

Night work and differential effects on diurnal rhythms of cortisol, melatonin, and testosterone among permanent night-working men

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Objectives Night work-induced circadian misalignment is associated with an increased risk of adverse health effects. It has been suggested that permanent night workers can entrain to being awake at night and therefore do not experience substantial circadian disruption in relation to night work. The aim of this study was to investigate the effects of night work on diurnal rhythms of cortisol, melatonin, and testosterone among permanent night workers.

Methods Twenty-three men with permanent night work participated in this observational field study. They collected saliva every 4 hours over a 24-hour period for assessment of diurnal rhythms (mesor, amplitude and acrophase) on three days: the 2nd and 4th consecutive night shifts, and a day off. Within-person regression analyses with repeated measures adjusted for age were used to evaluate differences between the 2nd and 4th night shifts and between night shifts taken together versus days off.

Results There were no statistically significant differences between diurnal rhythms on the 2nd and 4th consecutive night shifts. We observed differences in levels (mesor and amplitude) of cortisol and melatonin on night shifts compared to days off with the highest levels during work days. The timing (acrophase) of testosterone, but not cortisol and melatonin, differed between night shifts and days off. Thus, observed differences between the various hormonal diurnal rhythms to night work indicate internal desynchronization.

Conclusion Studies of how diurnal rhythms of multiple biological systems are affected by night work and the link to health effects are warranted to further elucidate the mechanisms linking night work to adverse health effects.

Key terms circadian rhythm; endocrine system; night shift work; night worker; permanent night work.

Night work is associated with increased risk of chronic disease such as diabetes, cardiovascular disease and cancer (1–3), and circadian disruption has been suggested as a mechanism linking night work to disease (4–6). Because night workers stay awake and eat during the night and sleep during the day, behavioral diurnal rhythms become misaligned with the internal circadian rhythms entrained by the external light-dark cycle (7). In addition to this circadian misalignment, a state of internal desynchronization between several levels of the circadian system occurs. Endogenous diurnal rhythms,

which result from the combined effects of the circadian system and behavioral and environmental effects, have also been observed to be desynchronized in field studies of shift workers (8). Additionally, the diurnal rhythms of cortisol, melatonin and testosterone in saliva depend on the number of consecutive night shifts. Thus they become increasingly desynchronized with more consecutive night shifts (8).

Most studies under real-life conditions have shown modest adaptation in diurnal rhythms of cortisol and melatonin in relation to night work (7). It has, however,

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been speculated that permanent night work allows for better adaptation of endogenous circadian rhythms compared to rotating night shift work. On the one hand, permanent night workers often have more consecutive night shifts, and their work schedule does not regularly alternate between shift types (9). As a consequence, it is hypothesized that permanent night workers have better adaptation of endogenous rhythms to night work (10) and therefore experience less circadian misalignment and internal desynchronization compared to shift workers with occasional night work. On the other hand, permanent night workers change their behavior on days off and are awake during day time to be with family and for other social reasons, and therefore they are also exposed to circadian misalignment (11). Thus, only a very small minority (<3%) of permanent night workers show “complete” adjustment or adaptation of their melatonin rhythm to night work (12).

Field studies of diurnal rhythms among permanent night workers include either melatonin or cortisol in saliva (13, 14), excreted 6-sulfatoxymelatonin in urine (11, 15, 16) whereas a single study includes both cortisol and melatonin in saliva (17). Most studies compare groups of permanent night workers to groups of non-permanent night workers, and those which compared diurnal rhythms during work and leisure time within the same persons include only one hormone eg, cortisol (14) or melatonin (16). To our knowledge, no previous studies have simultaneously analyzed diurnal rhythms of the three hormones: cortisol, melatonin and testosterone during work and time off within permanent night workers. The simultaneous study of multiple hormones provides knowledge on internal desynchronization, which may contribute to understanding the underlying mechanisms linking night work and adverse health effects, and may ultimately help inform strategies for disease prevention.

The aim of the present paper was to provide insights into potential internal desynchronization between multiple endocrine systems by investigating effects of night shifts on the diurnal rhythms of cortisol, melatonin and testosterone within permanent night workers under real-life conditions. We will first compare the 2nd and 4th consecutive night shift, and secondly night shifts and days off within the same persons.

Methods

This paper presents results from the within-person observational field study “*Accidents and health among permanent night workers*” (data collection period: January 2019 to September 2020). The study was conducted in accordance with the Declaration of Helsinki

and approved by the National Committee on Health Research Ethics in Denmark (H-18046118). All participants have given written consent to the inclusion of material pertaining to themselves.

Participants

We included 23 male employees working permanent night work in companies within wholesale and retail trade. Their weekly schedules entailed either 4 consecutive night shifts followed by 3 days off (4+3) or a 5+2 schedule. Night shifts were defined as ≥ 3 hours of work between 23:00 and 06:00 hours. Employees were initially informed about the project at the workplace either during a health examination or at information meetings. Employees were provided with contact details for a researcher on the project. Participants then received written and oral information by phone or in person.

Questionnaire

All participants completed a baseline questionnaire before the first saliva collection day. They were asked about tenure, night shift work experience, leisure-time physical activity, self-rated overall health, general job satisfaction, and diurnal type (single question).

