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Supplemental Information for

Sex Differences in the Circadian Misalignment Effects on Energy Regulation

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Supplementary Information Text.

Materials and Methods

Study design. "Minimization" was used to minimize imbalance—according to age, sex, and body mass index (BMI)—in the order of laboratory visits (4 male participants and 3 female participants undertook the circadian alignment protocol first, the others undertook the circadian misalignment protocol first).

Diet. On day 1 of both protocols, participants received an *ad libitum* lunch at ~12:00 PM. Caloric intake was prorated for the 12-h behavioral cycle on day 4 of the circadian misalignment protocol (i.e., they received 50% of the caloric content compared with the 24-h days). Identical test meals (33.3% of calculated daily calorie intake) were given at 1 h (breakfast) and 13 h (dinner) following scheduled wake time on the 1st and 3rd test days. Isocaloric lunch (identical within each participant) was given at 4.5h following wake time on test days. On non-test days, participants consumed three meals and one snack (each 30% and 10% of daily calorie intake, respectively).

Polysomnography. Sleep was recorded during sleep periods 1, 4, and 6 in the circadian alignment protocol and during sleep periods 1, 5, and 7 in the circadian misalignment protocol. Polysomnography recordings included electroencephalography (EEG), left and right electrooculography (EOG), bipolar submental electromyography (EMG), and bipolar electrocardiography (ECG). EEG was recorded from F3, C3, and O1 referenced to M2, and from F4, C4, and O2 referenced to M1. Digitized EEG, EOG, EMG, and EKG data were inspected using Vitascore software (TEMEC Instruments). Sleep recordings were scored visually in 30-s epochs according to the conventional criteria of the American Academy of Sleep Medicine (1).

Indirect calorimetry and physical activity. For each test meal, three sessions were sequentially measured at 30 min before the start of each test meal, and 30, 90 min after the start of the test meal. Each session lasted for 24 min. Oxygen uptake (VO2) and carbon dioxide (VCO2) production data were recorded every min and data from the first 5 min of each recording were discarded. Remaining data were used to calculate respiratory quotient (RQ; VCO2/VO2), and carbohydrate and lipid oxidation according to the formulae of Frayn (2), assuming negligible protein oxidation. Physical activity (n=12; 4 females) was estimated with wrist actigraphy [Actiwatch Spectrum (Philips-Respironics) or Actiwatch-L (Mini Mitter)] wore by participants throughout the in-lab stays except during shower. Technical difficulties precluded actigraphy measurements in 2 participants of the 14 participants.

Core body temperature was measured throughout using a rectal thermistor.

Heart rate variability. For assessment of heart rate variability, a three-lead electrocardiogram was recorded on a Vitaport (TEMEC Instruments). Participants underwent 7 min of voluntary paced breathing at 10:20AM and 10:20 PM in the circadian alignment protocol (wake period 5 and 7) and at 10:20 PM and 10:20 AM in the circadian misalignment protocol (wake period 6 and 8). Heart rate variability analyses were performed according to the standards of the Task Force (3).

Urinary catecholamines. Twenty-four-hour urinary epinephrine and norepinephrine levels were determined by collecting urine voids shortly after scheduled wake time until shortly after

scheduled wake time 24 h later: i.e., between ~7:00 AM and 7:00 AM in the circadian alignment protocol (wake period 5 and 6 and wake period 7 and 8) and between ~7:00 PM and 7:00 PM in the circadian misalignment protocol (wake period 6 and 7 and wake period 8 and 9). Urine voids were scheduled every 4 h during the wake episodes and once after the 8-h sleep opportunities. Any extra voids were stored at 4 °C and pooled with the subsequent scheduled voids. At each scheduled void time, 10-mL aliquots were collected in 50 μL of HCl and stored at −80 °C until assaying. The total volume and timing of each void was recorded. Urinary epinephrine and norepinephrine excretion rates were calculated as the total volume multiplied by the concentration divided by the time difference between the scheduled void and the previous scheduled void.

