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Journal

Journal of the Endocrine Society, 6(12)

ISSN

2472-1972

Authors

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Publication Date

2022-10-26

DOI

10.1210/jendso/bvac153

Peer reviewed

Journal of the Endocrine Society, 2022, **6**, 1–10
https://doi.org/10.1210/jendso/bvac153
Advance access publication 29 September 2022
Clinical Research Article

Endogenous Diurnal Patterns of Adrenal and Gonadal Hormones During a 24-Hour Constant Routine After Simulated Shift Work

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Abstract

Context: Night-shift work causes circadian misalignment, predicts the development of metabolic diseases, and complicates the interpretation of hormone measurements.

Objective: To investigate endogenous circadian rhythms, dissociated from behavioral and environmental confounds, in adrenal and gonadal steroids after simulated shift work.

Methods: Fourteen healthy adults (ages 25.8 ± 3.2 years) were randomized to 3 days of night or day (control) shift work followed by a constant routine protocol designed to experimentally unveil rhythms driven endogenously by the central circadian pacemaker. Blood was sampled every 3 hours for 24 hours during the constant routine to concurrently obtain 16 Δ4 steroid profiles by mass spectrometry. Cosinor analyses of these profiles provided mesor (mean abundance), amplitude (oscillation magnitude), and acrophase (peak timing).

Results: Night-shift work marginally increased cortisol by 1 μg/dL ($P = 0.039$), and inactive/weak derivatives cortisone ($P = 0.003$) and 18-hydroxycortisol ($P < 0.001$), but did not alter the mesor of potent androgens testosterone and 11-ketotestosterone. Adrenal-derived steroids, including 11-ketotestosterone ($P < 0.01$), showed robust circadian rhythmicity after either day- or night-shift work. In contrast, testosterone and progesterone showed no circadian pattern after both shift work conditions. Night-shift work did not alter the amplitude or acrophase of any of the steroid profiles.

Conclusion: Experimental circadian misalignment had minimal effects on steroidogenesis. Adrenal steroids, but not gonadal hormones, showed endogenous circadian regulation robust to prior shift schedule. This dichotomy may predispose night-shift workers to metabolic ill health. Furthermore, adrenal steroids, including cortisol and the main adrenal androgen 11-ketostosterone, should always be evaluated during the biological morning whereas assessment of gonadal steroids, particularly testosterone, is dependent on the shift-work schedule.

Key Words: circadian misalignment, healthy young adults, internal desynchrony, reproductive health, sex steroids

Abbreviations: 17OHP, 17α-hydroxyprogesterone.

Sleep loss increases cortisol (a key catabolic signal) and decreases testosterone (a major anabolic hormone), thereby imbalancing the hormonal signaling of whole-body metabolism [1-4]. This dysregulation would be expected to cause metabolic dysfunction, and experimental studies do in fact show that sleep loss induces insulin resistance, a major factor in the development of type 2 diabetes mellitus [3, 4]. Furthermore, we recently demonstrated that fixing cortisol and testosterone to prevent imbalance during sleep loss muted the development of insulin resistance [3-5]. This suggests that cortisol and testosterone signaling are important mechanisms by which sleep loss induces insulin resistance.

Both cortisol and testosterone also exhibit well-established temporal profiles that vary systematically across the 24-hour day. Alterations in signaling of these and other hormones could help explain why experimentally shifting the timing of

Received: 8 August 2022. **Editorial Decision:** 27 September 2022. **Corrected and Typeset:** 31 October 2022

behavioral rhythms relative to endogenous circadian rhythmicity (ie, circadian misalignment) induces insulin resistance, even in the absence of sleep loss [3, 6]. And it may ultimately explain why night-shift work, which induces circadian misalignment, is associated with the future development of common metabolic disorders such as obesity and type 2 diabetes mellitus [3, 7]. This is because the induction of insulin resistance is widely recognized as underpinning the development of type 2 diabetes mellitus, and controlled experimental trials show that 1 to 4 nights of in-laboratory simulated shift work, even in the absence of sleep loss, induces insulin resistance [3].

Although cortisol and testosterone are the main metabolically active hormones secreted from the adrenal gland and testis, respectively, a set of 11-oxygenated androgens of predominately adrenal origin have been recently implicated in the signaling of many physiological and pathophysiological processes that impact metabolic health. These processes occur across the human lifespan and in both sexes and include, for example, polycystic ovarian disease in younger women and castration-resistant prostate cancer in older men [8, 9]. Understanding the signaling characteristics of cortisol, testosterone, and these other hormones is a prerequisite to properly identify and interpret the clinical and regulatory implications of such hormone abnormalities [10].

The signaling and action of many hormones, including cortisol and testosterone, depends on their underlying pulsatile and diurnal (ie, 24-hour) rhythms [11-13]. Randomized controlled trials show that better mimicking the diurnal rhythmicity of cortisol further optimized weight, blood pressure and glucose metabolism [14], and replicating both pulsatile and diurnal rhythmicity further improved working memory and caused subtle differences in the neural processing of emotional input assessed by functional magnetic resonance imaging and psychological face expression recognition tasks [15].

The 24-hour patterns observed in these hormones could be driven centrally by the central circadian pacemaker in the suprachiasmatic nuclei of the hypothalamus and/or by external environmental or behavioral factors that have a diurnal pattern. Identifying the underlying 24-hour patterns of these hormones as driven specifically by the central circadian pacemaker, free of external influences, requires the use of the constant routine protocol as the gold standard method to remove or uniformly distribute external influences so that only the endogenous rhythm is expressed [16, 17]. The endogenous circadian rhythm of cortisol was established decades ago using the constant routine method [18, 19], and one of these studies also showed that circadian misalignment did not alter the timing of the cortisol rhythm [19]. However, the underlying endogenous rhythm of testosterone remains unknown because a constant routine has not been utilized to assess it, and the diurnal rhythms observed in prior studies may have been driven by external influences, including cycles of light/dark, sleep/wake, feeding/fasting, and activity/rest [20-22]. Prior studies examining the diurnal rhythm of 11-oxygenated androgens also did not remove the influence of these external confounds by utilizing a constant routine protocol [10]. Accordingly, the aim of this study was to determine the very nature of the underlying 24-hour pattern of secretion of clinically relevant androgenic hormones as driven by the central circadian pacemaker, and the effect of circadian misalignment on these endogenous rhythms.

