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Dalbram, Emilie; Basse, Astrid L.; Zierath, Juleen R.; Treebak, Jonas T.

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Voluntary wheel running in the late dark phase ameliorates diet-induced obesity in mice without altering insulin action

Emilie Dalbram[1, †], Astrid L. Basse[1, †], Juleen R. Zierath[1, 2] and Jonas T. Treebak[1*]

[1] *Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark*

[2] *Integrative Physiology, Department of Molecular Medicine and Surgery and Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden.*

[†] *These authors contributed equally to this work*

Running head: Time-of-day-restricted physical activity and insulin action

***Correspondence:**

Jonas T. Treebak, PhD

Novo Nordisk Foundation Center for Basic Metabolic Research

Faculty of Health and Medical Sciences, University of Copenhagen

Blegdamsvej 3B, 7.7.46

DK2200 Copenhagen

Denmark

jttreebak@sund.ku.dk

Abstract

Metabolic dysfunction and type 2 diabetes are associated with perturbed circadian rhythms. However, exercise appears to ameliorate circadian disturbances as it can phase-shift or reset the internal clock system. Evidence is emerging that exercise at a distinct time of day can correct misalignments of the circadian clock and influence energy metabolism. This suggests that timing of exercise training can be important for the prevention and management of metabolic dysfunction. In this study, obese high-fat diet-fed mice were subjected to voluntary wheel running (VWR) at two different periods of the day in order to determine the effects of time-of-day-restricted VWR on basal and insulin-stimulated glucose disposal. VWR in the late dark phase reduced body weight gain compared to VWR in the beginning of the dark phase. Conversely, time-of-day-restricted VWR did not influence insulin action and glucose disposal, since skeletal muscle and adipose tissue glucose uptake and insulin signaling remained unaffected. Protein abundance of the core clock proteins, Brain-muscle arnt-like 1 (BMAL1) and Circadian locomotor output control kaput (CLOCK), were increased in skeletal muscle after VWR, independent of whether mice had access to running wheels in the early or late dark phase. Collectively, we provide evidence that VWR in the late dark phase ameliorates diet-induced obesity without altering insulin action or glucose homeostasis.

Word count: 211

New and Noteworthy: Exercise appears to ameliorate circadian disturbances as it can entrain the internal clock system. We provide evidence that voluntary wheel running increases core clock protein abundance and influences diet-induced obesity in mice in a time-of-day-dependent manner. However, the effect of time-of-day-restricted voluntary wheel running on body weight gain is not associated with enhanced basal- and insulin-stimulated glucose disposal suggesting that time-of-day-restricted voluntary wheel running affects energy homeostasis rather than glucose homeostasis.

Keywords: Circadian rhythms, insulin action, skeletal muscle, glucose uptake, exercise training

Introduction

Almost all organisms ranging from single cell bacteria to plants and animals exhibit behavioral, physiological, and biochemical rhythms termed circadian rhythms. These ~24-hour biological cycles promote the fitness of organisms by improving their ability to adapt to daily changes in environmental factors such as light and food intake (57). However, in modern society we are increasingly ignoring natural time cues by changing our sleep-wake patterns, eating behavior, and periods of locomotor activity. Emerging evidence suggest that such environmental insults may be the underlying mechanism for metabolic disease progression. For example, perturbed circadian rhythms have been associated with metabolic dysfunction and type 2 diabetes pathogenesis (29, 50).

At the molecular level, cell-autonomous circadian rhythms are generated by interconnected transcriptional and translational feedback loops. The positive arm in this feedback loop is composed of the transcriptional activators Brain-muscle arnt-like 1 (BMAL1) and Circadian locomotor output control kaput (CLOCK), whereas the negative arm consist of the transcriptional repressors Period (PER) and Cryptochrome (CRY) (16, 28, 34). This internal clock system orchestrates the regulation of physiological functions required for the maintenance of normal glucose homeostasis (11, 27, 42). Correspondingly, phenotypical characteristics of many clock gene mutant mice recapitulate the pathophysiology commonly observed in patients with type 2 diabetes. Whole-body knockout of *Clock* or *Bmal1* predisposes mice to obesity and hyperglycemia (50, 54). Furthermore, mice with a liver-specific deletion of *Bmal1* exhibit decreased insulin tolerance (36), whereas skeletal muscle-specific ablation of *Bmal1* impairs insulin-stimulated skeletal muscle glucose uptake and whole-body glucose tolerance (11). Conversely, ectopic expression of CLOCK and BMAL1 ameliorates insulin resistance in myotubes and improves insulin tolerance in a mouse model of type 2 diabetes (38, 61). As such, therapeutic strategies designed to enhance circadian clock function may be beneficial for the prevention and treatment of type 2 diabetes.

Exercise training is a recognized strategy to prevent, manage and treat many chronic metabolic illnesses, including type 2 diabetes (13). A single bout of exercise increases skeletal muscle glucose uptake via an insulin-independent mechanism that bypasses defects associated with this condition, and repeated physical activity results in a persistent increase in insulin action in skeletal muscle from obese and insulin-resistant individuals (19, 22, 39). Exercise appears to ameliorate circadian disturbances as it entrains the internal clock both centrally in the brain and in peripheral tissues (58, 59). Evidence is emerging that exercise at a distinct time of day can affect energy metabolism. Voluntary wheel running (VWR) for 4 hours per day results in a greater attenuation of HFD-induced weight gain in mice when performed in the late dark phase compared to the early dark phase (48). Furthermore, the respiratory exchange ratio was lower during the light phase in mice subjected to exercise training in the late dark phase. This suggests that timing of exercise training can influence whole-body energy metabolism and be important for the prevention and management of metabolic diseases. However, the effect of time-of-day-restricted exercise training on glucose metabolism and insulin action has not been determined.

In this study, we determined whether time-of-day-restricted VWR affects insulin action and glucose disposal in obese mice. We hypothesized that timing of VWR relative to the circadian phase of the molecular clock may augment the beneficial effects of VWR on clock-regulated physiological outputs and improve glucose homeostasis in addition to diet-induced obesity.

Materials and methods

Animal experiments

All animal experiments were performed in compliance with the Directive 2010/63/EU of the European Parliament and were approved by the Danish Animal Experiments Inspectorate (#2015-15-201-792). C57BL/6JBomTac male mice (Taconic, Denmark) were initially group-housed and subsequently single-housed for the exercise-training experiments. Mice were maintained under controlled lighting conditions (12h light: 12h dark cycle) at 22±1°C with *ad libitum* access to food and water. Mice were acclimatized to the facility at least one week prior to the experiments.

Experimental design

Eight-week-old C57BL/6JBomTac male mice were fed a 60% high-fat diet (HFD) (#D12492, ResearchDiets, Inc) or a 10% low-fat diet (LFD) matched for sucrose (#D12450J, ResearchDiets, Inc.) for 8 weeks. After 4 weeks on their respective diets, mice were single-housed and divided into 4 groups (24 mice/group): two groups of sedentary control mice fed either LFD or HFD, and two groups of HFD-fed mice subjected to VWR at either an early or a late time of the dark phase. One running group had access to running wheels during the first 4 hours of the dark phase (ZT 12-16), and the other running group had access to running wheels during the last 4 hours of the dark phase (ZT 20-24). In order to restrict access to the running wheels, an electronic and automated braking system was constructed and installed. Mice were subjected to VWR for 4 weeks and daily running distance and velocity were recorded electronically. Food intake was measured by manually weighing the food after which total calorie intake was calculated, and body weight was recorded weekly. Following the VWR intervention, mice were subdivided into 3 groups (8 mice/group) and *in vivo* glucose uptake was performed under basal, submaximal and maximal insulin stimulation.

116 ***Exclusion of animals***

117 An exclusion criterion was established prior to the study to exclude mice that did not adapt to the
118 wheel running protocol. Mice running a total distance less than 1/3 of the average were excluded
119 from the study. Four mice (out of 24) with access to running wheels during the early dark phase (ZT
120 12-16) were excluded from the study based on this criterion.

