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Key Words:	Circadian rhythm, Glucose, Insulin, Melatonin, Mitochondria, Shift work

Four days of simulated shift work reduces insulin sensitivity in humans

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1 Abstract

2 **Aim:** The aim of this study was to investigate the effects of four consecutive simulated night shifts on
3 glucose homeostasis, mitochondrial function and central and peripheral rhythmicity compared with a
4 simulated day shift schedule.

5 **Methods:** Seventeen healthy adults (8M:9F) matched for sleep, physical activity, and dietary/fat intake
6 participated in this study (night shift work $n= 9$; day shift work $n= 8$). Glucose tolerance and insulin
7 sensitivity before and after 4 nights of shift work were measured by an intravenous glucose tolerance test
8 and a hyperinsulinemic euglycemic clamp, respectively. Muscles biopsies were obtained to determine
9 insulin signalling and mitochondrial function. Central and peripheral rhythmicity were assessed by
10 measuring salivary melatonin and expression of circadian genes from hair samples, respectively.

11 **Results:** Fasting plasma glucose increased (4.4 ± 0.1 vs. 4.6 ± 0.1 mmol·L⁻¹; $P=0.001$) and insulin sensitivity
12 decreased ($25\pm 7\%$, $P<0.05$) following the night shift, with no changes following the day shift. Night shift
13 work had no effect on skeletal muscle protein expression (PGC1 α , UCP3, TFAM and mitochondria
14 Complex II-V) or insulin-stimulated pAkt Ser473, pTBC1D4Ser318 and pTBC1D4Thr642. **Importantly the**
15 **metabolic changes after simulated night shifts occurred despite no changes in the timing of melatonin**
16 **rhythmicity or hair follicle cell clock gene expression across the wake period (*Per3*, *Per1*, *Nr1d1* and**
17 ***Nr1d2*).**

18 **Conclusion:** Only four days of simulated night shift work in healthy adults is sufficient to reduce insulin
19 sensitivity which would be expected to increase the risk of T2D.

20 **Key words:** circadian rhythm, glucose, insulin, melatonin, mitochondria, shift work

21 **Short title:** Simulated shift work and insulin Sensitivity

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1 Introduction

2 Exposure to shift work is common with more than 18% of the Australian workforce working outside the
3 “normal” working hours of 0800h to 1800h.¹ Shift workers have more health problems even after controlling
4 for lifestyle, body composition and socioeconomic status.²⁻⁴ Indeed, shift work increases all-cause
5 mortality,⁵ and is an independent risk factor for insulin resistance, type 2 diabetes (T2D) and metabolic
6 syndrome⁶.

7
8 Disruption to hormone and sleep rhythms, eating patterns and light exposure occur during shift work. Sleep
9 is effected by shift work,^{7,8} due in part to the failure to shift the circadian rhythm of sleep to match the work
10 schedule. However, it is unresolved whether the increased insulin resistance associated with shift work is
11 due to changes in circadian rhythmicity *per se* and/or sleep patterns ⁹ or to lifestyle changes that often
12 accompany shift work, such as meal timing or food choices.

13
14 Shift work may also change metabolism through skeletal muscle mitochondrial disturbances. The production
15 of reactive oxygen species (ROS) in mitochondria occurs in a circadian manner, ¹⁰ and increases in skeletal
16 muscle ROS production, ¹¹ and mitochondrial dysfunction are associated with insulin resistance.¹² While a
17 recent study investigated day-night rhythmicity in human skeletal muscle oxidative capacity ¹³, to date the
18 impact of shift work on skeletal muscle mitochondrial function and ROS production has not been directly
19 investigated. In addition, muscle changes at mitochondrial level can also affect insulin sensitivity, but no
20 previous study has examined the link between short term shift work and skeletal mitochondria and insulin
21 sensitivity in healthy humans.

22
23 Therefore, we investigated the effect of four consecutive simulated night shifts on glucose homeostasis,
24 mitochondrial function and central and peripheral rhythmicity compared with a simulated day shift schedule
25 (Figure 1). To minimise potential confounding effects all the participants were provided with the same sleep
26 opportunity (9 hours). Physical activity level was controlled before and during the study and participants

maintained their habitual dietary preferences and intake during the study. To simulate travel to and from work, participants left the controlled conditions of the laboratory to walk in daylight for 30 minutes before and after each shift. This was expected to prevent changes in the phase of central circadian rhythms since our previous field study indicated no entrainment to 12 hour night shifts.¹⁴ In addition, participants used a driving simulator before and after each shift to simulate commuting and prepared their own normal meals. We hypothesised that such night shift work simulation would reduce whole body insulin sensitivity and skeletal muscle insulin signalling, reduce skeletal muscle mitochondrial respiration and increase mitochondrial ROS in the absence of either significant sleep debt or changes in rhythmicity or diet.

Results

There were no differences between the groups in age, gender balance, initial body weight, height, body mass index, screening Morningness/eveningness (MEQ) score, Melatonin Onset (MOn) or systolic and diastolic blood pressures (Table 1). Neither body weight nor the blood pressure changed across the study (Table 1).

Fasting glucose, insulin and leptin

Fasting blood glucose and insulin did not vary ($P > 0.05$) with the day of testing (between the IVGTT or the HEC). Fasting glucose was unchanged after the day shift (4.6 ± 0.2 vs. 4.5 ± 0.1 mmol·L⁻¹; $P > 0.05$), but higher after the night shift (4.4 ± 0.1 vs. 4.6 ± 0.1 mmol·L⁻¹; $P = 0.001$). Fasting insulin was not affected by shift work (Figure 2). Plasma leptin levels obtained prior to the IVGTT did not change following either the day or night shifts (Supplementary Table 2).

Intravenous Glucose Tolerance Test

The glucose AUC was unchanged following the day shift ($P > 0.05$) but increased 13.7% following the night shift ($P = 0.008$; Figure 2). The insulin AUC was not different between the groups before shift work and did not change with shift work in either group (Figure 2).

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1 Hyperinsulinemic Euglycemic Clamp

2 Paired data was only available from 5 day shift and 8 night shift participants. Three day shift participants

3 and one night shift participant were excluded for different reasons. One day shift participant became unwell

4 and vomited during the clamp, in another the target glucose level of 4.8 - 5.2 mmol L⁻¹ during the clamp was

5 not achieved, while the third was diagnosed with insulin resistance after analysis of insulin levels post study.

6 For one night shift participant there were technical problems with the glucose analyser during the clamp.

7 Pre-shift glucose infusion rate (GIR) was not different between the two groups (Figure 3, Supplementary

8 Figure 1), however, it decreased following the night shift (P = 0.005). Insulin sensitivity (M value; Glucose

9 infusion rate / insulin concentration during the last 30 min) decreased following the night shift (-25.6 ±

10 7.4%; P = 0.03) but not the day shift (P > 0.05) (Figure 3).