Saliva samples

The participants collected saliva for assessment of cortisol, melatonin and testosterone while awake over a 24-hour period on three separate days: the 2nd and 4th consecutive night shifts and a day off. Participants were instructed to collect the first saliva sample immediately upon awakening after their primary sleep before the 2nd and 4th night shift and on a day off and the last sample right before bedtime regardless of time of day (figure 1). Participants were instructed to collect saliva samples continuously every 4 hours around 07:00, 11:00, 15:00, 19:00, 23:00, and 03:00 hours and so forth until they went to bed (starting and ending time could vary according to awakening time). To evaluate compliance, all days with saliva collection were validated against self-reported working hours from diaries. If saliva was not collected on the 2nd or 4th night shift, all saliva samples from that shift were excluded from the statistical analyses. Saliva was collected either on the first (32%) or the second (68%) day off. The majority of participants contributed with saliva on all three days (N=16), the rest on two or one day(s) (N=7). There were 6 days (10% of total days) with <5 saliva samples. A total of 37 (11%) saliva samples were collected around 11:00 after a night shift or around 03:00 hours on a day off.

Participants were instructed not to eat within half an hour before taking a saliva sample and to note the

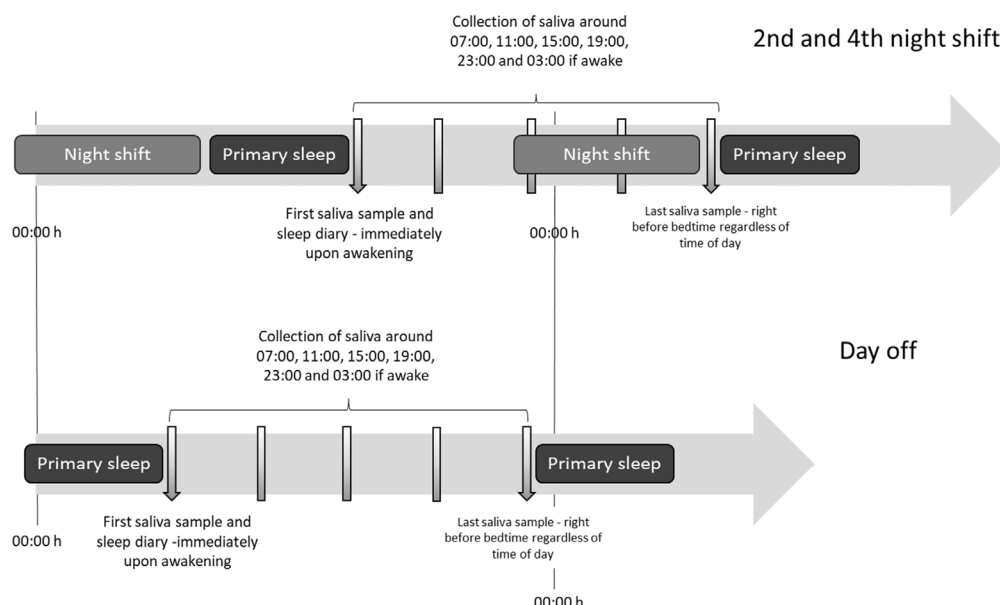


Figure 1. Study design. The participants collected saliva every 4 hours while awake over three 24-hour periods: the 2nd and 4th consecutive night shifts, and a day off. The first sample was collected immediately upon awakening after their primary sleep and the last sample right before bedtime regardless of time of day. During this period saliva samples were collected continuously every 4 hours at fixed clock times (relative to midnight) around 07:00, 11:00, 15:00, 19:00, 23:00, and 03:00 hours provided the participant was awake.

specific sampling time on a label on the saliva tube. If participants were unable to collect saliva samples at the specified time points, they were instructed to collect one as close to the specified times as possible; 78% of all samples were collected within ± 60 minutes of the bin center. The participants were given verbal and written instructions on how to collect the saliva samples and a phone number to the study's contact person in case of questions. The participants received a reminder the day before saliva sampling. Saliva samples were collected by drooling directly into a tube with no additives. The participants were instructed to keep the samples in the refrigerator or freezer until they were returned to the laboratory, where they were kept at -20°C until analysis. All samples were received within seven days, where cortisol, melatonin and testosterone in saliva are stable at room temperature (18).

Chemical analyses of melatonin, cortisol and testosterone

Analysis of hormones in the 342 saliva samples was done by liquid chromatography tandem mass spectrometry (LC-MS/MS) (18). The limit of detection (LOD) was 3.73 pmol/L, 0.26 nmol/L, and 6.63 pmol/L for melatonin (52% of samples had concentrations $<\text{LOD}$), cortisol (0.9% $<\text{LOD}$), and testosterone (7.3% $<\text{LOD}$), respectively. The distribution of samples $<\text{LOD}$ according to time of day and person was visually inspected and no systematic patterns were detected. The coefficient of variation (CV) was 4.5% for the intra-assay variation

and 7.1% for the inter-assay variation. Westgard control charts (19) were used to document that the LC-MS/MS method remained under statistical and analytical control. For concentrations $<\text{LOD}$, cortisol, melatonin and testosterone concentrations were imputed as a random number from a normal distribution with 2/3 of the mean LOD. Outliers according to Grubb's test (0.29%, 0.29% and 2.05% of samples for cortisol, melatonin and testosterone, respectively) were deleted before statistical testing.

Timing of sleep

Start and end times of sleep used for assessment of mid-sleep were obtained from sleep diaries. Participants were instructed to fill in their diary immediately after awakening from their primary sleep, ie, their main sleep period on that day (20).

