1 **Characterising 24-h skeletal muscle gene expression alongside metabolic &**
2 **endocrine responses under diurnal conditions.**

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1 **Abstract**

2 **Context:** Skeletal muscle plays a central role in the storage, synthesis, and breakdown
3 of nutrients, yet little research has explored temporal responses of this human tissue,
4 especially with concurrent measures of systemic biomarkers of metabolism.

5 **Objective:** To characterise temporal profiles in skeletal muscle expression of genes
6 involved in carbohydrate metabolism, lipid metabolism, circadian clocks, and autophagy
7 and *descriptively relate them to* systemic metabolites and hormones during a controlled
8 laboratory protocol.

9 **Methods:** Ten healthy adults (9M/1F, mean ± SD: age: 30 ± 10 y; BMI: 24.1 ± 2.7 $kg{\cdot}m^{-2}$)
10 rested in the laboratory for 37 hours with all data collected during the final 24 hours of
11 this period (i.e., 0800-0800 h). Participants ingested hourly isocaloric liquid meal
12 replacements alongside appetite assessments during waking before a sleep opportunity
13 from 2200-0700 h. Blood samples were collected hourly for endocrine and metabolite
14 analyses, with muscle biopsies occurring every 4 h from 1200 h to 0800 h the following
15 day to quantify gene expression.

16 **Results:** Plasma insulin displayed diurnal rhythmicity peaking at 1804 h. Expression of
17 skeletal muscle genes involved in carbohydrate metabolism (*Name* – Acrophase;
18 *GLUT4* - 1440 h; *PPARGC1A* –1613 h; *HK2* - 1824 h) and lipid metabolism (*FABP3* -
19 1237 h; *PDK4* - 0530 h; *CPT1B* - 1258 h) displayed 24 h rhythmicity that reflected the
20 temporal rhythm of insulin. Equally, circulating glucose (0019 h), NEFA (0456 h),
21 glycerol (0432 h), triglyceride (2314 h), urea (0046 h), CTX (0507 h) and cortisol
22 concentrations (2250 h) also all displayed diurnal rhythmicity.

23 **Conclusion:** Diurnal rhythms were present in human skeletal muscle gene expression
24 as well systemic metabolites and hormones under controlled diurnal conditions. The
25 temporal patterns of genes relating to carbohydrate and lipid metabolism alongside
26 circulating insulin are consistent with diurnal rhythms being driven in part by the diurnal
27 influence of cyclic feeding and fasting.

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1 **Key words:** Skeletal muscle, Gene expression, Circadian rhythms, Diurnal, Glucose,
2 Lipids

3 **Abbreviations:**

4 SCN – Suprachiasmatic nuclei

5 VLDL – Very low-density lipoprotein

6 CTX – C-terminal telopeptide

7

8 **Introduction**

9 The human circadian system consists of both central (suprachiasmatic nuclei; SCN) and
10 peripheral (e.g., muscle, liver, adipose) clocks. These allow for temporal coordination of
11 core physiological processes with cyclic environmental and behavioural events such as
12 light-dark, waking-sleeping, activity-rest, and feeding-fasting.

13

14 Daily variations in nutrient metabolism are apparent; glucose tolerance is generally
15 lower in the evening than in the morning, whereas lipid metabolism favours
16 progressively elevated circulating lipids later in the day and into the night (1-10). Diurnal
17 regulation of insulin secretion/clearance and sensitivity drives rhythmicity in both
18 carbohydrate and lipid metabolism (11), with lipid metabolism further dictated by
19 rhythmic intestinal triglyceride absorption, LPL activity, mitochondrial oxidative capacity,
20 and very low-density lipoprotein (VLDL) secretion (7,9,10,12-17). Equally, circulating
21 catabolic and anabolic markers, such as cortisol and testosterone, also exhibit
22 rhythmicity across the day, both peaking in the morning (18,19). Daily variation in these
23 hormones may contribute to day-night rhythms in muscle protein metabolism (20) but
24 may also further contribute to observed daily profiles in circulating glucose and lipids
25 (21-23). Despite possible interactions between these rhythms, there is limited human
26 data regarding temporal relationships between metabolic and endocrine markers of
27 carbohydrate, lipid, and protein metabolism.

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Skeletal muscle displays robust rhythmicity in transcriptomic regulation of the circadian clock, as well as carbohydrate, lipid, and protein metabolism; this may influence the central role of this tissue in the storage, synthesis, and breakdown of nutrients (13,24-28). Specifically, skeletal muscle is an important storage site for glucose (glycogen) (29,30) and lipids (intramyocellular lipids) (27,31), and is also the primary store of protein within the human body (32-35). The ability to readily dispose and mobilise these nutrients from this tissue is an important determinant of insulin sensitivity and therefore metabolic health (27,31,36-38). Furthermore, autophagy is a central process that regulates skeletal muscle protein turnover, as well as glucose and lipid metabolism and responds to a variety of stimuli, including, nutrient deprivation, and amino acid starvation (39,40). However, no studies have explored molecular regulation of this process within skeletal muscle across a 24-h period. Considering the importance of the skeletal muscle in facilitating the response to nutrient availability, it is remarkable that no studies to date have assessed rhythmicity in the molecular regulation of skeletal muscle metabolism alongside circulating metabolites and hormones involved in carbohydrate and lipid metabolism and bone resorption.

Previous studies employing constant-routine protocols to study daily variation in carbohydrate/lipid metabolism have provided valuable insight into endogenous circadian rhythmicity in the absence of behavioural rhythms. However, glucose and lipid metabolism are strongly modulated by diurnal behavioural factors, including: fasting duration (41), physical activity/exercise (42,43), sleep (44), and food/macronutrient intake/timing (45-48). During typical schedules, behavioural rhythms such as feeding-fasting are naturally aligned with cycles of light-dark and wake-sleep such that the majority of daylight hours are spent in the postprandial state, with the longest period of fasting across 24-hour period occurring at night (49). Given the divergent responses of circulating insulin to feeding and fasting, alongside the potent entrainment effect of insulin upon circadian clocks, it is vital to study such metabolic rhythms in the context of these diurnal influences (50-52).

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To enhance our knowledge of metabolic regulation across a 24-hour period of tightly controlled light-dark exposure and sleep-wake opportunity, it is now important to assess systemic hormonal and metabolite profiles alongside simultaneously collected skeletal muscle samples. To this end, the aim of this study was to characterise 24-h rhythms in skeletal muscle expression of genes involved in nutrient metabolism and autophagy alongside systemic metabolites and hormones, during a semi-constant routine whereby feeding-fasting was aligned with light-dark exposure and wake-sleep opportunity.

Materials and Methods

Approach to the research question

Given the protracted nature of this study, a single-arm time-series design was deemed appropriate. Whereas constant routine studies eliminate the influence of diurnal factors such as sleep-wake and fasting-feeding, the current study employed a semi-constant routine to study the diurnal influence of those factors. This protocol was characterised by designated wake and sleep opportunities that were aligned with feeding and fasting, respectively. Specifically, iso-caloric snacks were ingested by participants every hour during waking hours to align feeding-fasting with wake-sleep and light-dark, respectively Hourly feeds were prescribed to provide $6.66\%\cdot h^{-1}$ of estimated 24 h resting metabolic rate (RMR) across the 15 h waking period (i.e., 0800 – 2200 h), thus meeting individually-measured resting energy requirements and accounting for RMR as a driver of energy intake (53,54). This model of continuous (hourly) feeding was selected to facilitate characterisation of the underlying 24-h fed-fast rhythm in the absence of the acute meal responses that would occur with any particular meal pattern. Nonetheless, the overall 24-h pattern of nutrient availability with this model of continuous feeding is not dissimilar to that observed with a typical 3-square meal pattern (even without

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1 snacking) since, even though nutrients are commonly ingested only periodically by most
2 humans, there is a constant systemic appearance of nutrients from the gastrointestinal
3 tract for the entirely of waking hours (49).

4 Hourly blood sampling was deemed both sufficient and feasible to detect diurnal
5 rhythmicity in systemic hormones and metabolites (55,56). Conversely, a different
6 approach was required for muscle sampling due to the invasive nature of collecting
7 these samples. Four hourly sampling was deemed appropriate to assess rhythmic
8 expression of metabolic genes in this tissue while also minimising participant
9 discomfort.

10 Transcriptomic data from the same participants have been reported previously in an
11 untargeted analysis of rhythmicity (57). The aim of the current study was to analyse
12 skeletal muscle RNA levels in a targeted number of metabolic genes in order to contrast
13 with rhythms in circulating biomarkers. Plasma melatonin has also been reported
14 previously and is included in the current manuscript to illustrate 24-h profiles relative to
15 diurnal melatonin and melatonin onset (24,50). Likewise, cortisol from this protocol has
16 also been reported previously at 4-hourly resolution aligned with muscle biopsy samples
17 (24); updated biochemical analyses were therefore deemed necessary to increase
18 resolution and capture the profile of cortisol prior to the first biopsy at midday (0800-
19 1200 h).

20

21 *Research Design*

22 A time-series design was employed to investigate temporal rhythms in skeletal muscle
23 gene expression relating to carbohydrate metabolism, lipid metabolism, circadian
24 clocks, and autophagy, alongside plasma glucose, non-esterified fatty acids, insulin,
25 glycerol, triglycerides, and C-terminal telopeptide (CTX), as well as serum cortisol and
26 testosterone under conditions of semi-constant routine. Following a 7-day period of
27 standardised wake-sleep, meal-timing, and light exposure (a typical living pattern for
28 this population, thus serving to reduce between-participant variation in response to the
29 semi-constant routine), participants underwent a 37-hour in-patient visit to the resting

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laboratory at the University of Bath. During the final 24-hours of this visit, participants had a designated sleeping opportunity (2200 -0700 h) and hourly isocaloric feedings during waking periods (0800 - 2200 h) to preserve diurnal influences of sleep-wake and fasting-feeding. Hourly blood samples were collected throughout the day (whilst awake) and night (during sleep) for assessment of rhythms in the systemic concentrations of glucose, non-esterified fatty acids, and insulin, along with melatonin and cortisol to provide a validated internal phase marker. Skeletal muscle samples were collected every 4-h from 1200 h for the remainder of the trial for assessment of RNA expression.

Participants

Ten healthy participants (9M;1F, **Table 1**), who maintained a typical sleep-wake cycle (i.e. not of extreme chronotype and kept a consistent daily routine) and did not perform shift work, were recruited and screened via local advertisement. Participant screening was undertaken through completion of a general health questionnaire and validated chronotype questionnaires to assess habitual sleep patterns and diurnal preferences (58-60). Participants were excluded from participation if they had a habitual sleep duration not within 6-9 hours per night and/or a Pittsburgh Sleep Quality Index >5. With regards to shift work, exclusion criteria were in place for individuals who had participated in shift work or had travelled across more than two time zones within three weeks of the study. All volunteers were fully briefed on the requirements of the study prior to provision of written informed consent. Ethical approval for the experimental protocol was obtained from the Cornwall and Plymouth NHS research ethics committee (reference: 14/SW/0123). All procedures were performed in accordance with the Declaration of Helsinki.

[Table 1]

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1 *Pre-experimental standardisation week*

2 Participants adhered to a strict routine of feeding and sleeping in the 7-days prior to
3 entering the laboratory, waking between 0600 and 0700 h and going to sleep between
4 2200 and 2300 h, confirmed using time-stamped voicemail. The median (IQR) time that
5 those voicemails were received were 0653 h (0643-0722) for waking and 2245 h (2230-
6 2250) for lights-out, respectively.

7

8 Upon waking, participants ensured at least 15 minutes of natural light exposure within
9 1.5 hours of waking, affirmed by wrist actigraphy using a light sensor, further confirming
10 standardisation of sleep-wake patterns (Actiwatch™, Cambridge Neurotechnology;
11 Cambridge, UK). Self-selected meals were scheduled at 0800, 1200 and 1800 h, with
12 assigned snacking opportunities at 1000, 1500 and 2000 h. Participants also completed
13 a weighed record of all food and fluid intake on the final two days of this 7-day
14 standardisation period and verified that they had consumed the reported meals and
15 snacks at the prescribed times (Table 2).