11

12 Skeletal muscle insulin signalling

13 Phosphorylated Akt Ser473, TBC1D4 Ser318 and TBC1D4 Thr642 during the HEC increased similarly in

14 both groups before and after shift work (Figure 4).

15

16 Skeletal muscle mitochondrial respiration

17 Muscle protein expression of PGC1α, UCP3, TFAM and Complex II-V did not change in response to either

18 the day or night shifts (Supplementary Table 3). There was no effect (P > 0.05) of day or night shifts on

19 skeletal muscle mitochondrial respiration (Figure 5). Citrate synthase activity of skeletal muscle

20 mitochondria also did not change in any group (Pre Day shift 5.88 ± 0.61 μmol·min⁻¹ g⁻¹ w/w; Post Day

21 shift 6.57 ± 0.52 μmol·min⁻¹ g⁻¹ w/w; Pre Night shift 6.86 ± 0.51 μmol·min⁻¹ g⁻¹ w/w; Post Night shift 5.72

22 ± 0.68 μmol·min⁻¹ g⁻¹ w/w).

23

24 Skeletal muscle mitochondrial ROS (H₂O₂) emission

25 The data for 1 participant in each group was excluded from analysis for technical reasons with the

26 respirometer (Day shift n = 6; Night shift, n = 8). Following night shifts, the ROS emission (per mg muscle

wet-weight) was significantly reduced in Complex I+II_{Leak} ($P = 0.04$; Figure 5) with no other changes observed.

Dietary intake

There was no significant difference in dietary intake between the groups prior to entry into the study ($P > 0.05$, Figure 6). During the study, dietary energy, protein, total and saturated fat, available carbohydrates (starch and soluble sugars) and sugars during the shifts were similar in both groups (Figure 6). Those on night shift consumed more energy in the first meal opportunity during their “work period” at 2400h (2021 ± 248 kJ) than those on day shift at 1000h (1349 ± 90 kJ) and this was reversed at the second “work period” meal opportunities for night shift participants at 0300h and day shift participants at 1300h (Supplementary Figure 2). With respect to macronutrients, protein intake was decreased in both groups during the study compared with pre-study ($P = 0.04$) whereas other components did not change (Figure 6).

Melatonin rhythms

The onset of melatonin occurred after the last sample collection (2200h) on the night before the trial commenced in 6/8 day shift participants and this pattern continued throughout the study. In the morning the Melatonin Offset (MOff) occurred around 0800h throughout the study (Supplementary Figures S4 & S5, Supplementary Table 4). In the night shift group, the onset of melatonin occurred after 2200h in 6/9 of the participants prior to commencement of the shifts. During the night shifts melatonin was secreted and the MOn was delayed by approximately 1.6 hours between day 3 and day 7 ($P=0.02$; Supplementary Figures S4 & S5). There were no changes in the MOff times across the study for the night shift participants or between the two shift schedules.

Hair follicle cell gene expression

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The expression of *Per3*, *Per1*, *Nr1d1* and *Nr1d2* mRNA decreased across the day ($P=0.001$; Supplementary Figure S5) similarly before (day 1) and after (day 7) the day shift and night shifts with no differences ($P > 0.05$) between the two shifts.

Sleep

Subjective sleep duration prior to the shifts was similar for both groups (Supplementary Figure S6). During the simulated shifts there was a trend ($P = 0.06$) for day shift (7.1 ± 0.6 h) to sleep more than the night shift participants (6.2 ± 1.4 h). When the nap prior to commencing the night shift was included, the night shift group obtained 1.6 hours less total sleep than the day shift group over the 4 days ($P > 0.05$). Actigraph records (objective assessment) were only available for 3 day shift and 4 night shift participants due to technical difficulties. No difference in sleep duration, latency, percent sleep efficiency or wakefulness after sleep onset (WASO) was observed between the day and night shift participants ($P > 0.05$; Supplementary Figure S6, Supplementary Table 5).

Discussion

This study provides novel evidence showing that a roster of night shifts reduces glucose tolerance and insulin sensitivity in healthy non shift workers under controlled conditions. Importantly, this effect was observed despite no significant changes in the amount of sleep obtained or the diet in the day and the night shift participants. Thus, these results suggest that alterations in the times of wakefulness, eating and sleep *per se* may be sufficient for the previously observed decrease in insulin sensitivity in shift workers, independent of changes in circadian rhythm.

There have been few shift work simulation studies that were designed to eliminate or substantially reduce phase shifting as a variable that might explain the metabolic response. Al-Naimi, *et al.* assessed the postprandial glucose response to test meals at different times over a day shift and night shift.¹⁵ As

expected,¹⁶ the timing of the melatonin rhythm and overall melatonin production were not affected by the sustained wakefulness during that study. However, a significant increase in plasma glucose was observed when participants stayed awake and ate food at night (night shift) compared with the day shift. This change in the glycaemia at night did not change the plasma insulin levels, but it is a clear marker of lower glucose tolerance. Another study by Morris *et al.* imposed 8 hour sleep opportunities between 2300h and 0700h (control/day shift) or between 1100h and 1900h (night shift) for 3 days.¹⁷ Peak melatonin levels in the night shift condition were 52% and 59% lower than the day shift condition on days 1 and 3 of the study, respectively. Moreover, the time of peak melatonin levels shifted by 1h 19min and 3h 10min on days 1 and 3, respectively.¹⁷ In the current study peak melatonin levels were delayed, but more modestly, by approximately 1.6 h between day 3 and day 7 in the night shift group, however, unlike the Morris *et al.* study, we did not sample blood across 24 hours. Both studies found that simulated night shift impaired insulin sensitivity, even though Morris *et al.* used standard test meals to assess insulin sensitivity, while the current study used the gold standard hyperinsulinemic euglycemic clamp procedure. Interestingly, during the wake period the changes in insulin sensitivity were independent of changes in salivary melatonin onset, which is a marker of central rhythmicity or hair follicle cell clock gene mRNA expression which is a marker of peripheral central rhythmicity. In addition, the impairment of insulin sensitivity in the current study was not related to changes in the phosphorylation of the key insulin signalling proteins such as Akt, and TBC1D4.¹⁸⁻²⁰ Taking all these results together, we suggest that the reduction in insulin sensitivity observed after the night shift work may have been due to insulin resistance in other tissues such as the liver. Further studies using glucose tracers during the hyperinsulinaemic euglycaemic clamp are needed to assess the insulin sensitivity of the liver after night shifts to answer these questions.