Assays. Plasma leptin levels were measured by radioimmunoassay (Millipore, St. Charles, MO), with a detection limit of 0.1 ng/ml, an intra-assay coefficient of variation (CV) of 5.2-7.5%, and an inter-assay CV of 3.2-8.9%. For active ghrelin assay, plasma is acidified with HCl, preserved with Phenylmethylsulfonyl fluoride (PMSF), and stored at -80°C. Active ghrelin is measured by an ELISA assay from Linco Research (St. Louis, MO), with a detection limit of 3.2 pg/Ml, an intra-assay CV of 6.5 to 9.5%, and an inter-assay CV of 9.6 to 16.2%. Serum melatonin levels were measured by radioimmunoassay (Bühlmann Laboratories AG) with a detection limit of 0.3 pg/ml, an intra-assay CV of 7.9%, and an inter-assay CV of 11.7%. Serum cortisol levels were measured by chemiluminescent immunoassay (Beckman Coulter) with a dynamic range of 0.4 to 60 ug/dl, an intra-assay CV of 4.4 to 6.7%, and an inter-assay CV of 6.4 to 7.9%. Serum free fatty acid (FFA) levels were measured by enzymatic colorimetric method assay (Wako Pure Chemical Industries, Ltd.) with a detection limit of 0.001 mEq/l, an intra-assay CV of 0.6 to 0.8%, and an inter-assay CV of 0.8 to 4.9%. Serum triglyceride levels were measured by enzymatic colorimetric method assay (Roche Diagnostics) with a detection limit of 3.5 mg/dl, an intra-assay CV of 1.6%, and an inter-assay CV of 1.9%.

Supplemental Figures and Tables.

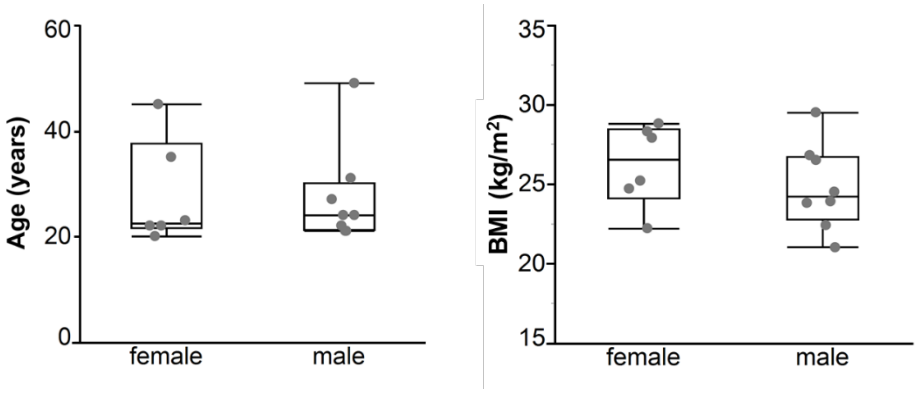

Fig. S1. No significant differences in age and BMI between female and male participants. Each dot represents individual value. The box plots display the full range of variation (from min to max, the lower and upper whiskers), the interquartile range (central rectangle), and the median (horizontal line in the central rectangle).