16

17 **[Table 2]**

18

19 *Experimental Protocol*

20 Following the standardisation week, participants reported to the laboratory at 1900 h on
21 experimental day 1 to acclimatise to the laboratory (**Figure 1**). Laboratory conditions
22 were standardised for the duration of their stay, with blackout-blinds to prevent the
23 penetration of natural light and room temperature maintained at 20-25°C. During waking
24 hours, artificial lighting was set at 800 lux in the direction of gaze (0700-2200 h) and
25 turned off (0 lux) during sleeping hours (2200-0700 h), during which time participants
26 wore an eye mask. Participants remained in a semi-recumbent position throughout (i.e.,
27 head-end of bed elevated to 30°). Upon arrival, participants were shown to their bed
28 and provided with a prescribed meal composed of a baked potato with butter and

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cheese, steamed vegetables (broccoli and mini corn), followed by a bowl of fresh strawberries, raspberries and blueberries (1245 kcal; 31% carbohydrate, 50% fat and 19% protein). An instant hot chocolate made with whole milk was then provided at 21:30 (242 kcal; 56% carbohydrate, 24% fat and 20% protein) before lights out at 2200 h.

On day 2, participants were woken at 0700 h and RMR was immediately measured over 15 minutes using indirect calorimetry via the Douglas bag technique (61). An intravenous cannula was inserted to an antecubital vein to allow for hourly 10 mL blood draws from 0800 h, alongside appetite VAS during waking hours (reported previously (50)). Muscle biopsies were collected every 4 hours from 1200 h on day 2 through to 0800 h on day 3. After each set of measurements, an hourly feed (commencing at 0800 h) was ingested in the form of a meal-replacement solution (1.25 kcal·mL^{-1}, 45% carbohydrate, 25% fat, 30% protein; Resource Protein, Nestlé; Vevey, Switzerland). Each hourly dose was prescribed to give 6.66%·h^{-1} of measured 24-h RMR across the 15 h wake period (118 ± 19 kcal·h^{-1}). Plain water was consumed *ad libitum* and participants had access to mobile devices, on-demand entertainment, music and reading material throughout waking hours only. Toilet breaks were permitted in the first half of each hour as required.

The final set of waking measurements were collected at 2200 h, along with ingestion of the final prescribed feed. Following this, the lights were switched-off and participants were asked to wear an eye mask throughout the lights-out period. Blood samples continued throughout the night at hourly intervals without intentionally waking the participants. Participants were woken at 0700 h, and a blood sample was immediately drawn. The final set of measurements were made at 0800 h.

[Figure 1]

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1 *Outcome Measures*

2 **Blood Sampling and Analysis –** At each time-point, 10 mL of whole blood was drawn
3 and immediately distributed into tubes treated with lithium heparin (for analysis of
4 melatonin) or ethylenediaminetetraacetic acid (EDTA; for analysis of glucose, insulin,
5 non-esterified fatty acids, glycerol-corrected triglycerides, glycerol and C-terminal
6 telopeptide) or left to clot at room temperature for 15 minutes (Serum; for analysis of
7 cortisol and testosterone). Blood collection tubes were centrifuged for 10 minutes (3466
8 x g, 4°C), after which the supernatants were removed and stored at -80°C.

9 Plasma melatonin concentration was measured in the heparinised samples using a
10 radioimmunoassay (Surrey Assays Ltd, UK; Assay performance reported elsewhere
11 (50)). Plasma insulin (Mercodia, Sweden; RRID: AB_2877672; Intra-Assay CV:
12 6%/Inter-Assay CV: 13%), C-terminal telopeptide (CTX; Immunodiagnostic systems,
13 UK; RRID: AB_2923399; Intra-Assay CV: 19%/Inter-Assay CV: 27%)) (ISD, UK),
14 glucose (Intra-Assay CV: 3%/Inter-Assay CV: 3%), non-esterified fatty acids (NEFA;
15 Intra-Assay CV: 6%/Inter-Assay CV: 6%), glycerol (Intra-Assay CV: 12%/Inter-Assay
16 CV: 18%) and triglycerides (Intra-Assay CV: 4%/Inter-Assay CV: 18%) (Randox, UK)
17 were quantified in EDTA-treated plasma, with cortisol (Tecan, CH; RRID: AB_2924715;
18 Intra-Assay CV: 6%/Inter-Assay CV: 7%) and testosterone (R&D Systems, Bio-Techne,
19 US; RRID: AB_2820244; Intra-Assay CV: 30%/Inter-Assay CV: 28%) quantified in
20 serum.

21

22 *Skeletal muscle sampling and analysis*

23 Skeletal muscle samples were collected from the *vastus lateralis* under local
24 anaesthesia (1% lidocaine: Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples
25 were collected at 4-hourly intervals from 1200 until 0800 h (i.e., 6 in total) from a 3-5
26 mm incision in the anterior aspect of the thigh using a Bergstrom needle adapted for
27 suction (62,63). Samples were taken from each leg in a randomly determined
28 alternating order between dominant and non-dominant leg, ascending up the leg with
29 skin incisions separated by 2–3 cm. Daytime biopsies were taken following the VAS and

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blood sample, but before the prescribed feed. Thirty minutes prior to sleep, incisions for the night-time biopsies were made to minimise disruption to participants' sleep. For night-time tissue biopsies (i.e. 0000 and 0400 h), participants were woken briefly but continued to wear the eye mask while samples were taken by torch-light (samples acquired and researchers left the laboratory within 3-5 minutes). Samples were immediately snap-frozen in liquid nitrogen for subsequent storage at -80°C.

Samples were later homogenised in 2 mL Trizol (Invitrogen, UK) and centrifuged 2500 x *g* for 5 min at 4°C. The top layer and pellet were removed and 200 µl of chloroform was added per 1 mL of Trizol and mixed vigorously for 15 s. Samples were subsequently incubated at room temperature for 3 min prior to centrifugation at 2500 x *g* for 5 min at 4°C. The aqueous phase was then removed and mixed with an equal volume of 70% ethanol prior to loading on a RNeasy mini column for extraction (Qiagen, Crawley, UK). All samples were quantified using spectrophotometry, with 2 µg of total RNA reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Taqman low density Custom Array using Micro Fluidic cards (Life Technologies, Thermo Fisher Scientific) was used for the relative quantification of expression of 45 genes listed in Table 3, as previously described (64,65). The geometric mean of 18S ribosomal RNA (*18S*) (Hs03003631_g1), Actin alpha 1, skeletal muscle (*ACTA1*) (Hs05032285_s1), and Hydroxymethylbilane synthase (*HMBS*) (Hs00609296_g1) was used as an endogenous control. The comparative threshold cycle (Ct) method was used to process the data where ∆Ct=Ct target gene – Ct endogenous control (Geometric mean of *18s, Actin, HMBS*); cosinor analysis on the raw ct values of *18S, ACTA1,* and *HMBS* did not indicate 24-h rhythmicity across the protocol, with mean ± SD ct values demonstrating high stability over all timepoints (10.2 ± 0.2, 15.4 ± 0.2 and 27.9 ± 0.1, respectively). Data were then normalised to an internal calibrator and finally 24-h mean expression. One gene (*OTX1*; Orthodenticle Homeobox 1) was undetectable and therefore data for 44 genes are presented.

[Table 3]

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Statistical Analysis

Concentrations for circulating metabolic and endocrine markers were adjusted to melatonin onset for each participant as determined by the 25% method (i.e., calculation of when 25% of the peak melatonin concentration occurred) (66). The time in minutes between melatonin onset and midnight was calculated for each participant and used to adjust 24-h profiles. The resulting x-values were binned around half past the hour with average y-values plotted at half past the hour (67-69). Muscle data were not adjusted for melatonin onset as 4-hourly sampling resolution was not deemed sufficient for this type of subtle adjustment.

Analysis of rhythmicity for all outcomes was conducted using the cosine method (Prism 9, Graphpad; CA, USA). In this approach, a cosine wave is fit to the 24-h profile of a given variable and compared against a horizontal line through the mean values (null). If a cosine wave provides a better fit (R^2) for the data than the horizontal line then the dataset characterises diurnal (or 24-h) rhythmicity, with the mesor (rhythm-adjusted mean), amplitude (magnitude of the difference between mesor and peak/trough values) and acrophase (timing of rhythmic peak) all identified and reported (56,70). Reported *p*-values are the output of the Extra sum-of-squares F test. This method was chosen *a priori* to provide a greater descriptive characterisation of temporal patterns compared to commonly used statistical approaches such as analyses of variance (e.g. 1-way ANOVA looking at effects of time or 2-way ANOVA for treatment*time interactions) but it must also be recognised that different analytical approaches may yield varied results (56). Whilst *post hoc* adjustment of *p*-values for multiple statistical tests is sometimes required to minimise inflation of type I error rates (i.e. false positives), it has been questioned whether such adjustment is always necessary (71) and it is rare to see such adjustment between separate outcome measures. Moreover, given the aims of the study to characterise rhythmicity in metabolic outcomes it was not deemed necessary to perform such adjustments. All data are presented as mean ± SD unless otherwise stated (e.g., figures are mean ± 95% Confidence Intervals).

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Results

Metabolites

All plasma metabolites displayed diurnal rhythmicity. Mean plasma glucose was rhythmic (p = 0.04, R^2 = 0.03, **Figure 2A**). The acrophase occurred at 0119 h and fell to the nadir in the afternoon, with a mean concentration of 4.83 ± 0.44 mmol·L^{-1} and amplitude of 0.17 mmol·L^{-1}. Plasma NEFA was also rhythmic peaking at 0456 h and falling to the nadir in the afternoon, with an amplitude of 0.15 mmol·L^{-1} and rhythm adjusted mean of 0.18 ± 0.05 mmol·L^{-1} (p < 0.01, R^2 = 0.38, **Figure 2B**). Likewise, diurnal rhythmicity was evident in plasma glycerol. Mean concentrations across the period were 0.02 ± 0.01 mmol·L^{-1} and the diurnal rhythm was characterised by an amplitude of 0.08 mmol·L^{-1}, peaking at 0432 h with lowest values in the afternoon (p < 0.01, R^2 = 0.14, **Figure 2C**). Plasma triglycerides were also rhythmic with the acrophase occurring at 2314 h and falling to a nadir in the afternoon, with an amplitude of 0.13 mmol·L^{-1} and 24-h mean of 0.94 ± 0.32 mmol·L^{-1} (p < 0.01, R^2 = 0.06, **Figure 2D**). Finally, plasma urea was rhythmic across the period, peaking at 0046 h with an amplitude of 0.66 mmol·L^{-1} and mean concentration of 7.45 mmol·L^{-1} (p < 0.01, R^2 = 0.08, **Figure 2E**).

[Figure 2]

Hormones and telopeptides

Plasma insulin was rhythmic, peaking at 1804 h before falling to an overnight nadir (p < 0.0001, R^2 = 0.08, **Figure 3A**). The diurnal rhythm occurred with an amplitude of 10.0 pmol·L^{-1}and a mean concentration of 43.4 ± 17.1 pmol·L^{-1}. Plasma CTX was also characterised by diurnal rhythmicity (p < 0.0001, R^2 = 0.19, **Figure 3B**); peak concentration occurred at 0507 h and fell to the nadir during the afternoon, with an

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1 amplitude of 0.16 ng·mL^{-1} and mean of 0.29 ± 0.20 ng·mL^{-1}. Serum cortisol was also
2 rhythmic, peaking at 1050 h with an amplitude of 22.3 nmol·L^{-1} ($p < 0.0001$, $R^2 = 0.12$,
3 **Figure 3C**). Average cortisol concentration across the 24-h period was 232 ± 55 nmol·L^{-1}
4 . Conversely, mean serum testosterone was not rhythmic with an average
5 concentration of 70.2 ± 54.8 nmol·L^{-1} ($p = 0.62$, **Figure 3D**). Melatonin data are reported
6 elsewhere (24,50). Briefly, peak plasma melatonin occurred at 0330 h and mean
7 melatonin onset occurred at 2318 h ± 46 min (**Figures 2 and 3**).

