Gestational Chronodisruption Impairs Circadian Physiology in Rat Male Offspring, Increasing the Risk of Chronic Disease

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Chronic exposure to light at night, as in shift work, alters biological clocks (chronodisruption), negatively impacting pregnancy outcome in humans. Actually the interaction of maternal and fetal circadian systems could be a key factor determining a fitting health in adults. We propose that chronic photoperiod shift (CPS) during pregnancy alter maternal circadian rhythms and impair circadian physiology in the adult offspring, increasing health risks. Pregnant rats were exposed to normal photoperiod (12 h light, 12 h dark) or to CPS until 85% of gestation. The effects of gestational CPS were evaluated on the mother and adult offspring. In the mother we measured rhythms of heart rate, body temperature, and activity through gestation and daily rhythms of plasma variables (melatonin, corticosterone, aldosterone, and markers of renal function) at 18 days of gestation. In adult offspring, we measured rhythms of the clock gene expression in the suprachiasmatic nucleus (SCN), locomotor activity, body temperature, heart rate, blood pressure, plasma variables, glucose tolerance, and corticosterone response to ACTH. CPS altered all maternal circadian rhythms, lengthened gestation, and increased newborn weight. The adult CPS offspring presented normal rhythms of clock gene expression in the SCN, locomotor activity, and body temperature. However, the daily rhythm of plasma melatonin was absent, and corticosterone, aldosterone, renal markers, blood pressure, and heart rate rhythms were altered. Moreover, CPS offspring presented decreased glucose tolerance and an abnormal corticosterone response to ACTH. Altogether these data show that gestational CPS induced long-term effects on the offspring circadian system, wherein a normal SCN coexists with altered endocrine, cardiovascular, and metabolic function. **(*Endocrinology* 157: 4654–4668, 2016)**

The most recent evolutionary challenge being faced by the human population worldwide is chronic exposure to artificial light at night as a result of our modern life (1). It is well established that light at night induces an alteration in our biological clocks, known as chronodisruption; however, the consequences of this insult are poorly understood. The internal temporal order of physiological systems depends on circadian biological clocks regulating a multitude of physiological functions, such as blood pressure (BP), metabolism, the immune response, sleep-wake cycles, adrenal response, and renal function (2). In fact, the circadian system acts as a supraphysiological system that coordinates a myriad of functions on a 24-hour basis, creating an internal temporal order. Actually it is clear that the proper organization of circadian rhythms is key for the normal function of several physiological systems in the adult (3–5). Chronodisruption caused by shift work is associated with a number of metabolic disturbances (meta-

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Abbreviations: BP, blood pressure; BUN, blood urea nitrogen; CPS, chronic phase shift; HDL, high-density lipoprotein; LD, light/dark photoperiod; RT-qPCR, quantitative real-time PCR; VLDL, very low-density lipoprotein.

bolic syndrome, obesity, and diabetes) and lower plasma melatonin levels, during night work and daytime sleep, when compared with daytime workers (1). In this context, an increased risk of miscarriage, preterm delivery, and low birth weight have been reported in shift-worker women (6–9), supporting that exposure to chronodisruption has negative effects in human pregnancy. Therefore, maternal chronodisruption could persistently alter different physiological responses in her offspring, likewise extensively reported in human and animal models subjected to the developmental origin of health and disease studying prenatal insults such as the alteration of food consumption, oxygen availability, or stress (10–14).

In the rat, experimental manipulation of the photoperiod to simulated shift work by exposure to chronic phase shifts (perinatal CPS) during pregnancy and early postpartum altered maternal rhythms of corticosterone, leptin, glucose, insulin, free fatty acids, triglycerides, and cholesterol concentrations as well as the expression of gluconeogenic and circadian clock genes in the maternal liver at 20 days of gestation. In the fetus, it disrupted the timing of clock gene expression in the fetal liver, supporting a specific alteration in liver circadian metabolism (15). Using a rat model of gestational chronodisruption by maternal exposure to constant light (from 50% gestation to delivery), we and others demonstrated that this treatment changes fetal growth trajectory (16), fetal physiology (16–18), and importantly the expression of clock genes in the fetal adrenal gland (16). Both models displayed long-term consequences in the offspring in different species.

Maternal exposure to constant light and to a normal photoperiod from birth resulted in postnatal morning-evening differences in clock gene expression in the adult heart (19), adrenal (16, 20), liver (21), hippocampus (17), and adipose tissue (22). In the rat, CPS exposure during pregnancy and the first week of life resulted in metabolic consequences in the adult offspring, including a decrease in glucose response to insulin and higher plasma insulin and leptin (23). Of note, these animals maintain a normal circadian temperature rhythm (23), suggesting normal function of the master clock, residing in the suprachiasmatic nucleus (SCN) because it is known that this rhythm is directly controlled by the SCN (24). It is unclear whether the effects observed in the offspring were induced during gestation or early in the neonatal development. Nevertheless, given that some fetal peripheral clocks are operating earlier than the fetal SCN (for example, adrenal, liver, and hippocampus (17, 25, 26), it is possible that the SCN is spared after an insult during gestation. In contrast, peripheral clocks already oscillating in fetal life may be impacted. Hypothetically, if it remains altered in the adult, these peripheral clocks could provide a mechanism by which maternal chronodisruption alters metabolic response in the offspring (15).

In the present study, we further investigated the effects of developmental chronodisruption secondary to chronic shifting of the photoperiod, during gestation, on offspring circadian rhythms and its effects on glucose regulation and BP, strong predictors of an increased risk of chronic diseases in adulthood. Our hypothesis is that gestational chronodisruption is translated in the offspring in a misalignment of the circadian system, inducing an anomalous physiological response and increased the risk of chronic diseases during adult life. To test our hypothesis, we used a gestational chronodisruption model (CPS up to 85% of gestation) in the rat. Our specific aims were to investigate the impact of maternal exposure to chronic photoperiod shifting on the following: 1) maternal circadian rhythms of heart rate, body temperature, locomotor activity, endocrine rhythms (melatonin, corticosterone, aldosterone), and markers of renal function; 2) the effect of gestational CPS on these rhythms in the 90-day-old offspring; and 3) the effects on BP and glucose tolerance in the offspring.

Materials and Methods

Animals

Animal handling and care was performed following the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research of the National Research Council. The protocols were approved by the Bioethics Commission from the Universidad Austral de Chile (CBA number 1120938 and ACT-1116).

The animals were maintained in a 12-hour light, 12-hour dark cycle (light on at 700 AM; ~400 lux at the head level) under controlled temperature (18°C-20°C), and food and water was available ad libitum. Sprague Dawley rats (obtained from Charles River Laboratories International Inc) were mated and raised in our animal facility. Timed-pregnant females were used in the study, and the day in which the spermatozoa was observed in the smear of the vaginal contents was considered embryonic day 0 and the pregnant females were randomly allocated to the following two photoperiods. The first was the light/dark (LD) control photoperiod (12 h light, 12 h dark cycle [lights on at 700 AM]). The second photoperiod was the CPS photoperiod, using a similar protocol to that reported by Varcoe et al (23). Briefly, pregnant females were exposed to lighting schedule manipulation every 3–4 days, reversing completely the photoperiod (Figure 1). The photoperiod reversal occurred on the night of day 0 of gestation so that rather than lights going off at 7:00 PM remained on until 7:00 AM of day 2. At 18 days of gestation, the mothers returned to a normal 24-hour photoperiod (12 h light, 12 h dark, lights on at 7:00 AM) and continued in this photoperiod thereafter. Cohorts of the animals were used in three different protocols. Exposure of pregnant females to CPS had no impact on food consumption and maternal weight.

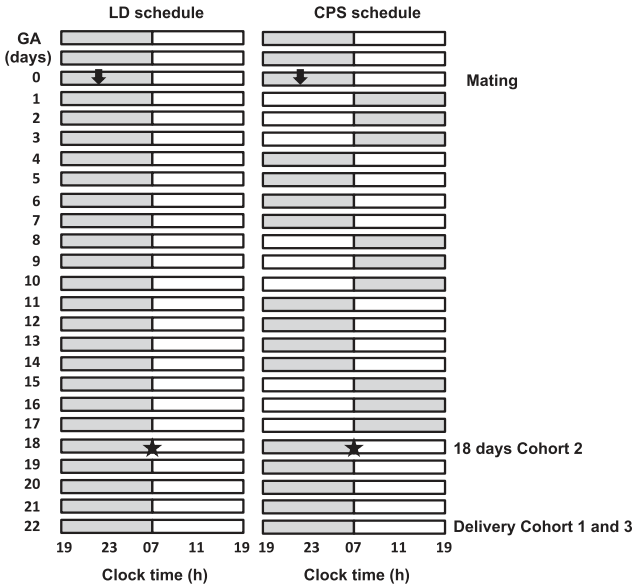

Figure 1. Schematic representation of the photoperiod protocols used throughout gestation. Three cohorts of female rats raised and maintained in photoperiod 12-hour light, 12-hour dark were mated and separated into two groups: 1) LD photoperiod and 2) CPS photoperiod. CPS dams had their photoperiod shifted by 12 hours for three consecutive cycles, after which the dark phase was extended for 12 hours to restore the original photoperiod for a further 4 days. GA, gestational age. Cohort 1 animals were instrumented for telemetric measurement of maternal activity, temperature, and heart rate during pregnancy and delivery. Cohort 2 mothers were euthanized to study plasma maternal circadian rhythms and fetal morphometry at 18 days of pregnancy (indicated by star in the figure). Cohort 3 animal offspring were raised to study the effects of CPS on the adult.

Cohort 1: effect of chronodisruption on activity, temperature, and heart rate during pregnancy

Adult female rats (n = 9) kept under control 12-hour light, 12-hour dark photoperiod (LD) were implanted with telemetric transponders in the abdominal cavity (ER-4000 energizer; Minimitter Co Inc), following the implantation procedures suggested by the manufacturers. Forty-eight hours before the surgery, the animals received a carprofen gel treatment that delivers an effective dose of 5 mg/kg·d (MediGel CPF; ClearH_2O). Transponders were implanted in the abdominal cavity under general anesthesia (isoflurane 2.0%–3.0%), and recordings of body temperature, locomotor activity, and heart rate were started immediately after the surgery. On day 7–10 after surgery, the implanted females were mated and separated into two groups: control (LD; n = 5) and CPS group (n = 4). Females were individually housed and noninvasive recordings were performed in specially conditioned rooms by placing home cages on top of the ER-4000 energizer/receivers. Data were collected at 15-second intervals during pregnancy. When the end of gestation approached, the pregnant females were observed continuously and the hour of delivery was recorded. Twenty-four hours after delivery, the mother and her offspring were euthanized (as described below), the transponder was recovered, newborn weights were recorded, and maternal and newborn tissues were dissected and stored in our tissue bank.