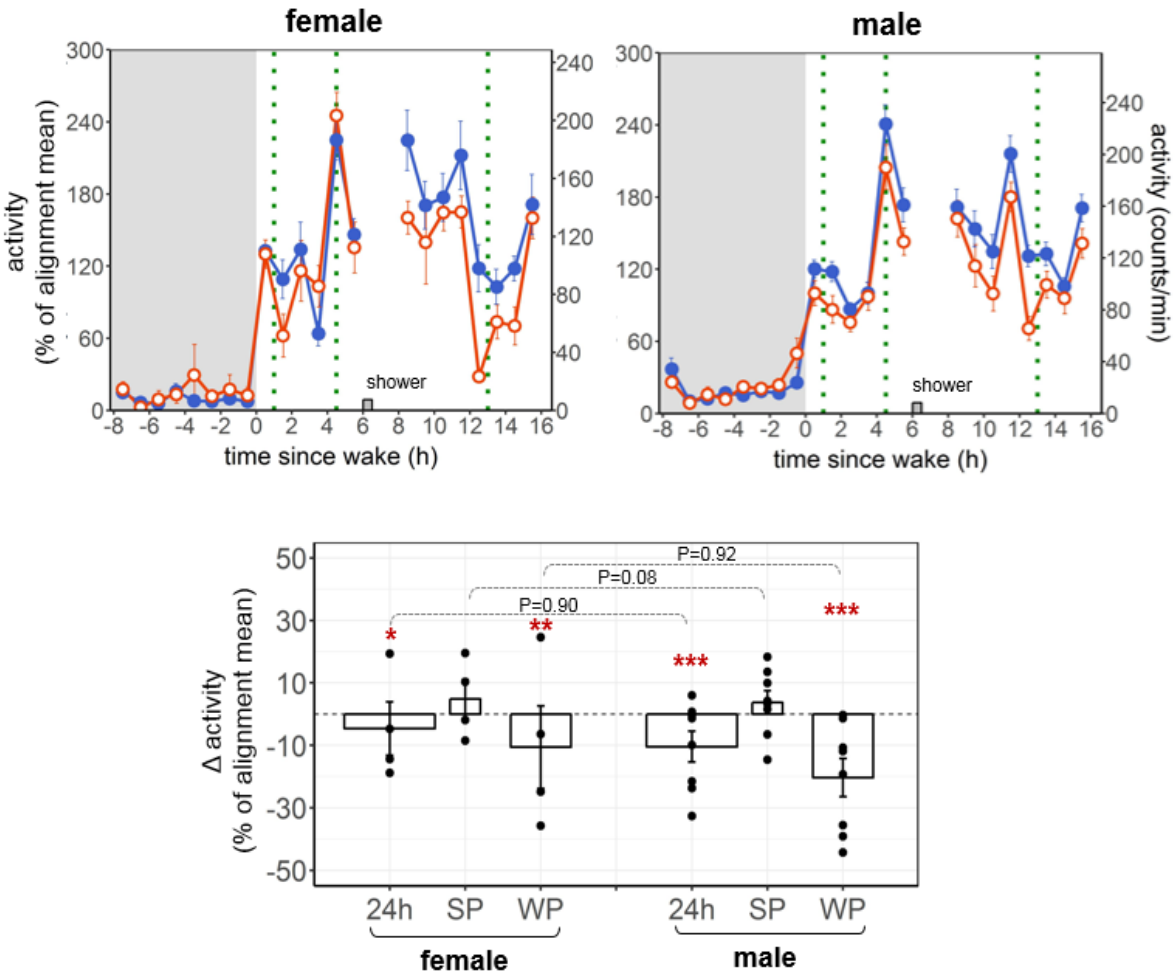

Fig. S2. Effects of circadian misalignment on physical activity counts in females (top left) and males (top right). Gray bar represents sleep opportunity; green dotted line represents a meal. Percentage changes are shown in the bar graph as described in Figure 2. *P-values*, statistical significance for interaction effect of misalignment and sex. *Adjusted P-values* for subgroup analysis by sex, * adj.P<0.05; *** adj.P<0.0001.