8

9 **[Figure 3]**

10

11 *Skeletal muscle gene expression*

12 Of the 44 genes assessed, 26 displayed rhythmicity (all $p < 0.05$) (**Figure 4**). This
13 diurnal rhythmicity was evident for core clock genes (Acrophase – h, Amplitude - %):
14 *ARNTL* (2218 h, 70%), *CLOCK* (2329 h, 11%), *CRY2* (1308 h, 23%), *NPAS2* (0012 h,
15 37%), *NR1D1* (0404 h, 63%), *NR1D2* (0804 h, 36%), *PER1* (1021 h, 48%), *PER2* (0821
16 h, 41%), *PER3* (0930 h, 57%), and *TP53* (0500 h, 20%). Genes relating to autophagy
17 and protein metabolism were also rhythmic: *MYOD1* (1914 h, 41%), *FOXO3* (0900 h,
18 26%), *FBXO32* (0716 h, 39%). Diurnal oscillations were also present in the expression
19 of genes involved in glucose and lipid metabolism; *GLUT4* (1440 h, 25%), HK2 (1828 h,
20 21%), *FABP3* (1237 h, 15%), *PDK4* (0530 h, 133%) and *CPT1B* (1258 h, 14%). Finally,
21 diurnal variation was apparent in genes involved in mitochondrial signalling;
22 *PPARGC1A* (1613 h, 15%) and *UCP3* (0659 h, 58%), *SIRT3* (1509 h, 10%) as well as
23 transcriptional/translational regulation and MAPK signalling; *CREB5* (0357 h, 19%),
24 *EIF4EBP1* (0741 h, 11%), and *HNRNPDL* (1317 h, 35%). Temporal relationships
25 between rhythmic circulating biomarkers and skeletal muscle genes are reported in
26 **Figure 5**.

27

28 **[Figure 4]**

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[Figure 5]

Discussion

This is the first study to report serial measures of human skeletal muscle alongside systemic markers of metabolic regulation under controlled diurnal conditions. Diurnal rhythmicity was apparent in skeletal muscle genes relating to carbohydrate, lipid and protein metabolism, autophagy and mitochondrial signalling as well as in circulating glucose, insulin, NEFA, glycerol, triglycerides, cortisol, and c-terminal telopeptide.

Plasma insulin was rhythmic, peaking in the evening (~1800 h) and falling to nadir overnight (~0400 h). This is consistent with previous research employing a continuous glucose clamp (72) and generally agrees with the notion of greater insulin sensitivity in the morning compared to the evening (11). However, the timing of peak insulin differs from that reported in Wehrens et al (73) in which the acrophase of insulin occurred ~8-11 hours after a melatonin onset similar to that reported currently, placing peak time at ~0700-1000h. Nevertheless, methodological differences between studies allow for greater understanding of behavioural factors that may influence such rhythms. The continuous feeding pattern during waking hours in the current study suggests rhythmicity in circulating insulin occurs at least partly independent of food intake (74-76). Nonetheless, insulin is highly responsive to nutrient intake, and the coincidence of the overnight fast with lower nocturnal insulin suggests nutrient intake could be producing some of the apparent diurnal responses. Plasma glucose concentrations were also rhythmic (peak ~0130 h), consistent with studies of circadian misalignment, constant routine, and forced desynchrony thus further highlighting robust regulation of rhythms in plasma glucose by the endogenous clock even under controlled diurnal conditions (2,3,5,77). Interestingly, whilst glucose and insulin concentrations might

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usually be expected correlate when comparing acute meal responses over the minutes following feeding, the current model of hourly feeding and sampling over 24-h may explain why variance in insulin may be sufficient to alter glucose kinetics/flux but without necessarily being reflected by changes in the systemic concentrations of glucose. At the tissue level, skeletal muscle *GLUT4* and *PPARGC1a* RNA were rhythmic, with peak levels occurring at ~1500 and ~1600 h, respectively (i.e., when insulin was rising), with the lowest levels at ~0400 h (i.e., when insulin was lowest). Peak *HK2* RNA occurred at ~1830 h, shortly after the rhythmic peak in plasma insulin and therefore in line with the regulatory effects of insulin on hexokinase activity (78,79). The observation of rhythms in the skeletal muscle expression of *GLUT4* and *HK2* is contrary to previous work in mice whereby no significant oscillations in these genes (80,81). Collectively, the broad alignment of the rhythms of these genes with rhythmic plasma insulin reflects their involvement in skeletal muscle glucose uptake and their potential to influence diurnal glucose metabolism (82,83).

The diurnal profiles of NEFA and glycerol were also broadly anti-phasic to the 24-h profile of insulin (**Figure 5**). Circulating NEFA and glycerol were generally supressed during waking hours, before rising to peak at ~0400-0500 h, consistent with the nocturnal rise reported in previous literature (84-86). Plasma triglycerides were also rhythmic under controlled diurnal conditions, whereby systemic concentrations were low during the morning before rising to a peak at ~2330 h (**Figure 2D**). The rhythmic profile of these circulating lipids is consistent with the regulatory effects of insulin on adipose tissue lipolysis (87-89) and circulating triglyceride levels (90). The anti-phasic relationship between insulin with NEFA and glycerol alongside the aligned rhythms in insulin and triglycerides could be reflective of feeding status and the subsequent changes in adipose tissue lipolysis in the overnight fasted state (45,46,49). Circulating melatonin is speculated to in part contribute towards the regulation of lipid metabolism (91,92), this may be reflected in the temporal similarity in acrophase among systemic melatonin NEFA and glycerol (Figure 5), however further work is required to better understand the effects of melatonin on lipid metabolism.

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2 Peak expression of skeletal muscle *PDK4* RNA (~0530 h) occurred proximally to the
3 peak in systemic NEFA (**Figure 5**). This is consistent with previous work demonstrating
4 an association between diurnal variation in *PDK4* and NEFA, which may be explained
5 by the role of this gene in stimulating fatty acid utilisation in response to a rise in NEFA
6 availability (93-97). This temporal pattern may be driven the diurnal feeding pattern
7 present in both the current and previous work (97). However, following peak RNA
8 levels, *PDK4* declined at ~0800 h, despite the continual fasted state and resultant
9 elevated NEFA availability, suggesting observed effects may not be solely due to the
10 imposed feeding pattern. The profile of genes involved in the regulation of solubility,
11 mobility, and transport of fatty acids (e.g., *CPT1B* and *FABP3*) did not align with
12 systemic concentrations of NEFA (98,99), but broadly mirrored the rhythm in insulin.
13 Furthermore, alignment between *UCP3* expression with the profile of systemic NEFA is
14 consistent with the involvement of this gene in mitochondrial fatty acid oxidation
15 (100,101).

16

17 Plasma urea concentration increased gradually through waking hours (Peak ~0046 h),
18 before declining overnight. This could be in response to the imposed feeding pattern,
19 reflecting a greater rate of nitrogen excretion later in the day once the total amount of
20 nutrients had been consumed and subsequent decrease in response to the withdrawal
21 of nutrition during sleep (102,103).

22

23 Numerous metabolic and endocrine responses relevant to tissue turnover show diurnal
24 rhythms under semi-constant routine. Cortisol displayed the expected rhythm, peaking
25 at (~1100 h) before falling to its lowest value in the evening, approximately coinciding
26 with melatonin onset (73). Peak expression of skeletal muscle *FBXO32* occurred during
27 the morning period while cortisol was rising; consistent with the related action of this
28 gene and hormone in catabolic processes, which may be driven by the diurnal overnight
29 fast (104-108). Following muscle breakdown, autophagy is a vital process to stimulate

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1 muscle regeneration (39). Expression of *FOXO3*, which promotes expression of
2 downstream targeted autophagy-related proteins, also peaked in the morning when
3 cortisol is rising, which may reflect the proposed regulatory effects of cortisol in
4 stimulating increased autophagic flux in skeletal muscle (109,110). Collectively the
5 temporal patterns of these skeletal muscle genes hint at diurnal fluctuations in tissue
6 turnover, which has previously been observed in non-human models (108,111).
7 However, serum testosterone did not display diurnal rhythmicity. Previous studies have
8 demonstrated rhythmicity in systemic testosterone, with highest values early in the
9 morning (~0800 h) and corresponding lowest values ~12 h later (18,112-114). This
10 typical rhythm was not observed in the current study, which could be explained by
11 several mechanisms, including daytime hourly nutrition (115,116), sleep fragmentation
12 (117), and the potential acute effect of muscle biopsies on systemic cortisol (118). The
13 lack of rhythmicity could also be due to the sensitivity of measurement through the use
14 of commercial enzyme-based immuno-assays rather than gold standard measurement
15 by liquid chromatography mass spectrometry (119,120). Equally, neither free
16 testosterone nor sex hormone-binding globulin were assessed as part of these
17 analyses, both of which have been reported to display clear daily rhythms (114,121).
18 Finally, *MYOD1*, an important myogenic regulatory factor, displayed a similar peak and
19 nadir to insulin. This is in line with the proposed effects of insulin on muscle protein
20 turnover, hinting at diurnal patterns in skeletal muscle turnover, which are plausibly
21 driven by patterns of feeding and fasting (78,122).

22

23 Plasma CTX was lowest during the day in the fed state and peaked during the biological
24 night in the fasted state (~0500 h) in a remarkably similar rhythm and amplitude to
25 previous literature (123-125). Feeding reliably suppresses bone resorption, and acute
26 fasting dampens typical rhythmicity (124). The current data therefore highlight the
27 influence of diurnal feeding-fasting cycles on the typical 24 h patterns of systemic CTX
28 (126,127). However, plasma CTX was higher at the end of the measurement period
29 than the beginning, suggesting that other factors, such as sleep and wake cycles, may

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also impact bone resorption and future work should seek to establish the contribution of sleep on bone resorption independent of nutritional status (128,129).

Despite the novelty of simultaneously collected plasma and muscle samples under controlled diurnal conditions in a 24 h period, the current data must be interpreted in light of several factors. Participants were fed relative to individualised requirements, to account for the role of resting metabolic rate as a driver of energy intake and appetite (53,54). However, 24-h bed rest eliminates the influence of physical activity on circadian clocks, glucose, lipid, and protein metabolism in skeletal muscle as well as bone turnover (42,130-132). This is especially pertinent given that muscle samples were collected from the legs, which typically sustain greater load bearing than upper limbs, so bed rest may elicit greater metabolic perturbation (133). The potential for multiple tissue biopsies on localised inflammation must also be acknowledged. However, biopsies were taken from alternating limbs with each following biopsy on the same limb being taken 3 cm proximally to the initial incision. This is in line with Van Thienin and colleagues (134), who reported inflammatory markers were upregulated at the distal, but not at the proximal site when taking sequential samples from the same limb. Equally, it is a limitation of this study that sleep duration and quality were not objectively measured, so it is not possible to comment on the impact of nocturnal sampling on those outcomes or their potential influence on the primary outcomes. It should also be considered that the bright light in the laboratory may have delayed the melatonin onset time and therefore suppressed the release of melatonin in the first part of the night (135).

The use of a "semi-constant" routine with alignment of the dark-light cycle with fasting/food intake and sleep/wakefulness can be viewed as both a strength and a limitation of the current study. The model has ecological validity since the semi-constant routine reflects free-living environmental and behavioural cycles that exist outside of the laboratory; however, the presence of such diurnal factors also make it more difficult to disentangle whether rhythms are truly circadian or driven by behavioural/environmental cycles.