We therefore conducted an in-laboratory study where healthy young men and women were assigned to 3 days of either a simulated day-shift (ie, control condition) or night-shift (ie, experimental condition) schedule at random. Following the 3 days of shift work, blood was collected every 3 hours through an intravenous catheter during a 24-hour constant routine protocol, in which subjects remained semirecumbent and awake, in a controlled environment with fixed ambient temperature and dim light, and ate identical small snacks regularly every hour. The blood samples were used for later simultaneous extraction and concurrent measurement of 16 Δ4 steroids that comprise the mineralocorticoid, glucocorticoid, and androgen pathways—including cortisol, testosterone, and certain 11-oxygenated androgens—using liquid chromatography triple quadrupole tandem mass spectrometry [23].

Materials and Methods

Participants and Experimental Design

A total of 14 individuals between the ages of 22 and 34 years (mean = 25.8 ± 3.2 years; 10 males, 4 females; body mass index = 25.7 ± 3.2 kg/m^2) completed the study at the Sleep and Performance Research Center at Washington State University Health Sciences Spokane. The study protocol was approved by the Washington State University Institutional Review Board, and all participants provided written informed consent. Additional details regarding study design and results have been previously reported [24].

In brief, participant subjects were physically and psychologically healthy with no medical or drug treatment, as verified by physical examination, blood chemistry, urinalysis, questionnaires, and in-laboratory polysomnography. One female participant had an etonogestrel implant inserted at least a year prior to study participation and had not had a menstrual period for at least 6 months. None of the other participants were taking any hormonal therapies. Two of the remaining 3 female participants were studied in the follicular phase of the menstrual cycle based on menstrual history, but menstrual phase could not be conclusively determined in the third due to incomplete documentation. All subjects reported good habitual sleep of 6 to 10 hours duration, wake between 6 AM and 9 AM, no extreme morning or evening chronotypes, and no sleep or circadian disorders. They were not involved in shift work within 3 months of entering the study and did not travel across time zones in the month preceding participation.

The 7-day study protocol included a 24-hour baseline period. Following baseline, participants were assigned at random in groups of 2 or 3 to 1 of 2 simulated shift-work conditions: day shift (n = 7, 4 males) or night shift (n = 7, 6 males). Day- and night-shift sleep/wake schedules and blood-draw timing are depicted in Figure 1. The 3-day day-shift condition included a sleep opportunity from 10 PM to 6 AM each day. The 3-day night-shift condition included a nap opportunity from 2 PM to 6 PM prior to the first simulated night shift, followed by a 12-hour behavioral cycle shift and a sleep opportunity from 10 AM to 6 PM on all subsequent days. Scheduled wake was sustained by continuous observation and interactive conversation when required. During the simulated shift days, meals were provided at 1.5 (breakfast), 7 (lunch), and 13.5 (dinner) hours after scheduled waking. Subjects were kept under low light (<50 lux) and fixed ambient temperature (21 ± 1°C). Scheduled sleep was recorded by polysomnography (Nihon Kohden, Foothill Ranch, CA, USA), and visual scoring was conducted in accordance with American

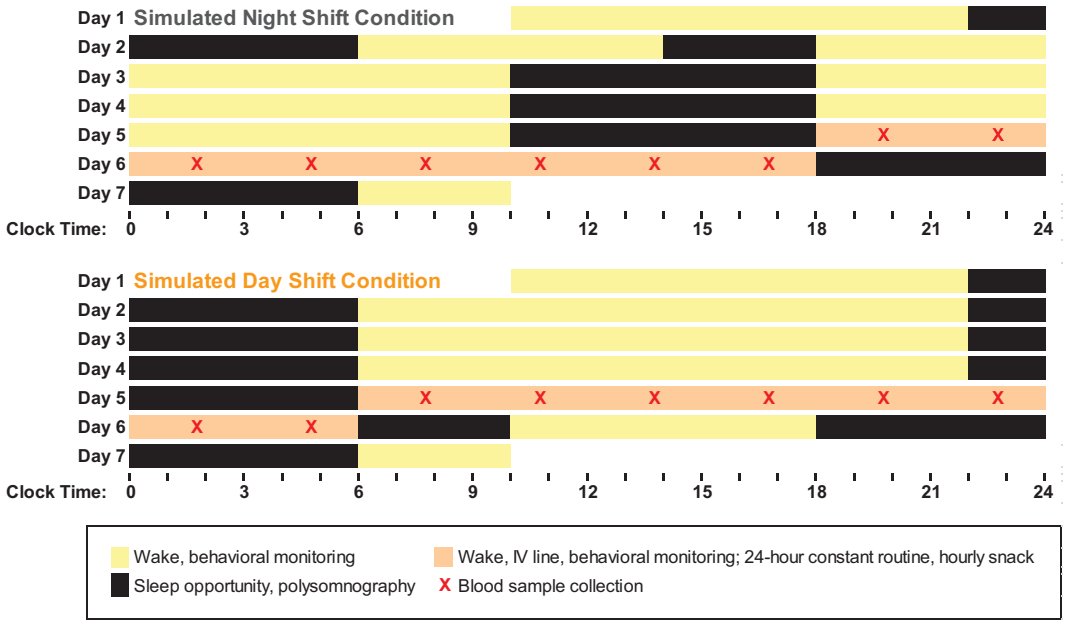

Figure 1. In-laboratory study design. The 7-day study comprised an adaptation period in both conditions (days 1 and 2), a transition nap in the simulated night-shift condition (2 PM-6 PM on day 2), and then in both conditions 3 days of a simulated day-shift or night-shift schedule (days 2-5), a 24-hour constant routine protocol (days 5, 6), and a recovery period (days 6, 7) [24]. The 24-hour constant routine protocol consisted of sustained wakefulness in constant ambient temperature and dim light, fixed posture, and hourly isocaloric snacks [24], such that the blood samples taken during this period reflected the endogenous circadian rhythmicity after the 3 days of simulated day or night shift.

