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

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Author Disclosure Statement

H.A.S. has received research grants from the Sleep Research Society Foundation and the Rank Prize Funds. J.A.B. is an investigator on research grants funded by BBSRC, MRC, British Heart Foundation, Rare Disease Foundation, EU Hydration Institute, GlaxoSmithKline, Nestlé, Lucozade Ribena Suntory, ARLA foods, Kennis Centrum Suiker and Salus Optima (L3M Technologies Ltd); has completed paid consultancy for PepsiCo, Kellogg's, SVGC and Salus Optima (L3M Technologies Ltd); is Company Director of Metabolic Solutions Ltd; receives an annual honorarium as a member of the academic advisory board for the International Olympic Committee Diploma in Sports Nutrition; and receives an annual stipend as Editor-in Chief of *International Journal of Sport Nutrition & Exercise Metabolism*. J.T.G. is an investigator on research grants funded by BBSRC, MRC, British Heart Foundation Summer Fruits, The Rank Prize Funds, The European Society for Clinical Nutrition and Metabolism (ESPEN), Lucozade Ribena Suntory, ARLA Foods Ingredients, Cosun Nutrition Center, and Clasado Biosciences; and has completed paid consultancy for PepsiCo, Tour Racing Ltd Violicom Medical Ltd, and 6d Sports Nutrition. LJJ is part of the National Institute for Health Research's Leicester Biomedical Research Centre, which is a partnership between University Hospitals of Leicester NHS Trust, Loughborough University, and the University of Leicester. This report is independent to the National Institute for Health Research. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research, or the Department of Health. LJJ has received funding for research from and/or acted as a consultant for Entrinsic Beverage Company LLC, Bridge Farm Nurseries, Decathlon SA, the Collagen Research Institute, PepsiCo, Inc., Volac International and Progressive Sports Technologies. LJJ has also received funding to attend conferences from PepsiCo Inc. and Danone Nutricia. In all cases, no personal payments were received by LJJ. DJC has received research funding from the British Nutrition Foundation and Society for Endocrinology.

29 **Abstract**

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

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

38 **Methods:** Ten healthy adults (9M/1F, mean ± SD: age: 30 ± 10 y; BMI: 24.1 ± 2.7
39 $kg \cdot m^{-2}$) rested in the laboratory for 37 hours with all data collected during the final 24
40 hours of this period (i.e., 0800-0800 h). Participants ingested hourly isocaloric liquid
41 meal replacements alongside appetite assessments during waking before a sleep
42 opportunity from 2200-0700 h. Blood samples were collected hourly for endocrine and
43 metabolite analyses, with muscle biopsies occurring every 4 h from 1200 h to 0800 h
44 the following day to quantify gene expression.

45 **Results:** Plasma insulin displayed diurnal rhythmicity peaking at 1804 h. Expression
46 of skeletal muscle genes involved in carbohydrate metabolism (*Name* – Acrophase;
47 *GLUT4* - 1440 h; *PPARGC1A* –1613 h; *HK2* - 1824 h) and lipid metabolism (*FABP3* -
48 1237 h; *PDK4* - 0530 h; *CPT1B* - 1258 h) displayed 24 h rhythmicity that reflected the
49 temporal rhythm of insulin. Equally, circulating glucose (0019 h), NEFA (0456 h),
50 glycerol (0432 h), triglyceride (2314 h), urea (0046 h), CTX (0507 h) and cortisol
51 concentrations (2250 h) also all displayed diurnal rhythmicity.