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Despite the aforementioned factors, diurnal rhythmicity was still observed in the majority of core clock genes, highlighting the robust rhythmic nature of skeletal muscle (57). Whilst the current findings hint at the possibility of diurnal influences of feeding patterns on circulating and tissue rhythms, direct comparison of divergent nutrient feeding patterns, especially where nutrition is provided through the night, is required to establish whether the observed rhythms are driven endogenously or by the imposed behavioural (feeding and sleep) factors (136).

In summary, this was the first study to measure diurnal rhythms in human skeletal muscle gene expression alongside systemic metabolites and hormones under controlled diurnal conditions. The diurnal pattern in genes relating to carbohydrate and lipid metabolism tended to reflect the pattern of insulin across 24 hours, which may in part be driven by the diurnal influence of cyclic feeding and fasting. This study provides novel context for metabolic regulation at both the tissue and systemic level.

Contributions Conceptualisation and Methodology; H.A.S., J.D.J., J-P.W., and J.A.B. Data Collection, Analysis, Visualisation and Interpretation; H.A.S., M.D., J-P.W., I.T., T.S., J.T.G., B.M., J.D.J., K.T., and J.A.B. Original Draft; H.A.S., and J.A.B. Review and Editing; M.D., J-P.W., I.T., T.S., J.T.G., D.J,C., I.V., L.J.J., B.M., J.D.J., and K.T. All authors read and approved the final manuscript.

Data Availability Statement

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

References

1. Van Cauter E, Polonsky KS, Scheen AJ. Roles of circadian rhythmicity and sleep in human glucose regulation. *Endocr Rev*. 1997;18(5):716-738.

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Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

2. Van Cauter E, Desir D, Decoster C, Fery F, Balasse EO. Nocturnal decrease in glucose tolerance during constant glucose infusion. *The Journal of clinical endocrinology and metabolism*. 1989;69(3):604-611.
3. Van Cauter E, Shapiro ET, Tillil H, Polonsky KS. Circadian modulation of glucose and insulin responses to meals: relationship to cortisol rhythm. *The American journal of physiology*. 1992;262(4 Pt 1).
4. Simon C, Brandenberger G, Saini J, Ehrhart J, Follenius M. Slow oscillations of plasma glucose and insulin secretion rate are amplified during sleep in humans under continuous enteral nutrition. *Sleep*. 1994;17(4):333-338.
5. Qian J, Scheer F. Circadian System and Glucose Metabolism: Implications for Physiology and Disease. *Trends in endocrinology and metabolism: TEM*. 2016;27(5):282-293.
6. Morgan LM, Aspostolakou F, Wright J, Gama R. Diurnal variations in peripheral insulin resistance and plasma non-esterified fatty acid concentrations: a possible link? *Ann Clin Biochem*. 1999;36(Pt 4):447-450.
7. Yoshino J, Almeda-Valdes P, Patterson BW, Okunade AL, Imai S, Mittendorfer B, Klein S. Diurnal variation in insulin sensitivity of glucose metabolism is associated with diurnal variations in whole-body and cellular fatty acid metabolism in metabolically normal women. *J Clin Endocrinol Metab*. 2014;99(9):2014-1579.
8. Zimmet PZ, Wall JR, Rome R, Stimmler L, Jarrett RJ. Diurnal variation in glucose tolerance: associated changes in plasma insulin, growth hormone, and non-esterified fatty acids. *Br Med J*. 1974;1(5906):485-488.
9. Ang JE, Revell V, Mann A, Mantele S, Otway DT, Johnston JD, Thumser AE, Skene DJ, Raynaud F. Identification of human plasma metabolites exhibiting time-of-day variation using an untargeted liquid chromatography-mass spectrometry metabolomic approach. *Chronobiology international*. 2012;29(7):868-881.
10. Pan X, Hussain MM. Diurnal regulation of microsomal triglyceride transfer protein and plasma lipid levels. *J Biol Chem*. 2007;282(34):24707-24719.
11. Saad A, Dalla Man C, Nandy DK, Levine JA, Bharucha AE, Rizza RA, Basu R, Carter RE, Cobelli C, Kudva YC, Basu A. Diurnal pattern to insulin secretion and insulin action in healthy individuals. *Diabetes*. 2012;61(11):2691-2700.
12. Pan X, Jiang XC, Hussain MM. Impaired cholesterol metabolism and enhanced atherosclerosis in clock mutant mice. *Circulation*. 2013;128(16):1758-1769.
13. van Moorsel D, Hansen J, Havekes B, Scheer F, Jörgensen JA, Hoeks J, Schrauwen-Hinderling VB, Duez H, Lefebvre P, Schaper NC, Hesselink MKC, Staels B, Schrauwen P. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. *Mol Metab*. 2016;5(8):635-645.
14. Lee A, Ader M, Bray GA, Bergman RN. Diurnal variation in glucose tolerance. Cyclic suppression of insulin action and insulin secretion in normal-weight, but not obese, subjects. *Diabetes*. 1992;41(6):750-759.
15. Sprenger RR, Hermansson M, Neess D, Becciolini LS, Sørensen SB, Fagerberg R, Ecker J, Liebisch G, Jensen ON, Vance DE, Færgeman NJ, Klemm RW, Ejsing CS. Lipid molecular timeline profiling reveals diurnal crosstalk between the liver and circulation. *Cell Rep*. 2021;34(5):108710.

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Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

16. Marrino P, Gavish D, Shafrir E, Eisenberg S. Diurnal variations of plasma lipids, tissue and plasma lipoprotein lipase, and VLDL secretion rates in the rat. A model for studies of VLDL metabolism. *Biochim Biophys Acta*. 1987;920(3):277-284.
17. Benavides A, Siches M, Llobera M. Circadian rhythms of lipoprotein lipase and hepatic lipase activities in intermediate metabolism of adult rat. *Am J Physiol*. 1998;275(3):R811-817.
18. Bremner WJ, Vitiello MV, Prinz PN. Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. *J Clin Endocrinol Metab*. 1983;56(6):1278-1281.
19. Debono M, Ghobadi C, Rostami-Hodjegan A, Huatan H, Campbell MJ, Newell-Price J, Darzy K, Merke DP, Arlt W, Ross RJ. Modified-release hydrocortisone to provide circadian cortisol profiles. *The Journal of clinical endocrinology and metabolism*. 2009;94(5):1548-1554.
20. Garlick PJ, Clugston GA, Swick RW, Waterlow JC. Diurnal pattern of protein and energy metabolism in man. *Am J Clin Nutr*. 1980;33(9):1983-1986.
21. Ottosson M, Lönnroth P, Björntorp P, Edén S. Effects of cortisol and growth hormone on lipolysis in human adipose tissue. *The Journal of clinical endocrinology and metabolism*. 2000;85(2):799-803.
22. Christiansen JJ, Djurhuus CB, Gravholt CH, Iversen P, Christiansen JS, Schmitz O, Weeke J, Jørgensen JO, Møller N. Effects of cortisol on carbohydrate, lipid, and protein metabolism: studies of acute cortisol withdrawal in adrenocortical failure. *The Journal of clinical endocrinology and metabolism*. 2007;92(9):3553-3559.
23. Kelly DM, Jones TH. Testosterone: a metabolic hormone in health and disease. *J Endocrinol*. 2013;217(3):R25-45.
24. Loizides-Mangold U, Perrin L, Vandereycken B, Betts JA, Walhin JP, Templeman I, Chanon S, Weger BD, Durand C, Robert M, Paz Montoya J, Moniatte M, Karagounis LG, Johnston JD, Gachon F, Lefai E, Riezman H, Dibner C. Lipidomics reveals diurnal lipid oscillations in human skeletal muscle persisting in cellular myotubes cultured in vitro. *Proc Natl Acad Sci U S A*. 2017;114(41):E8565-E8574.
25. Perrin L, Loizides-Mangold U, Chanon S, Gobet C, Hulo N, Isenegger L, Weger BD, Migliavacca E, Charpagne A, Betts JA, Walhin JP, Templeman I, Stokes K, Thompson D, Tsintzas K, Robert M, Howald C, Riezman H, Feige JN, Karagounis LG, Johnston JD, Dermitzakis ET, Gachon F, Lefai E, Dibner C. Transcriptomic analyses reveal rhythmic and CLOCK-driven pathways in human skeletal muscle. *eLife*. 2018;7.
26. Held NM, Wefers J, van Weeghel M, Daemen S, Hansen J, Vaz FM, van Moorsel D, Hesselink MKC, Houtkooper RH, Schrauwen P. Skeletal muscle in healthy humans exhibits a day-night rhythm in lipid metabolism. *Mol Metab*. 2020;37:100989.
27. Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *The Journal of clinical endocrinology and metabolism*. 2001;86(12):5755-5761.

ACCEPTED MANUSCRIPT

Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

28. DeFronzo RA, Gunnarsson R, Bjorkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J Clin Invest*. 1985;76(1):149-155.
29. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes*. 1981;30(12):1000-1007.
30. Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA, Shulman RG. Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. *The New England journal of medicine*. 1990;322(4):223-228.
31. van Loon LJ, Goodpaster BH. Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state. *Pflugers Arch*. 2006;451(5):606-616.
32. Poortmans JR, Carpentier A, Pereira-Lancha LO, Lancha A, Jr. Protein turnover, amino acid requirements and recommendations for athletes and active populations. *Braz J Med Biol Res*. 2012;45(10):875-890.
33. Felig P, Owen OE, Wahren J, Cahill GF, Jr. Amino acid metabolism during prolonged starvation. *J Clin Invest*. 1969;48(3):584-594.
34. Felig P, Wahren J. Amino acid metabolism in exercising man. *J Clin Invest*. 1971;50(12):2703-2714.
35. Biolo G, Fleming RY, Maggi SP, Wolfe RR. Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. *The American journal of physiology*. 1995;268(1 Pt 1):E75-84.
36. Johnson-Bonson DA, Narang BJ, Davies RG, Hengist A, Smith HA, Watkins JD, Taylor H, Walhin JP, Gonzalez JT, Betts JA. Interactive effects of acute exercise and carbohydrate-energy replacement on insulin sensitivity in healthy adults. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme*. 2021;46(10):1207-1215.
37. Taylor HL, Wu CL, Chen YC, Wang PG, Gonzalez JT, Betts JA. Post-Exercise Carbohydrate-Energy Replacement Attenuates Insulin Sensitivity and Glucose Tolerance the Following Morning in Healthy Adults. *Nutrients*. 2018;10(2).
38. Jensen J, Aslesen R, Ivy JL, Brørs O. Role of glycogen concentration and epinephrine on glucose uptake in rat epitrochlearis muscle. *The American journal of physiology*. 1997;272(4 Pt 1):E649-655.
39. Luk HY, Appell C, Levitt DE, Jiwan NC, Vingren JL. Differential Autophagy Response in Men and Women After Muscle Damage. *Front Physiol*. 2021;12:752347.
40. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008;451(7182):1069-1075.
41. Tsintzas K, Jewell K, Kamran M, Laithwaite D, Boonsong T, Littlewood J, Macdonald I, Bennett A. Differential regulation of metabolic genes in skeletal muscle during starvation and refeeding in humans. *J Physiol*. 2006;575(Pt 1):291-303.
42. Sylow L, Kleinert M, Richter EA, Jensen TE. Exercise-stimulated glucose uptake - regulation and implications for glycaemic control. *Nat Rev Endocrinol*. 2017;13(3):133-148.