121

122 ***In vivo glucose uptake***

123 *In vivo* glucose uptake was performed between ZT 5 and ZT 7. We chose this period in the middle
124 of the light phase to avoid possible time-specific alterations in glucose disposal at the time where
125 the mice were normally running. In order to determine the long-term effects of VWR and exclude
126 any acute running effects, mice did not have access to running wheels the night before the
127 experiment. Mice were fasted for 4 hours and anaesthetized with 8 µL/g body weight of
128 pentobarbital (10 mg/ml). Glucose clearance and uptake in skeletal muscle and adipose tissue was
129 assessed in response to a single retro-orbital injection of [2-^{3}H]DG [PerkinElmer, Waltham, MA,
130 12.32 MBq/kg body weight diluted in Gelofusine (B. Braun, Denmark)] without or with insulin (0.5
131 U/kg body weight or 1.2 U/kg body weight insulin). Blood was collected from the tail vein before,
132 5, 10, 15, 20 and 25 min after the injection. At the same time, a drop of blood was used to measure
133 glucose concentration with a handheld glucometer (Bayer Contour, Switzerland). Accumulation of
134 muscle radioactivity was assessed using a barium hydroxide/zinc sulfate and perchloric acid
135 precipitation procedure adapted from Ferre et al. (14), and the tissue-specific clearance of [2-^{3}H]DG
136 (K_g) and glucose uptake (R_g) were calculated as previously described (15, 32, 33, 53, 56) and shown
137 in Equation 1 and 2:

(Eq. 1)

$$K_g = \frac{[2-^3 H]DGP_{muscle}}{AUC[2-^3 H]DG_{plasma}}$$

$$R_g = \frac{[2-^3H]DGP_{muscle}}{(AUC[2-^3H]DG_{plasma}/AUC[Glucose]_{plasma})} \quad \text{(Eq. 2)}$$

where [2-^{3}H]DGP$_{muscle}$ is the accumulated [2-^{3}H]DGP radioactivity in the muscle over a 25 min period, AUC[2-^{3}H]DG$_{plasma}$ is the area under the plasma [2-^{3}H]DG disappearance curve, and AUC[Glucose]$_{plasma}$ is the area under the blood glucose disappearance curve. Both AUCs were calculated using the trapezoid method. K_g and R_g are used as concentration-independent and dependent indices of tissue-specific glucose uptake, respectively, since R_g takes the specific activity of the [2-^{3}H]DG tracer during the experimental period into account. The radioactivity in the lysates was determined by liquid scintillation counting (Hidex 300SL, Hidex, Finland) and related to total protein or tissue weight.

Quantitative polymerase chain reaction (qPCR)

Tissue samples were collected after the *in vivo* glucose uptake experiment between ZT 5 and ZT 7. RNA was purified from pulverized inguinal white adipose tissue (iWAT) using Trizol (#15596-026, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription and qPCR was performed as described (5). Gene expression of each sample was calculated and normalized to ssDNA input measured by Qubit ssDNA Assay Kit (#Q10212, Invitrogen, Carlsbad, CA). Primer sequences for *UCP1* were: FP: 5′- CTGCCAGGACAGTACCCAAG -3′; RP: 5'- TCAGCTGTTCAAAGCACACA -3'. *PGC-1α* primers used were: FP: 5′- CCCTGCCATTGTTAAGACC -3′; RP: 5'- TGCTGCTGTTCCTGTTTTC -3' and primer sequences for *Prdm16* were: FP: 5′- CAGCACGGTGAAGCCATTC -3′; RP: 5'- GCGTGCATCCGCTTGTG -3'.

Western blot analysis

Tissue samples were collected after the *in vivo* glucose uptake experiment between ZT 5 and ZT 7. Muscle protein extraction and Western blot analysis was performed as described (6). Protein concentration was determined by the bicinchoninic acid (BCA) assay (#23225, ThermoFischer, Waltham, MA). Samples were loaded according to protein concentration and resolved by SDS-PAGE. Ponceau S staining was performed to confirm that equal amounts of protein were transferred to each membrane. Western blot analysis was performed in a balanced design with samples from all experimental conditions represented on all gels. Two internal control samples were included on each gel and used for normalization to allow for a comparison of samples resolved on separate gels. Protein abundance was detected by immunoblot analysis using the antibodies listed in Table 1.

Statistical analysis

Statistical analyses were performed using paired (i.e., repeated measures)/unpaired analysis of variance (ANOVA) as appropriate. When unequal variance was detected between groups by a Brown-Forsythe test, the data was transformed to obtain equal variance. The Tukey *post hoc* test was used and statistical significance was set at $p<0.05$. Data were analyzed using SSPS software and expressed as mean ± standard error of mean (SEM).

Results

VWR in the late dark phase ameliorates HFD-induced obesity

To determine the effect of time-of-day-restricted VWR on HFD-induced obesity, we exposed HFD-fed mice to 4 hours of VWR in the early dark phase (ZT 12-16) or late dark phase (ZT 20-24) for 4 weeks. Although mice are naturally most active in the beginning of the dark phase (47), we observed no difference in running distance or velocity between the two running groups (Fig. 1A and B). Mice ran 92±6 min per day in the early dark phase and 92±8 min per day in the late dark phase. Moreover, total calorie intake was similar between the two running groups and the HFD-fed sedentary mice (Fig. 1C). VWR performed in the late dark phase had a greater effect on diet-induced obesity compared to VWR performed in the early dark phase (Fig. 1D). Body weight was reduced by 8% in mice that ran in the late dark phase compared with HFD-fed sedentary mice. Conversely, body weight was unaltered in mice that had access to running wheels in the early dark phase. Collectively, these results suggest that timing of VWR influences diet-induced obesity in mice, with VWR performed in the late dark phase being most efficacious in protecting against weight gain. A similar result has previously been reported (48), which highlights the suitability of this model for further studies on the effect of time-of-day-restricted VWR on obesity and metabolism.

Insulin dose-dependent glucose uptake in HFD-fed mice

To determine the correct insulin concentrations needed to obtain a submaximal and maximal insulin response in mice fed a HFD for 8 weeks an insulin dose-response experiment was performed. Tissue-specific [2-^{3}H]DG clearance (K_g) and glucose uptake (R_g) was measured *in vivo* using three different insulin concentrations (0.3, 0.5, and 1.2 U/kg). As expected, insulin injection resulted in a

dose-dependent decrease in blood glucose levels (Fig. 2A and B). Moreover, R_g and K_g increased with increasing insulin concentrations in the four different skeletal muscle types studied (i.e., extensor digitorum longus (EDL), soleus, gastrocnemius, and quadriceps; Fig. 2C-J). Since the lower dose of insulin (0.3 U/kg) failed to stimulate [2-^{3}H]DG clearance and glucose uptake significantly above baseline, subsequent experiments were performed using the higher insulin concentrations (0.5 U/kg and 1.2 U/kg) in order to determine submaximal and maximal glucose uptake.

HFD and time-restricted VWR does not alter insulin-stimulated blood glucose excursions in mice

We next determined whether time-of-day-restricted VWR influenced whole-body glucose homeostasis in HFD-fed mice. In general, fasting blood glucose levels were elevated in HFD-fed sedentary mice and mice subjected to VWR during the early dark phase compared to LFD-fed sedentary mice (Fig. 3A). Blood glucose levels in sedentary or running mice were not altered by an intravenous injection of saline (Fig. 3B). Conversely, insulin stimulation resulted in a parallel-shifted decrease in blood glucose levels between groups (Fig. 3C and D). To assess insulin responsiveness, the area over the curve (AOC) was calculated (Fig. 3E). AOC did not differ between the groups, but increased with insulin stimulation in a dose-dependent manner, indicating that a submaximal and maximal insulin response was achieved. Collectively, these results suggest that neither 8 weeks of HFD feeding nor time-restricted VWR affected insulin-stimulated blood glucose excursions, although differences in body weight gain were observed.

Time-of-day-restricted VWR does not alter tissue-specific glucose uptake

To determine whether timing of VWR influences insulin action in specific tissues, basal and insulin-stimulated [2-^{3}H]DG clearance and glucose uptake was assessed in four different skeletal muscle types and three different adipose depots (Fig. 4A-H). K_g increased upon insulin stimulation in a dose-dependent manner in the majority of muscle types studied (Fig. 4A-D), whereas R_g only showed an insulin dose-response in soleus muscle, with glucose uptake being higher in the presence of 1.2 versus 0.5 U/kg insulin (Fig. 4E). In EDL, gastrocnemius and quadriceps muscle, a maximal effect on glucose uptake was achieved in the presence of 0.5 U/kg insulin (Fig. 4F-H). Unexpectedly, R_g was increased in EDL, gastrocnemius, and quadriceps of HFD-fed sedentary mice compared to LFD-fed sedentary mice (Fig. 4F-H). However, VWR counteracted the effect of HFD-feeding by reducing R_g towards levels measured in LFD-fed sedentary mice independently of timing (Fig. 4F-H).