Mitochondrial function may also play a key role in insulin sensitivity, although whether mitochondrial dysfunction is a cause or consequence of insulin resistance remains controversial.^{12, 21-25} Interestingly, while circadian misalignment induced by sleep deprivation in mice has been found to impair brain mitochondrial function,²⁶ the impact of circadian misalignment on this in human skeletal muscle had not previously been

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examined. In the current study we measured skeletal muscle protein content and protein phosphorylation rather than gene expression as it provides a better indication of muscle function. To determine if any reduction in whole body insulin sensitivity was due, at least in part, to reductions in skeletal muscle insulin signalling we examined phosphorylation of Akt and TBC1D4 as markers of proximal and distal insulin signalling, respectively. PGC1 α , which is a master regulator of mitochondrial biogenesis was measured in case there was a decrease in citrate synthase (CS) activity (an indicator of mitochondrial volume). The mitochondrial complexes were measured as a way, along with CS activity, to determine if there was a change in mitochondrial volume with night shift that may explain any changes in mitochondrial function. TFAM (mitochondrial transcription factor A) indicates nucleus-mitochondrial interactions that would be of interest if there was a change in CS activity of PGC1 α . We also measured UCP3 as it is the main uncoupling protein in skeletal muscle with regards to understanding any changes in mitochondrial function. We found that four nights of shift work did not affect skeletal muscle mitochondrial respiration, mitochondrial content based on CS activity or protein expression of PGC1 α , TFAM and mitochondrial complexes. Our findings therefore indicate that the decreased insulin sensitivity after night shifts was not due to changes in mitochondrial respiration, volume or biogenesis. On the other hand, an intriguing finding of this study was the decrease in mitochondrial ROS (H₂O₂) emission in the CI+II_{Leak} state following night shifts. Currently, it is not possible to explain the reason of these intriguing results, but in contrast to the previous literature, recent evidence has shown that physiological levels of ROS generation are required for normal insulin responses²⁷. This question needs to be investigated more carefully in future studies, particularly in the context of chronic shift workers.

It has been proposed that predominant or additional drivers of poor metabolic health of shift workers may be poor nutrition and in particular altered appetite, increased total energy intake,²⁸ or increased fat and snack intake.²⁹ Previous shift work simulation studies have imposed strict dietary control on the participants which could in itself alter metabolic function.³⁰ We provided the participants with individualised ingredients to prepare their own normal meals and snacks in an attempt to make the simulation as close to a real work

1 situation as possible. An interesting consequence of this approach was that day shift participants voluntarily
2 had only a small snack on the first meal opportunity (1030h-1100h), whereas for the night shift participants
3 this small meal occurred in the second meal opportunity during their shift (0300h to 0330h). To what extent
4 this may have contributed to the change in insulin sensitivity observed in this study is not known. The
5 dietary intake of protein was slightly lower during both day and night shifts compared with pre-study levels,
6 but there was no difference in intake of any macronutrients between the day and night shifts. This would
7 suggest that the decrease in insulin sensitivity we report here in the night shift group was not due to altered
8 energy or fat intake.

10 Participants on night shift slept only an average of 25 minutes less per 24 h period than the day shift
11 participants which totalled 1.6h across the whole 4-day period (not statistically significant). This is less than
12 we have observed in the field, where the total sleep obtained on night shifts was 1.7 hours less *per night* than
13 the 7.4 hours of sleep obtained on days off.⁸ In a simulation study similar to that used in the current study,
14 the cumulative sleep debt accrued over 4 night shifts was modest at approximately 2.5 hours.³¹ This would
15 suggest that although the actigraphic analysis in current study lacked power, sleep debt is unlikely to have
16 had a major impact on glucose metabolism.

17 Despite the novel aspects of this study, the study had some limitations. For instance, the sample size was
18 relatively small, in part due to the challenging nature of the study which required participants undertaking
19 invasive procedures such as several venous cannulations and muscle biopsies while living under controlled
20 conditions in our research facility. While the repeated measures analysis indicated a robust change in insulin
21 sensitivity in the night shift group across the study, but not the day shift group, the study lacked the power to
22 detect a statistically significant change in insulin sensitivity between the day shift and night shift work
23 participants after simulated shift work. In addition, several technical problems meant that the full data set
24 was not available from some of the participants. We chose to use a non-randomised and non-cross over
25 design in order to match the two groups in terms of gender, age, physical activity levels and BMI, and to

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1 reduce the number of cannulations and muscle biopsies in each participant, respectively. Our determination
2 of central and peripheral clocks was based on only partial profiles of salivary melatonin and hair follicle
3 gene expression, respectively. This was because as we decided to not collect samples during the participants
4 sleep time, so as to avoid any disturbance on the sleep, which could affect the participant's metabolic
5 responses. Therefore, we do not know for certain that no changes occurred in central and/or peripheral
6 clocks following the night shift. However, despite these limitations, the study was able to demonstrate a
7 significant reduction in insulin sensitivity in the night shift group, with all participants (8/8) being lower
8 after the simulated night shift.

9 In conclusion, a 4 day roster of simulated night shift work is sufficient to reduce insulin sensitivity by
10 approximately 25%, independent of diet or changes in circadian rhythmicity. These results have important
11 implications for chronic shift workers who not only have changes in their work cycles but also tend to sleep
12 less and have unbalance diets, both of which can reduce insulin sensitivity. Our results provide important
13 mechanistic understanding of the negative effects of shift work on glucose metabolism and insulin action
14 that result in increased rates of T2D in chronic shift workers.

16 **Materials and methods**

17 *Participants*

18 Seventeen healthy non-smokers (9 females, 8 males; age 25.6 ± 5.1 years; Mean \pm SD) subjects completed
19 this study in an environmentally controlled facility within Victoria University (Melbourne, Australia).
20 Participants were eligible to take part in the study if they had intermediate morningness/eveningness
21 chronotype scores,³² and an intermediate melatonin onset measurement (assessed between 1800h and
22 2200h) collected under a light intensity of < 50 lux. Participants completed physical activity (7 days) and
23 dietary records (4 days) before entering the study and during the study. In addition, during the study the
24 heart rate of the participants was recorded during the awake period (data not shown). Participants were also
25 provided with all food and drinks during the study in agreement with the information provided in the dietary

records as well as the participants' personal preferences. Participants were excluded from participation if they had diabetes, currently smoked, were taking any medications affecting muscle or liver metabolism, exhibited disturbed sleep patterns, performed more than 1 hour of exercise per day, were current shift workers or who had travelled overseas within the last 2 months. Participants were assigned to either a simulated day shift group or a simulated night shift group, balanced for age, body weight, height, gender and physical activity. After an explanation of the experimental procedures all participants gave their written informed consent before commencing the study which was approved by the Human Ethics Committees of Victoria University and the University of Adelaide (Australia).