ACCEPTED MANUSCRIPT

Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

1 43. Edinburgh RM, Hengist A, Smith HA, Travers RL, Koumanov F, Betts JA, Thompson D, Walhin JP, Wallis GA, Hamilton DL, Stevenson EJ, Tipton KD, Gonzalez JT. Preexercise breakfast ingestion versus extended overnight fasting increases postprandial glucose flux after exercise in healthy men. *Am J Physiol Endocrinol Metab*. 2018;315(5):E1062-e1074.
6 44. Donga E, van Dijk M, van Dijk JG, Biermasz NR, Lammers GJ, van Kralingen KW, Corssmit EP, Romijn JA. A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways in healthy subjects. *J Clin Endocrinol Metab*. 2010;95(6):2963-2968.
10 45. Chowdhury EA, Richardson JD, Tsintzas K, Thompson D, Betts JA. Carbohydrate-rich breakfast attenuates glycaemic, insulinaemic and ghrelin response to ad libitum lunch relative to morning fasting in lean adults. *The British journal of nutrition*. 2015;114(1):98-107.
14 46. Chowdhury EA, Richardson JD, Tsintzas K, Thompson D, Betts JA. Effect of extended morning fasting upon ad libitum lunch intake and associated metabolic and hormonal responses in obese adults. *International journal of obesity (2005)*. 2016;40(2):305-311.
18 47. Meng H, Matthan NR, Ausman LM, Lichtenstein AH. Effect of prior meal macronutrient composition on postprandial glycemic responses and glycemic index and glycemic load value determinations. *Am J Clin Nutr*. 2017;106(5):1246-1256.
22 48. Yoshinaga MY, Quintanilha BJ, Chaves-Filho AB, Miyamoto S, Sampaio GR, Rogero MM. Postprandial plasma lipidome responses to a high-fat meal among healthy women. *The Journal of nutritional biochemistry*. 2021;97:108809.
25 49. Ruge T, Hodson L, Cheeseman J, Dennis AL, Fielding BA, Humphreys SM, Frayn KN, Karpe F. Fasted to fed trafficking of Fatty acids in human adipose tissue reveals a novel regulatory step for enhanced fat storage. *The Journal of clinical endocrinology and metabolism*. 2009;94(5):1781-1788.
29 50. Templeman I, Smith HA, Walhin JP, Middleton B, Gonzalez JT, Karagounis LG, Johnston JD, Betts JA. Unacylated ghrelin, leptin, and appetite display diurnal rhythmicity in lean adults. *J Appl Physiol (1985)*. 2021.
32 51. Tuvia N, Pivovarova-Ramich O, Murahovschi V, Lück S, Grudziecki A, Ost AC, Kruse M, Nikiforova VJ, Osterhoff M, Gottmann P, Gögebakan Ö, Sticht C, Gretz N, Schupp M, Schürmann A, Rudovich N, Pfeiffer AFH, Kramer A. Insulin Directly Regulates the Circadian Clock in Adipose Tissue. *Diabetes*. 2021;70(9):1985-1999.
37 52. Crosby P, Hamnett R, Putker M, Hoyle NP, Reed M, Karam CJ, Maywood ES, Stangherlin A, Chesham JE, Hayter EA, Rosenbrier-Ribeiro L, Newham P, Clevers H, Bechtold DA, O'Neill JS. Insulin/IGF-1 Drives PERIOD Synthesis to Entrain Circadian Rhythms with Feeding Time. *Cell*. 2019;177(4):896-909.e820.
41 53. Blundell JE, Caudwell P, Gibbons C, Hopkins M, Naslund E, King N, Finlayson G. Role of resting metabolic rate and energy expenditure in hunger and appetite control: a new formulation. *Dis Model Mech*. 2012;5(5):608-613.
44 54. Blundell JE, Finlayson G, Gibbons C, Caudwell P, Hopkins M. The biology of appetite control: Do resting metabolic rate and fat-free mass drive energy intake? *Physiol Behav*. 2015;152:473-478.

ACCEPTED MANUSCRIPT

Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

55. Rynders CA, Morton SJ, Bessesen DH, Wright KP, Jr., Broussard JL. Circadian Rhythm of Substrate Oxidation and Hormonal Regulators of Energy Balance. *Obesity (Silver Spring, Md)*. 2020;28 Suppl 1(Suppl 1):S104-s113.
56. Cornelissen G. Cosinor-based rhythmometry. *Theor Biol Med Model*. 2014;11:16.
57. Perrin L, Loizides-Mangold U, Chanon S, Gobet C, Hulo N, Isenegger L, Weger BD, Migliavacca E, Charpagne A, Betts JA, Walhin JP, Templeman I, Stokes K, Thompson D, Tsintzas K, Robert M, Howald C, Riezman H, Feige JN, Karagounis LG, Johnston JD, Dermitzakis ET, Gachon F, Lefai E, Dibner C. Transcriptomic analyses reveal rhythmic and CLOCK-driven pathways in human skeletal muscle. *Elife*. 2018;16(7):34114.
58. Horne JA, Ostberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol*. 1976;4(2):97-110.
59. Buysse DJ, Reynolds CF, 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry research*. 1989;28(2):193-213.
60. Roenneberg T, Wirz-Justice A, Merrow M. Life between clocks: daily temporal patterns of human chronotypes. *Journal of biological rhythms*. 2003;18(1):80-90.
61. Compher C, Frankenfield D, Keim N, Roth-Yousey L. Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *J Am Diet Assoc*. 2006;106(6):881-903.
62. Tarnopolsky MA, Pearce E, Smith K, Lach B. Suction-modified Bergstrom muscle biopsy technique: experience with 13,500 procedures. *Muscle & nerve*. 2011;43(5):717-725.
63. Bergstrom J. Muscle Electrolytes in Man - Determined by Neutron Activation Analysis on Needle Biopsy Specimens - Study on Normal Subjects, Kidney Patients, and Patients with Chronic Diarrhoea. *Scandinavian journal of clinical and laboratory investigation*. 1962;14.
64. Gonzalez JT, Richardson JD, Chowdhury EA, Koumanov F, Holman GD, Cooper S, Thompson D, Tsintzas K, Betts JA. Molecular adaptations of adipose tissue to 6 weeks of morning fasting vs. daily breakfast consumption in lean and obese adults. *Journal of Physiology*. 2017:1-14.
65. Tsintzas K, Norton L, Chokkalingam K, Nizamani N, Cooper S, Stephens F, Billeter R, Bennett A. Independent and combined effects of acute physiological hyperglycaemia and hyperinsulinaemia on metabolic gene expression in human skeletal muscle. *Clin Sci (Lond)*. 2013;124(11):675-684.
66. Benloucif S, Burgess HJ, Klerman EB, Lewy AJ, Middleton B, Murphy PJ, Parry BL, Revell VL. Measuring melatonin in humans. *Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine*. 2008;4(1):66-69.
67. Spiegel K, Tasali E, Leproult R, Scherberg N, Van Cauter E. Twenty-four-hour profiles of acylated and total ghrelin: relationship with glucose levels and impact of time of day and sleep. *J Clin Endocrinol Metab*. 2011;96(2):486-493.
68. Mantele S, Otway DT, Middleton B, Bretschneider S, Wright J, Robertson MD, Skene DJ, Johnston JD. Daily rhythms of plasma melatonin, but not plasma

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leptin or leptin mRNA, vary between lean, obese and type 2 diabetic men. *PLoS One*. 2012;7(5):e37123.
69. Otway DT, Mantele S, Bretschneider S, Wright J, Trayhurn P, Skene DJ, Robertson MD, Johnston JD. Rhythmic diurnal gene expression in human adipose tissue from individuals who are lean, overweight, and type 2 diabetic. *Diabetes*. 2011;60(5):1577-1581.
70. Refinetti R, Lissen GC, Halberg F. Procedures for numerical analysis of circadian rhythms. *Biol Rhythm Res*. 2007;38(4):275-325.
71. Perneger TV. What's wrong with Bonferroni adjustments. *Bmj*. 1998;316(7139):1236-1238.
72. Boden G, Ruiz J, Urbain JL, Chen X. Evidence for a circadian rhythm of insulin secretion. *The American journal of physiology*. 1996;271(2 Pt 1).
73. Wehrens SMT, Christou S, Isherwood C, Middleton B, Gibbs MA, Archer SN, Skene DJ, Johnston JD. Meal Timing Regulates the Human Circadian System. *Curr Biol*. 2017;27(12):1768-1775.e1763.
74. Ramracheya RD, Muller DS, Squires PE, Brereton H, Sugden D, Huang GC, Amiel SA, Jones PM, Persaud SJ. Function and expression of melatonin receptors on human pancreatic islets. *J Pineal Res*. 2008;44(3):273-279.
75. Stenvers DJ, Scheer F, Schrauwen P, la Fleur SE, Kalsbeek A. Circadian clocks and insulin resistance. *Nat Rev Endocrinol*. 2019;15(2):75-89.
76. Shea SA, Hilton MF, Orlova C, Ayers RT, Mantzoros CS. Independent circadian and sleep/wake regulation of adipokines and glucose in humans. *J Clin Endocrinol Metab*. 2005;90(5):2537-2544.
77. Van Cauter E, Blackman JD, Roland D, Spire JP, Refetoff S, Polonsky KS. Modulation of glucose regulation and insulin secretion by circadian rhythmicity and sleep. *J Clin Invest*. 1991;88(3):934-942.
78. Dimitriadis G, Mitrou P, Lambadiari V, Maratou E, Raptis SA. Insulin effects in muscle and adipose tissue. *Diabetes Res Clin Pract*. 2011;93 Suppl 1:S52-59.
79. Mandarino LJ, Printz RL, Cusi KA, Kinchington P, O'Doherty RM, Osawa H, Sewell C, Consoli A, Granner DK, DeFronzo RA. Regulation of hexokinase II and glycogen synthase mRNA, protein, and activity in human muscle. *The American journal of physiology*. 1995;269(4 Pt 1):E701-708.
80. Dyar KA, Ciciliot S, Wright LE, Biensø RS, Tagliazucchi GM, Patel VR, Forcato M, Paz MI, Gudiksen A, Solagna F, Albiero M, Moretti I, Eckel-Mahan KL, Baldi P, Sassone-Corsi P, Rizzuto R, Bicciato S, Pilegaard H, Blaauw B, Schiaffino S. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. *Mol Metab*. 2014;3(1):29-41.
81. Basse AL, Dalbram E, Larsson L, Gerhart-Hines Z, Zierath JR, Treebak JT. Skeletal Muscle Insulin Sensitivity Show Circadian Rhythmicity Which Is Independent of Exercise Training Status. *Front Physiol*. 2018;9(1198).
82. Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, Kelly DP, Spiegelman BM. Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci U S A*. 2001;98(7):3820-3825.