Academy of Sleep Medicine guidelines [25]. The night-shift condition did not cause any substantive sleep restriction [26] and had negligible impact on timing of the endogenous circadian rhythm [24].

At the end of the third day of either day or night shift, participants followed a 24-hour constant routine protocol. This involved participants staying in a room with fixed ambient temperature (21 ± 1°C) and dim light (<50 lux) while maintaining a fixed semirecumbent posture, with sustained wakefulness maintained as described previously, while receiving small isocaloric snacks on an hourly basis. Over the course of the 24-hour protocol, blood draws were conducted every 3 hours via intravenous catheter (see Fig. 1). Assayed blood samples were used to evaluate the endogenous 24-hour rhythms subsequent to circadian misalignment (night-shift condition) or alignment (day-shift condition). After completion of the 24-hour constant routine protocol, participants had a recovery day before going home.

Measurements

Blood was drawn through an intravenous catheter into EDTA-containing vacutainer tubes at 3-hour intervals throughout the 24-hour constant routine protocol. Samples were immediately centrifuged for 10 minutes at 2200 revolutions per minute at 4°C to separate plasma which was then frozen at −80°C until assayed. All samples from the same subject were assayed together. Liquid chromatography triple quadrupole tandem mass spectrometry was used to quantify 16 Δ4 steroids: cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, progesterone, 17α-hydroxyprogesterone (17OHP), 16 α-hydroxyprogesterone, and rostenedione, testosterone, 11-hydroxyandrostenedione, 11-ketoandrostenedione, 11-hydroxytestosterone, 11-keto testosterone, 18-hydroxycorticosterone, and 18-hydroxyco rtisol. Steroids were extracted and quantified simultaneously using spiked commercially available internal standards as previously described [23]. The sensitivity of measurement of all steroids was 5 pg/mL, and the inter and intra-assay coefficients of variation were <12% for each assay.

Statistical Analyses

Cosinor analysis was conducted by linear mixed-effects regression [24, 27] to assess for 24-hour rhythmicity in steroid values that were collected every 3 hours (8 timepoints for each subject) during the 24-hour constant routine protocol. Regression coefficients for mesor (mean abundance), acrophase (timing of peak), and amplitude (maximum extent of oscillation) for each sex were calculated simultaneously using a model that allowed for differences by sex. Overall mean and standard error of the mean were calculated by Cochrane's method to account for the distribution of sex [28]. As previously described, rhythm significance in each of the 2 conditions was determined by testing amplitude against zero by student's t-test, with a one-sided type I error threshold of 0.05, and 95% CI for acrophase were computed using the delta method [24]. Differences between conditions in acrophase, amplitude, and mesor were evaluated using student's *t*-test, with a 2-sided type I error threshold of 0.05, as previously described [24]. Finally, a sensitivity analysis was performed by repeating all analyses without the female participant who had received the etonogestrel implant, and findings were not changed; therefore, results for the full sample are reported. Analyses were conducted using the proc mixed procedure in the SAS statistical package version 9.4 (SAS Institute, Cary, NC).

Results

Participant characteristics are shown in Table 1. Figure 2 depicts the effects of prior simulated night-shift work on the mesor (mean abundance) of measured steroids that comprise

Table 1. Baseline characteristics

	Simulated day-shift condition (n = 7)	Simulated night-shift condition (n = 7)
Age (years); mean ± SD	24.0 ± 2.2	27.6 ± 3.2
Height (m); mean ± SD	1.8 ± 0.1	1.9 ± 0.1
Weight (kg); mean ± SD	81.1 ± 12.3	90.8 ± 19.6
BMI (kg/m^2); mean ± SD	25.7 ± 3.4	25.6 ± 3.3
Gender; n (%)		
Male	4 (57)	6 (86)
Female	3 (63)	1 (14)

Abbreviations: BMI, body mass index.

the mineralocorticoid, glucocorticoid, and androgen pathways. Table 2 shows circadian rhythm amplitude (maximum extent of oscillation), mesor, and acrophase (peak timing) after simulated day-shift work, night-shift work, and the difference between the 2 conditions after controlling for sex, for each of the 16 Δ4 steroids measured. Analogous amplitude, mesor, and acrophase estimates for men and for women separately are shown in Supplementary Tables S1 and S2, respectively [29]. Exemplar steroid profiles are shown in Figures 3 to 6, as discussed later.

Night-shift work did not alter the mesor of potent androgens such as testosterone and 11-ketotestosterone but did increase a weak/precursor androgen, 11-ketoandrostenedione ($t_{12} = 2.55$, $P = 0.025$): see Table 2 and Figure 3. Night-shift work slightly decreased the mesor of progesterone by 0.03 ng/mL ($t_{12} = -2.33$, $P = 0.038$) but had no effect on 17OHP: see Table 2 and Figure 4. Night-shift work marginally increased the mesor of the major glucocorticoid cortisol, by 1 μg/dL ($t_{12} = 2.32$, $P = 0.039$), as well the inactive glucocorticoid metabolite cortisone ($t_{12} = 3.64$, $P = 0.003$) and hybrid steroid 18-hydroxycortisol ($t_{12} = 4.75$, $P < 0.001$), but had no effect on other glucocorticoids, including corticosterone: see Table 2 and Figure 5. The associated amplitude and timing (acrophase) of steroids with endogenous circadian rhythmicity were not significantly affected by night-shift work for any of the measured steroids: see Table 2.

Significant endogenous circadian rhythmicity (as indicated by significant non-zero amplitudes) was observed for all adrenal hormones under both night- and day-shift conditions but not for gonadal hormones: see Table 2. In order to illustrate this, Figure 6 (upper panels) shows the individual participants' 24-hour profiles, as measured under constant routine, for the exemplar adrenal hormone cortisol. In contrast, individual rhythms were not present for the main testicular hormone testosterone: Figure 6 (lower panels).

