   demonstrated rhythmic core clock gene expression in ovine skeletal muscle, consistent with

24   the existence of a functioning transcription-translation feedback loop that responds to

25   changing photoperiods by phase shifting the profile of expression. Interestingly, we found

1 that sheep do not rhythmically express key metabolic genes that have been implicated in

2 responding to and participating in cellular rhythmicity, leading us to question the relationship

3 between the circadian timing system and cellular metabolism in this species. Finally, this

4 study has contributed towards a greater understanding of the physiological responses to

5 circadian rhythm disruption using an alternative animal model.

6

7

8 **COMPETING INTERESTS**

9 No conflicts of interest, financial or otherwise, are declared by the authors.

10

11 **AUTHOR CONTRIBUTIONS**

12 Contribution of each author was as follows:

13   1. Conception and design of the experiments (TJV, KLG, DJK)

14   2. Collection, analysis and interpretation of data (TJV, KLG, AV, MDS, MJB, LR, DJK)

15   3. Drafting the article or revising it critically for important intellectual content (TJV,

16   KLG, DJK)

17

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22
23

REFERENCES

Akashi M, Soma H, Yamamoto T, Tsugitomi A, Yamashita S, Yamamoto T, Nishida E, Yasuda A, Liao JK & Node K (2010). Noninvasive method for assessing the human circadian clock using hair follicle cells. *Proc Natl Acad Sci USA* **107,** 15643-15648.

Al-Naimi S, Hampton SM, Richard P, Tzung C & Morgan LM (2004). Postprandial metabolic profiles following meals and snacks eaten during simulated night and day shift work. *Chronobiol Int* **21,** 937-947.

Andersson H, Johnston JD, Messager S, Hazlerigg D & Lincoln G (2005). Photoperiod regulates clock gene rhythms in the ovine liver. *Gen Comp Endocrinol.* **142,** 357-363.

Arble DM, Bass J, Laposky AD, Vitaterna MH & Turek FW (2009). Circadian timing of food intake contributes to weight gain. *Obesity* **17,** 2100-2102.

Australian Bureau of Statistics (2012). Working time arrangements. **6342**.

Barclay JL, Husse J, Bode B, Naujokat N, Meyer-Kovac J, Schmid SM, Lehnert H & Oster H (2012). Circadian desynchrony promotes metabolic disruption in a mouse model of shiftwork. *PLoS One* **7,** e37150.

Beers TM (2000). Flexible Schedules and Shift Work: Replacing the '9-to-5' workday? . *Mon Lab Rev* **123,** 33-40.

Bergman EN (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* **70,** 567-590.

Boden MJ, Varcoe TJ & Kennaway DJ (2013). Circadian regulation of reproduction: From gamete to offspring. *Prog Biophys Mol Biol* **113,** 387-397.

Boivin DB, James FO, Wu A, Cho-Park PF, Xiong H & Sun ZS (2003). Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood* **102,** 4143-4145.

Borjigin J, Wang MM & Snyder SH (1995). Diurnal variation in mRNA encoding serotonin N-acetyltransferase in pineal gland. **378,** 783-785.

Canaple L, Rambaud J, Dkhissi-Benyahya O, Rayet B, Tan NS, Michalik L, Delaunay F, Wahli W & Laudet V (2006). Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock. *Mol Endocrinol* **20,** 1715-1727.

Coomans CP, van den Berg SA, Lucassen EA, Houben T, Pronk AC, van der Spek RD, Kalsbeek A, Biermasz NR, Willems van Dijk K, Romijn JA & Meijer JH (2013). The suprachiasmatic nucleus controls circadian energy metabolism and hepatic insulin sensitivity. *Diabetes* **62,** 1102-1108.

Dardente H, Fustin JM & Hazlerigg DG (2009). Transcriptional feedback loops in the ovine circadian clock. *Comp Biochem Physiol A Mol Integr Physiol* **153,** 391-398.