Statistical analyses

Saliva samples were categorized into six 4-hour time-of-day bins, each centered on the scheduled collection time. We included all samples in the 4-hour bins even, if they were delayed, and all days with ≥ 3 saliva samples in the main analyses. These choices were tested in sensitivity analyses. For the initial regression analyses, all hormone concentrations were log-transformed and treated as continuous variables. We used mixed effects models with fixed effects for night shifts/day off and time, and

a random intercept for each participant. Repeated saliva samples were specified with a compound symmetry covariance structure to account for within-subject correlations. In model 1, we included data from all six time slots during the 24-hour periods with night shifts to test whether diurnal rhythms differed with more consecutive night shifts (between the 2nd and 4th night shift). In model 2, we included data from all six time slots on all three 24-hour collection periods (2nd night, 4th night, and day off) to test whether diurnal rhythms across days with night shifts (2nd and 4th combined) differed from those on days off. In both models, the initial tests concerned the interaction term between time of day (six levels; 4-hour time slots) and 24-hour period (two levels; 2nd night and 4th night or night shift and day off). To reduce multiple analyses, it was *a priori* decided that only if there was an interaction effect, we would continue to describe any differences in diurnal rhythms by performance of cosinor analyses (see below). Hence, we do not report estimates from the initial analyses. Results from the cosinor analyses are presented in the text, if the P-value for the interaction term in the initial analyses was >0.10. For transparency, the P-values from the cosinor analyses are presented for both models in the tables. All analyses were adjusted for age. These analyses were performed using the statistical software SAS for Windows 9.4 (TS level 1M3, SAS Institute, Cary, NC).

Cosinor regressions were conducted using the R package *card*, which fits a model requiring a minimum of three data points. Based on concentrations of each of the three hormones, estimates were made for each 24-hour period for each individual. The model provides estimates for the mesor (mean level of the sinusoidal wave), amplitude (height of the oscillation from mesor to the peak), and acrophase (timing of the peak within the cycle) (21). The statistical analyses included only participants where $P < 0.20$ for the comparison between the linear model ($\text{Concentration} = \cos(24) + \sin(24)$) and the null model (constant hormone level) using a log-likelihood ratio test.

Acrophase was converted into hours since midnight using the formula: $\text{Time} = -\frac{\text{acrophase} * 24}{2 * \pi}$

To test the analytical choices and inform the discussion, a series of sensitivity analyses were performed by repeating the main analyses:

- after excluding measurements of hormones <LOD (rather than imputing)
- with adjustment for time of sampling as a continuous variable to adjust for deviations from the protocol
- with adjustment for self-reported diurnal type
- with exclusion of 4-hour time bins with few samples, ie, time slots centered around the scheduled 03:00 and 11:00 hour collections

- using three different criteria other than the log-likelihood test for the inclusion of sampling days in the analyses of cosinor measures: (i) all sampling days; (ii) only sampling days with ≥ 5 data points; (iii) only sampling days where $P < 0.20$ for the comparison between the linear model and the null model using an ANOVA.

Results

The 23 permanent night workers were on average 54.1 [standard deviation (SD) 10.5] years of age and had 19.8 (SD 14.2) years of tenure, of which 17.9 (SD 12.4) years were of night work experience. 48% reported excellent or quite good health, and 35% found that night work was not strenuous. There were 76% self-reported evening types and 24% morning types (table 1). Midsleep was between 10:00–10:30 hour in 24-hour periods with night shifts and at 04:42 hours on days off, indicating that they slept during the day after a night shift and during the night on days off (table 2).

Table 1. Description of the study population consisting of 23 men with permanent night work. [SD=standard deviation.]

	N	%	Mean	SD
Sex				
Female	0			
Male	23	100		
Age (years)			54.1	10.5
Tenure (years)			19.8	14.2
Night work experience (years)			17.9	12.4
Self-rated health				
Excellent/quite good	11	48		
Good/less good/bad	12	52		
Diurnal type				
Morning/more morning than evening	5	24		
Evening/more evening than morning	16	76		
Physical activity				
Physically inactive/light physical activity	6	26		
Moderate physical activity	9	39		
High physical activity	8	35		
Experience night shifts as strenuous (score)				
1–2 (not strenuous)	8	35		
3	9	39		
4–7 (very strenuous)	6	26		

Table 2. Average time of sleep start, sleep end and midsleep for 23 men with permanent night work on their 2nd and 4th night shift and a day off. [SD=standard deviation.]

	2 nd night shift		4 th night shift		Day off	
	Mean	SD	Mean	SD	Mean	SD
Sleep start	07:18	01:45	07:39	00:49	00:33	01:58
Sleep end	13:06	02:17	13:04	01:44	08:53	02:24
Mid sleep	10:12	01:53	10:22	01:09	04:42	02:00

Time profiles based on average concentrations of melatonin, cortisol and testosterone by 24-hour period and time slots are shown in figure 2 and data for mesor, amplitude and acrophase are given for all three hormones in table 3. Overall, mesor for cortisol rhythms ranged from 3.1 nmol/L (SD 1.0 nmol/L) on the day off to 4.6 nmol/L (SD 1.8 nmol/L) on the 4th night shift. The amplitudes were in the range of 2.2–3.1 pmol/L, and concentrations peaked between 13:24 and 14:02 hours. For melatonin, mesor varied between 22 (SD 10) pmol/L on the day off and 41 (SD 58) pmol/L on the 4th night shift, and the amplitudes were in the range of 20

and 48 pmol/L on the same days. Peak concentrations of melatonin occurred at 08:34 (SD 04:47) hours on the 2nd night shift and at 11:49 (SD 07:14) hours on the day off. For testosterone, mesor was in the range of 118–178 pmol/L with amplitudes in the range of 73–138 pmol/L, and the concentrations of testosterone peaked at 13:23 and 13:54 hours on the 2nd and 4th day with night shifts, respectively, and at 10:39 (SD 05:51) hours on days off.