Discussion

Prior studies have unequivocally established the diurnal rhythmicity of key metabolically active steroids such as cortisol and testosterone [18-22]. Recently, the diurnal rhythmicity of a novel set of adrenal 11-oxygenated steroids that are increasingly recognized to be important for human physiology and pathophysiology has also been documented [10]. However, the underlying rhythmicity of these 11-oxygenated steroids, as well as testosterone, as being driven by the central circadian pacemaker has not been unveiled previously. Here, by removing or uniformly distributing all external influences through a gold-standard constant routine protocol conducted in conjunction with an experimental and analytical paradigm where all blood steroids were treated equivalently and measured by state-of-the-art tandem mass spectrometry, we determined the origin of these diurnal rhythms. Specifically, we showed that 24-hour rhythms in steroids of predominately adrenal origin are robustly driven endogenously by the central circadian pacemaker, whereas steroids with a substantial gonadal origin generally did not show endogenous 24-hour rhythmicity.

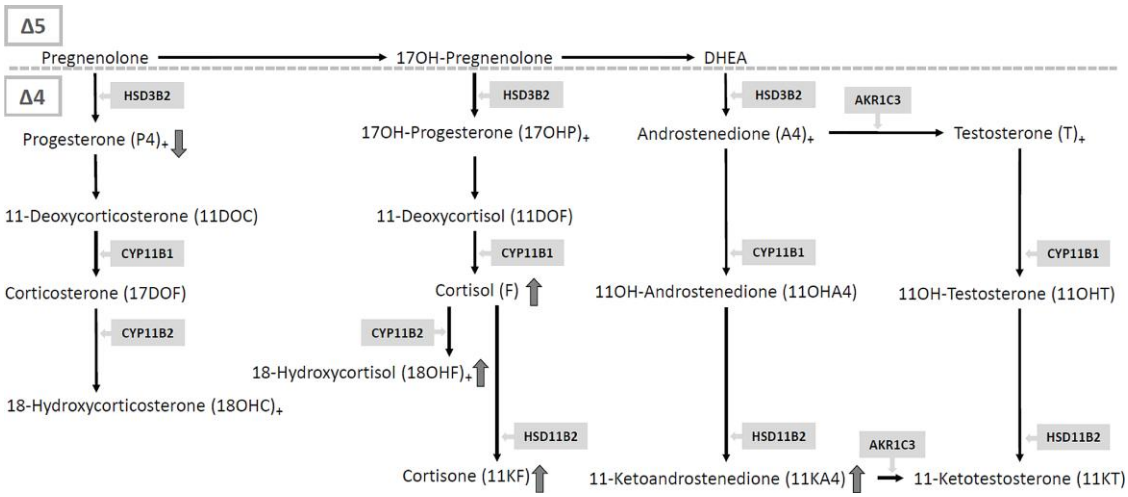

Figure 2. The Δ4 steroidogenesis pathway and the effects of prior simulated shift work on mesors calculated from each temporal steroid profile. The figure depicts the mineralocorticoid (left), glucocorticoid (middle), and androgen (right) pathways and the enzymes (gray) that catalyze the steroidogenic transformations. Thick gray upward/downward arrows indicate a significantly higher/lower mesor in the night-shift condition as compared to the day-shift condition for both sexes combined. Simulated shift-work effects for which significant sex differences were observed are marked with a + sign. The 3 Δ5 steroids shown above the dotted line were not measured but are included to illustrate how the steroidogenesis pathways are linked. The Δ4 steroid 16 α-hydroxyprogesterone is not shown since it does not have a position in the pathways depicted. Table 2 shows further details for 16 α-hydroxyprogesterone and all the other Δ4 steroids measured.

Table 2. Amplitude, mesor, and acrophase estimates by prior day- and night-shift conditions for men and women

	Amplitude ± SE (pg/mL)			Mesor ± SE (pg/mL)			Acrophase ± SE (HH:MM)		
	Day-shift condition	Night-shift condition	Night vs day difference	Day-shift condition	Night-shift condition	Night vs day difference	Day-shift condition	Night-shift condition	Night vs day difference
Corticosteroid pathway									
Progesterone[a,e]	**11.03 ± 5.65**[b]	4.22 5.85	−6.82 8.13	70.98 9.82	41.99 7.62	**−28.99 12.43**[c]	13:00 2:23	7:53 7:03	−5:06 7:26
11-Deoxycorticosterone	**6.34 2.12**[c]	**5.64 2.10**[c]	−0.70 2.98	22.34 1.44	19.91 1.48	−2.42 2.06	10:04 1:24	9:55 1:29	−0:09 2:03
Corticosterone	**1105.82 263.60**[d]	**1412.18 263.29**[e]	306.36 372.56	1544.49 187.32	1856.93 183.97	312.43 262.56	8:41 1:03	8:31 0:45	−0:10 1:17
18-Hydroxycorticosterone[a,c]	**58.57 23.78**[c]	**77.96 20.03**[d]	19.39 31.09	144.90 16.41	169.81 13.95	24.92 21.54	8:13 2:20	8:30 0:54	0:16 2:31
16 α-Progesterone	**36.27 9.19**[d]	**43.57 9.11**[d]	7.30 12.94	120.21 7.47	113.81 7.10	−6.41 10.31	7:59 1:03	7:36 0:49	−0:23 1:20
Glucocorticoid pathway									
17α-Hydroxyprogesterone[a,e]	40.52 35.51	42.13 36.95	1.61 51.25	568.09 72.34	546.64 54.57	−21.45 90.61	12:44 3:44	7:05 4:00	−5:38 5:28
11-Deoxycortisol	**55.56 16.86**[d]	**90.22 16.97**[e]	34.65 23.92	130.76 11.89	161.24 12.02	30.48 16.90	10:29 1:11	9:52 0:41	−0:37 1:22
Cortisol	**36 043.81 4705.42**[e]	**38 728.17 4869.28**[e]	2684.36 6771.33	62 903.27 3474.52	74 300.45 3482.08	**11 397.18 4919.06**[c]	8:43 0:31	9:44 0:28	1:00 0:42
18-Hydroxycortisol[a,c]	**155.15 38.13**[d]	**247.30 35.89**[e]	92.15 52.36	321.39 31.84	522.35 27.92	**200.96 42.35**[e]	9:52 0:59	9:47 0:32	−0:05 1:07
Cortisone	**5899.35 636.64**[e]	**5849.64 663.56**[e]	−49.71 919.58	13 550.24 450.83	15 908.18 463.98	**2357.95 646.94**[d]	9:53 0:27	11:02 0:26	1:08 0:37
Androgen pathway									
Androstenedione[a,e]	**130.26 39.41**[d]	**131.14 40.51**[d]	0.88 56.52	805.59 113.60	861.64 82.96	56.04 140.67	10:10 1:10	9:29 1:07	−0:40 1:37
11-Hydroxyandrostenedione	**328.59 66.08**[d]	**474.46 66.81**[e]	145.87 93.97	831.68 47.05	961.57 47.39	129.89 66.78	9:40 0:46	9:14 0:32	−0:25 0:56
11-Ketoandrostenedione	**50.05 16.74**[c]	**96.50 17.02**[e]	46.45 23.87	196.92 13.30	244.16 12.88	**47.24 18.52**[c]	10:12 1:15	9:00 0:40	−1:12 1:25
Testosterone[a,e]	84.75 154.01	123.32 160.00	38.57 222.08	3020.14 962.35	4502.19 685.51	1482.06 1181.54	16:27 7:03	20:40 5:45	4:12 9:06
11-Hydroxytestosterone	**41.36 11.90**[d]	**33.07 11.15**[c]	−8.29 16.31	106.19 9.03	93.52 8.23	−12.67 12.21	11:17 1:05	12:25 1:21	1:07 1:44
11-Ketotestosterone	**88.46 27.97**[d]	**102.92 28.26**[d]	14.47 39.76	303.72 22.01	286.62 21.22	−17.11 30.57	12:28 1:14	11:33 1:05	−0:54 1:38