Earl CR, D'Occhio MJ, Kennaway DJ & Seamark RF (1990). Temporal changes in the pattern of melatonin secretion in sheep held in constant darkness. *J Pineal Res* **8,** 115-121.

Elmahdi B, Sallmann HP, Fuhrmann H, von Engelhardt W & Kaske M (1997). Comparative aspects of glucose tolerance in camels, sheep, and ponies. *Comp Biochem Physiol A Physiol* **118,** 147-151.

Ferguson SA & Dawson D (2012). 12-h or 8-h shifts? It depends. *Sleep Med Rev* **16,** 519-528.

Ferguson SA, Kennaway DJ, Baker A, Lamond N & Dawson D (2012). Sleep and circadian rhythms in mining operators: limited evidence of adaptation to night shifts. *Appl Ergon* **43,** 695-701.

Figueiro MG & White RD (2013). Health consequences of shift work and implications for structural design. *J Perinatol* **33 Suppl 1,** S17-23.

Finck BN & Kelly DP (2006). PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* **116,** 615-622.

Fulkerson WJ & Tang BY (1979). Ultradian and circadian rhythms in the plasma concentration of cortisol in sheep. *J Endocrinol* **81,** 135-141.

Gamble KL, Motsinger-Reif AA, Hida A, Borsetti HM, Servick SV, Ciarleglio CM, Robbins S, Hicks J, Carver K, Hamilton N, Wells N, Summar ML, McMahon DG & Johnson CH (2011). Shift work in nurses: contribution of phenotypes and genotypes to adaptation. *PLoS One* **6,** e18395.

Gatford KL, De Blasio MJ, Thavaneswaran P, Robinson JS, McMillen IC & Owens JA (2004). Postnatal ontogeny of glucose homeostasis and insulin action in sheep. *Am J Physiol Endocrinol Metab* **286,** E1050-1059.

Gervois P, Chopin-Delannoy S, Fadel A, Dubois G, Kosykh V, Fruchart JC, Najib J, Laudet V & Staels B (1999). Fibrates increase human REV-ERBalpha expression in liver via a novel peroxisome proliferator-activated receptor response element. *Mol Endocrinol* **13,** 400-409.

Gomez-Abellan P, Diez-Noguera A, Madrid JA, Lujan JA, Ordovas JM & Garaulet M (2012). Glucocorticoids affect 24 h clock genes expression in human adipose tissue explant cultures. *PLoS One* **7,** e50435.

Grundy A, Tranmer J, Richardson H, Graham CH & Aronson KJ (2011). The influence of light at night exposure on melatonin levels among Canadian rotating shift nurses. *Cancer Epidemiol Biomarkers Prev* **20,** 2404-2412.

Guillaumond F, Dardente H, Giguere V & Cermakian N (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* **20,** 391-403.

Hampton SM, Morgan LM, Lawrence N, Anastasiadou T, Norris F, Deacon S, Ribeiro D & Arendt J (1996). Postprandial hormone and metabolic responses in simulated shift work. *J Endocrinol* **151,** 259-267.

Illnerova H & Vanecek J (1987). Entrainment of the circadian rhythm in the rat pineal N-acetyltransferase activity by prolonged periods of light. *Journal of Comparative Physiology A* **161,** 495-510.

Jaquiery AL, Oliver MH, Landon-Lane N, Matthews SJ, Harding JE & Bloomfield FH (2013). Unpredictable feeding impairs glucose tolerance in growing lambs. *PLoS One* **8,** e61040.

Karlsson B, Alfredsson L, Knutsson A, Andersson E & Toren K (2005). Total mortality and cause-specific mortality of Swedish shift- and dayworkers in the pulp and paper industry in 1952-2001. *Scand J Work Environ Health* **31,** 30-35.

Kaske M, Elmahdi B, von Engelhardt W & Sallmann HP (2001). Insulin responsiveness of sheep, ponies, miniature pigs and camels: results of hyperinsulinemic clamps using porcine insulin. *J Comp Physiol B* **171,** 549-556.