We next assessed K_g and R_g in epididymal white adipose tissue (eWAT), ingiuinal white adipose tissue (iWAT), and brown adipose tissue (BAT) (Fig. 5A-F). Basal [2-^{3}H]DG clearance was not affected by diet or VWR in eWAT, but HFD feeding reduced insulin-stimulated K_g in this adipose depot independent of VWR (Fig. 5A). In addition, basal and insulin-stimulated [2-^{3}H]DG clearance was reduced in iWAT and BAT of HFD-fed mice (Fig. 5B and C). Accordingly, glucose uptake was reduced in HFD-fed versus LFD-fed mice, indicating that 8 weeks of HFD impaired glucose disposal in adipose tissue. VWR did not restore glucose uptake in either eWAT, iWAT, or BAT (Fig. 5D-F). In addition, markers of browning (i.e. *Ucp1* and *Pgc-1α*) were not altered in iWAT in response to VWR (Fig. 6A and B). Moreover, expression of *Prdm16*, a transcriptional coregulator that controls the development of brown adipocytes, could not be detected (data not shown). Collectively, these data indicate that 4 weeks of time-of-day-restricted VWR does not affect glucose clearance and uptake in adipose tissue. Moreover, increased energy dissipation through thermogenic

adaptation is unlikely to explain the differential effects of time-of-day-restricted VWR on HFD-induced body weight development.

Insulin signaling in skeletal muscle of HFD-fed mice is not affected by time-of-day-restricted VWR

To investigate whether insulin signaling was affected by time-of-day-restricted VWR, Western blot analyses were performed on lysates from quadriceps muscle. Total AKT levels were unaltered by diet and VWR (Fig. 7A). Furthermore, AKT phosphorylation at S473 and T308 was unaffected by diet, VWR, and the low insulin dose (0.5 U/kg), but increased upon stimulation with the higher insulin dose (1.2 U/kg) in all groups (Fig. 7B and C). The lack of an insulin response upon stimulation with 0.5 U/kg insulin is contrary to the maximal glucose uptake obtained with this dose (Fig. 4D). A similar discordance between the insulin dose-response of AKT phosphorylation and glucose uptake has previously been reported in cell culture studies and ascribed to a lack of linearity between AKT phosphorylation and GLUT4 translocation (24, 51). Total TBC1D4 and GLUT4 abundance were not altered in response to either diet or VWR (Fig. 7D and E). Insulin increased TBC1D4 phosphorylation at T642 in a dose-dependent manner, indicating that a submaximal and maximal response was achieved at 0.5 U/kg and 1.2 U/kg insulin, respectively (Fig. 7F). However, VWR or diet did not affect phosphorylation at this site. A similar pattern of phosphorylation was observed at TBC1D4 S324 and S348 (Fig. 7G and H). Collectively, these data demonstrate that the canonical insulin signaling pathway is induced in a dose-dependent manner independent of diet and VWR.

VWR increases core clock protein abundance independently of time-of-day

Next, we examined the effects of 4 weeks of time-of-day-restricted VWR on protein abundance in quadriceps muscle. VWR increased Hexokinase 2 (HK2) and Sirtuin 3 (SIRT3) protein levels independent of timing (Fig. 8A and B). Moreover, VWR increased the levels of muscle glycogen, whereas liver glycogen remained unaffected by wheel running (Fig. 8C and D). Exercise training induces *Hk2* and *Sirt3* expression and increases muscle glycogen levels in rodents and humans (3, 5, 23, 40). Here we show that VWR increased the abundance of the core clock proteins, CLOCK and BMAL1 (Fig. 8E and F), independent of whether the mice ran in the early or late dark phase. However, VWR did not increase levels of Period 2 (PER2) and Cryptochrome 1 (CRY1) (Fig. 8G and H). Collectively, our results suggest that VWR increases protein abundance of the core clock components comprising the positive arm of the circadian transcription-translation feedback loop.

Discussion

Accumulating evidence suggest that HFD-feeding can disrupt normal circadian rhythmicity (12), whereas exercise training can correct misalignment of the circadian clock and influence energy metabolism (48, 58, 59). However, the mechanism by which exercise training influences the circadian clock and metabolism is incompletely known. Here, we explored the effects of time-of-day-restricted VWR on body weight gain, glucose homeostasis, and core clock protein abundance in skeletal muscle of obese mice. We provide evidence that daily VWR in the late dark phase results in greater attenuation of HFD-induced weight gain compared to VWR in the beginning of the dark phase. These findings confirm that VWR at the end of the active period is more effective for the prevention of diet-induced obesity, consistent with an earlier study (48). Nevertheless, timing of VWR did not affect tissue-specific [2-^{3}H]DG clearance, glucose uptake, or insulin signaling in skeletal muscle. Moreover, we found that VWR increases protein abundance of the core clock components, BMAL1 and CLOCK. The induction of BMAL1 and CLOCK did not differ between the running groups, suggesting that VWR influences the intrinsic muscle clock independently of timing.

Short-term HFD compromises insulin sensitivity and glucose disposal in mice (37, 55). Consistently, we found K_g to be reduced in response to HFD feeding in eWAT, iWAT and BAT. Furthermore, the HFD intervention increased basal blood glucose levels and decreased liver glycogen levels. Similar effects of HFD on liver glycogen have been observed in rats (10, 46). Exercise training-induced improvements in insulin sensitivity are partially mediated by weight loss and reduced adiposity (18, 45). However, we found no association between the effect of time-of-day-restricted VWR on [2-^{3}H]DG clearance and weight loss, since K_g in both skeletal muscle and

adipose tissue was unaltered in mice subjected to VWR in the late dark phase despite a significant reduction in body weight. In skeletal muscle, [2-^{3}H]DG clearance was not affected by HFD feeding, whereas glucose uptake measured by R_g was increased in EDL, quadriceps and gastrocnemius muscles of HFD-fed sedentary mice as compared with LFD-fed sedentary mice. The increased glucose uptake in skeletal muscle of HFD-fed mice could be a compensatory mechanism for the reduced insulin response in adipose tissue. HFD for 20 weeks decreases insulin-stimulated skeletal muscle glucose uptake, whereas VWR counteracts this effect (31). One reason for this discrepancy in the effects of HFD on insulin sensitivity could be the use of different mouse strains. A comparison between several sub-strains of C57BL/6 mice have shown that some mouse strains are more prone to develop insulin resistance on a HFD (25). Several other factors might also influence the experimental outcome (30). For example, single-housing of mice to obtain more accurate recordings of food intake and running distance may introduce a stress that in turn affects food intake and body weight gain. As such, the 8-week HFD and 4-week running protocol used in our study may not be sufficient to affect skeletal muscle insulin action in the present context.

Exercise training enhances glucose uptake and increases the activity of proteins involved in the insulin signaling pathway (1, 43). However, time-of-day-restricted VWR did not alter either [2-^{3}H]DG clearance, glucose uptake, or insulin signaling. Given that the animals were subjected to a moderate running protocol, a greater exercise stimulus may be necessary to enhance glucose uptake and insulin signaling. Nevertheless, we found that glycogen levels and abundance of the exercise-responsive proteins HK2 and SIRT3 were increased in quadriceps muscle after exercise training. Thus, the 4-week time-of-day-restricted wheel running protocol used in our study was sufficient to induce a training response, although insulin signaling, [2-^{3}H]DG clearance and glucose uptake in skeletal muscle remained unaffected. Contrary to our findings, exercise training fails to increase

muscle glycogen levels in HFD-fed rodents in some studies (20, 46). Different exercise regimens may have differential effects on metabolic outcomes. In humans, high-intensity interval training (HIIT) is an effective way to improve glucose and lipid metabolism (17). Moreover, HIIT is superior to continuous aerobic exercise training for the treatment of insulin resistance (52), and afternoon HIIT is more efficacious than morning HIIT at improving blood glucose levels in men with type 2 diabetes (49). The question of whether circadian timing of HIIT enhances insulin action would be of interest for future studies.