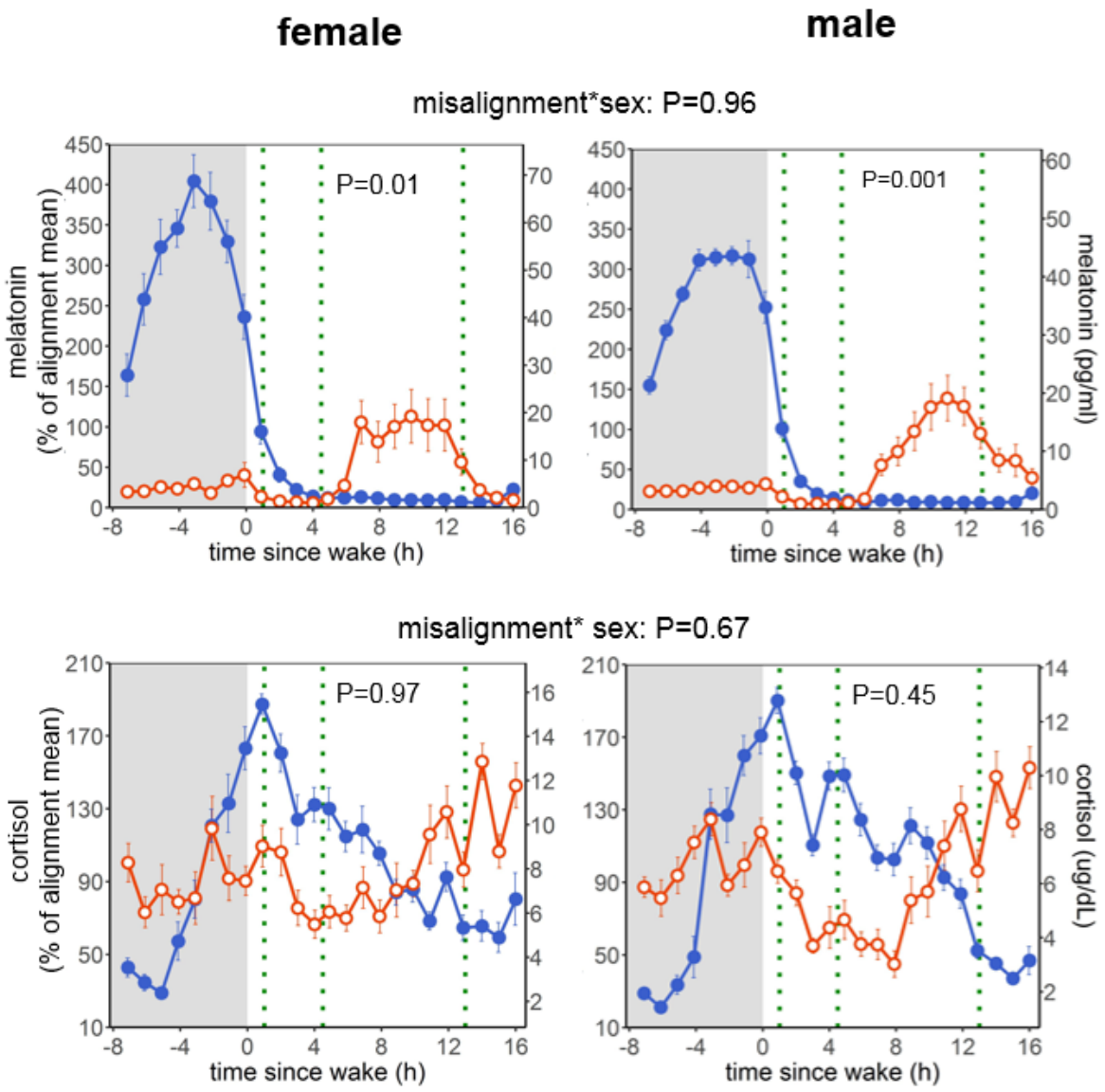

Fig. S3. Effects of circadian misalignment on 24-h melatonin and cortisol levels. Data are plotted relative to scheduled wake time. grey bar, sleep opportunity; green dotted lines, meals. Data are represented as mean ± SEM.