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83. Watkins SC, Frederickson A, Theriault R, Korytkowski M, Turner DS, Kelley DE. Insulin-stimulated Glut 4 translocation in human skeletal muscle: a quantitative confocal microscopical assessment. *Histochem J*. 1997;29(2):91-96.
84. Hagström-Toft E, Bolinder J, Ungerstedt U, Arner P. A circadian rhythm in lipid mobilization which is altered in IDDM. *Diabetologia*. 1997;40(9):1070-1078.
85. Schlierf G, Dorow E. Diurnal patterns of triglycerides, free fatty acids, blood sugar, and insulin during carbohydrate-induction in man and their modification by nocturnal suppression of lipolysis. *J Clin Invest*. 1973;52(3):732-740.
86. Boyle PJ, Avogaro A, Smith L, Bier DM, Pappu AS, Illingworth DR, Cryer PE. Role of GH in regulating nocturnal rates of lipolysis and plasma mevalonate levels in normal and diabetic humans. *Am J Physiol*. 1992;263(1 Pt 1):E168-172.
87. Arner P, Bolinder J, Engfeldt P, Ostman J. The antilipolytic effect of insulin in human adipose tissue in obesity, diabetes mellitus, hyperinsulinemia, and starvation. *Metabolism: clinical and experimental*. 1981;30(8):753-760.
88. Lafontan M, Langin D. Lipolysis and lipid mobilization in human adipose tissue. *Prog Lipid Res*. 2009;48(5):275-297.
89. Mahler R, Stafford WS, Tarrant ME, Ashmore J. The effect of insulin on lipolysis. *Diabetes*. 1964;13:297-302.
90. Scherer T, Lindtner C, O'Hare J, Hackl M, Zielinski E, Freudenthaler A, Baumgartner-Parzer S, Tödter K, Heeren J, Krššák M, Scheja L, Fürnsinn C, Buettner C. Insulin Regulates Hepatic Triglyceride Secretion and Lipid Content via Signaling in the Brain. *Diabetes*. 2016;65(6):1511-1520.
91. Hong F, Pan S, Xu P, Xue T, Wang J, Guo Y, Jia L, Qiao X, Li L, Zhai Y. Melatonin Orchestrates Lipid Homeostasis through the Hepatointestinal Circadian Clock and Microbiota during Constant Light Exposure. *Cells*. 2020;9(2).
92. Liu K, Yu W, Wei W, Zhang X, Tian Y, Sherif M, Liu X, Dong C, Wu W, Zhang L, Chen J. Melatonin reduces intramuscular fat deposition by promoting lipolysis and increasing mitochondrial function. *J Lipid Res*. 2019;60(4):767-782.
93. Pettersen IKN, Tusubira D, Ashrafi H, Dyrstad SE, Hansen L, Liu XZ, Nilsson LIH, Løvsletten NG, Berge K, Wergedahl H, Bjørndal B, Fluge Ø, Bruland O, Rustan AC, Halberg N, Røsland GV, Berge RK, Tronstad KJ. Upregulated PDK4 expression is a sensitive marker of increased fatty acid oxidation. *Mitochondrion*. 2019;49:97-110.
94. Zhang S, Hulver MW, McMillan RP, Cline MA, Gilbert ER. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutr Metab (Lond)*. 2014;11(1):10.
95. Lee FN, Zhang L, Zheng D, Choi WS, Youn JH. Insulin suppresses PDK-4 expression in skeletal muscle independently of plasma FFA. *American journal of physiology Endocrinology and metabolism*. 2004;287(1):E69-74.
96. Tsintzas K, Chokkalingam K, Jewell K, Norton L, Macdonald IA, Constantin-Teodosiu D. Elevated free fatty acids attenuate the insulin-induced suppression of PDK4 gene expression in human skeletal muscle: potential role of intramuscular long-chain acyl-coenzyme A. *J Clin Endocrinol Metab*. 2007;92(10):3967-3972.

ACCEPTED MANUSCRIPT

Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

97. Yamaguchi S, Moseley AC, Almeda-Valdes P, Stromsdorfer KL, Franczyk MP, Okunade AL, Patterson BW, Klein S, Yoshino J. Diurnal Variation in PDK4 Expression Is Associated With Plasma Free Fatty Acid Availability in People. *J Clin Endocrinol Metab*. 2018;103(3):1068-1076.
98. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov*. 2008;7(6):489-503.
99. Bonnefont JP, Djouadi F, Prip-Buus C, Gobin S, Munnich A, Bastin J. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Mol Aspects Med*. 2004;25(5-6):495-520.
100. Samec S, Seydoux J, Dulloo AG. Skeletal muscle UCP3 and UCP2 gene expression in response to inhibition of free fatty acid flux through mitochondrial beta-oxidation. *Pflugers Arch*. 1999;438(4):452-457.
101. Chrzanowski-Smith OJ, Edinburgh RM, Smith E, Thomas MP, Walhin JP, Koumanov F, Williams S, Betts JA, Gonzalez JT. Resting skeletal muscle PNPLA2 (ATGL) and CPT1B are associated with peak fat oxidation rates in men and women but do not explain observed sex differences. *Exp Physiol*. 2021;106(5):1208-1223.
102. Witard OC, Jackman SR, Breen L, Smith K, Selby A, Tipton KD. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *Am J Clin Nutr*. 2014;99(1):86-95.
103. Mackay EM, Mackay LL. The concentration of urea in the blood of normal individuals. *J Clin Invest*. 1927;4(2):295-306.
104. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science*. 2001;294(5547):1704-1708.
105. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci U S A*. 2001;98(25):14440-14445.
106. Hoberman HD. Endocrine regulation of amino acid protein metabolism during fasting. *Yale J Biol Med*. 1950;22(4):341-367.
107. Wing SS, Goldberg AL. Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *The American journal of physiology*. 1993;264(4 Pt 1):E668-676.
108. Shavlakadze T, Anwari T, Soffe Z, Cozens G, Mark PJ, Gondro C, Grounds MD. Impact of fasting on the rhythmic expression of myogenic and metabolic factors in skeletal muscle of adult mice. *Am J Physiol Cell Physiol*. 2013;305(1):C26-35.
109. Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S, Nakae J, Tagata Y, Nishitani S, Takehana K, Sano M, Fukuda K, Suematsu M, Morimoto C, Tanaka H. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. *Cell Metab*. 2011;13(2):170-182.
110. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, Burden SJ, Di Lisi R, Sandri C, Zhao J, Goldberg AL, Schiaffino S, Sandri M. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab*. 2007;6(6):458-471.

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1 111. Kelu JJ, Pipalia TG, Hughes SM. Circadian regulation of muscle growth
2 independent of locomotor activity. *Proc Natl Acad Sci U S A*.
3 2020;117(49):31208-31218.
4 112. Resko JA, Eik-nes KB. Diurnal testosterone levels in peripheral plasma of human
5 male subjects. *The Journal of clinical endocrinology and metabolism*.
6 1966;26(5):573-576.
7 113. Marrama P, Carani C, Baraghini GF, Volpe A, Zini D, Celani MF, Montanini V.
8 Circadian rhythm of testosterone and prolactin in the ageing. *Maturitas*.
9 1982;4(2):131-138.
10 114. Plymate SR, Tenover JS, Bremner WJ. Circadian variation in testosterone, sex
11 hormone-binding globulin, and calculated non-sex hormone-binding globulin
12 bound testosterone in healthy young and elderly men. *J Androl*. 1989;10(5):366-
13 371.
14 115. Gagliano-Jucá T, Li Z, Pencina KM, Beleva YM, Carlson OD, Egan JM, Basaria
15 S. Oral glucose load and mixed meal feeding lowers testosterone levels in
16 healthy eugonadal men. *Endocrine*. 2019;63(1):149-156.
17 116. Lehtihet M, Arver S, Bartuseviciene I, Pousette A. S-testosterone decrease after
18 a mixed meal in healthy men independent of SHBG and gonadotrophin levels.
19 *Andrologia*. 2012;44(6):405-410.
20 117. Luboshitzky R, Zabari Z, Shen-Orr Z, Herer P, Lavie P. Disruption of the
21 nocturnal testosterone rhythm by sleep fragmentation in normal men. *J Clin*
22 *Endocrinol Metab*. 2001;86(3):1134-1139.
23 118. Cumming DC, Quigley ME, Yen SS. Acute suppression of circulating
24 testosterone levels by cortisol in men. *The Journal of clinical endocrinology and*
25 *metabolism*. 1983;57(3):671-673.
26 119. Handelsman DJ, Hirschberg AL, Bermon S. Circulating Testosterone as the
27 Hormonal Basis of Sex Differences in Athletic Performance. *Endocr Rev*.
28 2018;39(5):803-829.
29 120. Handelsman DJ, Wartofsky L. Requirement for mass spectrometry sex steroid
30 assays in the Journal of Clinical Endocrinology and Metabolism. *The Journal of*
31 *clinical endocrinology and metabolism*. 2013;98(10):3971-3973.
32 121. Cooke RR, McIntosh JE, McIntosh RP. Circadian variation in serum free and
33 non-SHBG-bound testosterone in normal men: measurements, and simulation
34 using a mass action model. *Clin Endocrinol (Oxf)*. 1993;39(2):163-171.
35 122. Abdulla H, Smith K, Atherton PJ, Idris I. Role of insulin in the regulation of human
36 skeletal muscle protein synthesis and breakdown: a systematic review and meta-
37 analysis. *Diabetologia*. 2016;59(1):44-55.
38 123. Redmond J, Fulford AJ, Jarjou L, Zhou B, Prentice A, Schoenmakers I. Diurnal
39 Rhythms of Bone Turnover Markers in Three Ethnic Groups. *The Journal of*
40 *clinical endocrinology and metabolism*. 2016;101(8):3222-3230.
41 124. Schlemmer A, Hassager C. Acute fasting diminishes the circadian rhythm of
42 biochemical markers of bone resorption. *Eur J Endocrinol*. 1999;140(4):332-337.
43 125. Aoshima H, Kushida K, Takahashi M, Ohishi T, Hoshino H, Suzuki M, Inoue T.
44 Circadian variation of urinary type I collagen crosslinked C-telopeptide and free
45 and peptide-bound forms of pyridinium crosslinks. *Bone*. 1998;22(1):73-78.

ACCEPTED MANUSCRIPT

Downloaded from https://academic.oup.com/jcem/advance-article/doi/10.1210/clinem/dgae350/7680211 by University of Bern user on 24 May 2024

126. Henriksen DB, Alexandersen P, Bjarnason NH, Vilsbøll T, Hartmann B, Henriksen EE, Byrjalsen I, Krarup T, Holst JJ, Christiansen C. Role of gastrointestinal hormones in postprandial reduction of bone resorption. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2003;18(12):2180-2189.
127. Walsh JS, Henriksen DB. Feeding and bone. *Arch Biochem Biophys*. 2010;503(1):11-19.
128. Ochs-Balcom HM, Hovey KM, Andrews C, Cauley JA, Hale L, Li W, Bea JW, Sarto GE, Stefanick ML, Stone KL, Watts NB, Zaslavsky O, Wactawski-Wende J. Short Sleep Is Associated With Low Bone Mineral Density and Osteoporosis in the Women's Health Initiative. *J Bone Miner Res*. 2020;35(2):261-268.
129. Swanson CM, Kohrt WM, Buxton OM, Everson CA, Wright KP, Jr., Orwoll ES, Shea SA. The importance of the circadian system & sleep for bone health. *Metabolism*. 2018;84:28-43.
130. Saracino PG, Rossetti ML, Steiner JL, Gordon BS. Hormonal regulation of core clock gene expression in skeletal muscle following acute aerobic exercise. *Biochem Biophys Res Commun*. 2019;508(3):871-876.
131. Silva BSA, Uzeloto JS, Lira FS, Pereira T, Coelho ESMJ, Caseiro A. Exercise as a Peripheral Circadian Clock Resynchronizer in Vascular and Skeletal Muscle Aging. *International journal of environmental research and public health*. 2021;18(24).
132. van Loon LJC, Koopman R, Stegen J, Wagenmakers AJM, Keizer HA, Saris WHM. Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. *Journal of Physiology-London*. 2003;553(2):611-625.
133. Crossland H, Skirrow S, Puthucheary ZA, Constantin-Teodosiu D, Greenhaff PL. The impact of immobilisation and inflammation on the regulation of muscle mass and insulin resistance: different routes to similar end-points. *J Physiol*. 2019;597(5):1259-1270.
134. Van Thienen R, D'Hulst G, Deldicque L, Hespel P. Biochemical artifacts in experiments involving repeated biopsies in the same muscle. *Physiol Rep*. 2014;2(5):e00286.
135. Chang AM, Aeschbach D, Duffy JF, Czeisler CA. Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness. *Proc Natl Acad Sci U S A*. 2015;112(4):1232-1237.
136. Hengist A, Edinburgh RM, Davies RG, Walhin JP, Buniam J, James LJ, Rogers PJ, Gonzalez JT, Betts JA. Physiological responses to maximal eating in men. *The British journal of nutrition*. 2020;124(4):407-417.

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Table 1: Participant characteristics of the study cohort. Data are presented as mean ± SD.

Characteristic	Mean ± SD
Age (y)	30 ± 10
Height (m)	1.81 ± 0.06
Body Mass (kg)	78.7 ± 7.0
Body Mass Index (kg·m^{-2})	24.1 ± 2.7
Resting Metabolic Rate (kcal·day^{-1})	1724 ± 314
Midsleep time (hh:mm)*	03:42 ± 01:13
Horne-Östberg Score	57 ± 11
Pittsburgh Sleep Quality Index	3 ± 2

*Determined from the Munich Chronotype Questionnaire (60)

1

2

Table 2: Dietary intake in the 48-h prior to the laboratory visit. Data are presented as mean ± SD.