Data are listed as means ± standard error of the mean. Statistically significant difference from zero are bolded.
[a]Significant sex difference.
[b]$P = 0.049$.
[c]$P < 0.05$.
[d]$P < 0.01$.
[e]$P < 0.001$.

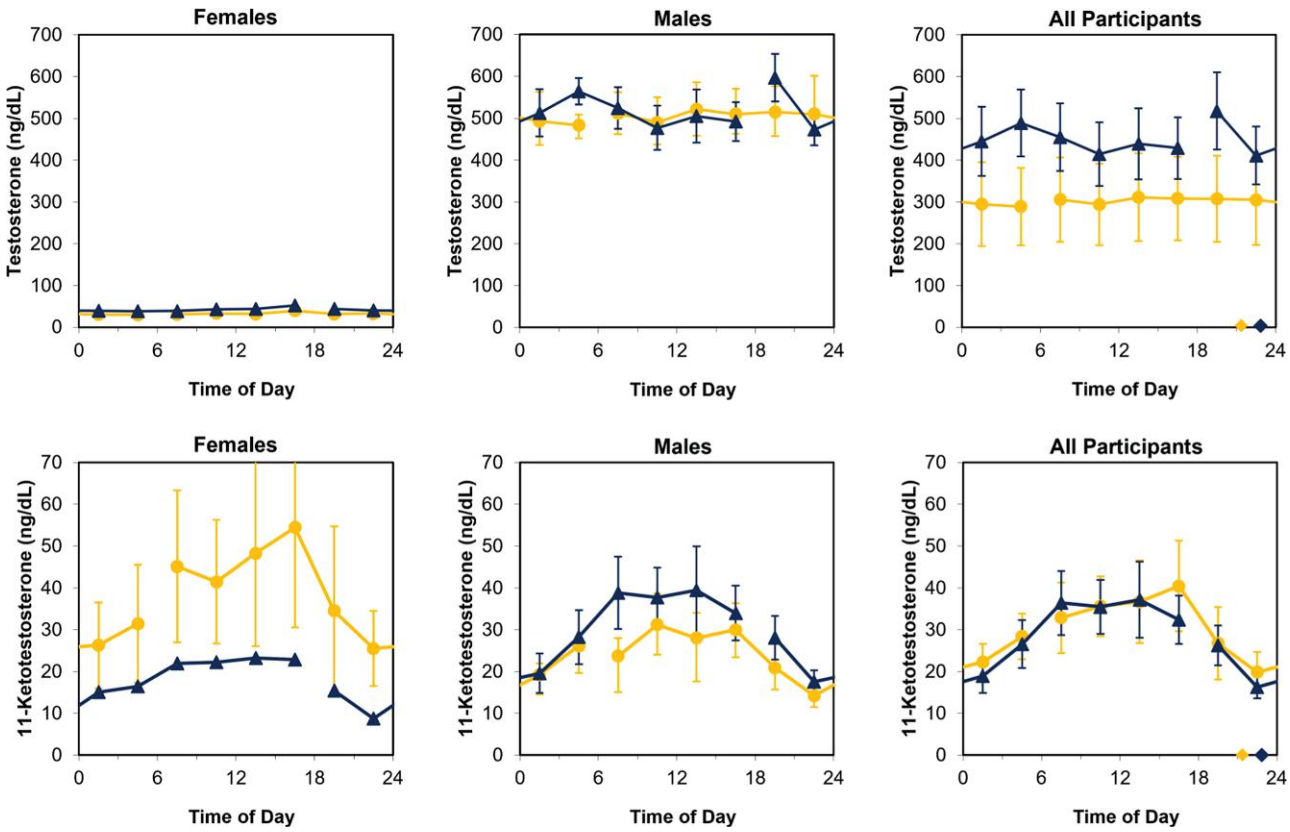

Figure 3. Temporal patterns of exemplar androgens measured under constant routine following 3 days of a simulated day-shift (yellow) or night-shift (blue) schedule. Data are shown as mean ± SE by time of day for the active gonadal androgen testosterone (top) and the active adrenal-derived androgen 11-ketotestosterone (bottom) in women (left), men (middle), and both (right). The gap in the line shows where the 24-hour constant routine protocol began/ended.

In particular, our finding of the robust endogenous circadian rhythmicity of cortisol is consistent with prior literature [18, 19] and supports the reliability and validity of our experimental and analytical procedures. However, the diurnal rhythm of testosterone as previously observed in the presence of sleep/wake and other behavioral rhythms [20-22] was not replicated under constant routine, and thus this rhythm is not attributable to endogenous circadian regulation but rather to environmental or behavioral influences, which are likely associated with the sleep/wake cycle. Although progesterone showed weak evidence of rhythmicity after the day-shift work condition only ($P = 0.049$), 17OHP also did not exhibit a circadian rhythm after either condition. These findings may be explained by prior studies that directly showed substantial, but not exclusive, gonadal production through cannulation of the testicular and ovarian veins [30, 31].