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

57

58 **Key words:** Skeletal muscle, Gene expression, Circadian rhythms, Diurnal, Glucose,
59 Lipids

Abbreviations:

SCN – Suprachiasmatic nuclei

VLDL – Very low-density lipoprotein

CTX – C-terminal telopeptide

84 **Introduction**

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

90

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

105

106 Skeletal muscle displays robust rhythmicity in transcriptomic regulation of the circadian
107 clock, as well as carbohydrate, lipid, and protein metabolism; this may influence the
108 central role of this tissue in the storage, synthesis, and breakdown of nutrients (13,24-
109 28). Specifically, skeletal muscle is an important storage site for glucose (glycogen)
110 (29,30) and lipids (intramyocellular lipids) (27,31), and is also the primary store of
111 protein within the human body (32-35). The ability to readily dispose and mobilise
112 these nutrients from this tissue is an important determinant of insulin sensitivity and
113 therefore metabolic health (27,31,36-38). Furthermore, autophagy is a central process
114 that regulates skeletal muscle protein turnover, as well as glucose and lipid
115 metabolism and responds to a variety of stimuli, including, nutrient deprivation, and

116 amino acid starvation (39,40). However, no studies have explored molecular
117 regulation of this process within skeletal muscle across a 24-h period. Considering the
118 importance of the skeletal muscle in facilitating the response to nutrient availability, it
119 is remarkable that no studies to date have assessed rhythmicity in the molecular
120 regulation of skeletal muscle metabolism alongside circulating metabolites and
121 hormones involved in carbohydrate and lipid metabolism and bone resorption.

122

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

135

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

144

145

146

147 **Materials and Methods**

148 *Approach to the research question*

149 Given the protracted nature of this study, a single-arm time-series design was deemed
150 appropriate. Whereas constant routine studies eliminate the influence of diurnal
151 factors such as sleep-wake and fasting-feeding, the current study employed a semi-
152 constant routine to study the diurnal influence of those factors. This protocol was
153 characterised by designated wake and sleep opportunities that were aligned with
154 feeding and fasting, respectively. Specifically, iso-caloric snacks were ingested by
155 participants every hour during waking hours to align feeding-fasting with wake-sleep
156 and light-dark, respectively Hourly feeds were prescribed to provide $6.66\% \cdot h^{-1}$ of
157 estimated 24 h resting metabolic rate (RMR) across the 15 h waking period (i.e., 0800
158 – 2200 h), thus meeting individually-measured resting energy requirements and
159 accounting for RMR as a driver of energy intake (53,54). This model of continuous
160 (hourly) feeding was selected to facilitate characterisation of the underlying 24-h fed-
161 fast rhythm in the absence of the acute meal responses that would occur with any
162 particular meal pattern. Nonetheless, the overall 24-h pattern of nutrient availability
163 with this model of continuous feeding is not dissimilar to that observed with a typical
164 3-square meal pattern (even without snacking) since, even though nutrients are
165 commonly ingested only periodically by most humans, there is a constant systemic
166 appearance of nutrients from the gastrointestinal tract for the entirely of waking hours
167 (49).

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

174 Transcriptomic data from the same participants have been reported previously in an
175 untargeted analysis of rhythmicity (57). The aim of the current study was to analyse
176 skeletal muscle RNA levels in a targeted number of metabolic genes in order to
177 contrast with rhythms in circulating biomarkers. Plasma melatonin has also been
178 reported previously and is included in the current manuscript to illustrate 24-h profiles

179 relative to diurnal melatonin and melatonin onset (24,50). Likewise, cortisol from this
180 protocol has also been reported previously at 4-hourly resolution aligned with muscle
181 biopsy samples (24); updated biochemical analyses were therefore deemed
182 necessary to increase resolution and capture the profile of cortisol prior to the first
183 biopsy at midday (0800-1200 h).

184

185 *Research Design*

186 A time-series design was employed to investigate temporal rhythms in skeletal muscle
187 gene expression relating to carbohydrate metabolism, lipid metabolism, circadian
188 clocks, and autophagy, alongside plasma glucose, non-esterified fatty acids, insulin,
189 glycerol, triglycerides, and C-terminal telopeptide (CTX), as well as serum cortisol and
190 testosterone under conditions of semi-constant routine. Following a 7-day period of
191 standardised wake-sleep, meal-timing, and light exposure (a typical living pattern for
192 this population, thus serving to reduce between-participant variation in response to the
193 semi-constant routine), participants underwent a 37-hour in-patient visit to the resting
194 laboratory at the University of Bath. During the final 24-hours of this visit, participants
195 had a designated sleeping opportunity (2200 -0700 h) and hourly isocaloric feedings
196 during waking periods (0800 - 2200 h) to preserve diurnal influences of sleep-wake
197 and fasting-feeding. Hourly blood samples were collected throughout the day (whilst
198 awake) and night (during sleep) for assessment of rhythms in the systemic
199 concentrations of glucose, non-esterified fatty acids, and insulin, along with melatonin
200 and cortisol to provide a validated internal phase marker. Skeletal muscle samples
201 were collected every 4-h from 1200 h for the remainder of the trial for assessment of
202 RNA expression.