	Mean ± SD
Energy (kcal)	3002 ± 726
Carbohydrate (kcal)	1279 ± 357
Protein (kcal)	551 ± 235
Fat (kcal)	520 ± 176
Alcohol (kcal)	0 ± 0

3

4

Table 3: – Gene expression assay targets in human skeletal muscle (*Vastus lateralis*)

Gene	Protein/enzyme	Assay ID
18S rRNA	18S ribosomal RNA	Hs03003631_g1
ACTA1	Actin alpha 1, skeletal muscle	Hs05032285_s1
HMBS	Hydroxymethylbilane synthase	Hs00609296_g1
ARNTL	Basic helix-loop-helix ARNT like 1	Hs00154147_m1
CLOCK	Circadian Locomotor Output Cycles Kaput	Hs00231857_m1
CRY1	Cryptochrome circadian regulator 1	Hs00172734_m1
CRY2	Cryptochrome circadian regulator 2	Hs00901393_m1
CSN1KE	Casein kinase 1 epsilon	Hs01095999_g1
NPAS2	Neuronal PAS domain protein 2	Hs00231212_m1
NR1D1	Nuclear receptor subfamily 1 group D member 1	Hs00253876_m1
NR1D2	Nuclear receptor subfamily 1 group D member 2	Hs00233309_m1
PER1	Period circadian protein 1	Hs00242988_m1
PER2	Period circadian protein 2	Hs01007553_m1
PER3	Period circadian protein 3	Hs00213466_m1

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TP53	Tumor protein p53	Hs01034249_m1
MYH1	Myosin heavy chain 1	Hs00428600_m1
MYOD1	Myogenic differentiation 1	Hs00159528_m1
FOXO3	Forkhead box O3	Hs00818121_m1
FBXO32	F-box protein 32	Hs01041408_m1
MTOR	Mechanistic target of rapamycin kinase	Hs00234508_m1
SIRT1	Sirtuin 1	Hs01009006_m1
AKT1	AKT serine/threonine kinase 1	Hs00178289_m1
B4GALT5	beta-1,4-galactosyltransferase 5	Hs00941041_m1
CS	Citrate synthase	Hs02574374_s1
HK2	Hexokinase 2	Hs00606086_m1
GLUT4	Solute carrier family 2-member 4	Hs00168966_m1
PDK4	Pyruvate dehydrogenase kinase 4	Hs01037712_m1
CPT1B	Carnitine palmitoyltransferase 1B	Hs00189258_m1
FABP3	Fatty acid binding protein 3	Hs00997362_m1
PPARD	Peroxisome proliferator activated receptor delta	Hs04187066_g1
PPARG	Peroxisome proliferator activated receptor gamma	Hs00173304_m1
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1	Hs01562315_m1
PRKAA2	Protein kinase AMP-activated catalytic subunit alpha 2	Hs00178903_m1
ALAS1	5'-aminolevulinate synthase 1	Hs00963537_m1
CYCS	Cytochrome c, somatic	Hs01588974_g1
PPARGC1A	PPARG coactivator 1 alpha	Hs00173304_m1
SIRT3	Sirtuin 3	Hs00953477_m1
TFAM	Transcription factor A, mitochondrial	Hs00273372_s1
UCP3	Uncoupling protein 3	Hs01106052_m1
MAPK1	Mitogen-activated protein kinase 1	Hs01046830_m1
MAPK3	Mitogen-activated protein kinase 3	Hs00385075_m1
MAPK14	Mitogen-activated protein kinase 14	Hs01051152_m1
MAL	Myelin and Lymphocyte T-cell differentiation protein	Hs00707014_s1
CREB5	cAMP responsive element binding protein 5	Hs00191719_m1
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	Hs00607050_m1
HNRNPDL	Heterogeneous nuclear ribonucleoprotein D like	Hs00943609_m1
RPS6	Ribosomal protein S6	Hs04195024_g1

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1 **Figure Legends Section**

2

3 **Figure 1 –** Schematic representation of the study protocol.

4

5 **Figure 2** – 24-hour profile for melatonin onset adjusted A) plasma glucose B) plasma
6 NEFA C) plasma glycerol D) plasma triglycerides E) plasma urea. Solid lines denote the
7 regression that best fits the data with the horizontal dotted line representing the 24-hour
8 mean concentration used for the null comparison. The dotted vertical line denotes
9 melatonin onset. The shaded areas represent 24-h melatonin profile.

10

11 **Figure 3** – 24-hour profile for melatonin onset adjusted A) plasma insulin B) plasma c-
12 terminal telopeptide (CTX) C) serum cortisol D) serum testosterone. Solid lines denote
13 the regression that best fits the data with the horizontal dotted line representing the 24-
14 hour mean concentration used for the null comparison. The dotted vertical line denotes
15 melatonin onset. The shaded areas represent 24-h melatonin profile.

16

17 **Figure 4 –** Relative changes in skeletal muscle RNA expression across the 24-h semi-
18 constant routine. Diurnal rhythmicity (as determined by cosinor analysis) are denoted by
19 a clock symbol.

20

21 **Figure 5 –** Peak (circles) and nadir (triangles) timings of circulating metabolites,
22 hormones, telopeptides, and skeletal muscle genes displaying significant diurnal
23 rhythmicity. The dark/fasted period is depicted in the shaded grey region.

24

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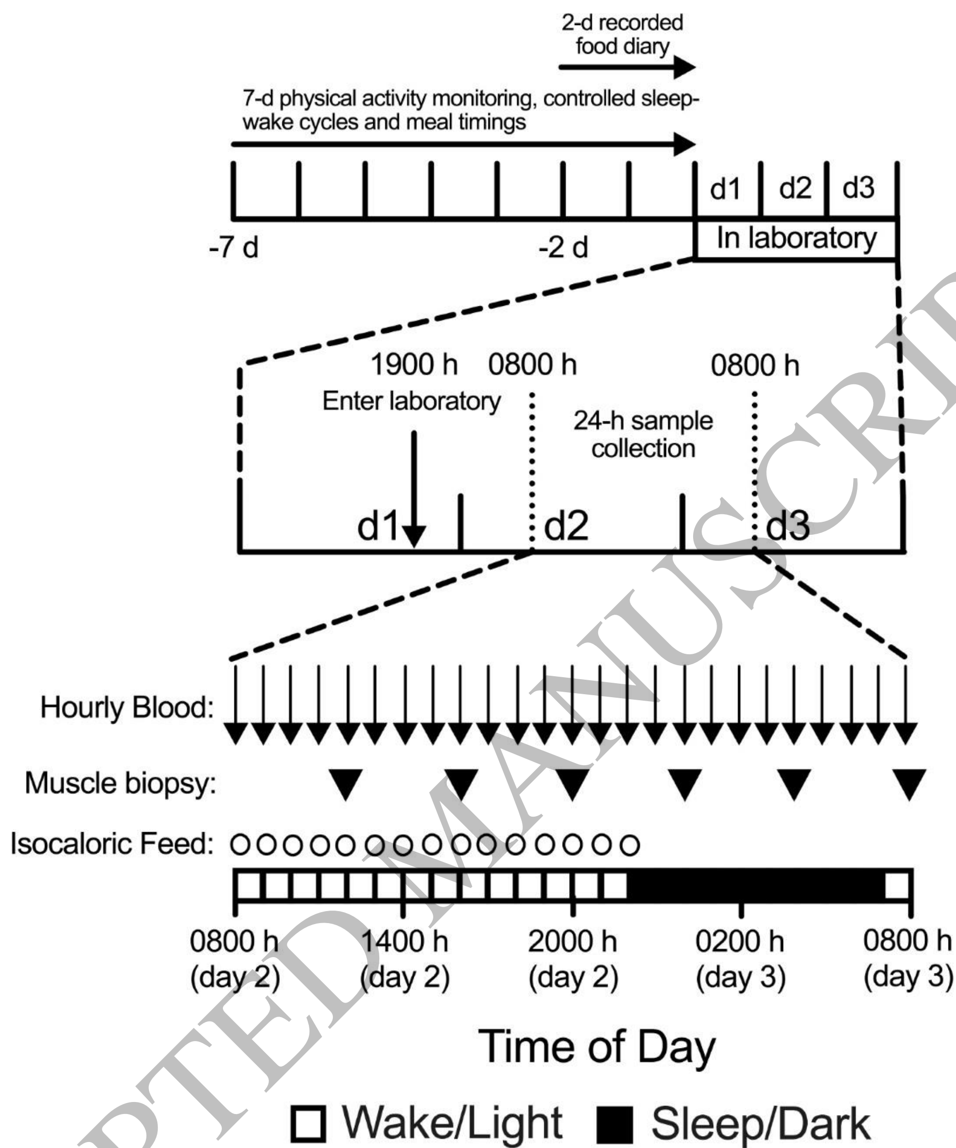

1

2 *Figure 1*
3 *125x150 mm (DPI)*

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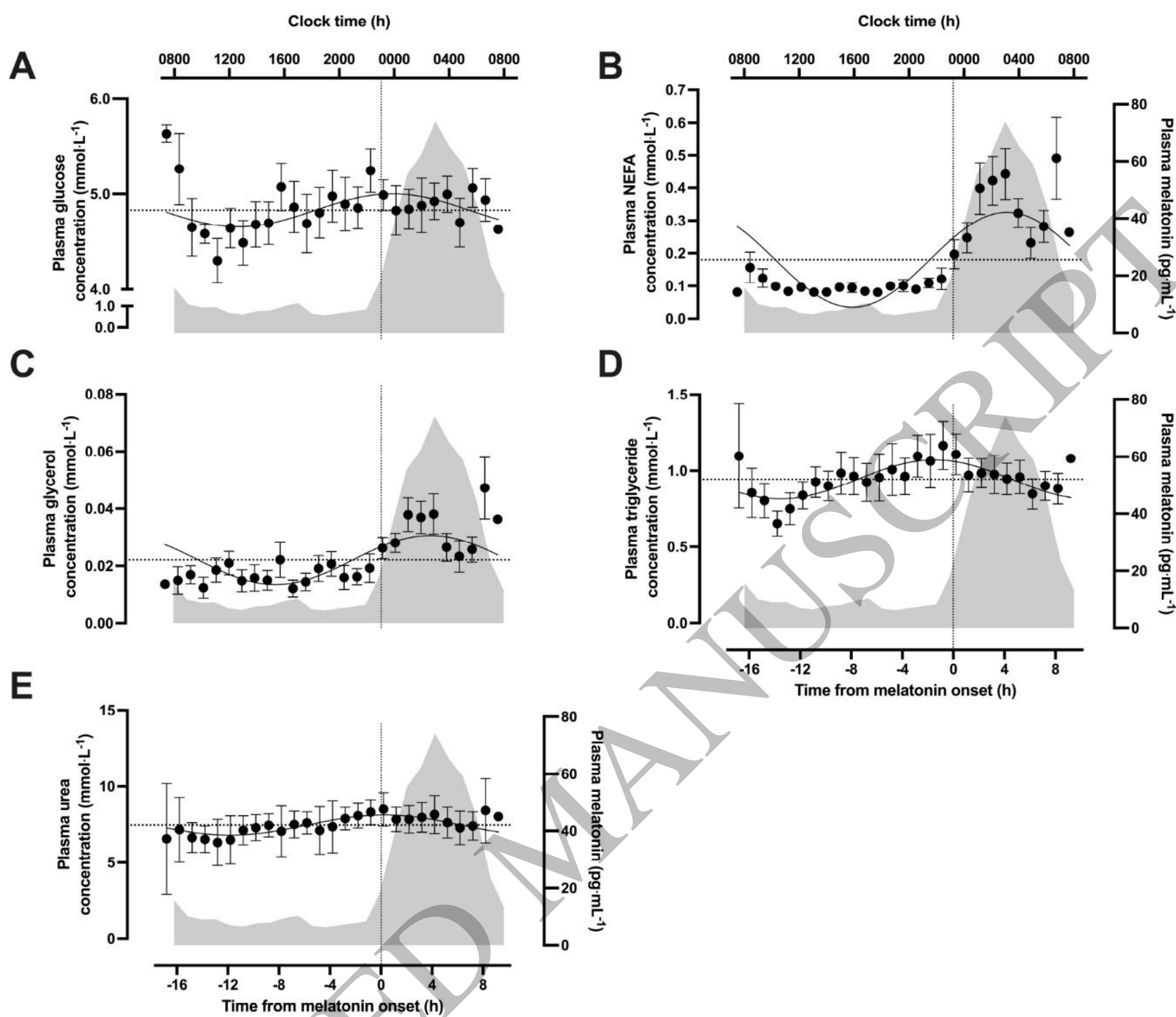