Kennaway DJ (1994). Effect of a phase advance of the light/dark cycle on pineal function and circadian running activity in individual rats. *Brain Res Bull* **33,** 639-644.

Kennaway DJ, Gilmore TA & Seamark RF (1982). Effects of melatonin implants on the circadian rhythm of plasma melatonin and prolactin in sheep. *Endocrinology* **110,** 2186-2188.

Kennaway DJ & Rowe SA (2000). Effect of stimulation of endogenous melatonin secretion during constant light exposure on 6-sulphatoxymelatonin rhythmicity in rats. *J Pineal Res* **28,** 16-25.

Kennaway DJ, Varcoe TJ, Voultsios A & Boden MJ (2013). Global loss of bmal1 expression alters adipose tissue hormones, gene expression and glucose metabolism. *PLoS One* **8,** e65255.

Kennaway DJ, Voultsios A, Varcoe TJ & Moyer RW (2003). Melatonin and activity rhythm responses to light pulses in mice with the Clock mutation. *Am J Physiol* **284,** R1231-1240.

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM & Baler R (1997). The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent Progress in Hormone Research* **52,** 307-357.

Lemberger T, Saladin R, Vazquez M, Assimacopoulos F, Staels B, Desvergne B, Wahli W & Auwerx J (1996). Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm. *J Biol Chem* **271,** 1764-1769.

Lemberger T, Staels B, Saladin R, Desvergne B, Auwerx J & Wahli W (1994). Regulation of the peroxisome proliferator-activated receptor alpha gene by glucocorticoids. *J Biol Chem* **269,** 24527-24530.

Li S, Chen XW, Yu L, Saltiel AR & Lin JD (2011). Circadian metabolic regulation through crosstalk between casein kinase 1delta and transcriptional coactivator PGC-1alpha. *Mol Endocrinol* **25,** 2084-2093.

Lincoln G, Messager S, Andersson H & Hazlerigg D (2002). Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: evidence for an internal coincidence timer. *Proc Natl Acad Sci USA* **99,** 13890-13895.

Liu C, Li S, Liu T, Borjigin J & Lin JD (2007). Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature* **447,** 477-481.

Liu T & Borjigin J (2005). Reentrainment of the circadian pacemaker through three distinct stages. *Journal of Biological Rhythms* **20,** 441-450.

Lowden A, Moreno C, Holmback U, Lennernas M & Tucker P (2010). Eating and shift work - effects on habits, metabolism and performance. *Scand J Work Environ Health* **36,** 150-162.

Martin AM, Elliott JA, Duffy P, Blake CM, Ben Attia S, Katz LM, Browne JA, Gath V, McGivney BA, Hill EW & Murphy BA (2010). Circadian regulation of locomotor activity and skeletal muscle gene expression in the horse. *J Appl Physiol (1985)* **109,** 1328-1336.

McPherson M, Janssen I, Grundy A, Tranmer J, Richardson H & Aronson KJ (2011). Physical activity, sedentary behavior, and melatonin among rotating shift nurses. *J Occup Environ Med* **53,** 716-721.

Mendel VE & Raghavan GV (1964). A study of diurnal temperature patterns in sheep. *J Physiol* **174,** 206-216.

Mongrain V, Ruan X, Dardente H, Fortier EE & Cermakian N (2008). Clock-dependent and independent transcriptional control of the two isoforms from the mouse rorgamma gene. *Genes Cells* **13,** 1197-1210.

Morris CJ, Yang JN & Scheer FA (2012). The impact of the circadian timing system on cardiovascular and metabolic function. *Prog Brain Res* **199,** 337-358.

Muhlhausler BS, Duffield JA & McMillen IC (2007). Increased maternal nutrition stimulates peroxisome proliferator activated receptor-gamma, adiponectin, and leptin messenger ribonucleic acid expression in adipose tissue before birth. *Endocrinology* **148,** 878-885.

Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP & Sassone-Corsi P (2008a). The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* **134,** 329-340.

Nakahata Y, Sahar S, Astarita G, Kaluzova M & Sassone-Corsi P (2009). Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. *Science* **324,** 654-657.

Nakahata Y, Yoshida M, Takano A, Soma H, Yamamoto T, Yasuda A, Nakatsu T & Takumi T (2008b). A direct repeat of E-box-like elements is required for cell-autonomous circadian rhythm of clock genes. *BMC. Mol Biol* **9,** 1.

National Health and Medical Research Council (2013). Australian code of practice for the care and use of animals for scientific practice, 8th edition.**,** 96.

Oishi K, Shirai H & Ishida N (2005). CLOCK is involved in the circadian transactivation of peroxisome-proliferator-activated receptor alpha (PPARalpha) in mice. *Biochem J* **386,** 575-581.

Oster H, Damerow S, Hut RA & Eichele G (2006). Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. *J Biol Rhythms* **21,** 350-361.

Otway DT, Mantele S, Bretschneider S, Wright J, Trayhurn P, Skene DJ, Robertson MD & Johnston JD (2011). Rhythmic diurnal gene expression in human adipose tissue from individuals who are lean, overweight, and type 2 diabetic. *Diabetes* **60,** 1577-1581.

Pan A, Schernhammer ES, Sun Q & Hu FB (2011). Rotating night shift work and risk of type 2 diabetes: two prospective cohort studies in women. *PLoS Med* **8,** e1001141.

Patel DD, Knight BL, Wiggins D, Humphreys SM & Gibbons GF (2001). Disturbances in the normal regulation of SREBP-sensitive genes in PPAR alpha-deficient mice. *J Lipid Res* **42,** 328-337.

Penning PD, Rook AJ & Orr RJ (1991). Patterns of ingestive behavior of sheep continuously stocked on monocultures of ryegrass or white clover. *Applied Animal Behaviour Science* **31,** 237-250.

Piccione G, Caola G & Refinetti R (2005). Temporal relationships of 21 physiological variables in horse and sheep. *Comp Biochem Physiol A Mol Integr Physiol* **142,** 389-396.

Piccione G, Gianesella M, Morgante M & Refinetti R (2013). Daily rhythmicity of core and surface temperatures of sheep kept under thermoneutrality or in the cold. *Res Vet Sci* **95,** 261-265.

Polidarova L, Sladek M, Novakova M, Parkanova D & Sumova A (2013). Increased sensitivity of the circadian system to temporal changes in the feeding regime of spontaneously hypertensive rats - a potential role for Bmal2 in the liver. *PLoS One* **8,** e75690.

Ramsey KM, Yoshino J, Brace CS, Abrassart D, Kobayashi Y, Marcheva B, Hong HK, Chong JL, Buhr ED, Lee C, Takahashi JS, Imai S & Bass J (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science* **324,** 651-654.

Salgado-Delgado R, Angeles-Castellanos M, Saderi N, Buijs RM & Escobar C (2010). Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work. *Endocrinology* **151,** 1019-1029.

Salgado-Delgado RC, Saderi N, Basualdo Mdel C, Guerrero-Vargas NN, Escobar C & Buijs RM (2013). Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats. *PLoS One* **8,** e60052.

Scheer FA, Hilton MF, Mantzoros CS & Shea SA (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci USA* **106,** 4453-4458.

Sebert SP, Hyatt MA, Chan LL, Yiallourides M, Fainberg HP, Patel N, Sharkey D, Stephenson T, Rhind SM, Bell RC, Budge H, Gardner DS & Symonds ME (2010). Influence of prenatal nutrition and obesity on tissue specific fat mass and obesity-associated (FTO) gene expression. *Reproduction* **139,** 265-274.

Simonetta G, Walker DW & McMillen IC (1991). Effect of feeding on the diurnal rhythm of plasma cortisol and adrenocorticotrophic hormone concentrations in the pregnant ewe and sheep fetus. *Exp Physiol* **76,** 219-229.