The presence of an implanted transponder did not modify the gestational length, newborn weight, and maternal weight gain in LD or CPS conditions (Supplemental Table 1).

Cohort 2: effect of chronodisruption on plasma maternal circadian rhythms and fetal morphometry at 18 days of gestation

At 18 days of gestation (Figure 1), a parallel cohort of dams from each pregnancy condition (LD and CPS) not subjected to telemetry were euthanized every 4 hours around the clock (n = 5 per clock time; LD, n = 30 mothers; CPS, n = 29; n = 4 at 12:00 AM), starting at 8:00 AM on day 18 and ending at 8:00 AM of day 19 of gestation, as reported previously (25). Briefly, pregnant rats were deeply anesthetized (isoflurane 3.0%–4.0%; Baxter Laboratories), a midline incision was done, and a blood sample was collected from the vena cava, and an overdose of sodium thiopental (150 mg/kg; Vetpharma) was administrated. The fetuses were delivered by hysterotomy. Three fetuses from each litter were preserved in formalin 4% to perform biometric studies. The remaining fetuses were dissected and the fetal organs were collected and stored in our tissue bank.

Cohort 3: effect of maternal chronodisruption on the adult offspring

Immediately after birth, parallel cohorts of dams from each pregnancy condition (LD mothers, n = 27; CPS mothers, n = 30) were kept under standard photoperiod (12 h light, 12 h dark, lights on at 7:00 AM). Pups were weaned at 21 days old, with the males being raised in standard photoperiod (12 h light, 12 h dark, lights on at 7:00 AM) to be studied at 90 days of age. These males were housed in pairs (brothers together in standard cages of 48 × 27 × 20 cm) under the LD cycle and controlled temperature (18°C-20°C), with food and water ad libitum. The studies described below were performed in offspring from different mothers to avoid litter effect.

Telemetric studies

Five males at 75–80 days old, from each pregnancy condition and from different mothers, were implanted with telemetric transponders in the abdominal cavity, as described above, to record body temperature, locomotor activity, and heart rate during 10 days in standard 12-hour LD photoperiod (lights on at 7:00 AM).

Daily rhythms

Males from each pregnancy condition (LD from nine mothers and CPS from 12 mothers) were euthanized every 4 hours around the clock (n = 5 per clock time: LD, n = 30 males; CPS, n = 28 males, n = 4 at 4:00 PM and 12:00 AM), starting at 8:00 AM and ending at 4:00 AM. To avoid litter effects, each clock time point contains animals from different mothers; thus, no siblings were used at the same time point. Briefly, male rats were deeply anesthetized (isoflurane 2.0%–3.0%), a midline incision was done, a blood sample was collected from the vena cava, and next an overdose of sodium thiopental (150 mg/kg) was given at the same site. Organs were collected, weighed, and stored in our tissue bank, and whole brain was stored in RNAlater (Ambion Inc) for SCN dissection. To dissect the SCN, a hypothalamic block that included the SCN was prepared first; then we followed a protocol based in our previous experience (20). We made a transverse cut

just rostral to the optic chiasm and another transverse cut about 1 mm at which the optic chiasm ended. Next, a horizontal cut 0.8–1.2 mm below the third ventricle as seen from the front and two parasagittal cuts lateral to the border of the chiasm completed the SCN block.

Serum was separated and stored at −20°C to measure corticosterone, aldosterone, melatonin, glucose, and renal (creatinine, blood urea nitrogen [BUN], urea, and uric acid) and hepatic markers (C3, C4, albumin, total protein, calcium, cholesterol, high-density lipoprotein [HDL], very low-density lipoprotein [VLDL] and total cholesterol, transaminases, and bilirubin).

RNA extraction and quantitative real-time PCR (RT-qPCR) analysis of SCN

Quantitative RT-qPCR was used to evaluate the mRNA expression of clock genes *Bmal1* and *Per2*. Total RNA was extracted using the SV total RNA isolation system (Promega) according to the manufacturer's instructions. This kit includes a digestion step with deoxyribonuclease to rule out contamination with genomic DNA. About 2.0 μg of total RNA was reversed transcribed using random primers (Promega) and Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp). RT-qPCR was performed using primers described in 2012 by Mendez et al (16) and KAPA SYBR FAST quantitative PCR master mix (Kapa Biosystems, Inc). The quantitative PCR was carried out in a Rotor-Gene Q real-time platform (QIAGEN). A melting curve analysis was performed on each sample after the final cycle to ensure that a single product was obtained. Relative amounts of all mRNAs were calculated by the comparative ΔΔcycle threshold method (27) using the equation $2^{-\Delta\Delta Ct}$. *18S-rRNA* was used as housekeeping gene.

Functional tests

Morning-afternoon adrenal response to ACTH

Male rats at 90- to 100-day-old (10 animals per each pregnancy condition) were tested, five at 8:00 AM and five at 8:00 PM. Two siblings from five CPS or LD mothers were used; a sibling was allocated to the morning experiment and the other to the afternoon experiment. Briefly, at 5:00 AM or 5:00 PM, the animals were anesthetized (isoflurane 2.0%–2.5%), a blood sample (−180 min sample) was collected from the tail vein, and immediately dexamethasone was injected im (Oradexon 300 μg/kg body weight; Novartis Laboratories) to suppress endogenous ACTH. At 8:00 AM or 8:00 PM, ACTH was injected ip (Synacthen depot, 100 μg/kg body weight; Novartis Laboratories), and blood samples were collected from the tail vein at 60 and 120 minutes after ACTH injection. Serum was prepared and stored at −20°C to measure corticosterone. The samples corresponding to the dark period were taken under red light.

Blood pressure

Systolic BP was measured every 4 hours around the clock in individual animals from each pregnancy condition (LD, n = 8; CPS, n = 8; from eight different mothers), using an ultrasonic Doppler flow detector 811-B (PARKS Electronics) placed in the tail. At each clock time, the mean of five readings was obtained. Animals were adapted to the procedure for at least 10–15 minutes over a period of 10 days before the 24-hour measurement.

Intraperitoneal glucose tolerance test

Male rats (n = 5 animals per pregnancy condition from five different mothers) were studied between 90 and 100 days of age. At 8:00 AM after 12 hours of fasting, the animals were anesthetized (isoflurane 2.0%–2.5%) and ip injected with glucose (1g/kg; glucose, Sanderson Laboratories). A blood drop was collected from the tail to measure glucose levels (Accu-Chek; Roche Diagnostics) at −15 and 0 minutes before glucose administration and 30, 60, 90, 120, and 180 minutes after injection.

Plasma determinations

Melatonin

Melatonin concentration in mothers and their male offspring were measured by a double RIA using a commercial kit (melatonin research RIA kit, catalog number 07L119102; MP Biomedical) following the manufacturer's instructions. The inter- and intraassay coefficients were 9.4% and 8.7%, respectively.

Corticosterone

The corticosterone concentration in the mothers and their male offspring were measure by a RIA using a commercial kit (Coat-a-Count rat corticosterone kit; Siemens Healthcare Diagnostics) following the manufacturer's instructions. The inter- and intraassay coefficients were 5.% and 6.3%, respectively.

Aldosterone

The aldosterone concentration in the mothers and their male offspring were measured by a RIA using a commercial kit (Coat-a-Count aldosterone kit, PITKAL-5; Siemens Healthcare Diagnostics) following the manufacturer's instructions. The inter- and intraassay coefficients were 3.9% and 4.1%, respectively.

Renal and hepatic markers

Creatinine, BUN, urea, uric acid, C3, C4, albumin, total protein, calcium, cholesterol (HDL, VLDL, and total), transaminases, and bilirubin were measured in an automatic blood analyzer from Clinica Alemana de Valdivia (Cobas c311 analyzer; Roche Diagnostics), and all the samples were analyzed in duplicate and all the values were in the analytical range allowed by the equipment.

Statistical analysis

Data are expressed as mean ± SEM. Telemetric recordings containing 15-minute data collections from each pregnant female or their male offspring were analyzed by cosinor using the El Temps program (developed by Toni Diez, Universidad de Barcelona, Barcelona, Spain [trial]). This method tests whether the data fit the cosine function with a period of 24 hours as follows: $Vt = M + A \cos 15\ (t\text{-}\phi)$, with Vt being the value of the variable (temperature, activity, or heart rate) at time t; ϕ the acrophase (hour at which the variable reaches the maximum value), M the mesor (average of the variable over 24 h), and A the amplitude (difference between the value of the variable at ϕ and the mesor). Also, the program performs actograms for each animal and each variable. Parameters (mesor amplitude and acrophase) of the cosinor equations fitting 24-hour rhythms ($P < .05$) in individuals were compared by an ANOVA and a Tukey's test.

The 24-hour changes in plasma corticosterone, aldosterone, SCN clock gene expression, melatonin, creatinine, BUN, urea,