Exercise training influences the intrinsic skeletal muscle clock (41, 58, 59). Here we report that VWR affects the core clock components at the protein level. We found that VWR increased the abundance of BMAL1 and CLOCK in quadriceps muscle, whereas abundance of PER2 and CRY1 was unaltered by VWR. Due to the experimental design, skeletal muscles were dissected at a single time-point, rather than over several time-points. In general, multiple time-points are needed to detect changes in the phase or amplitude of the molecular clock. Thus, we cannot exclude the possibility that VWR affects components comprising the negative transcriptional feedback loop of the circadian clock. When energy levels are low, as with fasting or exercise training, 5'-AMP-activated protein kinase (AMPK) phosphorylates CRY1 and targets it for degradation (35). AMPK also indirectly activates sirtuin 1 (SIRT1) via increases in NAD^+ levels (7). SIRT1 activation results in PER2 deacetylation, destabilization, and further degradation of the clock protein (2). Collectively, these findings suggest that CRY1 and PER2 can be affected by extrinsic stimuli. Whether the higher abundance of BMAL1 and CLOCK in skeletal muscle of mice subjected to VWR is due to increased expression, decreased turn-over, or a phase-shift of the circadian clock remains to be established. In general, the induction of core clock protein levels did not differ

between the two running groups, suggesting that VWR in the present context influences the intrinsic muscle clock independently of timing.

We demonstrate that 4 hours of daily VWR in the late dark phase results in greater attenuation of HFD-induced weight gain compared to 4 hours of VWR in the beginning of the dark phase. Furthermore, VWR in the late dark phase ameliorates the HFD-induced elevation of basal blood glucose levels. These findings suggest that repeated wheel running at the end of the active period is more effective for the treatment of diet-induced obesity. The changes in body weight gain are most likely driven by differences in energy balance between the groups. We hypothesized that the increased weight loss associated with exercise training in the late dark phase could be due to alterations in adiposity. Exercise training promotes browning of subcutaneous WAT in mice (4, 8, 44), which results in more metabolically active cells that potentially protect against obesity and associated disease (26). However, expression of *Ucp1* or *Pgc-1α*, genes associated with the transcriptional control of brown fat development, were unaltered in iWAT after VWR. Moreover, VWR did not affect glucose clearance and uptake in any of the adipose depots studied. This indicates that the reduction in body weight gain observed with VWR in the late dark phase is unlikely to occur from increased energy dissipation due to exercise-induced thermogenesis or exercise-mediated improvements in adipose tissue insulin action. A similar positive effect of late dark phase VWR on body weight gain has been reported (48). In that study, energy expenditure was unaltered between mice running in the early versus the late dark phase. This suggests that the effect of time-of-day-restricted VWR on body weight gain is due to a change in energy intake rather than energy expenditure. Since food intake was similar between groups in both studies, a potential explanation could be altered nutrient absorption. The previous study attributed the difference in body weight to an interaction between the order of meal- and exercise time, since mice fed before

running gained less body and fat mass compared to mice that ran before eating (48). Furthermore, time-restricted VWR changes the feeding pattern of mice with *ad libitum* access to food (47). VWR at the end of the dark phase advances the feeding phase, whereas VWR in the beginning of the dark phase delays the feeding phase. Time of feeding has been reported to have a significant impact on body weight gain (9, 21). The feeding pattern may affect body weight gain through changes in the microbiota, since time-restricted HFD-feeding is associated with an increase in beneficial microbes and a decrease in obesogenic bacteria (60). Thus, VWR performed in the late dark phase may protect against weight gain in HFD-fed mice through changed timing of food intake.

In conclusion, we report that VWR in the late dark phase ameliorates diet-induced obesity in mice without altering basal and insulin-stimulated tissue-specific glucose clearance and uptake. These findings suggest that time-of-day-restricted VWR influences energy homeostasis, rather than glucose homeostasis. Furthermore, we have demonstrated that VWR increases the abundance of core clock proteins in skeletal muscle of HFD-fed mice independent of the timing of exercise bouts. The molecular mechanism accounting for the greater protection against body weight gain associated with VWR in the late dark phase requires further elucidation. In the future, a better understanding of the interactions between VWR, energy homeostasis and circadian biology may refine intervention strategies to counteract the development of obesity and related diseases.

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Disclosures

The authors declare no conflict of interest.

Author contributions

411 ED, ALB, JRZ and JTT conceived and designed the study. ED and ALB performed the
412 experiments. ED, ALB and JTT analyzed and interpreted the data. ED and ALB prepared the
413 figures and drafted the manuscript. All authors read, revised and approved the final manuscript.

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Figure Captions

Figure 1: Effect of time-of-day-restricted VWR on HFD-induced obesity.

Male C57BL/6JBom mice on a high fat diet (HFD) underwent 4 weeks of VWR. Mice were allowed to run either the first 4 hours of the dark phase (early dark phase VWR) or the last 4 hours of the dark phase (late dark phase VWR) or kept as sedentary controls. Sedentary controls received either HFD (HFD sedentary) or low-fat diet (LFD sedentary). During the 4 weeks of VWR, **(A)** running distance, **(B)** average running intensity, **(C)** total calorie intake, and **(D)** weekly body weight were recorded. *p<0.05 vs. HFD sedentary, ***p<0.001 vs. LFD sedentary and #p<0.05 vs all other groups. Data are presented as mean ± SEM for n=20-24 mice.

Figure 2: Dose-dependent increases in insulin-stimulated blood glucose excursion and skeletal muscle glucose uptake in HFD-fed mice.

An insulin dose-response experiment was performed in 16-week-old C57BL/6JBom male mice fed a HFD for a total of 8 weeks. Mice were injected with either saline or insulin (0.3, 0.5 or 1.2 U/kg) and *in vivo* glucose uptake was measured using [2-^{3}H]DG. **(A)** Blood glucose concentrations were measured every 5 min for 25 min and **(B)** the area over the curve (AOC) was calculated. Glucose clearance (K_g) **(C-F)** and glucose uptake (R_g) **(G-J)** was measured in soleus, EDL, gastrocnemius, and quadriceps muscle. #p<0.05, ##p<0.01 and ###p<0.001 vs. saline, and *p<0.05, **p<0.01 and ***P<0.001 for insulin dose effect. Data are presented as mean ± SEM for n=6 mice.

Figure 3: Insulin-stimulated blood glucose excursions are unaffected by HFD and time-restricted VWR.

In vivo glucose uptake was determined under basal, submaximal and maximal insulin-stimulated conditions in LFD-fed and HFD-fed sedentary mice and HFD-fed mice subjected to 4 weeks of time-restricted VWR. **(A)** Basal blood glucose levels, (**B-D)** blood glucose measurements after administration of either saline or insulin (0.5 or 1.2 U/kg body weight), **(E)** area over the curve (AOC) calculated from Fig. B-D using the trapezoid method. #p<0.05 for main effect of exercise and diet, *p<0.05 vs. LFD sedentary, ***p<0.001 for main effect of insulin. Data are presented as mean ± SEM for n=6-8 mice.

Figure 4: Time-of-day-restricted VWR does not affect glucose clearance or uptake in skeletal muscle of HFD-fed mice.

Basal and insulin stimulated glucose clearance (K_g) **(A-D)** and glucose uptake (R_g) **(E-H)** was measured in soleus, EDL, gastrocnemius, and quadriceps muscle. **p<0.01 and ***p<0.001 for main effect of insulin, #p<0.05 and ##p<0.01 vs. LFD sedentary, and §p<0.05 vs. HFD-fed sedentary. Data are presented as mean ± SEM for n=6-8 mice.

Figure 5: Time-of-day-restricted VWR does not affect glucose clearance or uptake in adipose tissue of HFD-fed mice.

Basal and insulin-stimulated glucose clearance (K_g) **(A-C)** and glucose uptake (R_g) **(D-F)** was measured in epididymal white adipose tissue (eWAT)**,** inguinal white adipose tissue (iWAT), and brown adipose tissue (BAT). **p<0.01 and ***p<0.001 vs. saline, ##p<0.01 and ###p<0.001 vs. LFD sedentary, and §p<0.05 vs. HFD-fed sedentary. Data are presented as mean ± SEM for n=6-8 mice.

Figure 6: Time-of-day-restricted VWR does not induce browning of iWAT.