When comparing rhythms on the 2nd and 4th night shift (model 1), there were no statistically significant interaction effects between 24-hour periods and time slots in the regression analyses for cortisol ($P=0.196$), melatonin

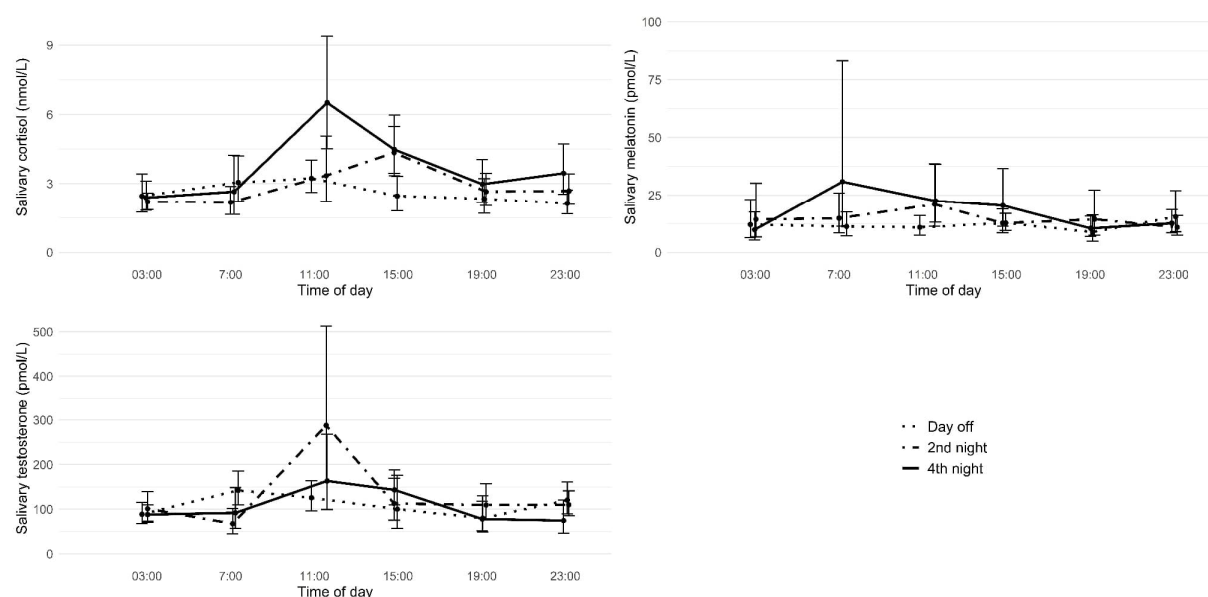


Figure 2. Concentrations of cortisol, melatonin and testosterone on the 2nd and 4th day with night shift and a day off (raw data). Bars represent 2*SEM. Concentrations are plotted against the average of the actual time of collection.

Table 3. Results from cosinor analyses. The analyses were based on analyses of cortisol, melatonin and testosterone measured in saliva collected every 4 hours from 23 men with permanent night work on their 2nd and 4th night shift and a day off. **Bolded text indicates overall significant statistical difference.** [SD=standard deviation.]

	2 nd night shift		4 th night shift		Day off		P value	
	Mean	SD	Mean	SD	Mean	SD	Model 1 ^a	Model 2 ^b
Acrophase ^c								
Cortisol (hh:mm)	14:00	04:33	14:02	04:50	13:24	06:01	0.683	0.642
Melatonin (hh:mm)	08:34	04:47	10:11	05:34	11:49	07:14	0.237	0.255
Testosterone (hh:mm)	13:23	04:33	13:54	03:39	10:39	05:51	0.985	0.048
Amplitude								
Cortisol (nmol/L)	2.5	1.8	3.1	1.7	2.2	1.4	0.145	0.247
Melatonin (pmol/L)	27	20	48	74	20	16	0.435	0.042
Testosterone (pmol/L)	116	123	73	56	138	154	0.502	0.149
Mesor								
Cortisol (nmol/L)	3.7	1.3	4.6	1.8	3.1	1.0	0.055	0.023
Melatonin (pmol/L)	28	18	41	58	22	10	0.563	0.035
Testosterone (pmol/L)	178	133	118	45	173	85	0.074	0.335

^a Difference between the 2nd and 4th night shift. Adjusted for age.

^b Difference between days with night work and days off. Adjusted for age.

^c Acrophase is represented as time relative to midnight.

($P=0.437$) or testosterone ($P=0.472$), indicating that the diurnal rhythms were similar on the two days with night shifts for all the hormones. The initial regression analysis of the interaction between 24-hour periods (2nd and 4th night shifts combined or days off) and time (model 2) showed that there were differences in the overall rhythms of cortisol ($P<0.001$), melatonin ($P=0.092$), and testosterone ($P=0.007$). The subsequent cosinor analyses are shown in table 3. They revealed that for cortisol, mesor was higher during 24-hour periods with night shifts (0.88 pmol/L, SE=0.37 pmol/L, $P=0.023$) compared to days off. For melatonin, the mesor (6.31 pmol/L, SE=2.73 pmol/L, $P=0.035$) and amplitude (8.76 pmol/L, SE=3.95 pmol/L, $P=0.042$) were higher during 24-hour periods with night shifts compared to days off. For testosterone, the acrophase was delayed during 24-hour periods with night shifts compared to days off (03:12, SE 01:31 hours, $P=0.048$). There were no other statistically significant differences in diurnal rhythms.