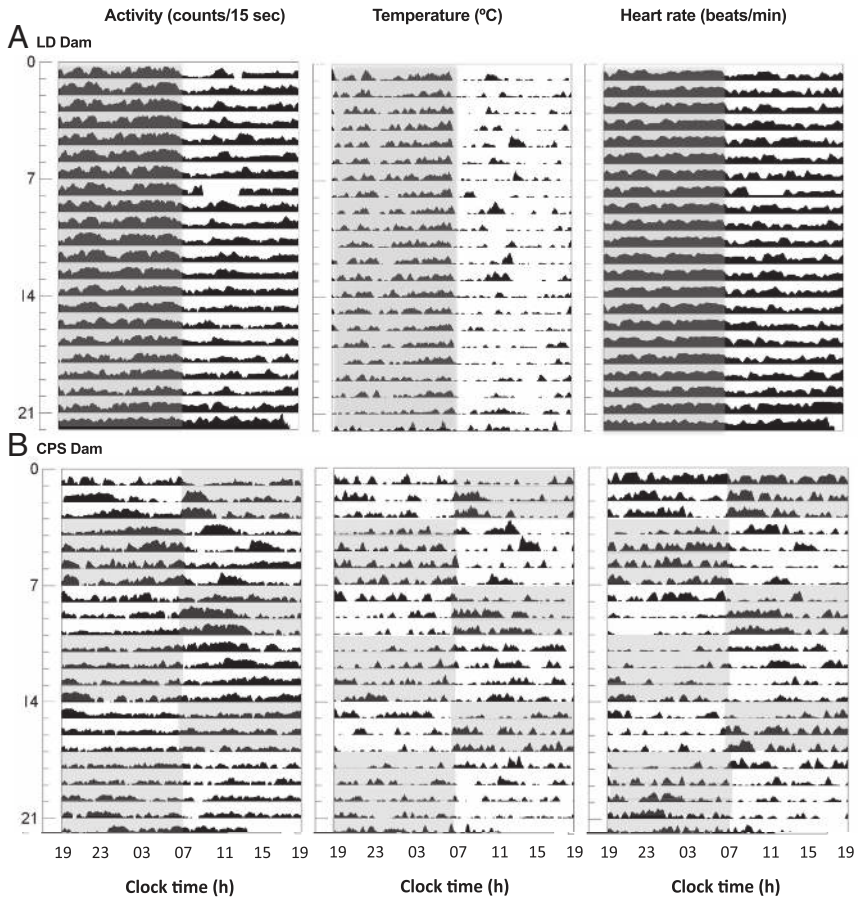

Figure 2. Representative actogram of daily rhythms of activity, temperature, and heart rate throughout pregnancy in a pregnant rat exposed to LD (A) and CPS (B) photoperiod. Adult female rats (n = 9) kept under control LD were implanted with telemetric transponders in the abdominal cavity. Recordings were started 1 week before mating. Data were collected at 15-second intervals until a few hours past birth. Gray bars indicate dark period. Each horizontal line represents a day of recording. Gestational age is indicated on the left side of the figure. Clock time hours are indicated on the bottom of each graph.

uric acid, C3, C4, albumin, total protein, calcium, cholesterol (HDL, VLDL, and total), transaminases, and bilirubin were analyzed by a one-way ANOVA using Newman-Keuls as a post hoc test. Additionally, mean data were fitted to a theoretical cosine function to calculate acrophases. The effect of the treatment in the 24-hour rhythms was analyzed by a two-way ANOVA using a Tukey's multiple comparisons test as a post hoc or an unpaired *t* test.

Statistical analyses were performed using GraphPad Prism version 6.00 for Mac (GraphPad Software, www.graphpad.com. Results were considered significant at $P < .05$.

Results

Impact of CPS photoperiod during pregnancy on maternal circadian rhythms and fetal development

Under LD conditions, pregnant rats displayed circadian oscillations of activity, temperature, and heart rate from the beginning of gestation (Figures 2 and 3 and Table 1). Acrophases of the three rhythms were always positioned in the dark period but advanced as pregnancy progressed (Table 1). Birth occurred at 21.64 ± 0.03 days of gestation (at about 9:00 AM), as indicated in the figures, and it was preceded by an increase in maternal temperature in the previous 24 hours. At 18 days of gestation, pregnant rats maintained in LD condition show plasma circadian rhythms in hormones (corticosterone, aldosterone, melatonin), glucose, and renal function markers (creatinine, BUN, urea, and uric acid; Figure 4).

Chronodisruption by exposure to CPS during gestation had profound effects on the maternal circadian physiology. CPS abolished circadian rhythms of activity, temperature, and heart rate, measured continuously during pregnancy, and altered hormonal and metabolic rhythms measured at 18 days of gestation, impacting fetal development, pregnancy duration, and newborn weight.

As shown in Figure 2 (representative actograms) and Figure 3, CPS had an immediate effect on the heart rate rhythm, whereas the circadian rhythms for temperature and activity disappeared at the end of the first week of gestation (Figure 3). As observed in the figures, CPS mothers had increased activity, temperature, and heart rate in the dark period, but the pattern did not adjust to 24 hours. As in control dams, temperature increased about 24 hours before birth. CPS had two notorious effects; one of them was lengthening of gestation by almost 12 hours, thus shifting birth clock time hours to 20.9 (at 22.08 d of gestation; Table 2). A second effect, most likely related to the previous one, was that CPS newborns were heavier at birth than LD newborns (Table 2). The effect of CPS on maternal weight gain and newborn weight were confirmed in animals of cohort 3, not subjected to telemetry (Supplemental Table 1).

In addition, CPS altered the maternal daily rhythms of plasma variables as well as fetal development. The 24-hour measurements done at 18 days of gestation show that CPS had no effect on total concentrations measured as the 24-hour means of corticosterone and aldosterone; however, the phase of these rhythms was shifted, such as the pattern at 24 hours was the opposite pattern to that in LD

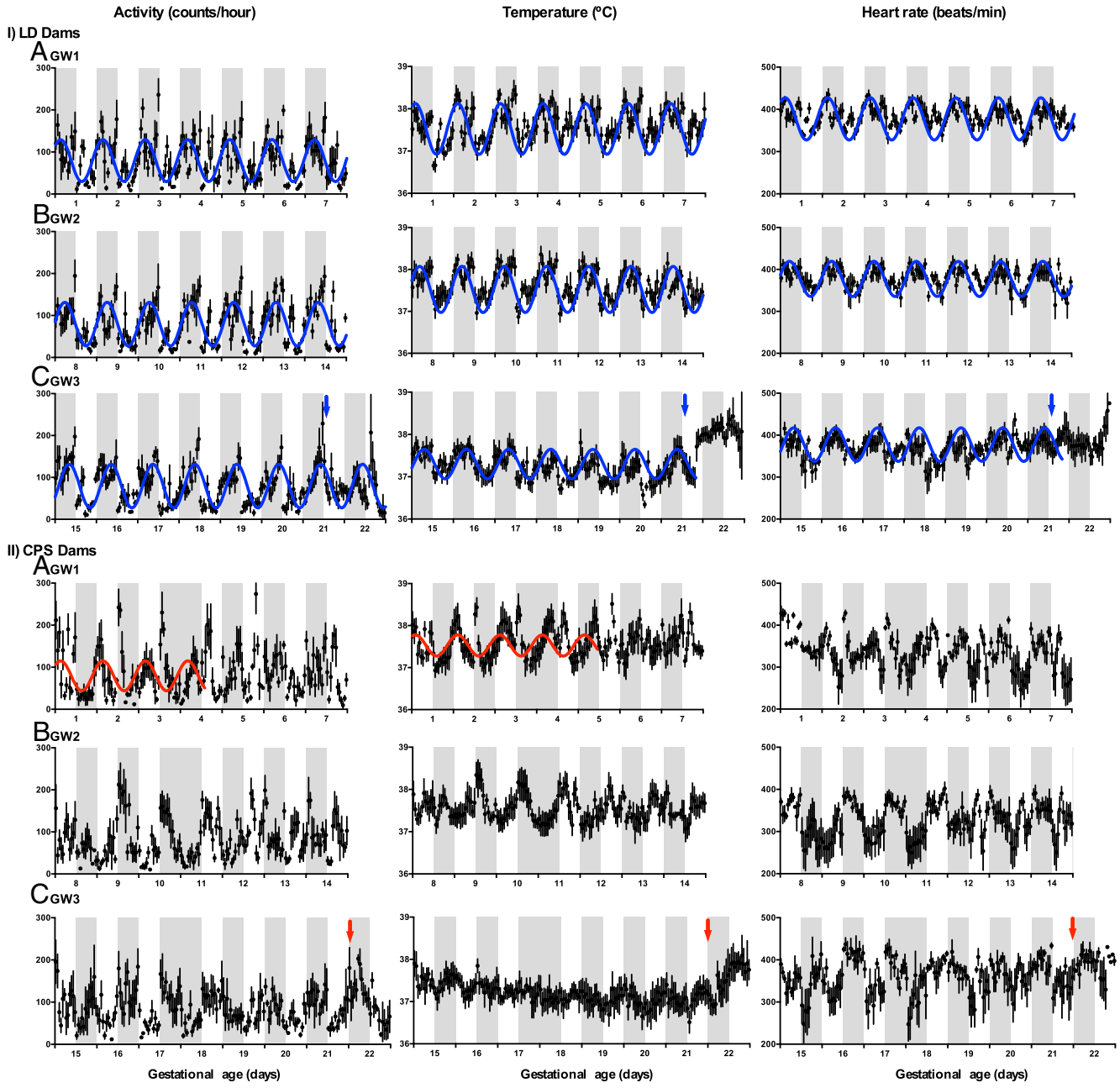

Figure 3. Effect of CPS on the daily maternal activity, temperature, and heart rate rhythms through weeks 1–3 of gestation (GW 1–3). Mean ± SEM activity (counts per hour), temperature (°C), and heart rate (beats per minute) were measured at 1-minute intervals by telemetry from day 0 until 24 hours past birth. LD, mothers maintained in 12-hour light, 12-hour dark (n = 5); CPS, mothers under chronic photoperiod shift (n = 4). Shaded bars represent the hours of lights off. The blue and red lines represent the theoretical 24-hour cosinor function fitting the data in the LD and CPS dams. The arrows indicate the hour of birth.

pregnant females (Figure 4, A and B). A similar occurrence was observed for the glucose circadian rhythms (Figure 4C), which presented a slight increase in the 24-hour mean (CPS: 128.4 ± 3.53 mg/dL vs LD: 119.9 ± 3.4 mg/dL; $P <$ .05, Student's *t* test). In contrast, there was a significant decrease in the total melatonin concentration as the 24-hour mean (CPS: 68.7 ± 10.8 pg/mL vs LD: 96.9 ± 8.9 pg/mL; $P <$ .05, Student's t test), although a circadian rhythm was still present in CPS dams, with an acrophase in the dark period, as found in pregnant females kept in the LD condition (Figure 4D). Moreover, CPS treatment induced a profound change in the daily rhythm of plasma variables associated with renal function: creatinine, BUN, and urea (Figure 4, E–G). Creatinine shows an increase in the 24-hour mean (CPS: 0.289 ± 0.021 mg/dL vs LD: 0.246 ± 0.007 mg/dL; $P <$ .05, Student's *t* test) and an increase in the amplitude of the 24-hour rhythm (Figure 4E), whereas the BUN and urea 24-hour rhythms show an opposite acrophase

Table 1. Effect of Gestation on the Acrophase (Clock Time Hours) of Maternal Circadian Rhythms Measured Telemetrically (Mean ± SEM) in LD Condition

	Nonpregnant (n = 10)	Pregnant (n = 5)		
		GW 1	GW2	GW3
Activity	0.54 ± 0.21	0.58 ± 0.37	1.25 ± 0.44	3.85 ± 0.48[a]
Temperature, °C	23.49 ± 0.13	23.60 ± 0.27	0.42 ± 0.51	2.75 ± 0.36[a]
Heart rate, beats/min	24.01 ± 0.16	23.44 ± 0.28	0.77 ± 0.41	3.11 ± 0.42[a]

Abbreviation: GW, gestational week.