Experimental protocol

The study was conducted in an environmentally controlled facility within the Institute of Sport, Exercise and Active Living (ISEAL) at Victoria University. On four consecutive days the control day shift group were assigned to have a 9 hour sleep opportunity between 2200h and 0700h, while the night shift group was assigned a 9 hour sleep opportunity between 0800h and 1700h (Figure 1). When two participants were studied simultaneously, they were allocated to separate rooms and were assigned to the same shift. Participants recorded their dietary intake for 4 days before entering the laboratory using a semi-quantitative 4-day weighed food diary and were provided with food and beverages similar to this throughout the study since changing the diet can have its own effects on insulin sensitivity.

The study was completed in 2 stages with an intervening break for operational reasons.

Stage 1: After arriving at the research facility, the participants prepared their dinner at 2000h and then were confined to their bed in a completely darkened room from 2200h until 0700h to sleep. Then, after an overnight fast, an intravenous glucose tolerance test (IVGTT, see below) commenced at ~0830h. Participants stayed for the rest of the day in the research facility. A meal opportunity was provided after the IVGTT (~12:30h) and again at 1730h and 2000h. Participants could leave the facility for 30 min each day

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(1730h - 1800h) and were able to undertake low to moderate intensity walking, but not exercise training.

Participant heart rates were recorded during the awake period (data not shown). Saliva was collected at 1800h, 1900h, 2000h, 2100h and 2200h, after which participants were confined to their bedrooms to sleep.

The next morning (Day 2) after an overnight fast, muscle biopsies (see below) were collected before and after a hyperinsulinaemic euglycaemic clamp (HEC, see below). All participants performed the tests at the same time of day (\pm 1h). Upon completing the second biopsy, the participants were free to leave the research facility.

8

Stage 2: This stage began 2.6 ± 1.2 days and 1.8 ± 0.5 days after Stage 1 for the day shift and night shift participants, respectively. Participants returned to the facility at approximately 1400h and those assigned to the night shift were given a nap opportunity between 1500h and 1800h. Hourly saliva sampling commenced at 1800h, with those on the day shift ceasing sampling at 2200h and recommencing sampling at 0700h, 0800h and 0900h. Those on the night shift continued hourly saliva collection until the commencement of their sleep opportunity at 0800h. The maximum light intensity recorded in the night shift was 290 lux.

Participants spent their time in the facility completing questionnaires, using a driving simulator, watching TV, playing games, reading, studying or cooking. All these activities kept them active, however, they were not permitted to engage in any formal exercise. The day shift group maintained their sleep opportunity between 2200h and 0700h throughout the study. The night shift participants had 3 consecutive days of sleep opportunity between 0800h and 1700h, followed by a transition sleep opportunity from 0800h to 1500h on Day 6 during the last 2 days of the stage 2 (Day 7 and 8) to help them return to night sleep (2200h to 0700h).

On Day 7 and Day 8 after an overnight fast, both groups were subjected to the IVGTT and muscle biopsies/HEC as above (Figure 1). To simulate commuting to work, during stage 2 participants left the facility and were encouraged to walk outside (but not engage in moderate or high intensity exercise) for 30 min between 0800h - 0830h and 0700h - 0730h for the day shift and night shift groups, respectively and again in the afternoon between 1730h - 1800h for both groups. Adherence to these activity restrictions were monitored with a heart rate monitor. Both groups were assigned five eating opportunities. For the day shift

these were 0730h - 0800h, 1030h – 1100h, 1300h – 1400h, 1730h – 1800h and 2000h – 2100h. For the night shift they were 1700h – 1730h, 2000h - 2100h, 2400h -0030h, 0300h – 0330h and 0600h-0700h. No food or beverages (except water) were allowed outside of these times. No caffeinated beverages were allowed.

Specific methods

Intravenous glucose tolerance test (IVGTT): Teflon catheters (20 G) were inserted into an antecubital vein of both arms. A bolus dose of $0.3\text{g}\cdot\text{kg}^{-1}$ of glucose (25% dextrose solution in saline, Baxter Healthcare Corporation, Toongabbie, NSW, Australia) was injected into a vein. Then, blood (3 ml) was collected from the contralateral vein into fluoride/potassium oxalate and lithium heparin tubes 20, 10 and 1 min before and 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160 and 180 min after the bolus. Whole blood glucose was analysed using a glucose analyser (YSI 2300 Stat, Yellow Springs Instrument, Yellow Springs). Heparinised blood was centrifuged and plasma stored at -80°C for subsequent insulin analysis.

Hyperinsulinaemic euglycaemic clamp (HEC): A 20 G Teflon catheter was inserted into antecubital vein on each arm. The arm to be used for blood collection was placed within an electrical heated cylinder, for arterialization of the venous blood. Five minutes after administration of a bolus dose of insulin ($9\text{ mU}\cdot\text{kg}^{-1}$; Actrapid; Novo Nordisk, Bagsvaerd, Denmark), insulin was infused at a rate of $40\text{ mU}\cdot\text{m}^2\cdot\text{body surface area}\cdot\text{min}^{-1}$ together with 25% D-glucose for 115 min. Blood was collected every 5 minutes from the contralateral vein, assayed for glucose and the infusion rate adjusted accordingly to maintain blood glucose levels between 4.8 and 5.2 mM. The steady state glucose infusion rate (GIR) was calculated as the average over the last 30 min of the clamp and expressed as $\text{mg}\cdot\text{kg}\cdot\text{min}^{-1}$. Blood samples collected before the start of the HEC and at 30, 60, 90 and 120 min of the HEC were assayed for plasma insulin.

Muscle biopsies: Vastus lateralis muscle biopsies (approximately 150 mg) were obtained by a qualified medical doctor before and immediately after each HEC from separate incisions separated by $\sim 1\text{ cm}$. The site

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of the biopsies was anaesthetised using lignocaine without epinephrine. Samples were used to assess mitochondrial respiration on fresh tissue and the rest snap frozen in liquid nitrogen and then stored at -80C.