None of the sensitivity analyses changed the overall conclusions from the primary analyses when considering the lower number of participants and therefore expected lower statistical power (see supplementary material, www.sjweh.fi/article/4332, tables S1–S4).

Discussion

In this within-person observational field study, permanent night workers had higher mesor of cortisol and melatonin during 24-hour periods with night shifts compared to days off. For melatonin, the amplitude was also higher during days with night shifts compared to days off. Testosterone was the only hormone for which there was a difference in the acrophase between night shifts and days off. We observed no differences between the 2nd and the 4th consecutive night shift in the diurnal rhythms of the three hormones.

Night shifts versus days off

Levels (mesor and amplitude) of melatonin and cortisol were higher during days with night shifts compared to days off among permanent night workers in the present within-person study, indicating that the levels are suppressed on days off. This appears to be in contrast to previous studies within permanent night workers, where either no differences were observed in diurnal cortisol rhythms between night shifts and days off (14) or urinary 6-sulphatoxy-melatonin concentrations were lowest during the 24-hour periods with night shifts (16). Exposure to light suppresses the melatonin excretion (22). Therefore, shift workers typically have lower levels of melatonin during night shifts compared to day

shifts or days off, because they are exposed to light when working at night, where concentrations of melatonin concentrations normally peak (8). As discussed later, the permanent night workers in the present study exhibit a phase-delayed melatonin rhythm compared to non-night workers. Accordingly, they are awake and therefore exposed to light when their melatonin peaks during the day on days off, which may explain why melatonin was most blunted on the days off.

The acrophase of testosterone was 03:12 hours delayed on night shifts compared to days off. Similarly, the acrophase for testosterone was delayed in a study of shift workers after three consecutive weeks with night work (23). This aligns well with testosterone being released during deep sleep (24), since the participants have a 5–6 hours delayed mid sleep time after a night shift compared to days off. Acrophases for cortisol and melatonin on days off did not differ from night shifts within the permanent night workers, which is in contrast to findings among other free-living shift workers (8, 23) and in a comparable laboratory study of simulated night work (25). Due to few days off, saliva was collected on the 1st or 2nd day off, which may be insufficient time to phase shift in real-life settings before returning to (night) work.

Number of consecutive night shifts

Increasing number of consecutive night shifts has been associated with a phase delay of cortisol and melatonin in within-person study designs among rotating shift workers (8, 26). We observed no differences in diurnal rhythms of cortisol and melatonin from the 2nd and 4th night shift among permanent night workers. The lack of difference in acrophase with increasing number of consecutive night shifts may be interpreted as adaptation to night work. Alternatively, the individual differences and differences in light exposure cause some participants to advance and others to delay their phase (17, 27–29). Among shift-working police officers scheduled to work seven consecutive night shifts on patrol, only ~25% rhythms of cortisol/aMT6s substantially delayed (partial adaptation to night schedule) (26). Stratified analyses were not feasible due to the small sample size, but future studies on night work and diurnal rhythms could benefit from including a sufficient number of participants to perform subgroup analyses by stratification between those who advance and those who delay their diurnal rhythms.

Taken together, there are differential effects of night work on diurnal rhythms of the hormones in response to night work within the group of permanent night workers. These differences indicate that the diurnal rhythms become desynchronized among permanent night workers in real-life settings.

Permanent and non-permanent night workers

In general, between-person studies find lower levels in terms of average or peak concentrations of cortisol and melatonin in groups of night shift compared to day workers (7). In the present study, peak levels (mesor plus amplitude) of melatonin in saliva averaged 42 pmol/L on days off, which is substantially lower than previously reported peak values (17.6 pg/mL corresponding to 76 pmol/L) among non-night working employees (30). Among shift workers, we have previously observed higher average peak levels of around 60 pmol/L on days off (8). Thus, permanent night workers in the present study have considerably lower levels of melatonin compared to both non-night and rotating night shift workers. This aligns with previous studies, where it has been found that both rotating shift workers and permanent night workers have lower levels and fluctuation of melatonin than day workers (7, 13, 31). Our results also align with a study on excretion of 6-sulfatoxy melatonin among nurses with different shift schedules, where it was found that nurses with permanent night work excreted less 6-sulfatoxy melatonin on both 24-hour periods with night shifts and days off compared to nurses working all other shift schedules (16).

Among healthy non-night working individuals, salivary melatonin peaks between 24:00 and 03:00 hours (30) and cortisol in the early morning around awakening (4, 32–34). Later peak times of melatonin have been observed among both rotating night shift workers (13, 31) and permanent night workers (13, 15) compared to day workers. This was corroborated in the present study, where the timing of the peak concentration for cortisol and melatonin among the permanent night workers was 13:24 hours for cortisol and 11:49 hours for melatonin on days off. Thus, there is a substantial phase delay in cortisol (~06:30 hours) and particularly melatonin (~09:00 hours) rhythms compared to non-night workers. In addition, the phase angle between the acrophases of diurnal rhythms of melatonin and cortisol is smaller (01:35 hours) among permanent night workers compared to non-night workers (04:00 hours) (34).