[a] Different from first week of gestation ($P < 0.05$, two way ANOVA).

to that of control animals (Figure 4, F and G) while maintaining the 24-hour mean concentration.

CPS affected neither the daily rhythm nor the 24-hour mean of ureic acid (Figure 4H). The other metabolic variables measured, C3, C4, albumin, total protein, calcium, cholesterol (HDL. VLDL, and total), transaminases, and bilirubin did not present daily rhythm in the LD condition and were not affected by CPS (Supplemental Table 2). As shown in Table 3, maternal CPS exposure affected fetal development as indicated by a lower placental and fetal weight at 18 days of gestation (Table 3). The latter is contrary to the previous observation that CPS newborns are heavier than LD newborns at birth (Table 2). An explanation of this apparent discrepancy may reside in that CPS treatment extended pregnancy duration, allowing for more time for fetal growth.

Thus, gestational chronodisruption by exposure to CPS altered the maternal circadian physiology, impacting fetal

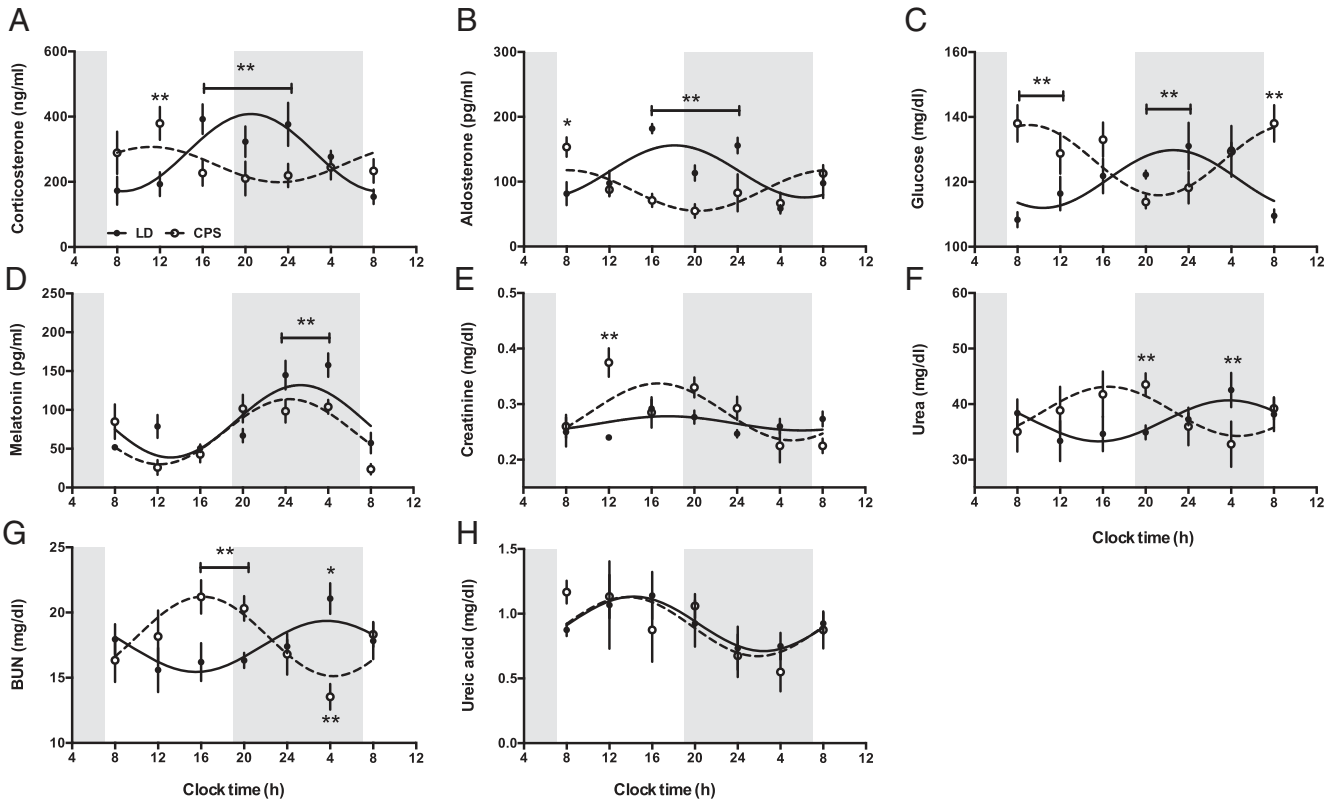

Figure 4. Effect CPS on 24-hour daily rhythms of maternal corticosterone, aldosterone, melatonin, glucose, and renal markers (creatinine, urea, BUN, and uric acid) at day 18 of gestation. Plasma was collected every 4 hours around the clock (n = 5/clock time; LD, n = 30 mothers, CPS, n = 29; at 12;00 PM, n = 4), starting at 8:00 PM on day 18 and ending at 8:00 PM of day 19 of gestation. Data are means ± SEM. Solid and dotted lines represent the theoretical 24-hour cosinor function in LD and in CPS dams, respectively. Gray bars indicate the clock time of lights off. *, Different from other time points ($P < .05$; ANOVA and Newman-Keuls). F-value (F) and degrees of freedom (DF1, DF2) for LD mothers were as follows: corticosterone: F (6, 28) = 9.3; aldosterone: F (6, 28) = 15.2; glucose: F (6, 28) = 7.4; melatonin: F (6, 28) = 25.4; creatinine: F (6, 28) = 4.1; urea: F (6, 27) = 3.2; BUN: F (6, 26) = 3.2; ureic acid: F (6, 28) = 1.3. F-value and degree of freedom for CPS mothers were as follows: corticosterone: F (6, 27) = 3.2; aldosterone: F (6, 27) = 5.3; glucose: F (6, 27) = 4.7; melatonin: F (6, 27) = 8.5; creatinine: F (6, 26) = 8.7; urea: F (6, 26) = 2.5; BUN: F (6, 25) = 5.7; ureic acid: F (6, 22) = 2.5. **, Different from LD ($P < .05$; two way ANOVA and Tukey).

Table 2. Effect of Gestational Chronodisruption on Gestation Length, Maternal Weight Gain, and Newborn Variables (Mean ± SEM)

	LD (n = 5)	CPS (n = 5)
Gestation length, d	21.64 ± 0.03	22.08 ± 0.08[a]
Maternal weight gain, g	131.0 ± 10.2	125.0 ± 8.4
Liter size, n	13.2 ± 1.0	14.0 ± 1.1
Newborn body weight, g	6.50 ± 0.10	7.41 ± 0.09[a]
Sex distribution, males/females	28/32	20/28

Abbreviation: GW, gestational week.
[a] Different from LD ($P < 0.001$,unpaired *t* test).

development and newborn weight. Next, we studied whether the altered pregnancy environment had effects on circadian physiology in the adult offspring

Impact of gestational CPS on circadian rhythms in the adult offspring

As described previously, after birth, the mothers and their pups were maintained in the regular photoperiod schedule (12 h1 light, 12 h dark, lights on at 7:00 AM). The male offspring was separated and studied at 90 days of age. Both groups showed similar food consumption between weaning (21 d old) and 90 days of age; CPS offspring were slightly heavier, but the difference was not significant (529.8 ± 9.2 g for LD vs 545.0 ± 9.5 g; $P = .25$, unpaired *t* test).

As shown in Figure 5 and Table 4, regular circadian rhythms of activity, temperature, and heart rate were present in the CPS and control group, with acrophases positioned in the dark period. There were no differences in activity and temperature rhythms between CPS and control animals. However, we detected a major effect on heart rate circadian rhythm, wherein the mesor was lower in animals gestated in CPS and the acrophase was advanced in almost 1 hour vs LD offspring (Table 4). Thus, we analyzed whether maternal chronodisruption modified heart rate variability because the amplitude of the heart rate circadian rhythm was increased in the CPS offspring. We found that those animals gestated in the CPS condition had an increase in the variation of heart rate (measured as SD) during day and night (Figure 6, A and B). These findings suggest circadian disturbances on heart rate that may affect the regulation of BP. To assess this issue further, we investigated the effect of the maternal exposure to CPS during gestation on systolic BP. We measured systolic BP using the tail-cuff method. We found a daily rhythm of systolic pressure in both groups of males, with an increase during the dark period; however, those males gestated under CPS presented higher systolic BP during the night as well as increased amplitude of the rhythm, relative to LD control animals (Figure 6C). Similar to the observation for heart rate, there was an increase in the variation of BP at both night and day (Figure 6D).

Table 3. Effect of Maternal Chronodisruption in Maternal and Fetal Variables at 18 Days of Gestation (24 Hour Mean ± SEM)

	18 Days of Gestation	
	LD (n = 30)	CPS (n = 28)
Maternal weight gain, g	121.0 ± 3.70	121.6 ± 4.00
Liter size, n	14.8 ± 0.40	14.0 ± 0.60
Fetal body weight, g	1.92 ± 0.40	1.84 ± 0.35[a]
Placental weight, g	0.50 ± 0.07	0.48 ± 0.08[a]
Biparietal diameter, mm	7.10 ± 0.05	7.16 ± 0.05
Crown-to-rump length, mm	22.4 ± 0.30	22.4 ± 0.23
Femur length, mm	3.70 ± 0.05	3.80 ± 0.04

[a] Different from LD ($P < 0.05$, unpaired *t* test).

In marked contrast to the presence of synchronized rhythms of activity, temperature, and heart rate, CPS young adults did not show synchronized daily rhythms of endocrine or most metabolic variables in plasma. Additionally, several of these variables showed alterations in total 24-hour concentrations. The most striking effects were observed upon plasma melatonin (Figure 7, A and B), which, in addition to the absence of a rhythm, presented plasma concentration in 24 hours much lower than in the control animals (Figure 7B). An absence of daily rhythm was observed also for corticosterone and aldosterone (Figure 7, C and D). Furthermore, corticosterone levels (as 24 h mean) were higher than those of control young adults (CPS: 190.7 ± 13.9 ng/mL vs LD: 152.7 ± 8.5 ng/mL; $P < .05$, Student's *t* test). Renal markers were also affected by the treatment during gestation, as indicated by the absence of a 24-hour rhythm for creatinine, BUN, urea, and ureic acid (Figure 8), with an increase in the 24-hour mean for creatinine (CPS: 0.241 ± 0.018 mg/dL vs LD: 0.191 ± 0.009 mg/dL; $P < .05$, Student's *t* test). Similar to the findings in the pregnant females (with or without CPS), the other metabolic variables measured, C3, C4, albumin, total protein, calcium, cholesterol (HDL. VLDL, and total), transaminases, and bilirubin, were not rhythmic and were not affected by maternal exposure to CPS (Supplemental Table 3). CPS animals showed a circadian rhythm of glucose; however, 24-hour glucose concentrations were higher than in the control animals, displaying a higher response to glucose loading than control offspring (Supplemental Figure 1C), suggesting impaired glucose regulation (Supplemental Figure 1A) as reported previously by us (17).