Western blot analysis: Protein (4-6 µg per well) along with a 4 point calibration curve was separated by gel electrophoresis at 150V for 75 min for OXPHOS proteins on 4-20% Criterion Stain-Free precast gels (Bio-Rad) and for all other proteins 200V for 45 min with 4-15% or 7.5% Criterion Stain-Free precast gels (Bio-Rad). Gels were UV activated for total protein visualisation on the membrane after transfer by ChemiDoc™ MP Imaging System (Bio-Rad). The membrane was blocked with 5% (w/v) skim milk powder dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h. The primary antibodies described below were diluted in 5% (w/v) BSA and 0.1% Sodium Azide in TBST or blocking buffer and incubated overnight at 4°C. The next day following a 1 hour incubation with anti-mouse or anti-rabbit HRP-secondary antibodies and a series of washes in TBST, chemiluminescent signal was developed (SuperSignal West Femto, Pierce) and imaged by the ChemiDoc™ MP Imaging System (Bio-Rad).

Mitochondria respirometry: Skeletal muscle fibres were permeabilised after collection by agitation with saponin (0.005% wt/vol) for 30 min at 4°C, then washed in respiratory buffer (MiR05) for respiration analysis. Tissue (2-3 mg) was added in duplicate to each chamber of a high-resolution respirometer (Oroboros Oxygraph-2K; Oroboros Instruments, Austria) containing MiR05 at 37°C. Oxygen flux (leak) was assessed by adding malate (final concentration 2 mM) and pyruvate (10 mM) for complex I (CI_{Leak}) and succinate (10 mM) for complex I+II in the absence of ADP (CI+II_{Leak}). Maximal oxidative phosphorylation (OXPHOS) was quantified by the addition of ADP (5 mM). Then, cytochrome *c* (10 mM) was added to assess for possible outer mitochondrial membrane defect (10% increase in flux rate). Subsequently, carbonylcyanide-4-(trifluoromethoxy)-phenyl-hydrazone was titrated (0.5 µM steps) to achieve maximum flux through the electron transfer system (CI+CII_{ETS}). Finally, electron transport through complex I and III was inhibited by the sequential addition of rotenone (1 µM) and antimycin A (5 µM), respectively.

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2 *Mitochondrial hydrogen peroxide (H_2O_2) emission:* H_2O_2 emission was measured simultaneously with
3 mitochondrial respiration in each chamber via the reaction with Amplex UltraRed (5 μ M; Life
4 Technologies), horseradish peroxidase (10 U·ml⁻¹) and superoxide dismutase (5 U·ml⁻¹) at 37°C. The rate of
5 fluorescent product appearance was monitored using fluorospectrometers (O2k-Fluo LED2-Module,
6 Oroboros Instruments, Austria).

7
8 *Citrate synthase activity:* Skeletal muscle tissue (15 - 20 mg) was placed in 300 μ L buffer (175 mM KCl and
9 2 mM EDTA, pH 7.4), mechanically homogenized for 30 s at 30 Hz (TissueLyser, Qiagen, Hilden,
10 Germany), freeze-thawed three times, then centrifuged at 4°C. Supernatant (5 μ L) was loaded in a 96-well
11 plate in duplicate in order to measure the citrate synthase activity spectrophotometrically at 412 nm and
12 37°C (iMark, Bio-Rad).

13
14 *Dietary intake:* Dietary records were used to provide the subjects with similar food and drinks during the
15 study. Additionally, participants provided semi-quantitative food diaries during the study that were analysed
16 using a computerised database (Foodworks 7 Professional Edition, Xyris Software, Australia).

17
18 *Saliva melatonin:* To examine central circadian rhythmicity, saliva was collected using Salivettes (Sarstedt
19 Australia, Technology Park, South Australia) and melatonin analyses were performed by radioimmunoassay
20 ³³ (Buhlmann Laboratories, Allschwil, Switzerland). Onset of melatonin secretion (MOn) was defined as the
21 time of day that the melatonin concentration exceeded 10 pM. In cases where the levels had not exceeded 10
22 pM by the time sampling ceased, the onset was recorded as occurring 1 hour later than the last sample
23 collection. Similarly, for determining the offset time (MOff), this was the time that the melatonin decreased
24 below 10 pM; if it was already below 10 pM before the first sample of the morning it was deemed to have
25 occurred 1 hour earlier than that time

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Hair follicle cell mRNA: Hair samples (~20) obtained from different regions of the scalp at 0800h, 1200h, 1600h and 2000h on day 1 and 7 were placed into tubes containing 100 µl of lysis buffer and stored at -80°C.³⁴ The RNA was extracted and DNAs treated using RNAqueous Micro Kits (Life Technologies Australia, Mulgrave, Victoria). RNA was reverse transcribed to cDNA using Turbo Superscript III (Life Technologies), random hexamers (Geneworks, Thebarton, SA) and DNTPs (GE Healthcare, North Sydney, NSW). Gene expression was quantified using Power Sybr Green master mix and a 7500 Real Time PCR System (Life Technologies). All primers used (Supplementary Table 1) were sourced from GeneWorks. Analysis of *Nr1d1*, *Nr1d2*, *Per1* and *Per3* mRNA expression was normalised to the expression of β-actin using the -ΔΔCt method.³⁵

Sleep: Participants wore an Actiwatch activity monitor (Phillips Respironics, Bend, OR) over the 4-7 days before the experimental period to ensure the required sleep schedule was maintained, and during the study. However, due to equipment failure, objective sleep information was available for only 7 of the 17 participants (3 day shift and 4 night shift). Each participant, however, provided in a sleep diary the estimated time of sleep onset and wake up times, from which subjective sleep duration was calculated.

Hormone analysis: Plasma insulin was analysed by double antibody RIA (HI-14K, EMD Millipore, Billerica, Massachusetts, USA). Plasma leptin was analysed in baseline samples for the IVGTT and HEC by double antibody RIA (HL-81K, EMD Millipore).

Statistical Analysis

Area under the glucose and insulin curves during the IVGTT was calculated using GraphPad Prism 7. All data were analyzed by repeated measures 2-way ANOVA (IBM SPSS Statistics Version 20). When the ANOVA revealed a significant interaction specific differences were identified using a least significant difference post hoc test. Percentage change between pre- and post- shift periods for the day and night shift groups were compared using paired samples t-tests. Actigraphic sleep data was first analysed using Phillips

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4 1 Respironics Actiware software (version 6.0) and then repeated measures ANOVA across the 3 day lead in
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6 2 and the 4 days of simulated day or night shifts. Friedman's non-parametric analysis of variance was used to
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8 3 determine if the time of onset varied within the groups across the duration of the study. Friedman's non-
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10 4 parametric analysis of variance was used to determine if the time of onset varied within the groups across
11
12 5 the duration of the study. Data are presented as means \pm SEM. A P value of ≤ 0.05 was considered
13
14 6 significant.
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13 of Australia. D.J.K. takes full responsibility for the work as a whole, including the study design, access to
14 data, and the decision to submit and publish the manuscript.
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18 **Author contributions**