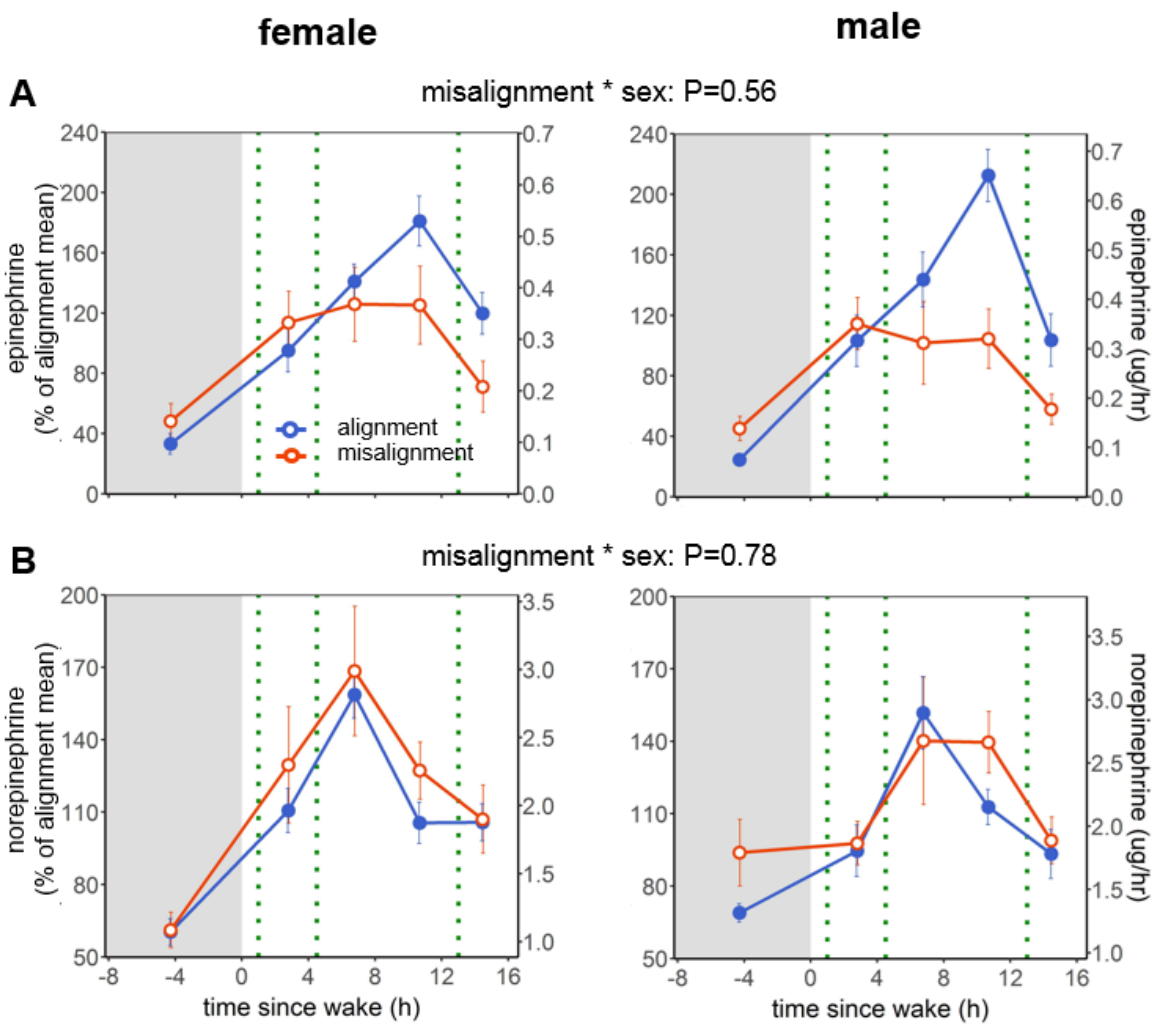

Fig. S4. Effects of circadian misalignment on urinary epinephrine (A) and norepinephrine (B) excretion rates in females (left) and males (right). Gray bar represents sleep opportunity; green dotted line represents a meal. *P-values*, statistical significance for interaction effect of misalignment and sex.