To determine whether the discrepancy between activity and temperature rhythms and corticosterone rhythm reside in changes in clockwork machinery of the offspring's SCN, we investigated the expression of the core clock genes *Per2* and *Bmal1* around the clock. Both clock genes pre-

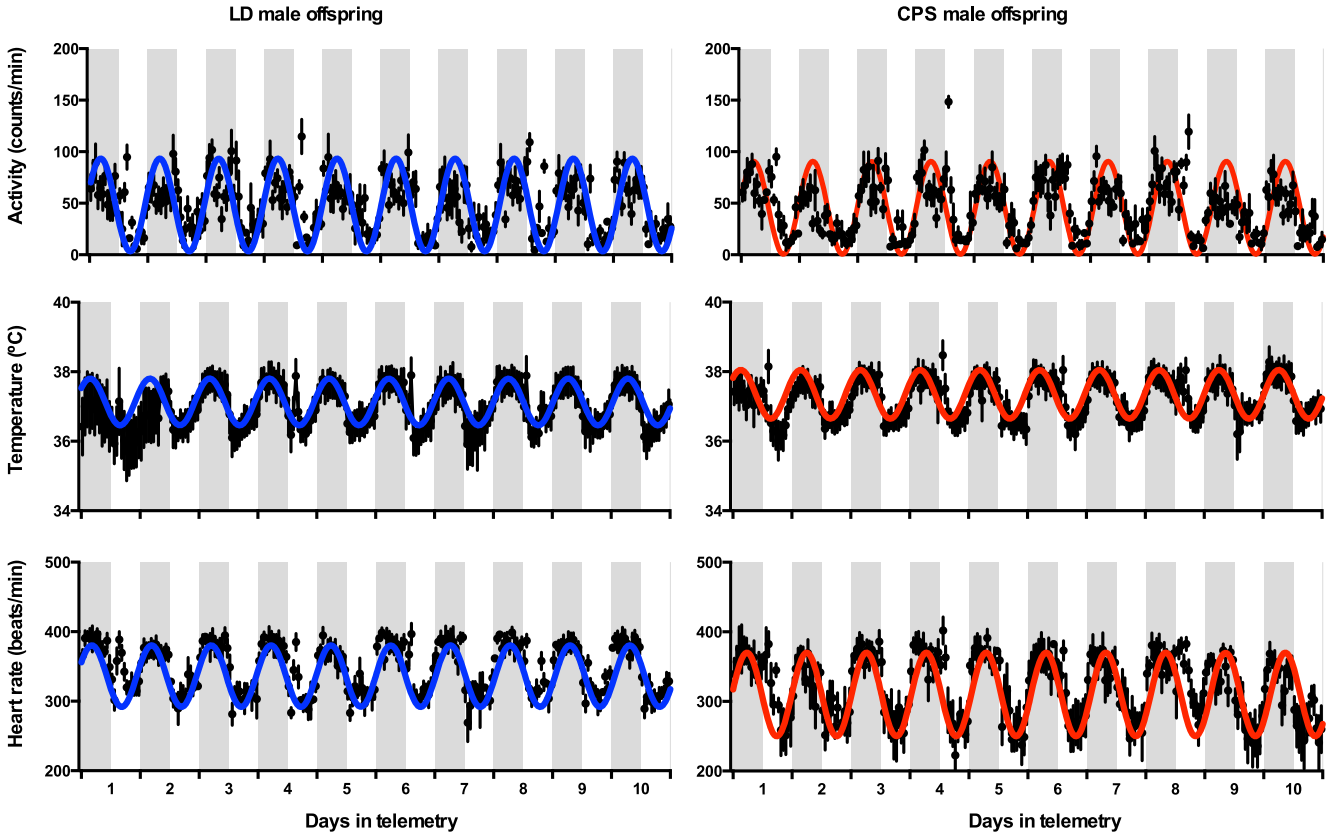

Figure 5. Effect of gestational CPS on the daily rhythms of activity, temperature, and heart rate in the 90-day-old offspring. Mean ± SEM activity, temperature, and heart rate during 10 days in 90-day-old males gestated in control condition (LD, n = 5, from different mothers) or CPS (n = 5, from different mothers). Both groups of males were raised in LD photoperiod from birth. Activity, temperature, and heart rate were measured at 1-minute intervals by telemetry. Shaded bars represent lights off. Blue and red lines represent the theoretical 24-hour cosinor function fitting the data in LD and CPS males, respectively.

sented a synchronized pattern in LD and CPS males (Figure 9, A and B), with the acrophase at clock times similar to those reported in the adult rat (28). We next investigated whether the flat corticosterone rhythm was associated with disturbances in the hypothalamo-pituitary-adrenal axis at the central or adrenal level. To test these possibilities, we suppressed endogenous ACTH by dexamethasone treatment and measured the response to a bolus of ACTH at two different clock times. As shown in Figure 10, A and B, dexamethasone treatment effectively suppressed corticosterone and both groups had similar concentrations before ACTH administration. ACTH induced a prompt increase in plasma corticosterone in both groups of animals, at the two hours tested. However, although control animals showed the known higher response to ACTH in the evening than the morning, such a difference was not observed in the CPS offspring, suggesting differences at the adrenal level with control animals (Figure 10, A and B).

Discussion

In the present work, we investigated whether chronic shifting of maternal photoperiod during pregnancy (CPS) al-

Table 4. Effect of Gestational Chronodisruption on the Parameters Describing the Circadian Rhythms of Activity, Heart Rate, and Temperature of Adult Male Offspring (Mean ± SEM)

	Activity		Heart Rate		Temperature	
	LD	CPS	LD	CPS	LD	CPS
Mesor	6.4 ± 0.4	6.1 ± 0.4	324.6 ± 15.6	267.6 ± 17.6[a]	36.9 ± 0.4	37.2 ± 0.29
Amplitude	3.0 ± 0.8	3.1 ± 0.2	53.5 ± 3.3	59.0 ± 5.5	0.7 ± 0.1	0.6 ± 0.1
Acrophase	1.3 ± 0.7	01.8 ± 0.2	0.9 ± 0.3	1.8 ± 0.2	0.8 ± 0.2	1.4 ± 0.3

Mesor and amplitude comprise activity (counts per minute), heart rate (beats per minute), temperature (degrees Celsius), and acrophase (clock time hours).

[a] Different from LD ($P < 0.05$, unpaired t test).

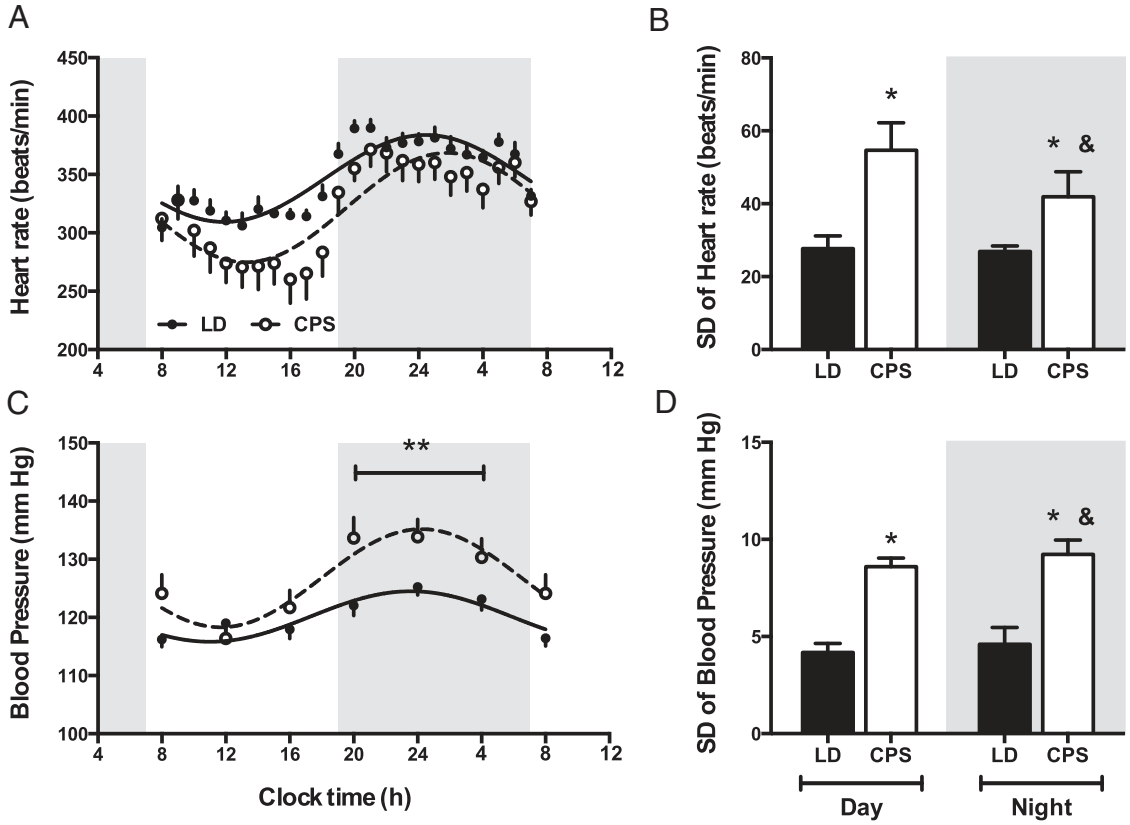

Figure 6. Effect of gestational CPS on daily changes of heart rate and systolic BP in the 90-day-old offspring. Data are means ± SEM. Black symbols represent males gestated in control condition (LD), white symbols represent males gestated in CPS. Solid and dotted lines represent the theoretical 24-hour cosinor function in LD and CPS males, respectively. Shaded bars represent lights off. Panels A and C, Twenty-four-hour daily rhythm in heart rate measured telemetrically for 10 days (n = 5) and BP measured every 4 hours for 24 hours (n = 8). **, Different from LD ($P <$ .05 (two way ANOVA and Tukey's test). Panels B and D, Mean ± SEM SD of heart rate and BP during the day and at nighttime. *, different from LD; &, different from daytime ($P <$ .05; ANOVA and Tukey's test). F-value and degree of freedom were as follows: heart rate: F (3, 36) = 59.2; BP: F (3, 26) = 163.6.

ters the circadian system organization in the adult offspring, potentially increasing the risk of chronic diseases. We tested the hypothesis, using an experimental rat model of gestational chronodisruption (chronic photoperiod shifting up to 85% of gestation, CPS), relative to a normal photoperiod (12 h light, 12 h dark, LD). Altogether these data show that altering the maternal circadian environment during gestation by exposure to CPS causes a misalignment of circadian physiological functions in the adult offspring, namely hyperglycemia and a higher nocturnal BP. In summary, our findings support a role of gestational chronodisruption in pathophysiological processes in the adult.