The expression of genes related to browning of white adipose tissue was measured in iWAT obtained from saline-treated mice during the *in vivo* glucose uptake experiment. **(A)** *Ucp1* expression and **(B)** *Pgc-1α* expression. A main effect of diet was detected, where **p<0.01 vs. LFD sedentary. Data are presented as mean ± SEM for n=6-8 mice.

Figure 7: Skeletal muscle insulin signaling is unaltered by 4 weeks of time-of-day-restricted VWR.

Total abundance and phosphorylation state of proteins involved in insulin signaling was measured in lysates from quadriceps muscle. **(A)** Total AKT, **(B)** AKT-pS473/AKT, **(C)** AKT-pT308/AKT, **(D)** GLUT4, **(E)** total TBC1D4, **(F)** TBC1D4-pT642/TBC1D4, **(G)** TBC1D4-pS324/TBC1D4, and **(H)** TBC1D4-pS348/TBC1D4. ***p<0.001 for main effect of insulin dose. Data are presented as mean ± SEM for n=6-8 mice.

Figure 8: Time-of-day-restricted VWR induces a training response and increases core clock protein abundance in skeletal muscle of HFD-fed mice.

Protein levels of exercise responsive genes and glycogen was measured in exercised and sedentary mice. **(A)** HK2, **(B)** SIRT3, **(C)** muscle glycogen, and **(D)** liver glycogen. Moreover, abundance of the core clock components **(E)** CLOCK, **(F)** BMAL1, **(G)** PER2, and **(H)** CRY1 was measured in quadriceps by Western blot analyses. *p<0.05, **p<0.01 and ***p<0.001 vs. sedentary groups, and #p<0.05 for main effect of diet. Data are presented as mean ± SEM for n=6-8 mice.

Table 1. Antibodies used for Western blot analyses

Target	Company	Catalog number
AKT total	Cell Signaling	9272S
AKT-pS473	Cell Signaling	9271L
AKT-pT308	Cell Signaling	9275L
GLUT4	Termo Scientific	PA1-1065
TBC1D4 total	Millipore	07-714
TBC1D4-pT642	Cell Signaling	8881
TBC1D4-pS324	Capra Science	Custom made
TBC1D4-pS348	Capra Science	Custom made
HK2	Cell Signaling	2867
SIRT3	Cell Signaling	5490
CLOCK	Sanat Cruz Biotechnology	sc-6927
BMAL1	Abcam	ab3350
PER2	Alpha Diagnostic	PER21-A
CRY1	Bethyl	A302-614A

Figure 1

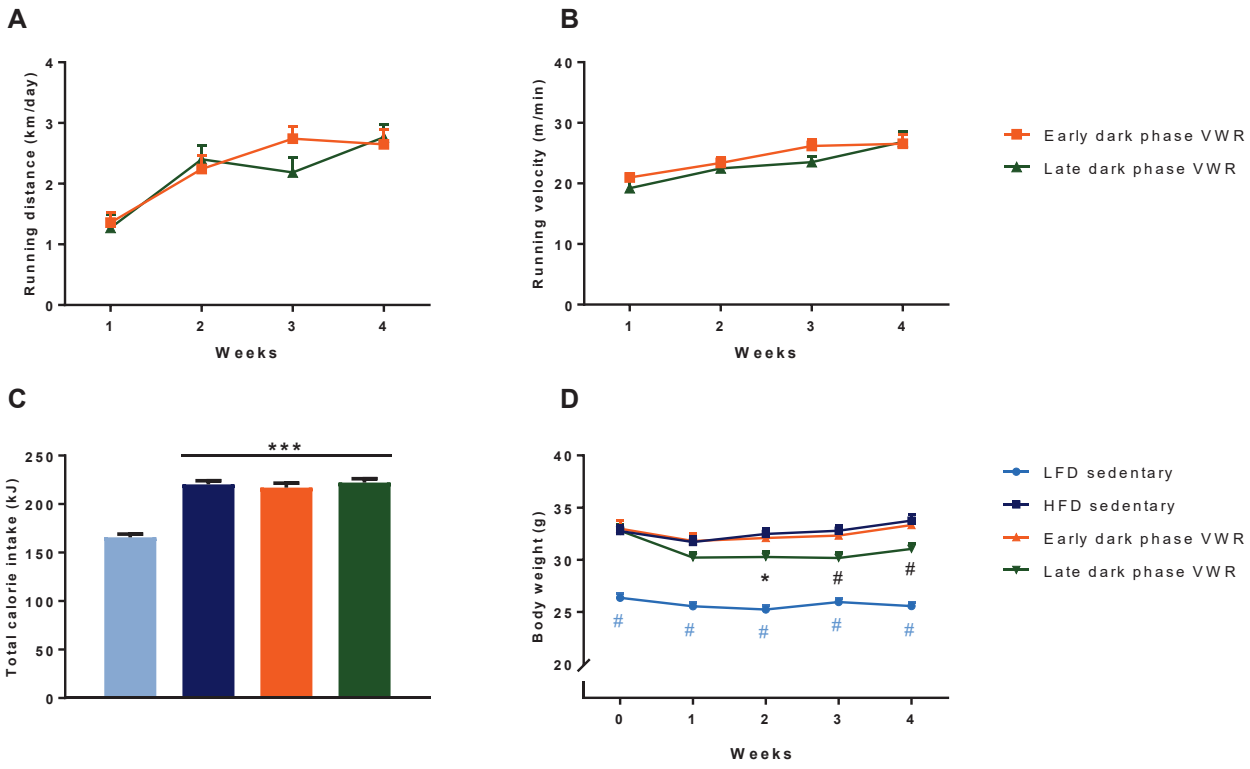

A
Running distance (km/day)
Weeks
B
Running velocity (m/min)
Weeks
Early dark phase VWR
Late dark phase VWR
C
Total calorie intake (kJ)
***
D
Body weight (g)
Weeks
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR

Figure 2

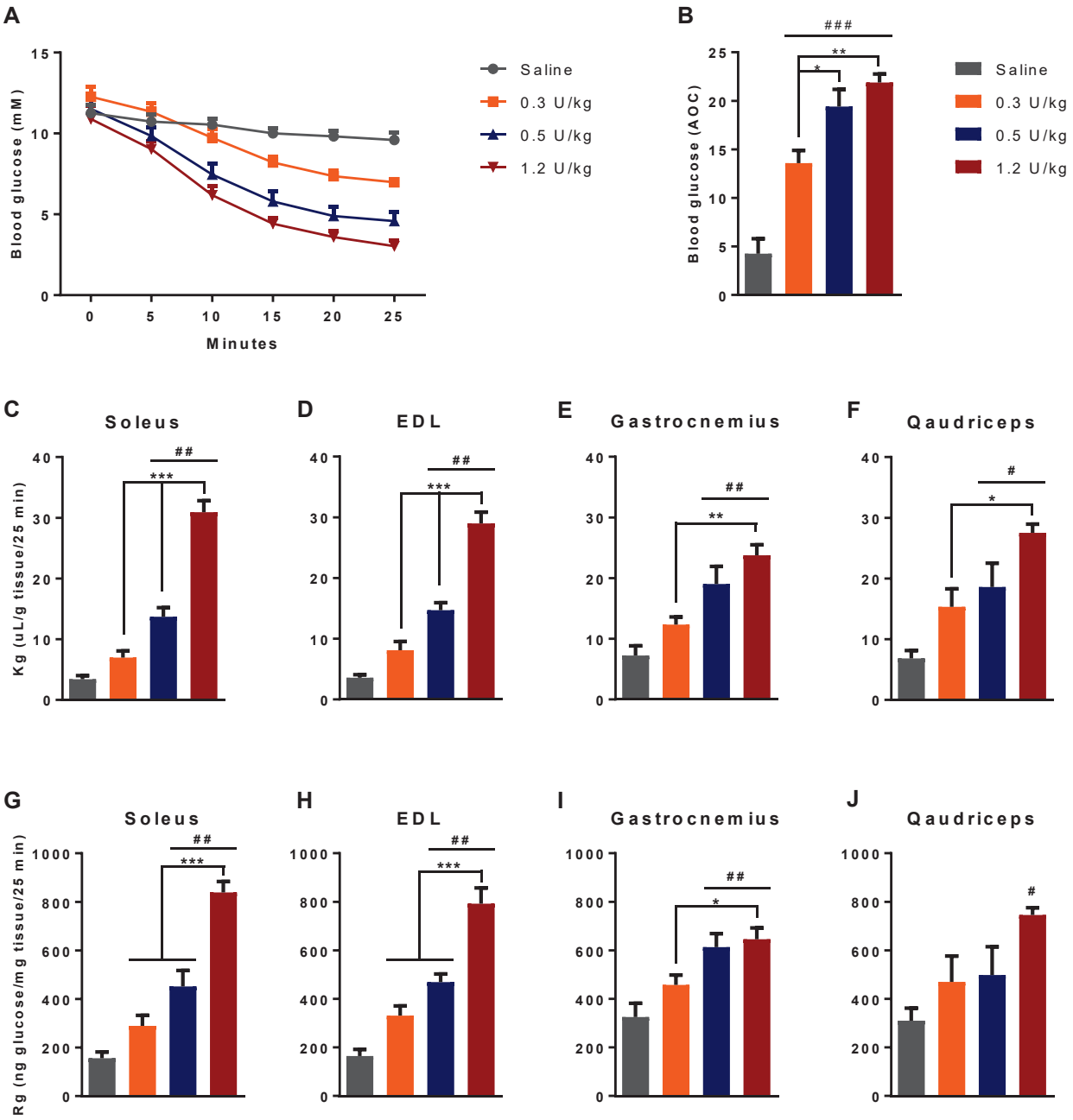

A
Blood glucose (mM)
Minutes
Saline
0.3 U/kg
0.5 U/kg
1.2 U/kg
B
Blood glucose (AOC)
###
**
*
C
Soleus
Kg (uL/g tissue/25 min)
D
EDL
E
Gastrocnemius
F
Qaudriceps
G
Soleus
Rg (ng glucose/mg tissue/25 min)
H
EDL
I
Gastrocnemius
J
Qaudriceps

Figure 3

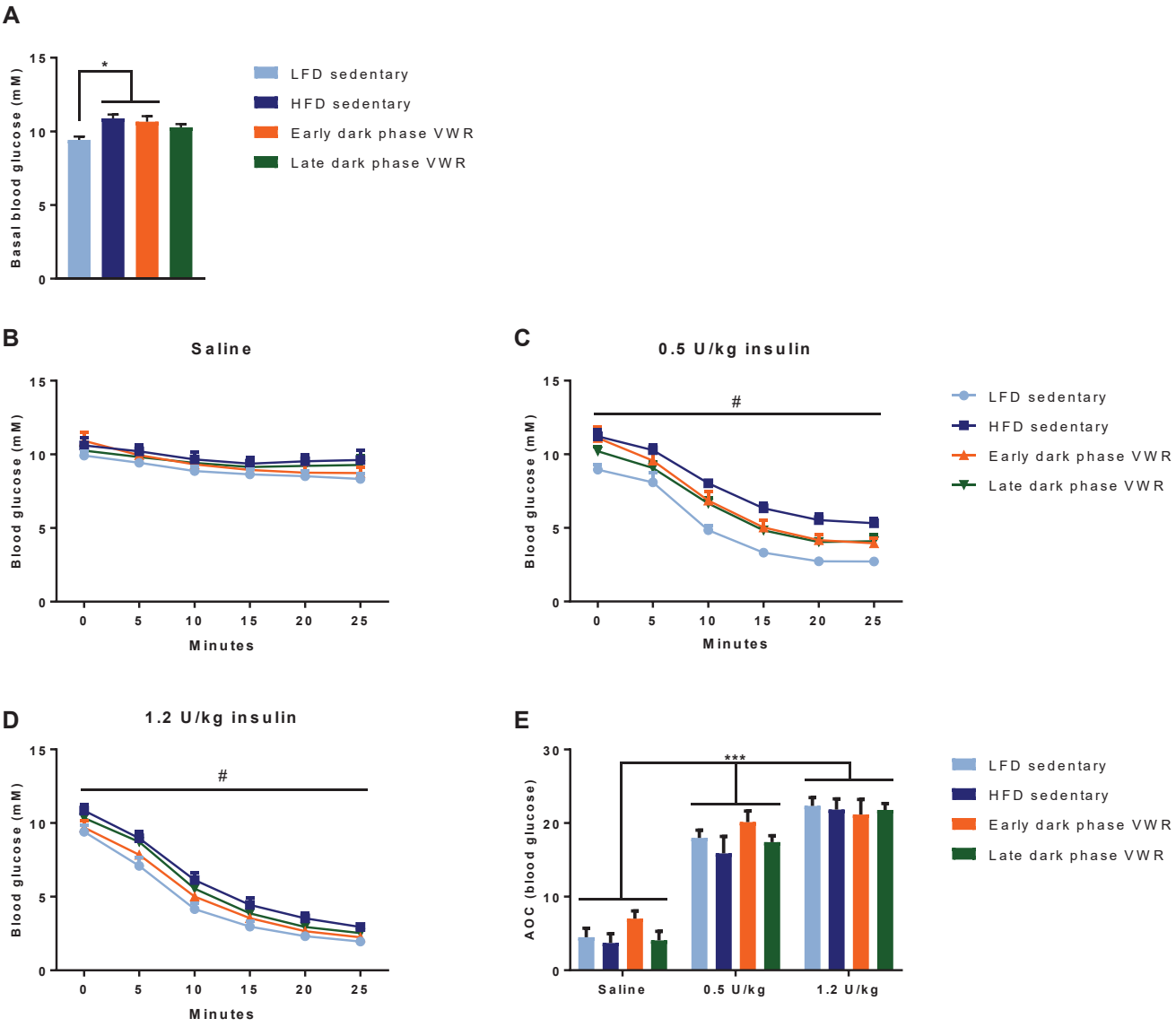
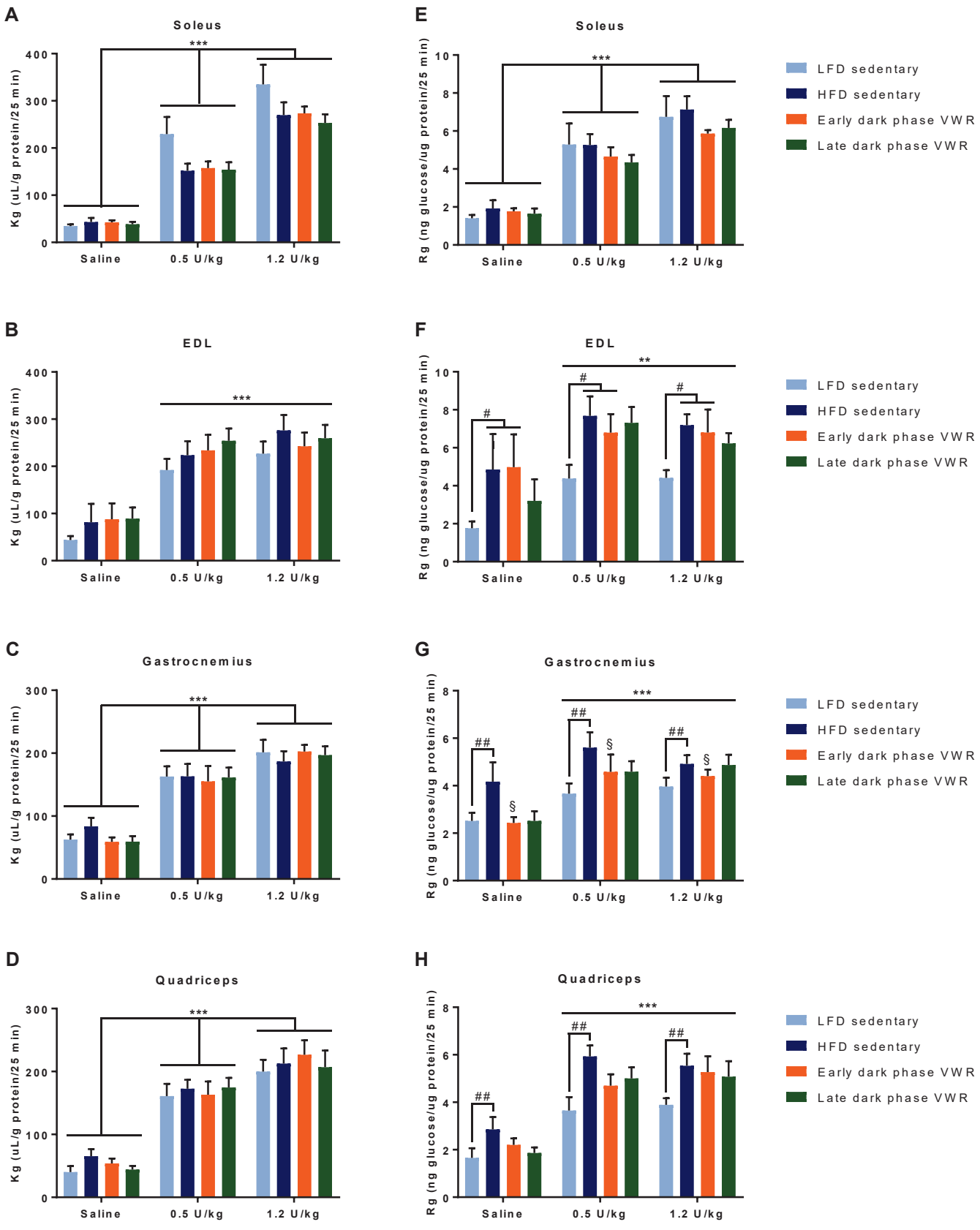