19 D.J.K., J.A.O., G.K.M., R.B., M.J.B. and M.L.J. designed research;
20 R.B., E.C.M., I.L., A.G., F.F., F.C., M.J.B. and G.K.M. performed the research;
21 D.J.K., M.J.B., G.K.M., R.B., I.L., A.J.T., D.S.H. and M.L.J. analyzed data and interpretation;
22 D.J.K., G.K.M. and R.B. wrote the paper.
23

24 **Conflict of interest**

25 The authors declare no conflict of interest
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For Peer Review

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1 **Legends to figures**

2 **Figure 1.** Schematic of the trial design for the day shift (left panel) and night shift participants (right panel).
3 The black bars indicate the times of the sleep opportunities. The vertical hashed bars indicate the times of
4 day when the participants were permitted to briefly leave the facility, to simulate a commute to and from
5 work. The gap between stage 1 and stage 2 ranged from 2.6 ± 1.2 days and 1.8 ± 0.5 days for the day shift
6 and night shift participants, respectively. IVGTT = Intravenous glucose tolerance test. HEC =
7 hyperinsulinemic euglycemic clamp (muscle biopsies were obtained before the commencement and
8 immediately before the end of each HEC). **A indicates the approximate time of arrival at the facility, L**
9 **indicates when they left the facility and M indicates the meal and snack opportunities.**

11 **Figure 2.** Glucose tolerance of the subjects in the night shift protocol was significantly impaired compared
12 to the subjects in the day shift protocol. Blood glucose ($\text{mmol}\cdot\text{L}^{-1}$) and plasma insulin ($\mu\text{U}\cdot\text{ml}^{-1}$) measured
13 before and after a bolus injection of glucose ($0.3\text{ g}\cdot\text{kg}^{-1}$) into participants (a, c) before (●) and after (○) the
14 day shift (mean \pm SEM; N = 7) and (b, d) before (●) and after (○) the night shift (N = 9) and the percentage
15 change (mean \pm SEM) in the glucose (e) and insulin (f) AUC for the day (●) and night (○) shift
16 participants. Data were compared by 2-way repeated measures ANOVA. Note that some SEM bars are
17 obscured by the symbols. * $P < 0.05$ versus Day shift.

19 **Figure 3.** Insulin sensitivity significantly decreased in the subjects following the night shift protocol while
20 no changes were observed in the day shift subjects. The (a) glucose infusion rate and (b) insulin sensitivity
21 (M value, glucose infusion rate divided by the insulin concentration) measured during the last 30 minutes of
22 the hyperinsulinaemic euglycaemic clamps determined pre- and post- shift work for the day shift (N=5) and
23 night shift (N=8). Data were compared by a paired sample t-test. The data are the mean \pm SEM. * $P < 0.05$
24 vs Pre (paired t test).

Figure 4. The night shift protocol did not induce changes in insulin signal transduction proteins in skeletal muscle. Representative blots and Western blot analysis of pAkt Ser473, pTBC1D4 Ser318 and pTBC1D4 Thr642 protein relative to total Akt2 and TBC1D4 from biopsies obtained at the start (B; Basal) and end (I; Insulin) of the hyperinsulinemic euglycemic clamp, before and after participants had completed their day shift or night shifts. Data are the mean \pm SEM for 5 day shift and 7 night shift participants. * indicates main effect for insulin, $P < 0.05$.

Figure 5. Skeletal muscle mitochondrial respiration (a) did not differ in the night and day shift groups before and after each protocol, but subjects in the night shift group showed a significant reduction in the emission of Reactive Oxygen Species (ROS) in Complex I+II_{Leak} after 4 days of night shift. H₂O₂ emission (b) from skeletal muscle biopsies from participants before (open columns) and after (cross hatched columns) the day shifts, and before (horizontal hatched columns) and after (filled columns) the night shifts. Measurements were made during state-4 (Leak), ADP stimulated state-3 oxidative phosphorylation (OXPHOS) and FCCP induced uncoupled (ETS) respiration, which was supported by pyruvate+malate (CI) and succinate (CII) oxidation; rotenone was added for CII_{ETS}. The O₂ flux data are mean \pm SEM (pmol·s·mg⁻¹ wet weight; N = 7 day and 9 night shift participants). H₂O₂ flux data are mean \pm SEM (pmol·s·mg⁻¹ wet weight; N = 6 day and 8 night shift participants). Data were compared by 2-way repeated measures ANOVA. * $P < 0.05$ vs Pre night shift.

Figure 6. Dietary energy intake and total fat and carbohydrate consumption did not differ between groups before and after the night and day shifts, respectively. However, subjects in the night shift group consumed less protein during the 4-day shifts than before the start of the study. Summary of the daily dietary intake of energy (a), protein (b), total fat (c), saturated fat (d), available carbohydrate (e) and sugars (f) calculated from 4 days of diet recordings at home before the study and on days 3-6 during the shifts. Data were compared by 2-way repeated measures ANOVA. * $P < 0.05$ vs Pre night shift. Data are the mean \pm SEM for 6 day and 8 night shift participants.

Table 1

Characteristics of the participants

	Day shift	Night shift	Probability
Gender	4F 4M	5F 4M	
Age (years)	25.0 ± 1.7	26.2 ± 1.9	P > 0.05
Height (m)	1.72 ± 0.02	1.72 ± 0.03	P > 0.05
Screening MEQ score	49.1 ± 1.1	52.3 ± 2.4	P > 0.05
Screening DLMO	2153h ± 33 m	2135h ± 20 m	P > 0.05
Pre study body weight (kg)	67.3 ± 3.13	65.0 ± 5.3	P > 0.05
Post study body weight (kg)	67.4 ± 3.2	65.3 ± 5.4	P > 0.05
Pre study BMI (kg·m ⁻²)	22.7 ± 1.0	21.7 ± 1.2	P > 0.05
Post study BMI (kg·m ⁻²)	22.8 ± 1.0	21.8 ± 1.2	P > 0.05
Pre study systolic blood pressure (mmHg)	115.9 ± 2.9	113.9 ± 4.7	P > 0.05
Pre study diastolic blood pressure (mmHg)	70.4 ± 1.9	70.9 ± 2.2	P > 0.05
Post study systolic blood pressure (mmHg)	115.9 ± 2.7	112.7 ± 5.8	P > 0.05
Post study diastolic blood pressure (mmHg)	69.4 ± 2.5	70.9 ± 2.6	P > 0.05

Screening MEQ = Screening Morningness/eveningness score. Screening DLMO = Screening Dim Light Melatonin Onset