Maternal exposure to CPS disrupted the biological clocks in the mother, altering locomotor activity, body temperature, and heart rate circadian rhythms throughout gestation. In addition, it modified endocrine rhythms (glucocorticoids, aldosterone, and melatonin) and the rhythms of selected plasma metabolic variables measured at 18 days of gestation. The overall effect was a misalignment of maternal circadian rhythms, suggesting that CPS altered internal temporal order of a wide range of functions in the mother. At 18 days of gestation, CPS fetuses presented growth restriction, as found previously by us using a model of maternal chronodisruption by exposure to constant light (16), supporting that maternal disarray affected fetal development. However, as shown by Varcoe (15), we found that weight at birth was higher than in control newborns. Furthermore, CPS lengthened gestation about 12 hours. These changes in fetal growth trajectory, with a low weight at 18 days of gestation, followed by accelerated growth, resulting in a heavier newborn at birth, are consistent with maternal chronodisruption acting as an insult during gestation. Altogether our current data and those reported by Varcoe et al (15, 23) support that CPS, by altering maternal internal temporal order, disturbed fetal physiology, acting as an adverse intrauterine condition, which may impose a long-term trade-off, eventually increasing adult disease prevalence, analogous to other reported pregnancy insults (10–14).

Young adults gestated under CPS (but raised and tested under LD conditions), had higher systolic BP during the

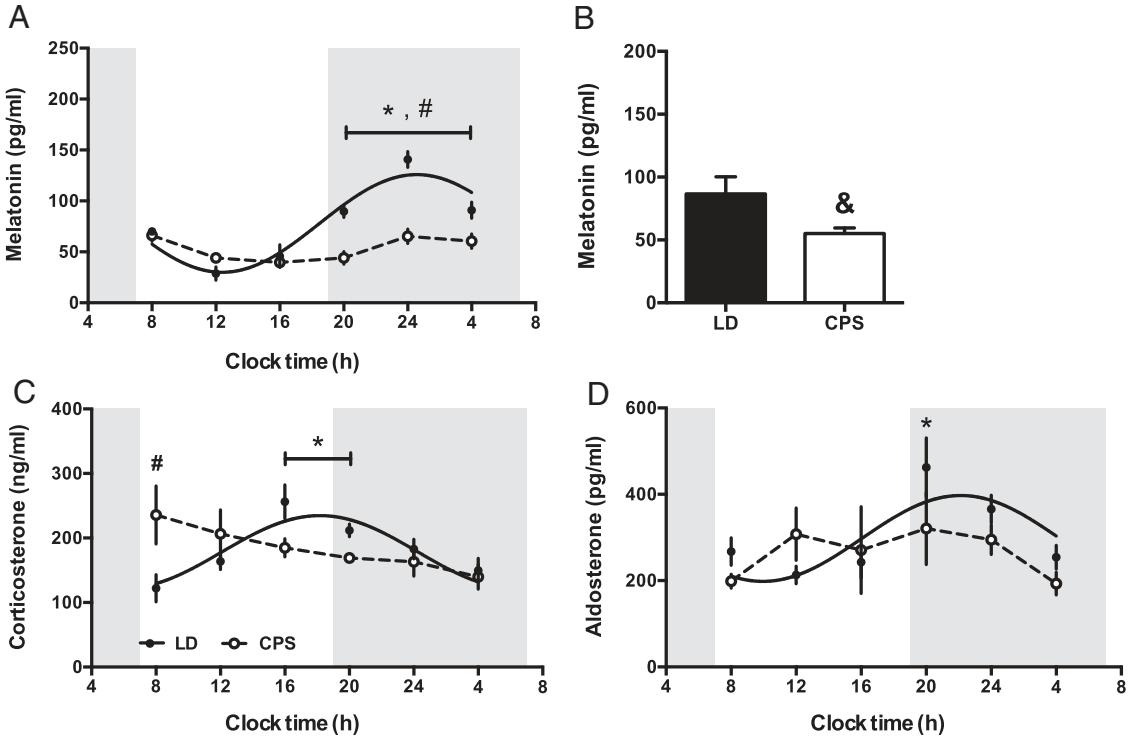

Figure 7. Effect of gestational CPS on daily rhythm of plasma melatonin, corticosterone, and aldosterone in the 90-day-old offspring. Data are means ± SEM. Black symbols represent males gestated in control condition (LD), and white symbols represent males gestated in CPS. Males from each pregnancy condition (LD: n = 9 mothers and CPS: n = 12 mothers) were euthanized every 4 hours around the clock (n = 5/clock time; LD: n = 30 males, CPS: n = 28 males at 4:00 PM and 12:00 AM, n = 4), starting at 8:00 AM and ending at 4:00 AM. A, Plasma melatonin concentration. B, Twenty-four-hour melatonin concentration. C and D, Plasma concentration of corticosterone and aldosterone. &, Different from LD ($P < .05$, Student's *t* test); *, different from other time points ($P < .05$; ANOVA and Newman-Keuls). F-value (F) and degree of freedom for LD offspring were as follows: melatonin: F (5, 23) = 29.4; corticosterone: F (5, 23) = 6.9; aldosterone: F (5, 23) = 5.5. #, Different from CPS ($P < .05$; two way ANOVA and Tukey). Solid and dotted lines represent the theoretical 24-hour cosinor function in LD and CPS males, respectively. The gray bars indicate lights off.

night, and had an increased heart rate variability in the rest period, demonstrating a negative effect of gestational chronodisruption in the offspring. Moreover, CPS animals presented indications of metabolic disturbances, ie, alteration in the glucose tolerance test as reported in models of maternal and early postnatal CPS (23) and maternal exposure to constant light (17). Additionally, when comparing mean 24-hour daily rhythms in adult CPS males with control LD males, we found differences suggesting modification of some specific circadian system outputs. Of note, SCN clock gene expression and the rhythms of locomotor activity, body temperature, heart rate, BP, and plasma glucose were similar to those of LD offspring. In contrast, no 24-hour daily rhythms in melatonin, corticosterone, aldosterone, and renal function were detected.

CPS adult animals maintained normal daily rhythms of activity and temperature adjusted to the LD cycle, as reported for temperature in older CPS offspring (23). Because these rhythms are directly controlled by the main circadian clock residing in the SCN (24), their integrity is consistent with the fact that SCN clock gene expression in CPS males was similar to that of LD males. Maintenance of diurnal changes in plasma glucose and BP is also consistent with a normal SCN function because these rhythms receive a strong SCN neural component mediated by the autonomic nervous system (29, 30). In contrast, endocrine rhythms such as melatonin and corticosterone, which require neural connections from the SCN via the autonomic nervous system to the pineal and adrenal, respectively, were impaired (31–33). The melatonin rhythm was clearly suppressed, as indicated by the low plasma concentrations observed at all clock times tested. However, the lack of corticosterone and aldosterone rhythms may represent a true absence of rhythm or desynchronization with the LD cycle. Overall, these findings suggest defective communication between the pineal and adrenal with the SCN or defects in these target organs. Given that the rhythms of glucocorticoids and melatonin provide synchronization signals for specific organs like the liver and kidney (4, 34, 35), their derangement may contribute to the observed changes in plasma variables related to renal function. These alterations added to an abnormal aldosterone rhythm may participate in the increases in BP observed in CPS young adults.

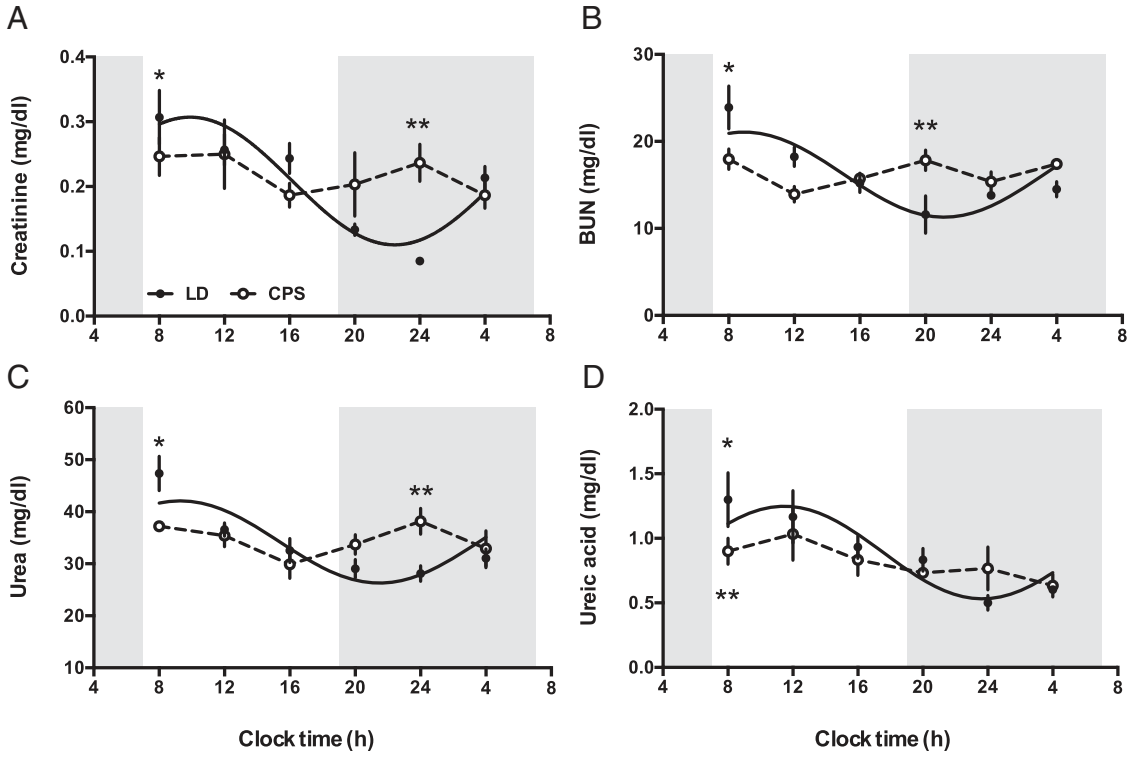

Figure 8. Effect of gestational CPS on daily rhythm of creatinine, BUN, urea, and uric acid in the 90-day-old offspring. Data are means ± SEM. Black symbols represent males gestated in control condition (LD), and white symbols represent males gestated in CPS. Males from each pregnancy condition (LD; n = 9 mothers and CPS; n = 12 mothers) were euthanized every 4 hours around the clock (n = 5/clock time; LD, n = 30 males, CPS, n = 28 males at 400 PM and 12:00 AM, n = 4), starting at 8:00 AM and ending at 4:00 AM. Solid and dotted lines represent the theoretical 24-hour cosinor function in LD and in CPS males, respectively. The gray bars indicate the clock time of lights off. *, Different from other time points ($P < .05$; ANOVA and Newman-Keuls). F-value (F) and degree of freedom for LD offspring were as follows: creatinine: F (5, 21) = 31.2; urea: F (5, 22) = 34.1; BUN: F (5, 21) = 18.8; ureic acid: F (5, 22) = 16.0. **, Different from LD ($P < .05$; two way ANOVA and Tukey).