203

204 *Participants*

205 Ten healthy participants (9M;1F, **Table 1**), who maintained a typical sleep-wake cycle
206 (i.e. not of extreme chronotype and kept a consistent daily routine) and did not perform
207 shift work, were recruited and screened via local advertisement. Participant screening
208 was undertaken through completion of a general health questionnaire and validated
209 chronotype questionnaires to assess habitual sleep patterns and diurnal preferences

210 (58-60). Participants were excluded from participation if they had a habitual sleep
211 duration not within 6-9 hours per night and/or a Pittsburgh Sleep Quality Index >5.
212 With regards to shift work, exclusion criteria were in place for individuals who had
213 participated in shift work or had travelled across more than two time zones within three
214 weeks of the study. All volunteers were fully briefed on the requirements of the study
215 prior to provision of written informed consent. Ethical approval for the experimental
216 protocol was obtained from the Cornwall and Plymouth NHS research ethics
217 committee (reference: 14/SW/0123). All procedures were performed in accordance
218 with the Declaration of Helsinki.

219

220 **[Table 1]**

221

222 *Pre-experimental standardisation week*

223 Participants adhered to a strict routine of feeding and sleeping in the 7-days prior to
224 entering the laboratory, waking between 0600 and 0700 h and going to sleep between
225 2200 and 2300 h, confirmed using time-stamped voicemail. The median (IQR) time
226 that those voicemails were received were 0653 h (0643-0722) for waking and 2245 h
227 (2230-2250) for lights-out, respectively.

228

229 Upon waking, participants ensured at least 15 minutes of natural light exposure within
230 1.5 hours of waking, affirmed by wrist actigraphy using a light sensor, further
231 confirming standardisation of sleep-wake patterns (Actiwatch^{TM}, Cambridge
232 Neurotechnology; Cambridge, UK). Self-selected meals were scheduled at 0800,
233 1200 and 1800 h, with assigned snacking opportunities at 1000, 1500 and 2000 h.
234 Participants also completed a weighed record of all food and fluid intake on the final
235 two days of this 7-day standardisation period and verified that they had consumed the
236 reported meals and snacks at the prescribed times (Table 2).

237

238 **[Table 2]**

239

240 *Experimental Protocol*

241 Following the standardisation week, participants reported to the laboratory at 1900 h
242 on experimental day 1 to acclimatise to the laboratory (**Figure 1**). Laboratory
243 conditions were standardised for the duration of their stay, with blackout-blinds to
244 prevent the penetration of natural light and room temperature maintained at 20-25°C.
245 During waking hours, artificial lighting was set at 800 lux in the direction of gaze (0700-
246 2200 h) and turned off (0 lux) during sleeping hours (2200-0700 h), during which time
247 participants wore an eye mask. Participants remained in a semi-recumbent position
248 throughout (i.e., head-end of bed elevated to 30°). Upon arrival, participants were
249 shown to their bed and provided with a prescribed meal composed of a baked potato
250 with butter and cheese, steamed vegetables (broccoli and mini corn), followed by a
251 bowl of fresh strawberries, raspberries and blueberries (1245 kcal; 31% carbohydrate,
252 50% fat and 19% protein). An instant hot chocolate made with whole milk was then
253 provided at 21:30 (242 kcal; 56% carbohydrate, 24% fat and 20% protein) before lights
254 out at 2200 h.