Simulated night-shift work had relatively minor effects on steroidogenesis in our study. Effects detected include a small increase in the mean abundance of cortisol of 1 μg/dL, a negligible decrease in progesterone of 0.03 ng/mL, and similarly inconsequential changes in steroid precursors that are inactive (cortisone and 18-hydroxycortisol) or marginally active (11-ketoandrostenedione) [8, 32, 33]. Amplitude and acrophase of all steroid profiles with endogenous circadian rhythmicity were not affected by prior night-shift work. These minor hormonal changes are in contrast with marked changes to the metabolome and lipidome that we have previously observed using the identical 3-day simulated shift-work paradigm [24, 34]. These data suggest that endocrine networks are robust to circadian misalignment relative to rhythms in metabolites and lipids that change dramatically.

The aforementioned findings have repercussions for the evaluation of the endocrine health of shift workers—a sizable group that comprises about 20% of the US working population. Millions more are likely involved because circadian misalignment is often experienced due to caregiver duties, medical conditions, and/or lifestyle [3, 35]. The relatively minor effects on steroidogenesis unveiled in our study suggest that shift work per se is unlikely to cause hypogonadism or clinically relevant hypercortisolemia—so simply changing working time arrangements may not improve hormonal dysfunction that is already present. The problem of how to assess hypogonadism in this population is amplified because symptoms consistent with hypogonadism are common in shift workers and especially so in those with shift-work sleep disorder [36-38].

As such, our data provide important information regarding how testosterone reference ranges should be applied to evaluate hypogonadism in this population. Normative reference ranges for testosterone and cortisol were developed in the morning among healthy young non-shift workers. Because of the marked 24-hour rhythmicity of these hormones [21, 39-41], these normative reference ranges cannot be applied indiscriminately to other times of day. The impact of the diurnal variation on the clinical measurement of serum testosterone in particular has been recognized for decades [42], but how this knowledge should be applied to shift workers has not been conclusively established until now. Our data,

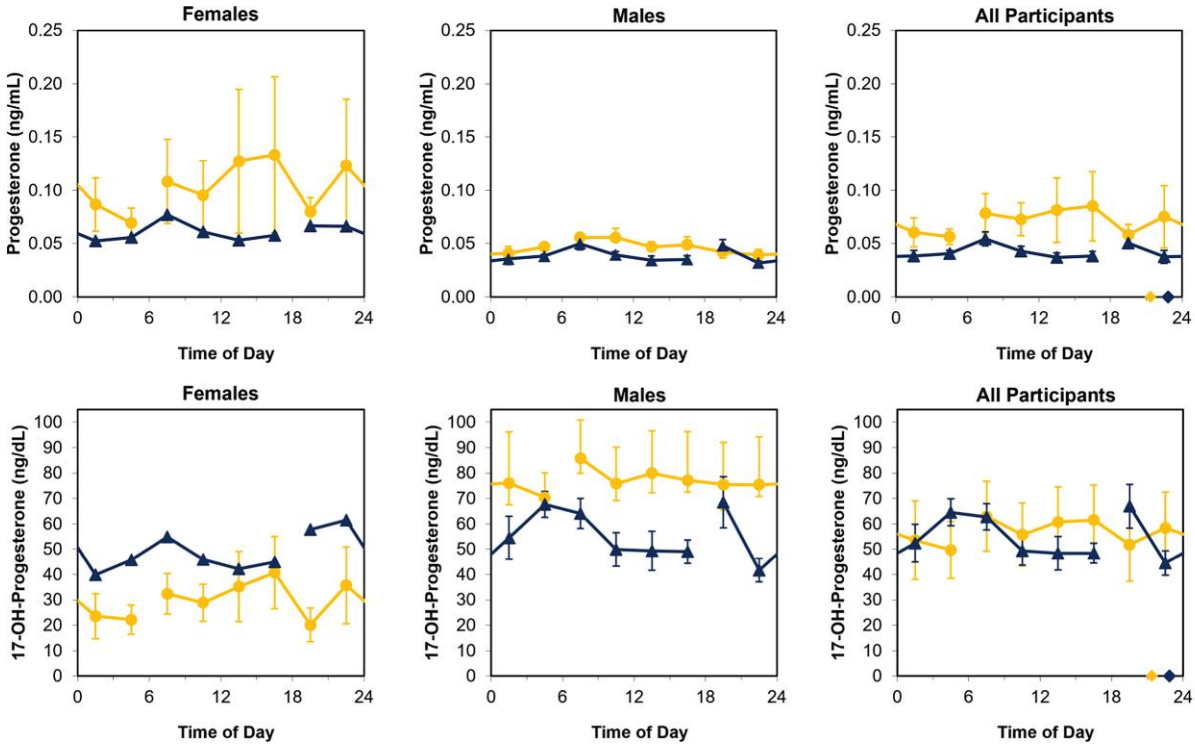

Figure 4. Temporal patterns of progesterone and 17OHP measured under constant routine following 3 days of a simulated day-shift (yellow) or night-shift (blue) schedule. Data are shown as mean ± SE by time of day for progesterone (top) and 17α-hydroxyprogesterone (bottom) in women (left), men (middle), and both (right). The gap in the line shows where the 24-hour constant routine protocol began/ended.