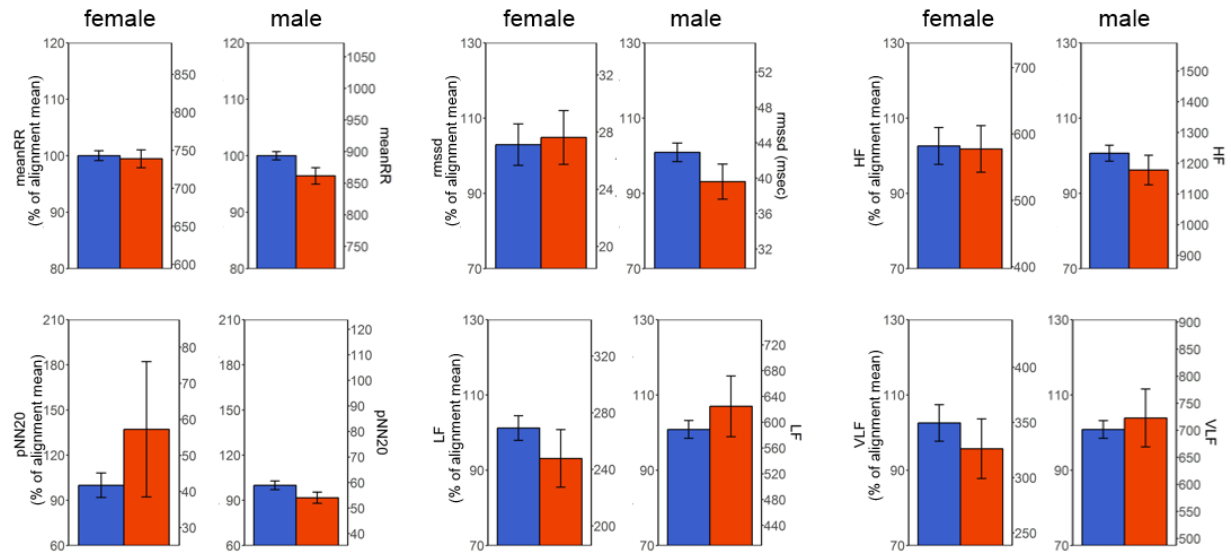

Fig. S5. Effects of circadian misalignment on wake period cardiac parasympathetic markers in females (left) and males (right). Blue bar represents circadian alignment, red bar as circadian misalignment. meanRR, average length of beat-to-beat interval; pNN20, percentage of consecutive heartbeat intervals differing by >20 ms; RMSSD, root mean square differences of consecutive heartbeat intervals; HF, High frequency power (0.15-0.4 Hz); LF, Low frequency power (0.04-0.15 Hz); VLF, very low-frequency power (≤0.04 Hz).