Taken together permanent night workers as a group have lower levels and delayed rhythms of cortisol and melatonin both during night shifts and days off compared to rotating night shift workers and day workers. The delayed peak of cortisol and melatonin could indicate that permanent night workers are to some degree adapted to sleep during the day rather than night. Yet, the degree of adaptation differs between cortisol and melatonin, as indicated by the phase angle. Thus, permanent night workers are similar to shift workers in that their diurnal rhythms are affected by the night work. Yet, the effects differ from those of shift workers with night work in that the permanent night workers have higher

levels of cortisol and melatonin on work days, and lower levels than shift workers on days off. Our study extends the finding from laboratory studies where the underlying physiological mechanisms are well studied eg, using forced desynchronization protocols (6, 33). To fully understand the health implications of night work, it is relevant to know in future studies what the effects are in real life, where behavioral and environmental exposures add to the total effect, as illustrated by the observation that permanent night workers have the lowest levels of cortisol and melatonin on days off rather than on days with night work.

Strengths and limitations

Major strengths of the present study are the simultaneous analyses of diurnal rhythms of three different hormones among permanent night workers in a real-life setting and the within-person design leading to reduced risk of confounding due to unmeasured individual time-invariant factors. A limitation is the assumed selection into (versus out of) permanent night work by individuals who thrive on (versus experience problems with) night work (35). The result is a population of permanent night workers who can better handle frequent night shifts. This is supported by the fact that the present study group had long-term experience with night work and only 26% found it strenuous. Selection mechanisms into and out of permanent night work could have important consequences for the generalizability and implications of the findings of this study. It is worth noting that despite likely selection effects, there are still differences in acute changes in diurnal rhythms of the three hormones. Yet possible long-term health consequences remain to be settled.

All participants in this study were men. While we assume that the underlying physiological mechanisms are similar for both men and women, there are notable differences in hormone levels, particularly for testosterone (36). Using samples from men only, makes it challenging to compare our findings directly with studies involving women. Nonetheless, by restricting to men in a small-scale study, we enhance the internal validity by reducing variability attributable to sex-specific physiological differences. The sample size was relatively small compared to previous studies, but given the within-person design, still sufficient to observe relevant effects. Saliva on days off was collected either on the first or second day off after a series of night shift, and we cannot disentangle possible effects related to this. In addition, we do not have records of what the participants did in their spare time. However, their sleep patterns indicate that they tend to revert to a regular day-time routine sleeping during the (late) night (between 00:33 to 08:53). For participants with a 4+3 schedule, the 4th night shift was the last night shift in a sequence of

consecutive night shifts; in the case of a 5+2 schedule, it was not. While it has been shown that sleep after the last night shift is truncated in order for the employee to return to day orientation (37), it is not assumed to affect the diurnal rhythms of hormones, since the saliva is collected during the 24-hour period with the night shift and therefore before the primary sleep after the last night shift. To evaluate the robustness of our findings and assess potential sources of bias due to deviations from the protocol, we performed a range of sensitivity analyses. Chronotype could confound the results, since permanent night workers are more often evening types (38), and diurnal patterns of cortisol have been shown to differ between morning and evening types (39, 40). Yet, chronotype did not appear to confound the analyses in the present study as adjustment for diurnal preference did not largely alter the results. Neither did the results from initial analyses appear to be driven by unstable estimates in underrepresented time windows, since the results were similar in analyses where time windows with low participation were excluded. Similar results were observed in other sensitivity analyses adjusted for time of sampling as a continuous variable to account for deviations from the protocol, and with different criteria than the log-likelihood test for the cosinor analyses. The use of saliva collected in a real-world setting implies that the exposure includes changes in multiple other exposures, that influence circadian rhythms eg, light exposures, eating time and compositions, and time of physical activity. Yet, it is important that we perform these challenging field studies to get a clearer view on the complexity of internal desynchronization in hormonal secretion. Taken together, it is our evaluation that the results are robust that potential biases are limited. Yet, future studies should further investigate the effect of eg, shift layout, measurement protocol, and markers used for the observed differences.

Concluding remarks

Among permanent night workers, we observed differences in levels (mesor and amplitude) of cortisol and melatonin on days with night shifts compared to days off, and with higher levels during the work days. In addition, the timing (acrophase) of testosterone, but not cortisol and melatonin, differed between night shifts and days off. Thus, the differences in the hormonal diurnal rhythms during night shifts and days off depend on the hormone, and the diurnal rhythms of these hormones are desynchronized among permanent night workers. Future studies of how diurnal rhythms of multiple biological systems are affected by night work and how these effects are linked to health effects are warranted to further elucidate the underlying mechanisms linking night work to adverse health effects.

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Disclosure of interest

The authors have no conflicting interests to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Torquati L, Mielke GI, Brown WJ, Kolbe-Alexander T. Shift work and the risk of cardiovascular disease. A systematic review and meta-analysis including dose-response relationship. *Scand J Work Environ Health* 2018 May;44(3):229–38. <https://doi.org/10.5271/sjweh.3700>.
2. Vyas MV, Garg AX, Iansavichus AV, Costella J, Donner A, Laugsand LE et al. Shift work and vascular events: systematic review and meta-analysis. *BMJ* 2012 Jul;345:e4800. <https://doi.org/10.1136/bmj.e4800>.
3. IARC. Night shift work. *IARC Monogr Identif Carcinog Hazards Hum* 2020;124:1–371.
4. Haus E, Smolensky M. Biological clocks and shift work: circadian dysregulation and potential long-term effects. *Cancer Causes Control* 2006 May;17(4):489–500. <https://doi.org/10.1007/s10552-005-9015-4>.
5. Morris CJ, Yang JN, Scheer FA. The impact of the circadian timing system on cardiovascular and metabolic function. *Prog Brain Res* 2012;199:337–58. <https://doi.org/10.1016/B978-0-444-59427-3.00019-8>.
6. Chellappa SL, Vujovic N, Williams JS, Scheer FA. Impact of Circadian Disruption on Cardiovascular Function and