255 On day 2, participants were woken at 0700 h and RMR was immediately measured
256 over 15 minutes using indirect calorimetry via the Douglas bag technique (61). An
257 intravenous cannula was inserted to an antecubital vein to allow for hourly 10 mL blood
258 draws from 0800 h, alongside appetite VAS during waking hours (reported previously
259 (50)). Muscle biopsies were collected every 4 hours from 1200 h on day 2 through to
260 0800 h on day 3. After each set of measurements, an hourly feed (commencing at
261 0800 h) was ingested in the form of a meal-replacement solution (1.25 kcal·mL^{-1}, 45%
262 carbohydrate, 25% fat, 30% protein; Resource Protein, Nestlé; Vevey, Switzerland).
263 Each hourly dose was prescribed to give 6.66%·h^{-1} of measured 24-h RMR across the
264 15 h wake period (118 ± 19 kcal·h^{-1}). Plain water was consumed *ad libitum* and
265 participants had access to mobile devices, on-demand entertainment, music and
266 reading material throughout waking hours only. Toilet breaks were permitted in the
267 first half of each hour as required.

268 The final set of waking measurements were collected at 2200 h, along with ingestion
269 of the final prescribed feed. Following this, the lights were switched-off and participants
270 were asked to wear an eye mask throughout the lights-out period. Blood samples
271 continued throughout the night at hourly intervals without intentionally waking the

272 participants. Participants were woken at 0700 h, and a blood sample was immediately
273 drawn. The final set of measurements were made at 0800 h.

274

275 **[Figure 1]**

276

277 *Outcome Measures*

278 **Blood Sampling and Analysis –** At each time-point, 10 mL of whole blood was drawn
279 and immediately distributed into tubes treated with lithium heparin (for analysis of
280 melatonin) or ethylenediaminetetraacetic acid (EDTA; for analysis of glucose, insulin,
281 non-esterified fatty acids, glycerol-corrected triglycerides, glycerol and C-terminal
282 telopeptide) or left to clot at room temperature for 15 minutes (Serum; for analysis of
283 cortisol and testosterone). Blood collection tubes were centrifuged for 10 minutes
284 (3466 x g, 4°C), after which the supernatants were removed and stored at -80°C.

285 Plasma melatonin concentration was measured in the heparinised samples using a
286 radioimmunoassay (Surrey Assays Ltd, UK; Assay performance reported elsewhere
287 (50)). Plasma insulin (Mercodia, Sweden; RRID: AB_2877672; Intra-Assay CV:
288 6%/Inter-Assay CV: 13%), C-terminal telopeptide (CTX; Immunodiagnostic systems,
289 UK; RRID: AB_2923399; Intra-Assay CV: 19%/Inter-Assay CV: 27%)) (ISD, UK),
290 glucose (Intra-Assay CV: 3%/Inter-Assay CV: 3%), non-esterified fatty acids (NEFA;
291 Intra-Assay CV: 6%/Inter-Assay CV: 6%), glycerol (Intra-Assay CV: 12%/Inter-Assay
292 CV: 18%) and triglycerides (Intra-Assay CV: 4%/Inter-Assay CV: 18%) (Randox, UK)
293 were quantified in EDTA-treated plasma, with cortisol (Tecan, CH; RRID:
294 AB_2924715; Intra-Assay CV: 6%/Inter-Assay CV: 7%) and testosterone (R&D
295 Systems, Bio-Techne, US; RRID: AB_2820244; Intra-Assay CV: 30%/Inter-Assay CV:
296 28%) quantified in serum.

297

298 *Skeletal muscle sampling and analysis*

299 Skeletal muscle samples were collected from the *vastus lateralis* under local
300 anaesthesia (1% lidocaine: Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples
301 were collected at 4-hourly intervals from 1200 until 0800 h (i.e., 6 in total) from a 3-5

302 mm incision in the anterior aspect of the thigh using a Bergstrom needle adapted for
303 suction (62,63). Samples were taken from each leg in a randomly determined
304 alternating order between dominant and non-dominant leg, ascending up the leg with
305 skin incisions separated by 2–3 cm. Daytime biopsies were taken following the VAS
306 and blood sample, but before the prescribed feed. Thirty minutes prior to sleep,
307 incisions for the night-time biopsies were made to minimise disruption to participants'
308 sleep. For night-time tissue biopsies (i.e. 0000 and 0400 h), participants were woken
309 briefly but continued to wear the eye mask while samples were taken by torch-light
310 (samples acquired and researchers left the laboratory within 3-5 minutes). Samples
311 were immediately snap-frozen in liquid nitrogen for subsequent storage at -80°C.