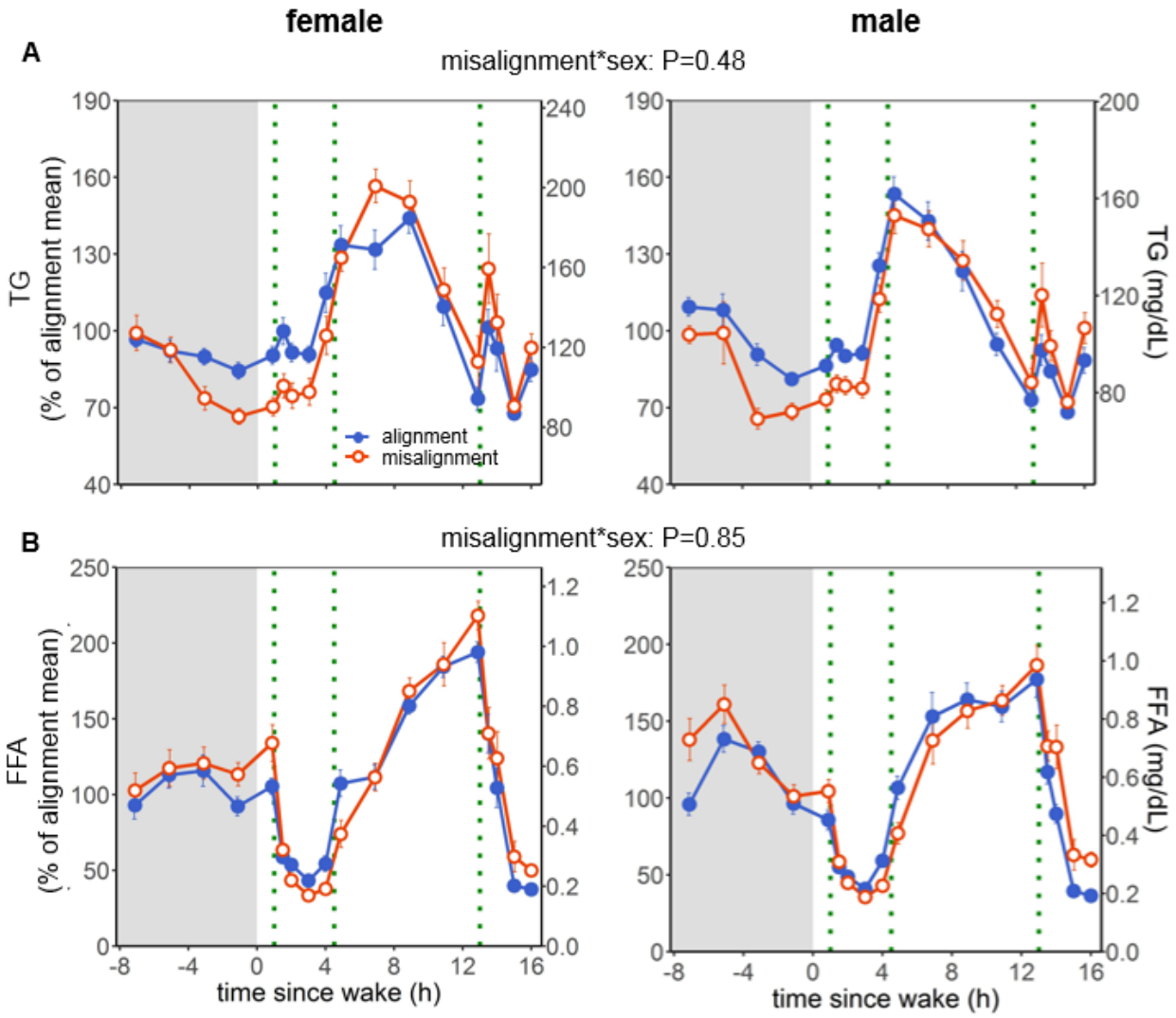

Fig. S6. Effects of circadian misalignment on triglycerides (A) and free fatty acids (B) in females (left) and males (right). Gray bar represents sleep opportunity; green dotted line represents a meal. *P-values*, statistical significance for interaction effect of misalignment and sex on 24-h circulating levels.

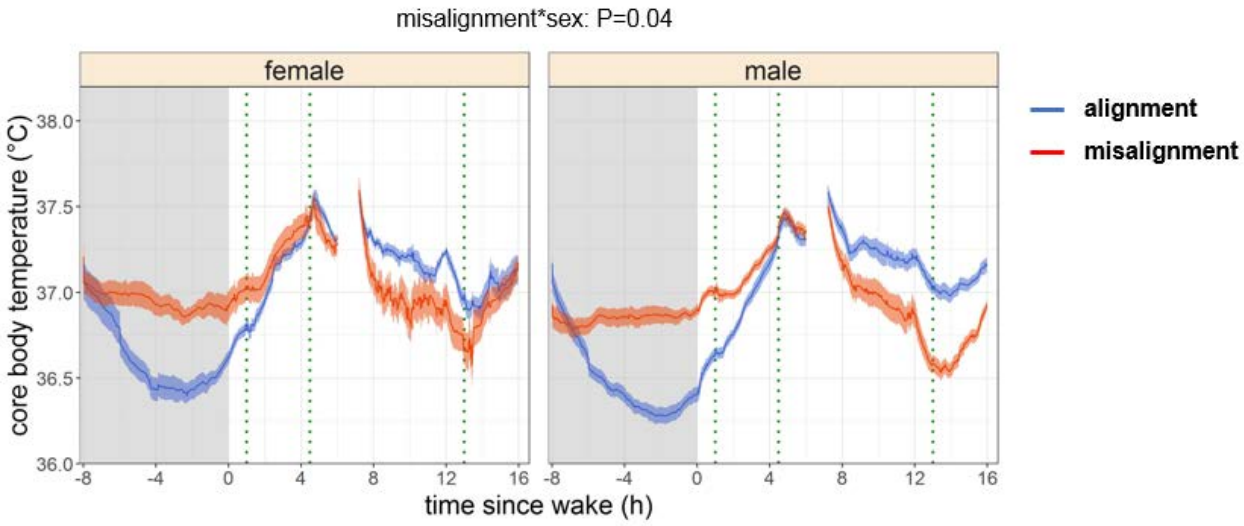

Fig. S7. Effects of circadian misalignment on core body temperature in females (left) and males (right). Gray bar represents sleep opportunity; green dotted line represents a meal. *P-values*, statistical significance for interaction effect of misalignment and sex.