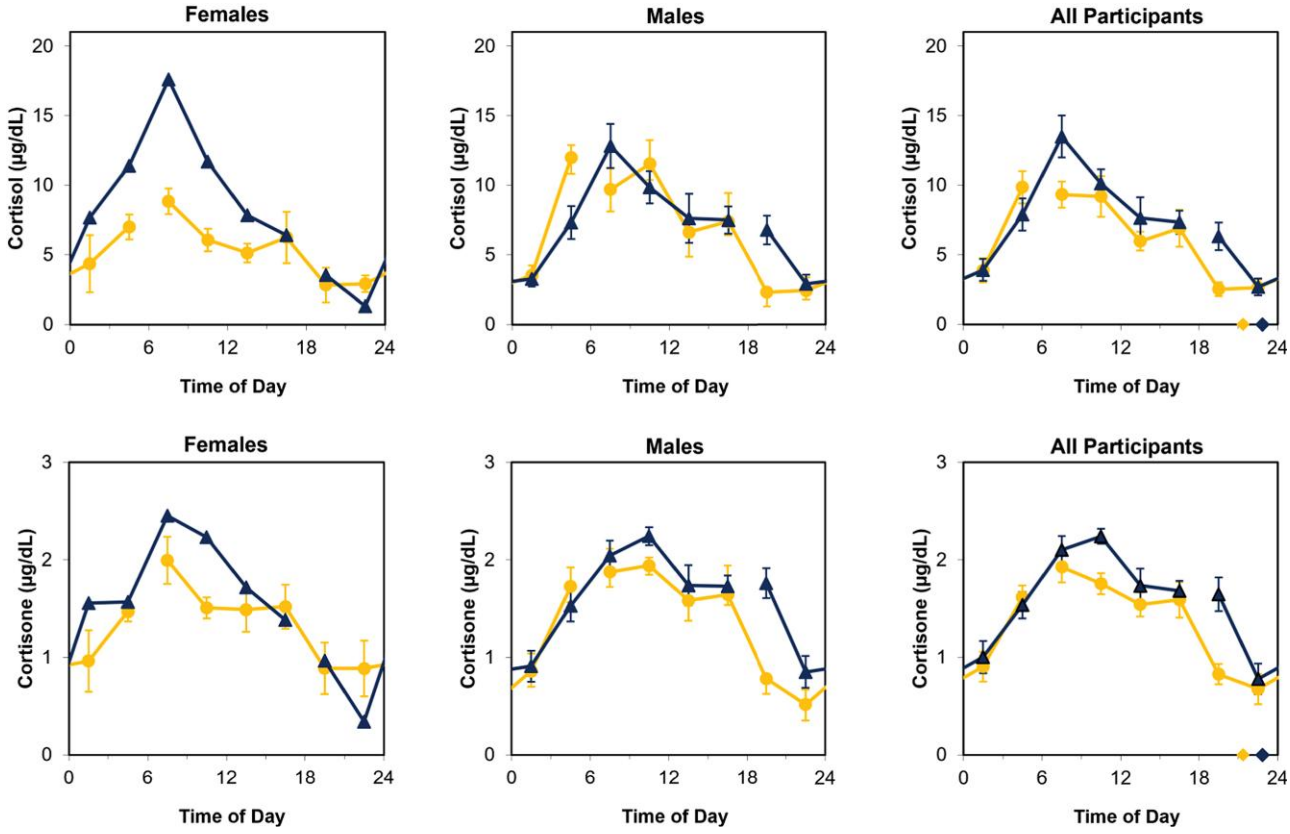

Figure 5. Temporal patterns of exemplar glucocorticoids measured under constant routine following 3 days of a simulated day-shift (yellow) or night-shift (blue) schedule. Data are shown as mean ± SE by time of day for cortisol (top) and cortisone (bottom) in women (left), men (middle), and both (right). The gap in the line shows where the 24-hour constant routine protocol began/ended.