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

334

335 **[Table 3]**

336

337

338 *Statistical Analysis*

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

347 Analysis of rhythmicity for all outcomes was conducted using the cosine method
348 (Prism 9, Graphpad; CA, USA). In this approach, a cosine wave is fit to the 24-h profile
349 of a given variable and compared against a horizontal line through the mean values
350 (null). If a cosine wave provides a better fit (R^2) for the data than the horizontal line
351 then the dataset characterises diurnal (or 24-h) rhythmicity, with the mesor (rhythm-
352 adjusted mean), amplitude (magnitude of the difference between mesor and
353 peak/trough values) and acrophase (timing of rhythmic peak) all identified and
354 reported (56,70). Reported *p*-values are the output of the Extra sum-of-squares F test.
355 This method was chosen *a priori* to provide a greater descriptive characterisation of
356 temporal patterns compared to commonly used statistical approaches such as
357 analyses of variance (e.g. 1-way ANOVA looking at effects of time or 2-way ANOVA
358 for treatment*time interactions) but it must also be recognised that different analytical
359 approaches may yield varied results (56). Whilst *post hoc* adjustment of *p*-values for
360 multiple statistical tests is sometimes required to minimise inflation of type I error rates
361 (i.e. false positives), it has been questioned whether such adjustment is always
362 necessary (71) and it is rare to see such adjustment between separate outcome
363 measures. Moreover, given the aims of the study to characterise rhythmicity in
364 metabolic outcomes it was not deemed necessary to perform such adjustments. All
365 data are presented as mean ± SD unless otherwise stated (e.g., figures are mean ±
366 95% Confidence Intervals).

367

368

369

370 **Results**

371 *Metabolites*

372 All plasma metabolites displayed diurnal rhythmicity. Mean plasma glucose was
373 rhythmic ($p = 0.04$, $R^2 = 0.03$, **Figure 2A**). The acrophase occurred at 0119 h and fell
374 to the nadir in the afternoon, with a mean concentration of 4.83 ± 0.44 mmol·L^{-1} and
375 amplitude of 0.17 mmol·L^{-1}. Plasma NEFA was also rhythmic peaking at 0456 h and
376 falling to the nadir in the afternoon, with an amplitude of 0.15 mmol·L^{-1} and rhythm
377 adjusted mean of 0.18 ± 0.05 mmol·L^{-1} ($p < 0.01$, $R^2 = 0.38$, **Figure 2B**). Likewise,
378 diurnal rhythmicity was evident in plasma glycerol. Mean concentrations across the
379 period were 0.02 ± 0.01 mmol·L^{-1} and the diurnal rhythm was characterised by an
380 amplitude of 0.08 mmol·L^{-1}, peaking at 0432 h with lowest values in the afternoon (p
381 < 0.01, $R^2 = 0.14$, **Figure 2C**). Plasma triglycerides were also rhythmic with the
382 acrophase occurring at 2314 h and falling to a nadir in the afternoon, with an amplitude
383 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**
384 **2D**). Finally, plasma urea was rhythmic across the period, peaking at 0046 h with an
385 amplitude of 0.66 mmol·L^{-1} and mean concentration of 7.45 mmol·L^{-1} ($p < 0.01$, $R^2 =$
386 0.08, **Figure 2E**).