Table S1. Individual demographics

ID	Sex	Age (years)	BMI (kg/m^2)	Body fat (%)	Race	Ethnicity	BMI categories	Chronotype	Menstrual cycle on admission
1	male	24	24.5	22	White, Asian	Non-hispanic	Normal	Intermediate	NA
2	male	31	26.5	33.5	White	Non-hispanic	Overweight	Intermediate	NA
3	male	22	21	19.1	Native Hawaiian	Non-hispanic	Normal	Moderate Evening	NA
4	female	45	28.3	42	Black or African American	Non-hispanic	Overweight	Intermediate	Luteal
5	female	22	28.8	44.9	White	Non-hispanic	Overweight	Intermediate	Follicular
6	female	35	24.7	36.1	White	Non-hispanic	Normal	Moderate Morning	Follicular
7	male	24	23.9	30.1	White	Non-hispanic	Normal	Intermediate	NA
8	male	27	22.4	21.1	White	Non-hispanic	Normal	Moderate Morning	NA
9	female	23	25.2	35.4	Asian	Non-hispanic	Overweight	Intermediate	Luteal
10	female	20	27.9	47.2	White	Hispanic	Overweight	Intermediate	Luteal
11	male	21	26.8	26	Black or African American	Non-hispanic	Overweight	Intermediate	NA
12	male	21	29.5	23.9	White	Non-hispanic	Overweight	Moderate Morning	NA
13	male	49	23.8	27	White	Non-hispanic	Normal	Moderate Morning	NA
14	female	22	22.2	28.4	White	Non-hispanic	Normal	Moderate Morning	Luteal

Note: NA represents “not applicable”.

Table S2. Effects of circadian misalignment on the peak time and magnitude of melatonin and cortisol profiles in females and males.

		melatonin		cortisol	
		peak time shift (h)	magnitude (% change)	peak time shift (h)	magnitude (% change)
test day 1	female	2.8 ± 2.2	-66.0 ± 10.8	2.6 ± 1.1	-31.9 ± 11.3
	male	1.3 ± 0.3	-49.5 ± 10.8	2.4 ± 1.1	-22.0 ± 4.3
	P value	*0.54*	*0.30*	*0.89*	*0.44*
test day 3	female	4.4 ± 2.1	-64.2 ± 15.8	6.5 ± 2.3	-21.5 ± 15.8
	male	3.5 ± 0.7	-57.5 ± 11.2	5.9 ± 1.9	-44.8 ± 5.6
	P value	*0.72*	*0.73*	*0.84*	*0.21*

Note: phase data are calculated as misalignment - alignment; amplitude data are calculated as (misalignment - alignment) / alignment; data are represented as mean ± SEM. *P values*, significance of group comparison (female *vs.* male).

Table S3. Effects of circadian misalignment on total sleep time and sleep stages in females and males.

		P value (misalignment *sex)	**female**		*adj. P value* (misalignment)	**male**		*adj. P value* (misalignment)
			mean	SEM		mean	SEM	
sleep	alignment	0.89	449.2	4.4	**0.02**	437.3	5.9	**0.001**
	misalignment		390.8	17.3		381.7	11.1	
S1	alignment	0.47	15.5	3.4	0.54	24.4	2.4	0.96
	misalignment		18.9	3.2		27.6	4.0	
S2	alignment	0.73	186.4	6.6	**0.03**	215.3	9.7	**0.02**
	misalignment		150.7	10.5		174.0	8.7	
S3	alignment	0.11	123.0	7.2	1	92.5	7.3	0.06
	misalignment		120.4	8.6		101.1	6.4	
REM	alignment	0.72	124.3	7.3	0.06	105.1	6.9	**0.02**
	misalignment		100.7	9.6		79.0	6.5	

Note: all data calculated as minutes. N1, stage 1 sleep; N2, stage 2 sleep; N3, stage 3 sleep; REM, rapid eye movement sleep.

References

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