- Disease. *Trends Endocrinol Metab* 2019 Oct;30(10):767–79. <https://doi.org/10.1016/j.tem.2019.07.008>.
7. Boivin DB, Boudreau P, Kosmadopoulos A. Disturbance of the Circadian System in Shift Work and Its Health Impact. *J Biol Rhythms* 2022 Feb;37(1):3–28. <https://doi.org/10.1177/07487304211064218>.
 8. Jensen MA, Hansen AM, Kristiansen J, Nabe-Nielsen K, Garde AH. Changes in the diurnal rhythms of cortisol, melatonin, and testosterone after 2, 4, and 7 consecutive night shifts in male police officers. *Chronobiol Int* 2016;33(9):1280–92. <https://doi.org/10.1080/07420528.2016.1212869>.
 9. Costa G. Shift work and health: current problems and preventive actions. *Saf Health Work* 2010 Dec;1(2):112–23. <https://doi.org/10.5491/SHAW.2010.1.2.112>.
 10. Garde AH, Begtrup L, Bjorvatn B, Bonde JP, Hansen J, Hansen AM et al. How to schedule night shift work in order to reduce health and safety risks. *Scand J Work Environ Health* 2020 Nov;46(6):557–69. <https://doi.org/10.5271/sjweh.3920>.
 11. Quera-Salva MA, Defrance R, Claustrat B, De Lattre J, Guilleminault C. Rapid shift in sleep time and acrophase of melatonin secretion in short shift work schedule. *Sleep* 1996 Sep;19(7):539–43.
 12. Folkard S. Do permanent night workers show circadian adjustment? A review based on the endogenous melatonin rhythm. *Chronobiol Int* 2008 Apr;25(2):215–24. <https://doi.org/10.1080/07420520802106835>.
 13. Daugaard S, Garde AH, Bonde JP, Christoffersen J, Hansen AM, Markvart J et al. Night work, light exposure and melatonin on work days and days off. *Chronobiol Int* 2017;34(7):942–55. <https://doi.org/10.1080/07420528.2017.1327867>.
 14. Kudielka BM, Buchtal J, Uhde A, Wüst S. Circadian cortisol profiles and psychological self-reports in shift workers with and without recent change in the shift rotation system. *Biol Psychol* 2007 Jan;74(1):92–103. <https://doi.org/10.1016/j.biopsycho.2006.08.008>.
 15. Papantoniou K, Pozo OJ, Espinosa A, Marcos J, Castaño-Vinyals G, Basagaña X et al. Circadian variation of melatonin, light exposure, and diurnal preference in day and night shift workers of both sexes. *Cancer Epidemiol Biomarkers Prev* 2014 Jul;23(7):1176–86. <https://doi.org/10.1158/1055-9965.EPI-13-1271>.
 16. Marie Hansen A, Helene Garde A, Hansen J. Diurnal urinary 6-sulfatoxymelatonin levels among healthy Danish nurses during work and leisure time. *Chronobiol Int* 2006;23(6):1203–15. <https://doi.org/10.1080/07420520601100955>.
 17. Koller M, Härma M, Laitinen JT, Kundi M, Piegler B, Haider M. Different patterns of light exposure in relation to melatonin and cortisol rhythms and sleep of night workers. *J Pineal Res* 1994 Apr;16(3):127–35. <https://doi.org/10.1111/j.1600-079X.1994.tb00092.x>.
 18. Jensen MA, Hansen AM, Abrahamsson P, Nørgaard AW. Development and evaluation of a liquid chromatography tandem mass spectrometry method for simultaneous determination of salivary melatonin, cortisol and testosterone. *J Chromatogr B Analyt Technol Biomed Life Sci* 2011 Sep;879(25):2527–32. <https://doi.org/10.1016/j.jchromb.2011.07.005>.
 19. Westgard JO, Barry PL, Hunt MR, Groth T. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem* 1981 Mar;27(3):493–501. <https://doi.org/10.1093/clinchem/27.3.493>.
 20. Nabe-Nielsen K, Larsen AD, Arup AE, Sallerup M, Schlünssen V, Hansen ÅM et al. Sleep duration and quality in permanent night work: an observational field study. *Int Arch Occup Environ Health* 2024 Sep;97(7):733–43. <https://doi.org/10.1007/s00420-024-02080-0>.
 21. Cunningham PS, Jackson C, Chakraborty A, Cain J, Durrington HJ, Blaikley JF. Circadian regulation of pulmonary disease: the importance of timing. *Clin Sci (Lond)* 2023 Jun;137(11):895–912. <https://doi.org/10.1042/CS20220061>.
 22. Tähkämö L, Partonen T, Pesonen AK. Systematic review of light exposure impact on human circadian rhythm. *Chronobiol Int* 2019 Feb;36(2):151–70. <https://doi.org/10.1080/07420528.2018.1527773>.
 23. Harding BN, Castaño-Vinyals G, Palomar-Cros A, Papantoniou K, Espinosa A, Skene DJ et al. Changes in melatonin and sex steroid hormone production among men as a result of rotating night shift work - the HORMONIT study. *Scand J Work Environ Health* 2022 Jan;48(1):41–51. <https://doi.org/10.5271/sjweh.3991>.
 24. Wittert G. The relationship between sleep disorders and testosterone. *Curr Opin Endocrinol Diabetes Obes* 2014 Jun;21(3):239–43. <https://doi.org/10.1097/MED.0000000000000069>.
 25. James FO, Cermakian N, Boivin DB. Circadian rhythms of melatonin, cortisol, and clock gene expression during simulated night shift work. *Sleep* 2007 Nov;30(11):1427–36. <https://doi.org/10.1093/sleep/30.11.1427>.
 26. Kosmadopoulos A, Boudreau P, Kervezee L, Boivin DB. Circadian Adaptation of Melatonin and Cortisol in Police Officers Working Rotating Shifts. *J Biol Rhythms* 2024 Feb;39(1):49–67. <https://doi.org/10.1177/07487304231196280>.
 27. Dijk DJ, Duffy JF, Silva EJ, Shanahan TL, Boivin DB, Czeisler CA. Amplitude reduction and phase shifts of melatonin, cortisol and other circadian rhythms after a gradual advance of sleep and light exposure in humans. *PLoS One* 2012;7(2):e30037. <https://doi.org/10.1371/journal.pone.0030037>.
 28. St Hilaire MA, Lammers-van der Holst HM, Chinoy ED, Isherwood CM, Duffy JF. Prediction of individual differences in circadian adaptation to night work among older adults: application of a mathematical model using individual sleep-wake and light exposure data. *Chronobiol Int* 2020;37(9-10):1404–11. <https://doi.org/10.1080/07420528.2020.1813153>.