A
Basal blood glucose (mM)
15
10
5
0
*
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR
B
Saline
Blood glucose (mM)
Minutes
0
5
10
15
20
25
C
0.5 U/kg insulin
#
D
1.2 U/kg insulin
#
E
AOC (blood glucose)
30
20
10
***
Saline
0.5 U/kg
1.2 U/kg

Figure 4


A
Soleus
Kg (uL/g protein/25 min)
400
300
200
100
0
***
Saline
0.5 U/kg
1.2 U/kg
E
Soleus
Rg (ng glucose/ug protein/25 min)
10
8
6
4
2
0
***
Saline
0.5 U/kg
1.2 U/kg
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR
B
EDL
Kg (uL/g protein/25 min)
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Saline
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Quadriceps
Kg (uL/g protein/25 min)
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Quadriceps
Rg (ng glucose/ug protein/25 min)
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Saline
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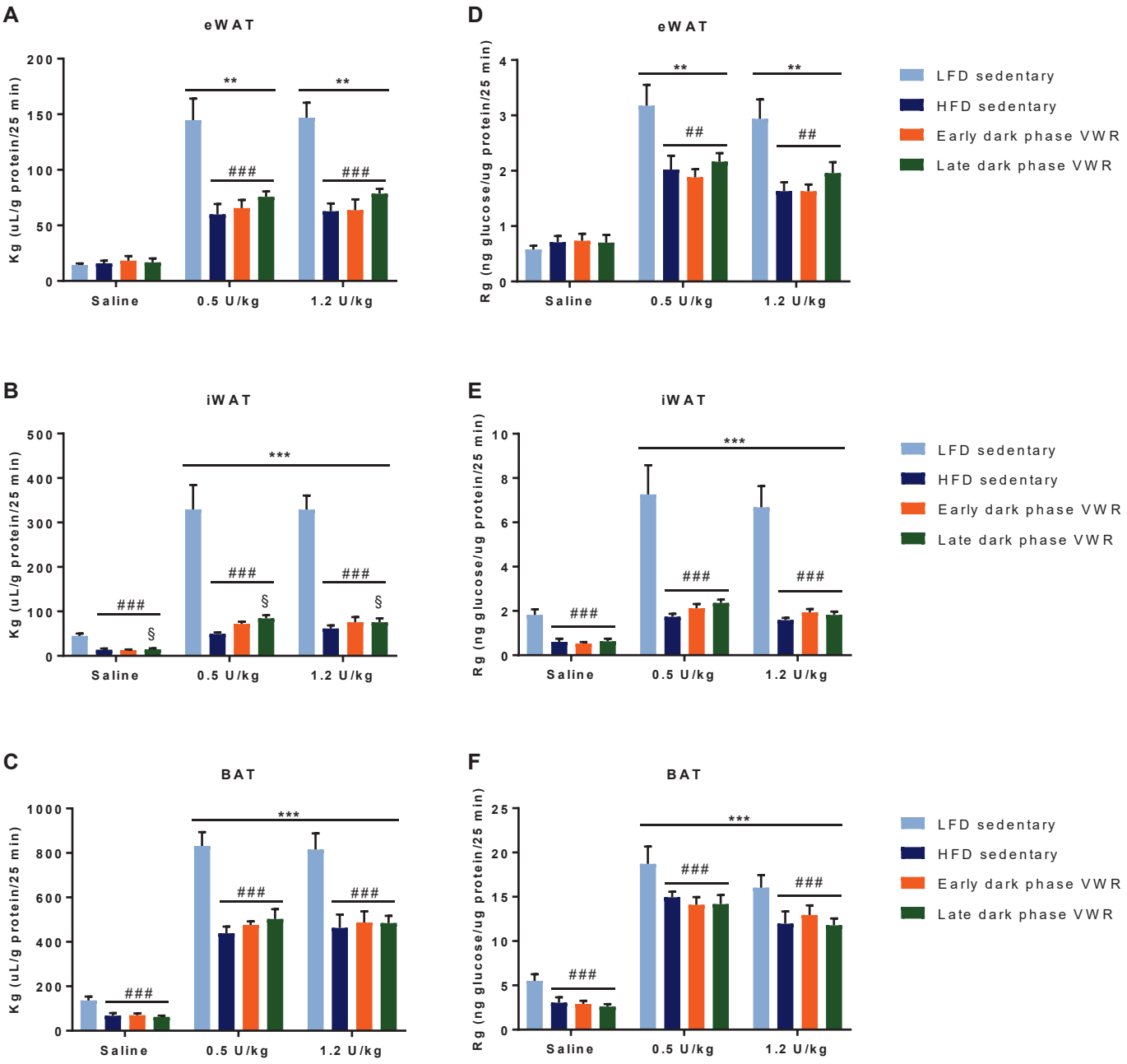
1.2 U/kg
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR

Figure 5


A
eWAT
Kg (uL/g protein/25 min)
200
150
100
50
0
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Saline
0.5 U/kg
1.2 U/kg
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eWAT
Rg (ng glucose/ug protein/25 min)
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Saline
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1.2 U/kg
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR
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iWAT
Kg (uL/g protein/25 min)
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Saline
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iWAT
Rg (ng glucose/ug protein/25 min)
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Saline
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1.2 U/kg
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR
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BAT
Kg (uL/g protein/25 min)
1000
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Saline
0.5 U/kg
1.2 U/kg
F
BAT
Rg (ng glucose/ug protein/25 min)
25
20
15
10
5
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###
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###
Saline
0.5 U/kg
1.2 U/kg
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR

Figure 6

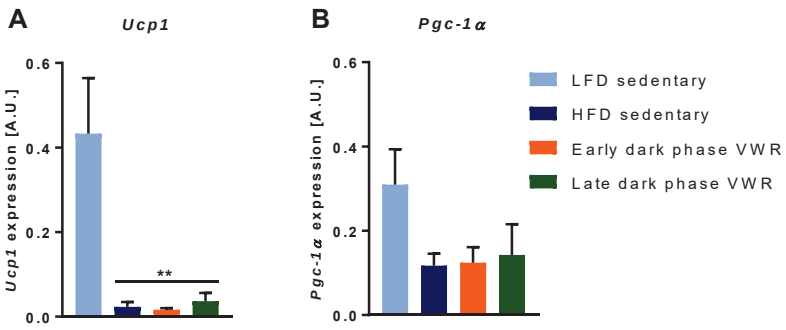

A
Ucp1
Ucp1 expression [A.U.]
0.6
0.4
0.2
0.0
**
B
Pgc-1α
Pgc-1α expression [A.U.]
0.6
0.4
0.2
0.0
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR