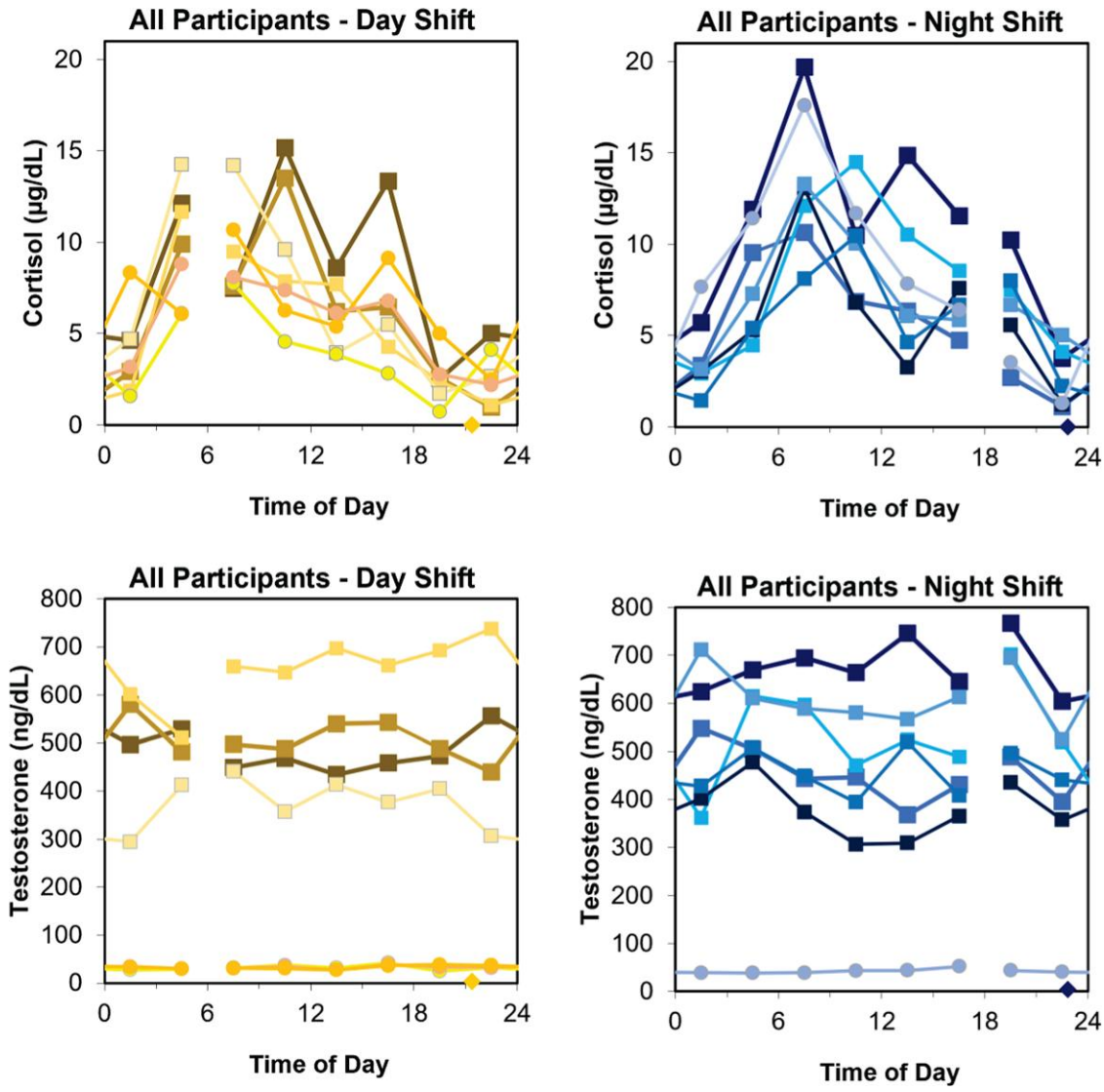

Figure 6. Temporal patterns of cortisol (top) and testosterone (bottom) in individual subjects measured under constant routine following 3 days of a simulated day-shift (left, yellow shades, n = 7) or night-shift (right, blue shades, n = 7) schedule in men (square) and women (circles). The gap in the line shows where the 24-hour constant routine protocol began/ended.

showing that the morning peak in testosterone is driven by exogenous factors, now provides strong evidence that the hormonal evaluation of hypogonadism in night-shift workers must account for the behavioral cycle of sleep/wake, feeding/fasting, and activity/inactivity. Since we and others have shown that sleep, in particular, strongly modifies testosterone [1, 43], the hormonal evaluation of hypogonadism should occur soon after waking, irrespective of time of day. In contrast, the strong circadian regulation of cortisol and its robustness to prior shift work indicates that assessment of the adrenal axis should occur in the biological morning.

Given the predominance of males, our study was unable to evaluate the effects of shift work on estradiol because of the detection limit of the assay. Although derivatization can improve detection [44], this usually requires extra plasma that was not available and could render comparisons between nonderivatized and derivatized steroids invalid. Another limitation was that we were not powered to examine sex differences, but we did control for them to provide data that is valid across both sexes. Interestingly, our analyses correctly identified 5 steroids that have previously been identified to show sex differences: progesterone, 17OHP, androstenedione, and testosterone as well as 18-hydroxycortisol [41, 45]. As expected, our results showed progesterone and androstenedione were higher in women [45], whereas 17OHP and testosterone were higher in men [41]. Our findings that 18-hydroxycorticosterone and 18-hydroxycortisol were higher in women will need verification in future studies because we are unaware of prior studies examining sex effects of 18-hydroxycorticosterone, and data are conflicting regarding sex differences on 18-hydroxycortisol [41].

Our study also had important strengths. We utilized gold-standard experimental methods including constant routine after simulated shift work in a dedicated sleep/circadian research laboratory with a high level of experimental control. We also applied gold-standard analytical methods such as tandem mass spectrometric measurement of temporal steroid patterns followed by mixed-effects cosinor analyses allowing for sex differences. Because our experimental and analytical methods were identically applied, comparisons across a range

of different steroids could be made in an unbiased fashion. Our experimental paradigm of 3 consecutive days of simulated night-shift work (compared to 3 consecutive days of simulated day-shift work) shifted the behavioral cycle by 12 hours, which is the maximum misalignment that can be achieved. It is also ecologically valid with regard to real-world work schedules, including those in many hospital settings [46] where 3 consecutive days of night work flanked by day work or days off are common (eg, in nurses). When not on night work, real-world night-shift workers routinely revert back to a day-aligned schedule (to interact with family and friends, address domestic duties, attend daytime events, etc.), so that circadian misalignment in bursts of 3 consecutive days is ecologically representative. Our study was not designed to address adjustment or accommodation to a consistently shifted schedule over weeks to months, and our data do not address this separate though related question.

In conclusion, we identified the presence/absence of underlying circadian regulation for a set of important steroids and the impact of circadian misalignment through simulated night- and day-shift conditions. We showed that the rhythmicity of cortisol—but not of testosterone—is under strong endogenous circadian regulation and is robust to prior shift schedule. Furthermore, since normative reference ranges for cortisol and testosterone were developed specifically in the morning among healthy young non-shift workers, we now show that the assessment in night-shift workers of the adrenal axis should occur in the biological morning, whereas hypogonadism in men should be determined according to the behavioral cycle, soon after waking irrespective of time of day.

Acknowledgments

We thank the study participants; the staff of the Sleep and Performance Research Center at Washington State University Health Sciences Spokane for their help conducting the clinical study; Matthew Layton for serving as physician of record; and Patrick O'Day for technical assay assistance.

Author Contributions

P.Y.L., R.J.A., S.G. and H.P.A.V.D. designed research; B.C.S., S.G. and H.P.A.V.D. performed research; R.J.A. conducted mass spectrometry measurements; H.P.A.V.D. and P.Y.L supervised the study; P.Y.L, M.R.K., F.Y., and H.P.A.V.D. analyzed data; M.R.K., F.Y., and P.Y.L. interpreted the initial results and drafted the manuscript; all authors reviewed and edited the manuscript.

Disclosures

This work was supported by start-up funds from Washington State University and North Carolina State University to S.G. and in part by United States Army Medical Research and Development Command award W81XWH-18-1-0100 to H.P.A.V.D.; National Institutes of Health Grant R01ES030113 to S.G., K24HL13632 and R01HL124211 to P.Y.L., F32HD097932 to F.Y., and K23HL157754; and the Veterans Affairs Greater Los Angeles Healthcare System Geriatric Research, Education and Clinical Center to M.R.K.. R.J.A. has received consulting fees from Janssen Pharmaceuticals and contracted research and consulting fees from Strongbridge Biopharma unrelated to the study, and H.P.A.V.D. has received contracted research and consulting fees from Jazz Pharmaceuticals and consulting fees from Eisai, unrelated to the study. The funding sources had no role in the study. The content of this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, Veterans Administration, or US federal government.

Clinical Trials

This is an investigator-initiated mechanistic study without clinical trial content or implications. It does not qualify as a clinical trial.

Data Availability

Data sets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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