29. Isherwood CM, Chinoy ED, Murphy AS, Kim JH, Wang W, Duffy JF. Scheduled afternoon-evening sleep leads to better night shift performance in older adults. *Occup Environ Med* 2020 Mar;77(3):179–84. <https://doi.org/10.1136/oemed-2019-105916>.
30. Burgess HJ, Fogg LF. Individual differences in the amount and timing of salivary melatonin secretion. *PLoS One* 2008 Aug;3(8):e3055. <https://doi.org/10.1371/journal.pone.0003055>.
31. Gómez-Acebo I, Dierssen-Sotos T, Papantoniou K, Garcia-Unzueta MT, Santos-Benito MF, Llorca J. Association between exposure to rotating night shift versus day shift using levels of 6-sulfatoxymelatonin and cortisol and other sex hormones in women. *Chronobiol Int* 2015 Feb;32(1):128–35. <https://doi.org/10.3109/07420528.2014.958494>.
32. Clow A, Hucklebridge F, Thorn L. The cortisol awakening response in context. *Int Rev Neurobiol* 2010;93:153–75. [https://doi.org/10.1016/S0074-7742\(10\)93007-9](https://doi.org/10.1016/S0074-7742(10)93007-9).
33. Scheer FA, Hilton MF, Mantzoros CS, Shea SA. Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci USA* 2009 Mar;106(11):4453–8. <https://doi.org/10.1073/pnas.0808180106>.
34. Fidan V, Alp HH, Gozeler M, Karaaslan O, Binay O, Cingi C. Variance of melatonin and cortisol rhythm in patients with allergic rhinitis. *Am J Otolaryngol* 2013;34(5):416–9. <https://doi.org/10.1016/j.amjoto.2013.03.004>.
35. Åkerstedt T, Sallinen M, Kecklund G. Shiftworkers' attitude to their work hours, positive or negative, and why? *Int Arch Occup Environ Health* 2022 Aug;95(6):1267–77. <https://doi.org/10.1007/s00420-022-01831-1>.
36. Keevil BG, Clifton S, Tanton C, Macdowall W, Copas AJ, Lee D et al. Distribution of Salivary Testosterone in Men and Women in a British General Population-Based Sample: The Third National Survey of Sexual Attitudes and Lifestyles (Natsal-3). *J Endocr Soc* 2017 Jan;1(1):14–25. <https://doi.org/10.1210/js.2016-1029>.
37. Garde AH, Nabe-Nielsen K, Jensen MA, Kristiansen J, Sørensen JK, Hansen ÅM. The effects of the number of consecutive night shifts on sleep duration and quality. *Scand J Work Environ Health* 2020 Jul;46(4):446–53. <https://doi.org/10.5271/sjweh.3885>.
38. Nabe-Nielsen K, Arup AE, Sallerup M, Harmsen R, Ginty AS, Nielsen MT et al. The 1001 nights-cohort - paving the way for future research on working hours, night work, circadian disruption, sleep, and health. *Eur J Epidemiol* 2025 Mar;40(3):371–82. <https://doi.org/10.1007/s10654-025-01201-4>.
39. Randler C, Schaal S. Morningness-eveningness, habitual sleep-wake variables and cortisol level. *Biol Psychol* 2010 Sep;85(1):14–8. <https://doi.org/10.1016/j.biopsycho.2010.04.006>.
40. Oginska H, Fafrowicz M, Golonka K, Marek T, Mojsa-Kaja J, Tucholska K. Chronotype, sleep loss, and diurnal pattern of salivary cortisol in a simulated daylong driving. *Chronobiol Int* 2010 Jul;27(5):959–74. <https://doi.org/10.3109/07420528.2010.489412>.

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