2 *Figure 2*
3 *156x134 mm (DPI)*

4

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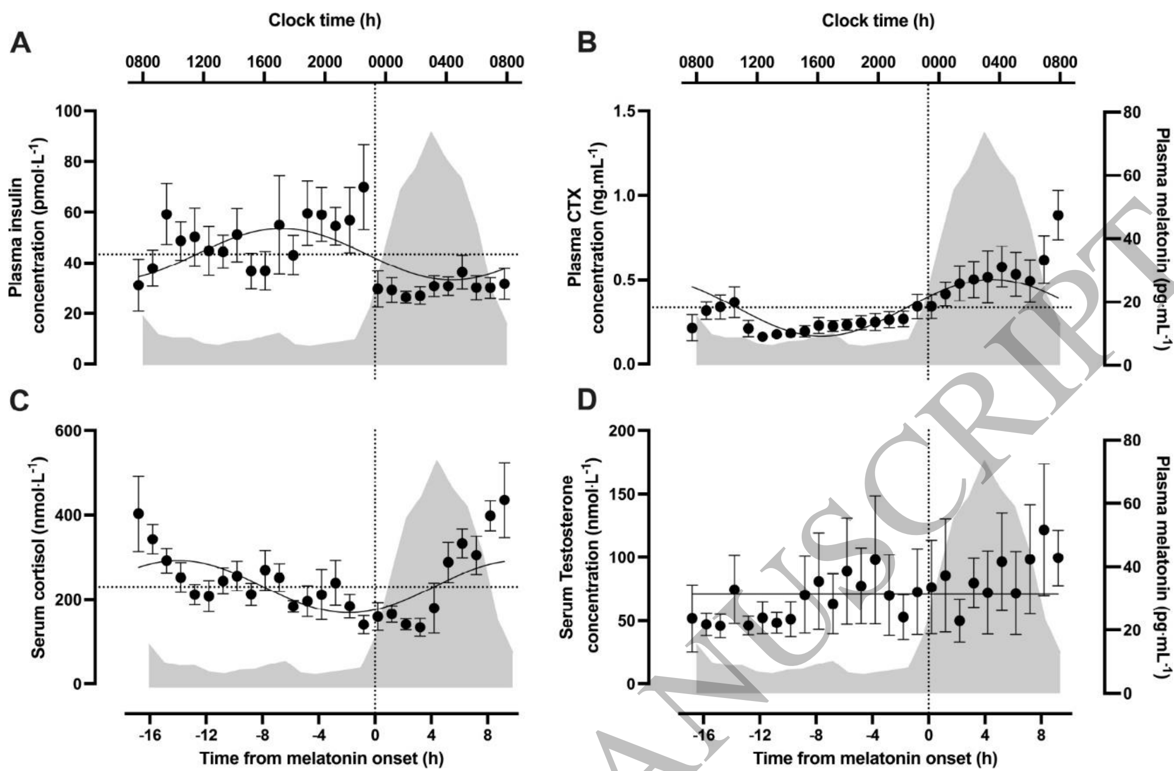

2 *Figure 3*
3 *154x100 mm (DPI)*

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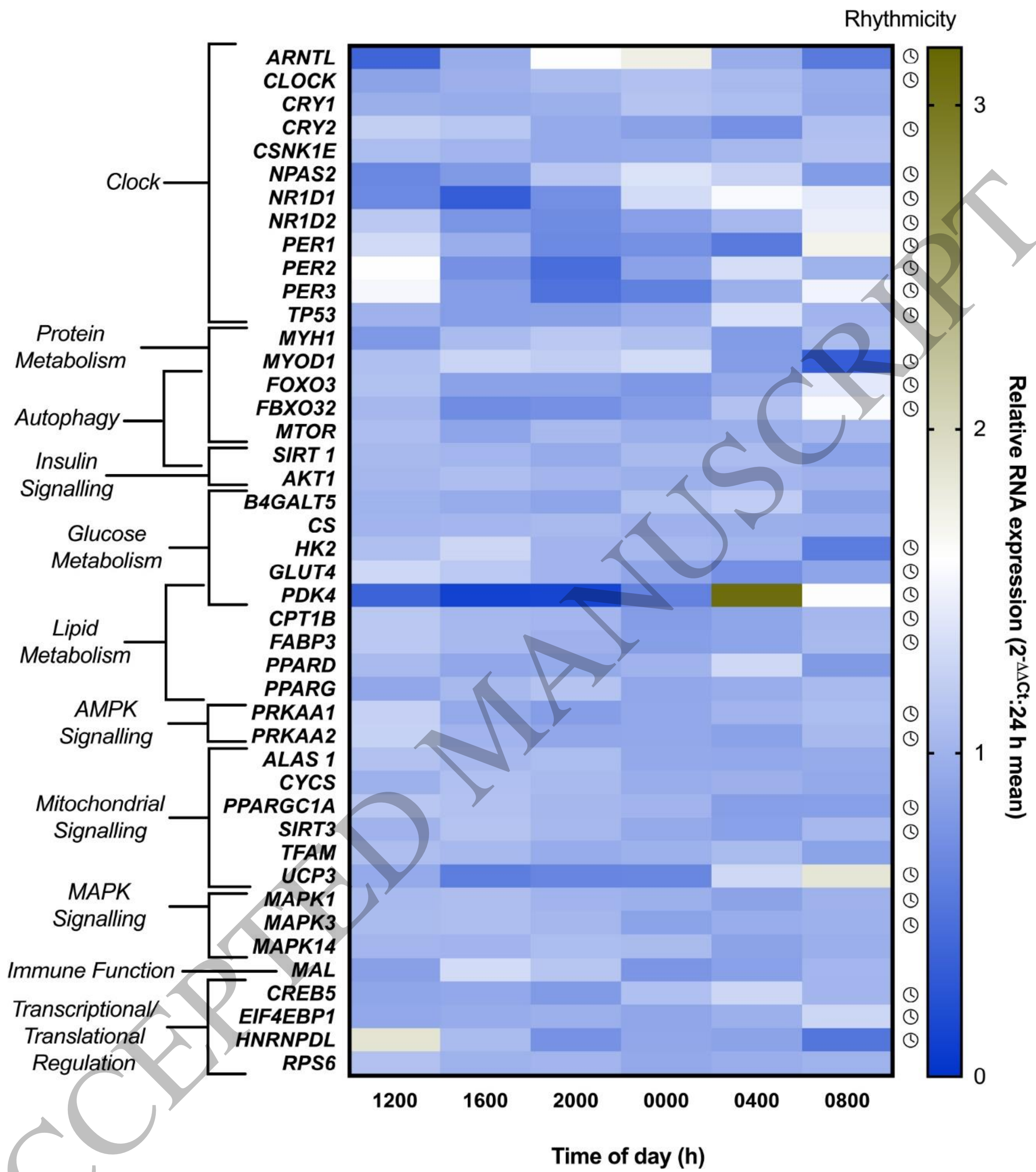

1

2 *Figure 4*
3 *155x176 mm (DPI)*

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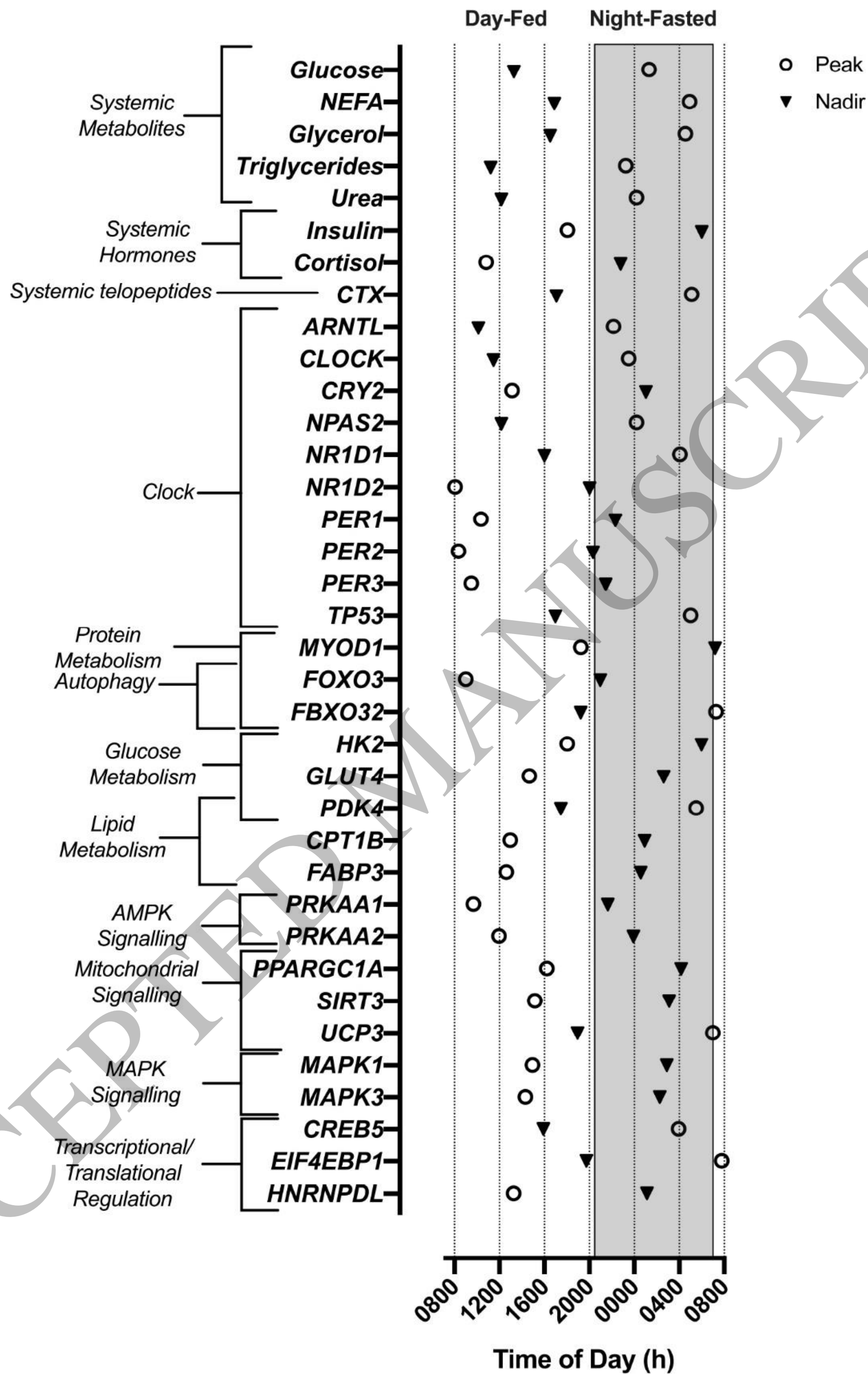

1

2 *Figure 5*
3 *123x196 mm (DPI)*

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