Figure 7

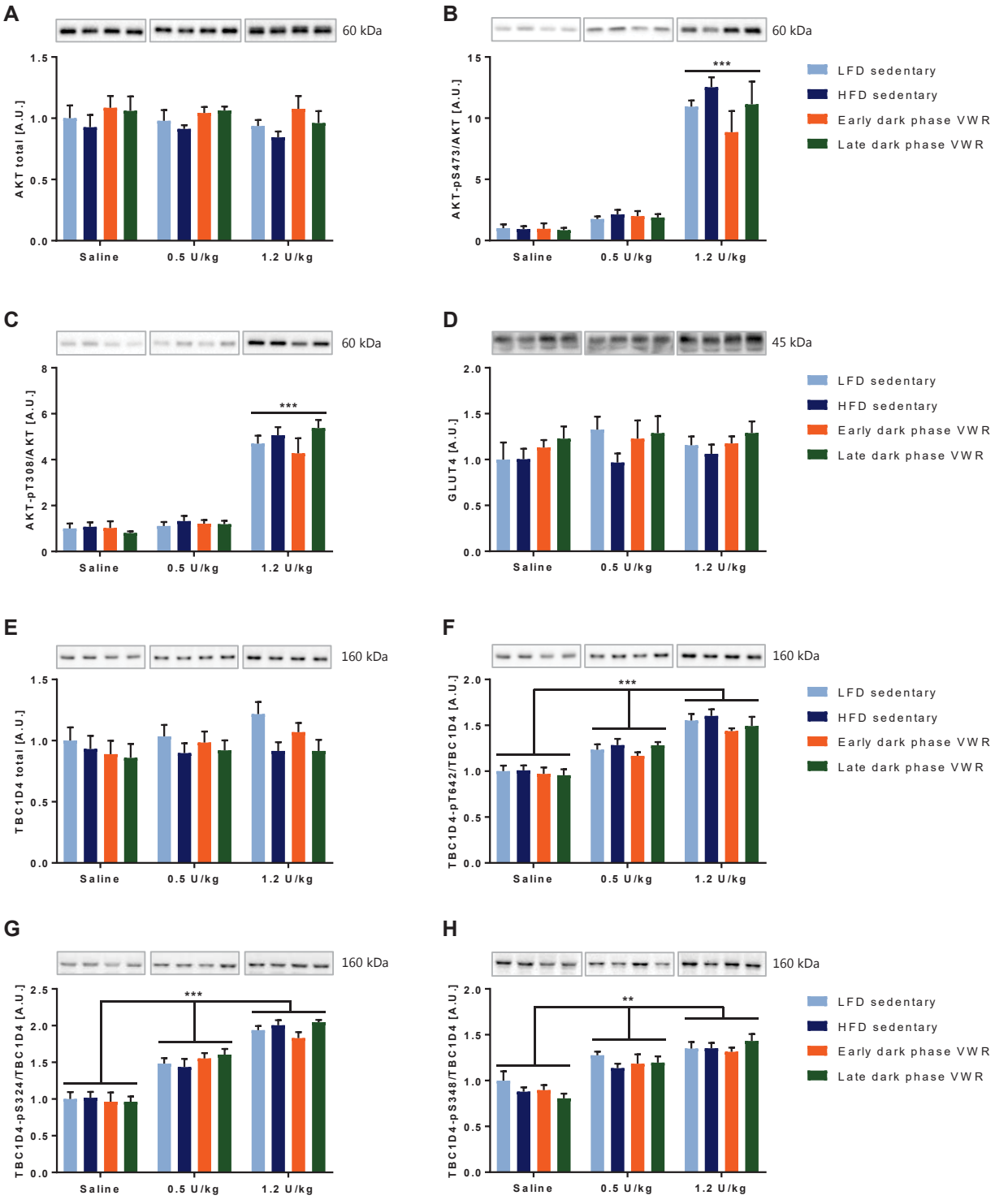
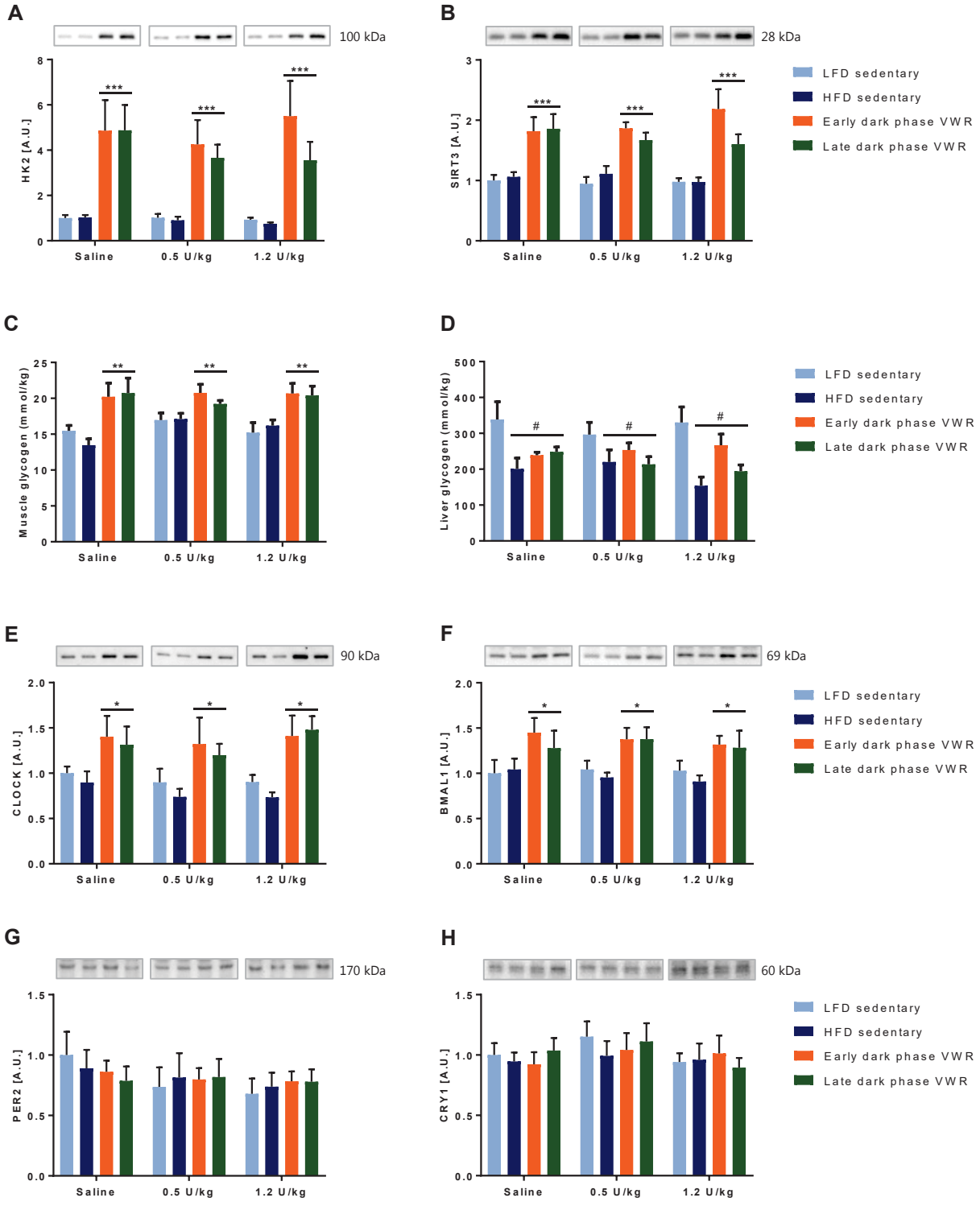

A
60 kDa
AKT total [A.U.]
Saline
0.5 U/kg
1.2 U/kg
B
60 kDa
AKT-pS473/AKT [A.U.]
***
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR
C
60 kDa
AKT-pT308/AKT [A.U.]
***
D
45 kDa
GLUT4 [A.U.]
E
160 kDa
TBC1D4 total [A.U.]
F
160 kDa
TBC1D4-pT642/TBC1D4 [A.U.]
***
G
160 kDa
TBC1D4-pS324/TBC1D4 [A.U.]
***
H
160 kDa
TBC1D4-pS348/TBC1D4 [A.U.]
**

Figure 8


A
100 kDa
HK2 [A.U.]
Saline
0.5 U/kg
1.2 U/kg
B
28 kDa
SIRT3 [A.U.]
LFD sedentary
HFD sedentary
Early dark phase VWR
Late dark phase VWR
C
Muscle glycogen (mmol/kg)
D
Liver glycogen (mmol/kg)
E
90 kDa
CLOCK [A.U.]
F
69 kDa
BMAL1 [A.U.]
G
170 kDa
PER2 [A.U.]
H
60 kDa
CRY1 [A.U.]