Stehle JH, von Gall C, Schomerus C & Korf HW (2001). Of rodents and ungulates and melatonin: creating a uniform code for darkness by different signaling mechanisms. *Journal of Biological Rhythms* **16,** 312-325.

Steineger HH, Sorensen HN, Tugwood JD, Skrede S, Spydevold O & Gautvik KM (1994). Dexamethasone and insulin demonstrate marked and opposite regulation of the steady-state mRNA level of the peroxisomal proliferator-activated receptor (PPAR) in hepatic cells. Hormonal modulation of fatty-acid-induced transcription. *Eur J Biochem* **225,** 967-974.

Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS & Bass J (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* **308,** 1043-1045.

Um JH, Pendergast JS, Springer DA, Foretz M, Viollet B, Brown A, Kim MK, Yamazaki S & Chung JH (2011). AMPK regulates circadian rhythms in a tissue- and isoform-specific manner. *PLoS One* **6,** e18450.

Varcoe TJ, Boden MJ, Voultsios A, Salkeld MD, Rattanatray L & Kennaway DJ (2013). Characterisation of the maternal response to chronic phase shifts during gestation in the rat: implications for fetal metabolic programming. *PLoS One* **8,** e53800.

Varcoe TJ & Kennaway DJ (2008). Activation of 5-HT2C receptors acutely induces Per1 gene expression in the rat SCN in vitro. *Brain Res* **1209,** 19-28.

Voultsios A, Kennaway DJ & Dawson D (1997). Salivary melatonin as a circadian phase marker: Validation and comparison with plasma melatonin. *J Biol Rhythms* **12,** 457-466.

Wang XS, Travis RC, Reeves G, Green J, Allen NE, Key TJ, Roddam AW & Beral V (2012). Characteristics of the Million Women Study participants who have and have not worked at night. *Scand J Work Environ Health* **38,** 590-599.

Watanabe M, Akamatsu Y, Furui H, Tomita T, Watanabe T & Kobayashi F (2004). Effects of changing shift schedules from a full-day to a half-day shift before a night shift on physical activities and sleep patterns of single nurses and married nurses with children. *Ind Health* **42,** 34-40.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M & Tei H (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288,** 682-685.

Zambon AC, McDearmon EL, Salomonis N, Vranizan KM, Johansen KL, Adey D, Takahashi JS, Schambelan M & Conklin BR (2003). Time- and exercise-dependent gene regulation in human skeletal muscle. *Genome Biol* **4,** R61.

1   Table 1 Primers used for quantitative RT-PCR analysis.

Primer Name	Accession Number	Forward sequence 5' to 3'	Reverse sequence 5' to 3'
RPLP0	AF013214	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA
Bmal1	NM_001129734	ACAGGATAAGAGGGTCATCGC	CCCTCCATTTAAAATCTTCTTGCC
Clock	NM_001130932	CAGTCAGTCTCAAGGAAGCG	CTTTGTTGGTGTAGAGGAAGGG
Per1	AF044911	ACAACCCTTCTACCAGTGGC	TGAGCTCTCGCAGTGCC
Per2	AY253914	AGTGTGAAGCAAGTGAAAGCC	TGGGTGCCGCAAGGG
Per3	XM_004014022	GTTACCAAGCTCCTCAAATCCC	GGTCCTGAGGTAGGTAACCC
Cry1	NM_001129735	GCATTTGGAAAGAAAAGCTTGGG	GACATGACAAACAACCAAATCGG
Cry2	NM_001129736	GAGGCTGTTCAAGGAATGGG	CCCGTTCTTTCCCAAAGGG
Dec1	NM_001129741	AAGCAAGAATCCGAAGAACCC	AGGTCAGTGCTAGTGAAAGGG
Nr1d1 (Rev-erbα)	NM_001131029	TAACAACAACACAGGTGGCG	ACTGTACAGGGATTCAGGGC
Nr1d2 (Rev-erbβ)	XM_004021888	GACTTTTGAGGTTTTAATGGTACGG	TCAAACATAGAGTTTAGCAGATCCC
Nr1f1 (RORα)	XM_004011005	TACCTGGACATCCAGCCTTC	GGAAGTCTCTCCGTTGGTGA
Nr1f3 (RORγ)	XM_004003638	CCTCGTGCTCATCAATGCC	TGCAGAGATGATGATGAAAGGC
Pparα	XM_004007050.1	CGTGTGAACATGACCTAGAAG	ACGAAGGGCGGATTGTTG
Pgc1α	XM_004009738.1	TCTGGAACTGCAGGCCTAACTC	GCAAGAGGGCTTCAGCTTTG
Nampt	XM_004007842.1	GTTCCTGAGGGCTCTGTCAT	TTTGTGGCCACTGTGATTGG