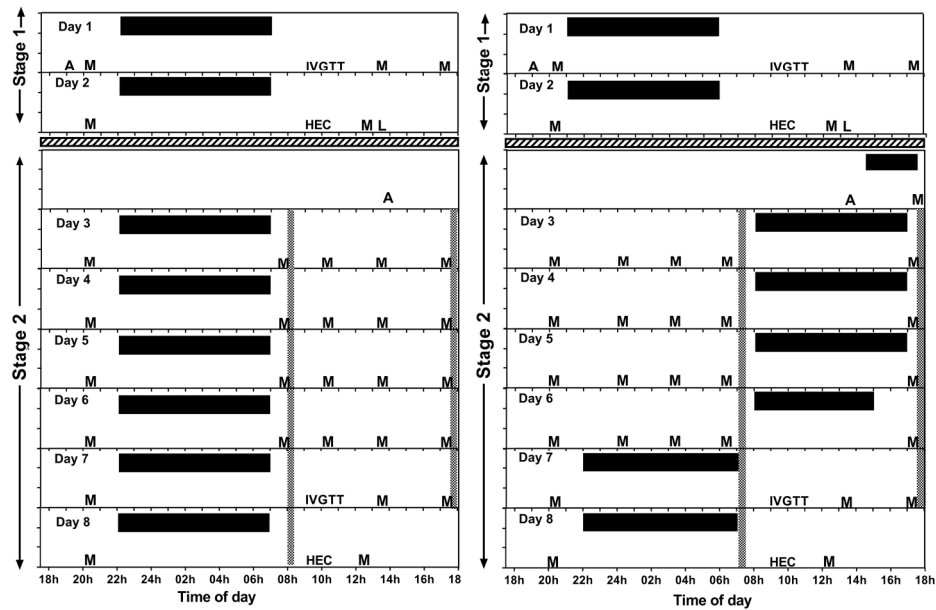


Figure 1

189x126mm (300 x 300 DPI)

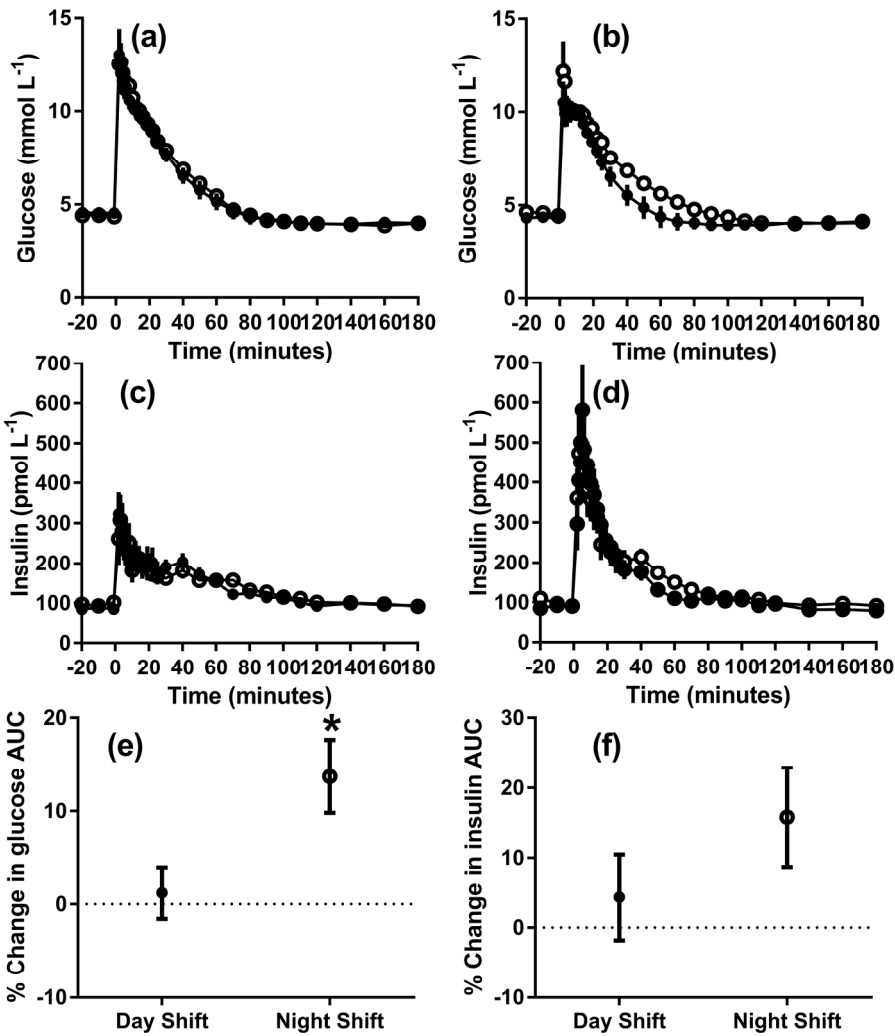


Figure 2

198x221mm (300 x 300 DPI)

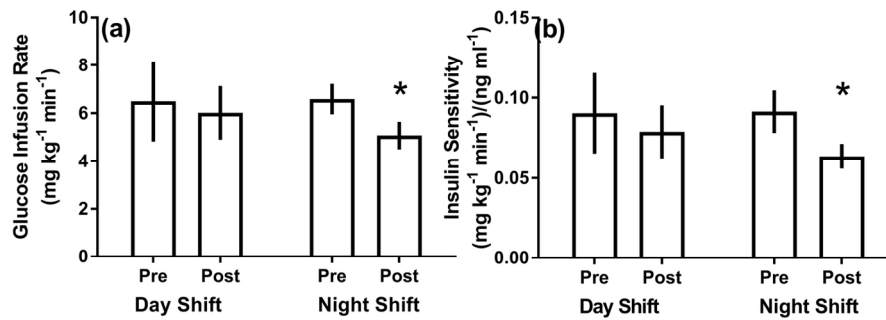


Figure 3

73x28mm (600 x 600 DPI)