At this point, it is difficult to demonstrate the actual relevance of each circadian rhythm in the BP alteration observed in CPS adult offspring. Regulation of BP involves many process (vasoconstriction, heart rate, heart contractility, body temperature, and renal function (revised by reference 36) and alteration of the circadian rhythms in any of these levels translates into an alteration of the BP (36). Nevertheless, there is evidence that glucocorticoids and melatonin also contribute to BP. As example, an increase of glucocorticoids levels augments BP (36). On the other hand, melatonin suppression also increases BP, whereas treatment with melatonin decreased BP (37, 38).

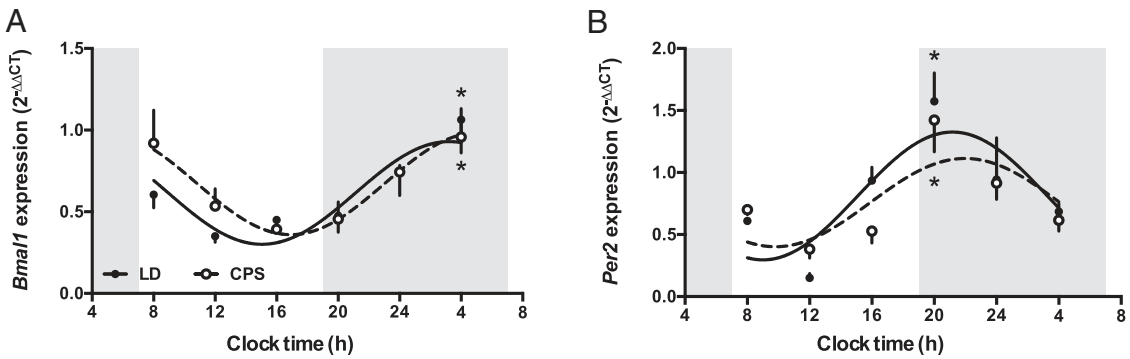

Figure 9. Effect of gestational CPS on daily rhythm of clock gene expression in SCN in the 90-day-old offspring. Data are means ± SEM. Black symbols represent males gestated in control condition (LD), and white symbols represent males gestated in CPS. Males from each pregnancy condition (LD; n = 9 mothers and CPS; n = 12 mothers) were euthanized every 4 hours around the clock *Bmal1* and *Per2* expression in LD (n = 3/clock time) and CPS offspring (n = 5/ clock time, except at 4:00 PM and 12:00 AM, n = 4). *, Different from other time points ($P < .05$; ANOVA and Newman-Keuls). F-value (F) and degree of freedom for LD offspring were as follows: *Bmal1*: F (5, 12) = 4.9; *Per2*: F (5, 12) = 7.3. F-value and degree of freedom for CPS offspring were as follows: *Bmal1*: F (5, 22) = 3.1; *Per2*: F (5, 22) = 7.6. Solid and dotted lines represent the theoretical 24-hour cosinor function in LD and CPS males, respectively. The gray bars indicate lights off.

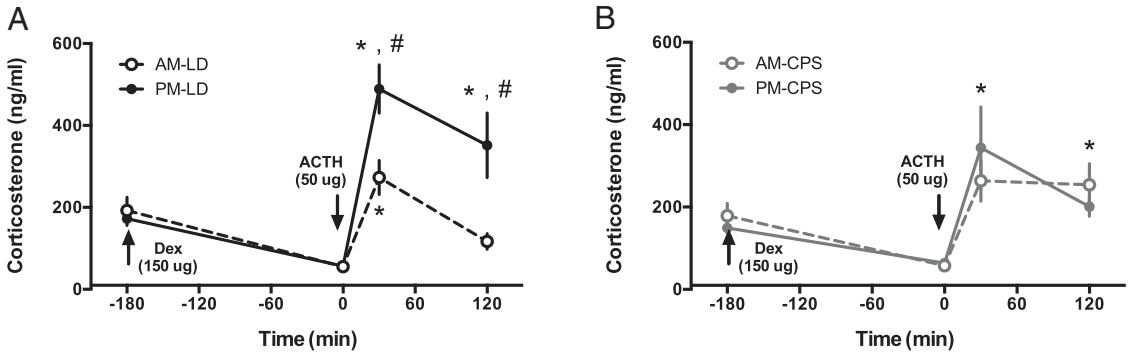

Figure 10. Effect of gestational CPS on corticosterone response to ACTH in the 90-day-old offspring. Data are means ± SEM of morning (AM; open symbols) and afternoon (PM; closed symbols) of corticosterone response to ACTH in LD (A) and CPS (B) male offspring (n = 5 /pregnancy condition from five different mothers; pair of sibling were used, one at AM and the other to PM experiments). *, Different from postdexamethasone administration ($P < .05$; two way ANOVA and Tukey); #, different from AM response ($P < .05$; two way ANOVA and Tukey).

Thus, consistent with our observation, the absence of nocturnal melatonin increase and the high mean plasma corticosterone levels may originate the observed cardiovascular alteration detected here and in a model of exposure to chronodisruption in the adult rat (39, 40). Worth mentioning, increased BP and heart rate variability, as found in the present study, are predictors of cardiovascular risk in humans.

Whether the absence of a melatonin rhythm did influence plasma corticosterone levels or vice versa remains unknown. The available data so far do not provide evidence that the circadian rhythm of glucocorticoids entrains the rhythm of melatonin in mammals. Moreover, chronic immobilization stress that augments plasma corticosterone increases plasma melatonin levels (41). In contrast, there is strong evidence for melatonin acting on the adrenal gland. Melatonin has been termed a multitasking hormone because of its myriads of actions (42). However, one of its better-known functions, although the precise mechanisms are unknown, is as a chronobiotic. Indeed, direct effects in pars tuberalis, retina, skin, and pancreas have been reported, in which melatonin acts as a chronobiotic signal in these tissues through direct action in clock gene expression (43–47). Along the same lines, previous work by our group shows that melatonin treatment in vitro shifted clock gene expression and corticosterone production in the rat fetal adrenal gland and cortisol in the nonhuman primate adrenal gland (20, 25) and inhibited ACTH induced *Per1* expression in the human and sheep adrenal gland (48, 49). We propose that part of the mechanism altering adrenal function observed at 90 days in CPS offspring could be the absence of nocturnal increase of melatonin.

Previous work from our laboratory demonstrated a direct inhibitory effect of melatonin on adrenal glucocorticoid production in the rat and other species (20, 25, 50), an effect that is also clock time dependent (51), consistent with the fact that melatonin nocturnal increase contributed to the known decrease of glucocorticoids at the end of the dark period. A similar mechanism could be involved in the lack of clock time response to ACTH observed here. In the rat, the presence of an ACTH rhythm is not required for a corticosterone rhythm because the latter persists in hypophysectomized rats treated with a constant level of ACTH (52). The absence of a melatonin rhythm, impacting in glucocorticoid production either by abolishing the rhythm or uncoupling the rhythm with the master clock may play a role in the abnormal corticosterone rhythm and in the lack of clock time response to ACTH observed in CPS offspring.

Chronodisruption exerted up to 85% gestation, an age at which the fetal SCN is not established as a master clock, affected fetal organs like the adrenal gland that are strong peripheral oscillators (25). Intriguingly although melatonin production has not started yet, pineal also shows clock gene expression in rat at 18 days of gestation (53). We propose that maternal chronodisruption, by acting on the peripheral oscillators active during gestation, preclude the development of normal circadian organization in the adult offspring. The overt effect is the misalignment of the function of these oscillators with the photoperiod, altering cardiovascular and metabolic function.

A major challenge as a society is to reduce the increasing tendency of the onset of diseases associated with cardiovascular alteration (hypertension, myocardial ischemia, stroke, and renal failure) and those related with metabolic syndrome (overweight and diabetes). The paramount issues in this matter are to prevent/reduce/treat the effects of disturbances during pregnancy on the risk of diseases during adult life. Whether the impact on the offspring of severe photoperiod shifts in the pregnant rat (references 15 and 23 and present study) extends to women exposed to light at night, ie, shift work, nocturnal work, and so on, must be investigated. Confidently, through the under-

standing of circadian dysfunction during gestation, as well as the impact of the specific timing of this insult, we can begin to prevent/treat health events that currently impinge on a large percentage of the world population and result in a familial and social burden and higher health costs.

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Author contributions included the following: N.M., D.H., H.G.R., J.M.S., M.S.-F., and C.T.-F. conceived and designed the research; N.M., D.H., C.S., E.R.S., K.V., P.A.-V., and P.C. performed the experiments; and N.M., D.H., C.T.-F., and M.S.-F. interpreted the results of the experiments, prepared the figures, and prepared the manuscript.