2

3

1   Table 2 Weight gain and food consumption of sheep during control and RAP conditions.

	Control	CPS
Body weight (kg)	80.3 ± 1.7	87.1 ± 1.3***
Daily weight gain (g)	170 ± 52	189 ± 23
Chaff (g/day)	1363 ± 134	1444 ± 101
Chaff (g/day/kg b.w.)	17.0 ± 1.6	17.7 ± 1.7
Nuts (g/day)	368 ± 67	456 ± 33*
Nuts (g/day/kg b.w.)	4.6 ± 0.8	5.6 ± 0.5
Values are mean ± SE $* P < 0.5$, $***P < 0.001$ relative to control condition.		

2
3

1   Fig.1 Timeline and design of the experiment. (A) From arrival into the animal facility until

2   week 4, ewes (n = 7) were exposed to a 12L:12D photoperiod with lights on at 1000h for 3

3   weeks. Blood sampling and muscle biopsies were collected and glucose tolerance tests

4   performed. Thereafter, the photoperiod was reversed every Thursday through to Sunday for 4

5   weeks and the blood sampling, biopsies and tolerance test repeated. (B) The pattern of

6   light/dark and the timing of the blood collections (arrows) and muscle biopsies (triangles) are

7   shown for the control and Chronic Phase Shifting (RAP) conditions. Numbers above each

8   arrow represent the number of hours since the last photoperiod shift. GTT- Glucose

9   Tolerance Test

10

11 Fig.2 Plasma melatonin (A), glucose (B) and insulin (C) in sheep exposed to a control

12 photoperiod (open circles) and after chronic phase shifts 12-72 hours following the last

13 photoperiod shift (closed squares), with the x axis of each figure displaying the zeitgeber time

14 (ZT), and the time in hours since the last phase shift across the bottom panel. The data are the

15 Mean +/- SEM; (n = 7). Periods of darkness are indicated by shading. Significance is

16 indicated by *, $P < 0.05$ compared to control at same ZT, and #, $P < 0.05$ within RAP group

17 compared to same ZT in last 24 h of collections (48-72 h after last phase shift).

18

19 Fig.3 Clock and metabolic gene mRNA expression in sheep muscle biopsies collected under

20 control conditions (open circles) and RAP conditions, 48-66 hours after the last shift

21 photoperiod shift (closed squares). The data are the Mean +/- SEM; (n = 7). ZT24 data are the

22 ZT0 data re-plotted, periods of darkness indicated by shading, * $P < 0.05$

23

24 Fig. 4 The glucose and insulin levels before and after the intra venous administration of

25 glucose (0.25 g/kg) in sheep during the control photoperiod (open circles) or following 4

1   weeks of chronic phase shifts (closed squares). The data are the Mean +/- SEM; (n = 7). * P <

2   0.05.

3

4   Fig. 5 Core body temperature changes (continuous line) in a representative sheep during the

5   first week (top panel) and fourth week of phase shifting (bottom panel). The grey areas show

6   the average core body temperature of the sheep under the control 12L:12D photoperiod.

7   Periods of darkness are indicated across the bottom of each figure.

8