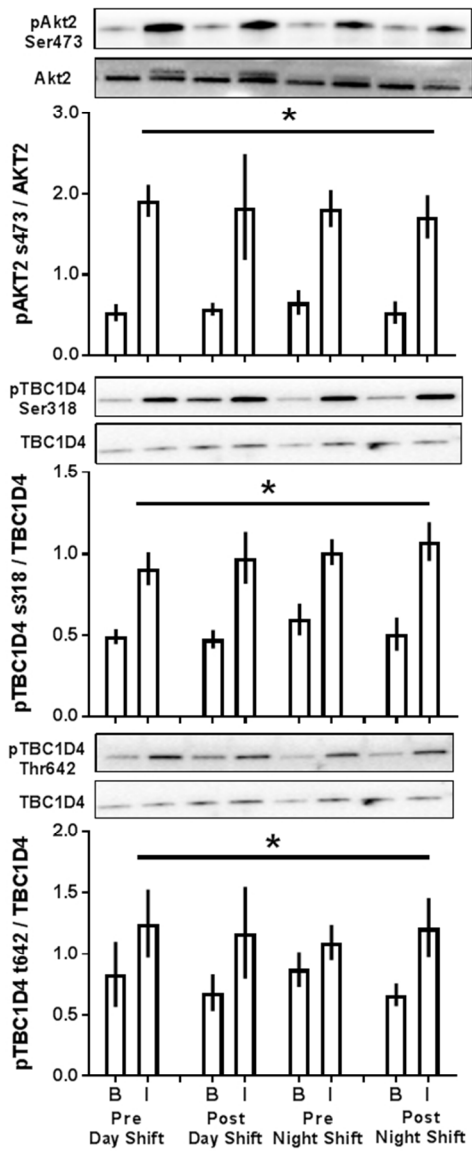


Figure 4

190x275mm (96 x 96 DPI)

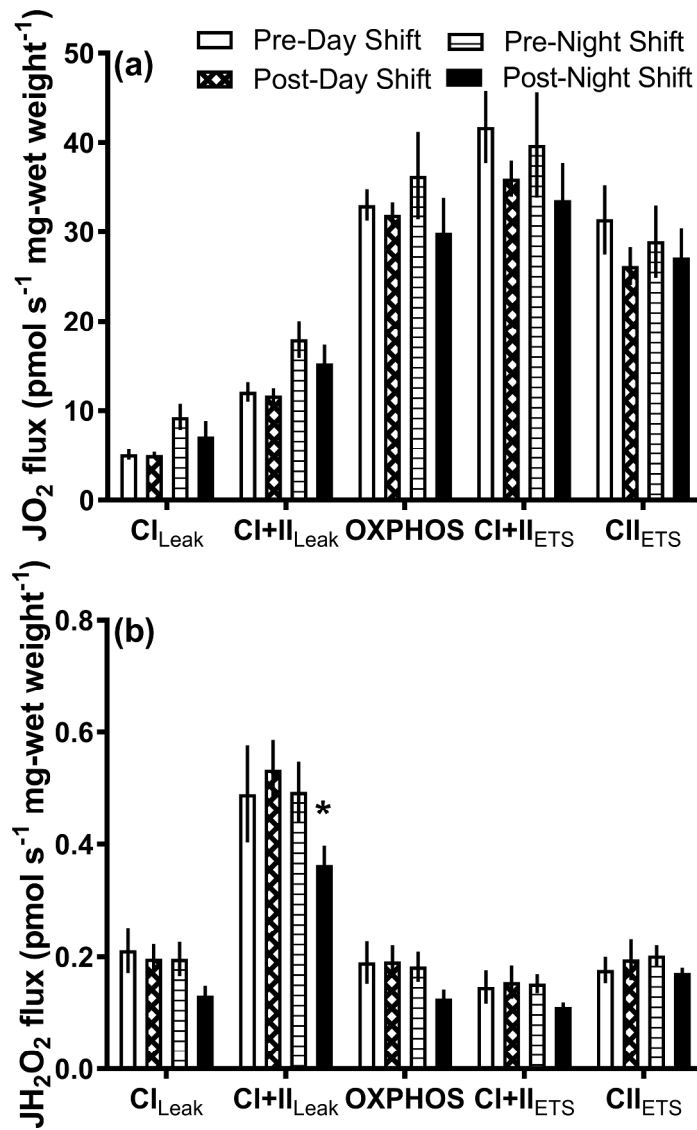


Figure 5

252x360mm (300 x 300 DPI)

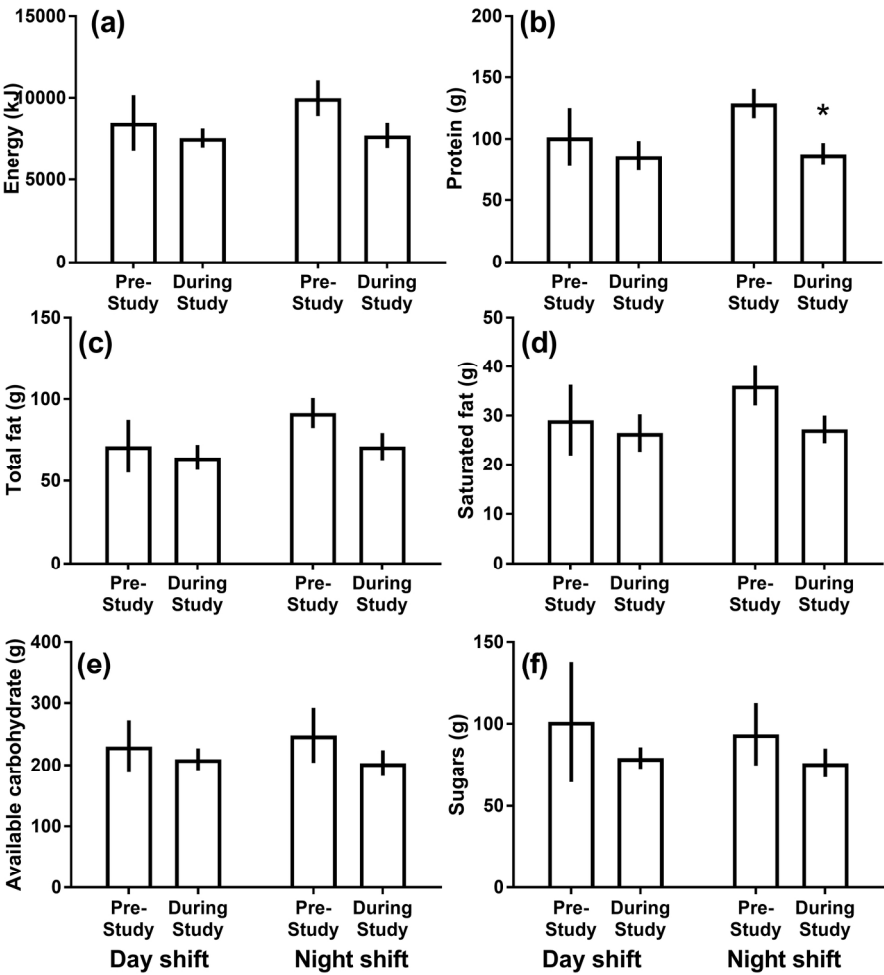


Figure 6

183x204mm (300 x 300 DPI)