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References

1. **American Medical Association.** *Health Effects Light at Night*. Chicago: American Medical Association; 2012:1–27.
2. **Richards J, Gumz ML.** Advances in understanding the peripheral circadian clocks. *FASEB J*. 2012;26(9):3602–3613.
3. **Bray MS, Shaw CA, Moore MWS, et al.** Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. *Am J Physiol Heart Circ Physiol*. 2008;294(2):H1036–H1047.
4. **Leliavski A, Dumbell R, Ott V, Oster H.** Adrenal clocks and the role of adrenal hormones in the regulation of circadian physiology. *J Biol Rhythms*. 2015;30(1):20–34.
5. **Sei H, Oishi K, Chikahisa S, Kitaoka K, Takeda E, Ishida N.** Diurnal amplitudes of arterial pressure and heart rate are dampened in clock mutant mice and adrenalectomized mice. *Endocrinology*. 2008; 149(7):3576–3580.
6. **Zhu JL, Hjollund NH, Andersen A-MN, Olsen J.** Shift work, job stress, and late fetal loss: the National Birth Cohort in Denmark. *J. Occup. Environ. Med*. 2004;46(11):1144–1149.
7. **Croteau A, Marcoux S, Brisson C.** Work activity in pregnancy, preventive measures, and the risk of delivering a small-for-gestational-age infant. *Am J Public Health*. 2006;96(5):846–855.
8. **Abeysena C, Jayawardana P, DE A Seneviratne R.** Maternal sleep deprivation is a risk factor for small for gestational age: a cohort study. *Aust N Z J Obstet Gynaecol*. 2009;49(4):382–387.
9. **Knutsson A.** Health disorders of shift workers. *Occup Med*. 2003; 53(2):103–108.
10. **Osmond C, Barker DJP.** Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women. *Environ Health Perspect*. 2000;108:545.
11. **Fowden AL, Giussani DA, Forhead AJ.** Intrauterine programming of physiological systems: causes and consequences. *Physiology (Bethesda, Md)*. 2006;21(1):29–37.
12. **Nathanielsz PW.** Animal models that elucidate basic principles of the developmental origins of adult diseases. *ILAR J*. 2006;47(1): 73–82.
13. **Nuyt AM.** Mechanisms underlying developmental programming of elevated blood pressure and vascular dysfunction: evidence from human studies and experimental animal models. *Clin Sci*. 2008; 114(1):1–17.
14. **Richter V, Briffa JF, Moritz KM, Wlodek ME, Hryciw DH.** The role of maternal nutrition, metabolic function and the placenta in developmental programming of renal dysfunction. *Clin Exp Pharmacol Physiol*. 2016;43(1):135–141.
15. **Varcoe TJ, Boden MJ, Voultsios A, Salkeld MD, Rattanatray L, Kennaway DJ.** Characterisation of the maternal response to chronic phase shifts during gestation in the rat: implications for fetal metabolic programming. *PLoS One*. 2013;8(1):e53800.
16. **Mendez N, Abarzua-Catalan L, Vilches N, et al.** Timed maternal melatonin treatment reverses circadian disruption of the fetal adrenal clock imposed by exposure to constant light. *PLoS One*. 2012; 7(8):e4271–e42719.
17. **Vilches N, Spichiger C, Mendez N, et al.** Gestational chronodisruption impairs hippocampal expression of NMDA receptor subunits Grin1b/Grin3a and spatial memory in the adult offspring. *PLoS One*. 2014;9(3):e91313.
18. **Torres-Farfan C, Valenzuela FJ, Germain AM, et al.** Maternal melatonin stimulates growth and prevents maturation of the capuchin monkey fetal adrenal gland. *J Pineal Res*. 2006;41(1):58–66.
19. **Galdames HA, Torres-Farfan C, Spichiger C, et al.** Impact of gestational chronodisruption on fetal cardiac genomics. *J Mol Cell Cardiol*. 2014;66:1–11.
20. **Torres-Farfan C, Rocco V, Monsó C, et al.** Maternal melatonin effects on clock gene expression in a nonhuman primate fetus. *Endocrinology*. 2006;147(10):4618–4626.
21. **Spichiger C, Torres-Farfan C, Galdames HA, Mendez N, Alonso-Vazquez P, Richter HG.** Gestation under chronic constant light leads to extensive gene expression changes in the fetal rat liver. *Physiol Genom*. 2015:47(12):621–633.
22. **Seron-Ferre M, Reynolds H, Mendez NA, et al.** Impact of maternal melatonin suppression on amount and functionality of brown adipose tissue (BAT) in the newborn sheep. *Front Endocrinol*. 2014; 5:232.
23. **Varcoe TJ, Wight N, Voultsios A, Salkeld MD, Kennaway DJ.** Chronic phase shifts of the photoperiod throughout pregnancy programs glucose intolerance and insulin resistance in the rat. *PLoS One*. 2011;6(4):e18504.
24. **Dibner C, Schibler U, Albrecht U.** The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol*. 2010;72:517–549.
25. **Torres-Farfan C, Mendez N, Abarzua-Catalan L, Vilches N, Valenzuela GJ, Seron-Ferre M.** A circadian clock entrained by melatonin is ticking in the rat fetal adrenal. *Endocrinology*. 2011;152(5): 1891–1900.
26. **Wharfe MD, Mark PJ, Waddell BJ.** Circadian variation in placental and hepatic clock genes in rat pregnancy. *Endocrinology*. 2011; 152(9):3552–3560.
27. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-δδC[T]) method. *Methods*. 2001;25(4):402–408.
28. **Girotti M, Weinberg MS, Spencer RL.** Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. *Am J Physiol Endocrinol Metab*. 2009;296(4):E888–E897.

29. **Buijs FN, Cazarez F, Basualdo MC, et al.** The suprachiasmatic nucleus is part of a neural feedback circuit adapting blood pressure response. *Neuroscience*. 2014;266:197–207.
30. **Kalsbeek A, Scheer FA, Perreau-Lenz S, et al.** Circadian disruption and SCN control of energy metabolism. *FEBS Lett*. 2011;585(10):1412–1426.
31. **Klein DC, Smoot R, Weller JL, et al.** Lesions of the paraventricular nucleus area of the hypothalamus disrupt the suprachiasmatic leads to spinal cord circuit in the melatonin rhythm generating system. *Brain Res. Bull*. 1983;10(5):647–652.
32. **Meyer-Bernstein EL, Jetton AE, Matsumoto SI, Markuns JF, Lehman MN, Bittman EL.** Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters. *Endocrinology*. 1999;140(1):207–218.
33. **Kalsbeek A, van der Spek R, Lei J, Endert E, Buijs RM, Fliers E.** Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol*. 2012;349(1):20–29.
34. **Soták M, Bryndová J, Ergang P, et al.** Peripheral circadian clocks are diversely affected by adrenalectomy. *Chronobiol Int*. 2016:33(5):520–529.
35. **Owino S, Contreras-Alcantara S, Baba K, Tosini G.** Melatonin signaling controls the daily rhythm in blood glucose levels independent of peripheral clocks. *PLoS One*. 2016;11(1):e0148214.
36. **Scheer FA, Kalsbeek A, Buijs RM.** Cardiovascular control by the suprachiasmatic nucleus: neural and neuroendocrine mechanisms in human and rat. *Biol Chem*. 2003;384(5):697–709.
37. **Simko F, Reiter RJ, Pechanova O, Paulis L.** Experimental models of melatonin-deficient hypertension. *Front Biosci (Landmark Ed)*. 2013;18:616–625.
38. **Pechanova O, Paulis L, Simko F.** Peripheral and central effects of melatonin on blood pressure regulation. *IJMS*. 2014;15(10):17920–17937.
39. **Molcan L, Vesela A, Zeman M.** Repeated phase shifts in the lighting regimen change the blood pressure response to norepinephrine stimulation in rats. *Physiol Res*. 2014;63(5):567–575.
40. **Molcan L, Teplan M, Vesela A, Zeman M.** The long-term effects of phase advance shifts of photoperiod on cardiovascular parameters as measured by radiotelemetry in rats. *Physiol Meas*. 2013;34(12):1623–1632.
41. **Dagnino-Subiabre A, Orellana JA, Carmona-Fontaine C, et al.** Chronic stress decreases the expression of sympathetic markers in the pineal gland and increases plasma melatonin concentration in rats. *J Neurochem*. 2006;97(5):1279–1287.
42. **Reiter RJ, Tan D-X, Fuentes-Broto L.** Melatonin: a multitasking molecule. *Prog Brain Res*. 2010;181:127–151.
43. **Dardente H, Menet JS, Poirel VJ, et al.** Melatonin induces Cry1 expression in the pars tuberalis of the rat. *Brain Res Mol Brain Res*. 2003;114(2):101–106.
44. **Johnston JD, Tournier BB, Andersson H, Masson-Pévet M, Lincoln GA, Hazlerigg DG.** Multiple effects of melatonin on rhythmic clock gene expression in the mammalian pars tuberalis. *Endocrinology*. 2006;147(2):959–965.
45. **Hiragaki S, Baba K, Coulson E, Kunst S, Spessert R, Tosini G.** Melatonin signaling modulates clock genes expression in the mouse retina. *PLoS One*. 2014;9(9):e106819.
46. **Nishiyama K, Hirai K.** The melatonin agonist ramelteon induces duration-dependent clock gene expression through cAMP signaling in pancreatic INS-1 β-cells. *PLoS One*. 2014;9(7):e102073.
47. **Sandu C, Liu T, Malan A, Challet E, Pévet P, Felder-Schmittbuhl M-P.** Circadian clocks in rat skin and dermal fibroblasts: differential effects of aging, temperature and melatonin. *Cell Mol Life Sci*. 2015;72(11):2237–2248.
48. **Valenzuela FJ, Reynolds HE, Torres-Farfan C, Rojas A.** Melatonin inhibition of the cortisol response to ACTH may be exerted through period circadian protein homolog 1 (Per1). *Rev Chil Endocrinol Diabetes*. 2012;5(1):6–12.
49. **Campino C, Valenzuela FJ, Torres-Farfan C, et al.** Melatonin exerts direct inhibitory actions on ACTH responses in the human adrenal gland. *Horm Metab Res*. 2011;43(05):337–342.
50. **Torres-Farfan C, Valenzuela FJ, Mondaca M, et al.** Evidence of a role for melatonin in fetal sheep physiology: direct actions of melatonin on fetal cerebral artery, brown adipose tissue and adrenal gland. *J Physiol*. 2008;586(16):4017–4027.
51. **Richter HG, Torres-Farfan C, Garcia-Sesnich J, et al.** Rhythmic expression of functional MT1 melatonin receptors in the rat adrenal gland. *Endocrinology*. 2008;149(3):995–1003.
52. **Meier AH.** Daily variation in concentration of plasma corticosteroid in hypophysectomized rats. *Endocrinology*. 2009;98(6):1475–1479.
53. **Seron-Ferre M, Mendez N, Abarzua-Catalan L, et al.** Circadian rhythms in the fetus. *Mol Cell Endocrinol*. 2012;349(1):68–75.