387

388 **[Figure 2]**

389

390 *Hormones and telopeptides*

391 Plasma insulin was rhythmic, peaking at 1804 h before falling to an overnight nadir (p
392 < 0.0001, $R^2 = 0.08$, **Figure 3A**). The diurnal rhythm occurred with an amplitude of
393 10.0 pmol·$L^{-1}$and a mean concentration of 43.4 ± 17.1 pmol·L^{-1}. Plasma CTX was also
394 characterised by diurnal rhythmicity ($p < 0.0001$, $R^2 = 0.19$, **Figure 3B**); peak
395 concentration occurred at 0507 h and fell to the nadir during the afternoon, with an
396 amplitude of 0.16 ng·mL^{-1} and mean of 0.29 ± 0.20 ng·mL^{-1}. Serum cortisol was also

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

403

404 **[Figure 3]**

405

406 *Skeletal muscle gene expression*

407 Of the 44 genes assessed, 26 displayed rhythmicity (all $p < 0.05$) (**Figure 4**). This
408 diurnal rhythmicity was evident for core clock genes (Acrophase – h, Amplitude - %):
409 *ARNTL* (2218 h, 70%), *CLOCK* (2329 h, 11%), *CRY2* (1308 h, 23%), *NPAS2* (0012 h,
410 37%), *NR1D1* (0404 h, 63%), *NR1D2* (0804 h, 36%), *PER1* (1021 h, 48%), *PER2*
411 (0821 h, 41%), *PER3* (0930 h, 57%), and *TP53* (0500 h, 20%). Genes relating to
412 autophagy and protein metabolism were also rhythmic: *MYOD1* (1914 h, 41%),
413 *FOXO3* (0900 h, 26%), *FBXO32* (0716 h, 39%). Diurnal oscillations were also present
414 in the expression of genes involved in glucose and lipid metabolism; *GLUT4* (1440 h,
415 25%), HK2 (1828 h, 21%), *FABP3* (1237 h, 15%), *PDK4* (0530 h, 133%) and *CPT1B*
416 (1258 h, 14%). Finally, diurnal variation was apparent in genes involved in
417 mitochondrial signalling; *PPARGC1A* (1613 h, 15%) and *UCP3* (0659 h, 58%), *SIRT3*
418 (1509 h, 10%) as well as transcriptional/translational regulation and MAPK signalling;
419 *CREB5* (0357 h, 19%), *EIF4EBP1* (0741 h, 11%), and *HNRNPDL* (1317 h, 35%).
420 Temporal relationships between rhythmic circulating biomarkers and skeletal muscle
421 genes are reported in **Figure 5**.

422

423 **[Figure 4]**

424

425 **[Figure 5]**

426

427

428 **Discussion**

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

434

435 Plasma insulin was rhythmic, peaking in the evening (~1800 h) and falling to nadir
436 overnight (~0400 h). This is consistent with previous research employing a continuous
437 glucose clamp (72) and generally agrees with the notion of greater insulin sensitivity
438 in the morning compared to the evening (11). However, the timing of peak insulin
439 differs from that reported in Wehrens et al (73) in which the acrophase of insulin
440 occurred ~8-11 hours after a melatonin onset similar to that reported currently, placing
441 peak time at ~0700-1000h. Nevertheless, methodological differences between studies
442 allow for greater understanding of behavioural factors that may influence such
443 rhythms. The continuous feeding pattern during waking hours in the current study
444 suggests rhythmicity in circulating insulin occurs at least partly independent of food
445 intake (74-76). Nonetheless, insulin is highly responsive to nutrient intake, and the
446 coincidence of the overnight fast with lower nocturnal insulin suggests nutrient intake
447 could be producing some of the apparent diurnal responses. Plasma glucose
448 concentrations were also rhythmic (peak ~0130 h), consistent with studies of circadian
449 misalignment, constant routine, and forced desynchrony thus further highlighting
450 robust regulation of rhythms in plasma glucose by the endogenous clock even under
451 controlled diurnal conditions (2,3,5,77). Interestingly, whilst glucose and insulin
452 concentrations might usually be expected correlate when comparing acute meal
453 responses over the minutes following feeding, the current model of hourly feeding and
454 sampling over 24-h may explain why variance in insulin may be sufficient to alter
455 glucose kinetics/flux but without necessarily being reflected by changes in the
456 systemic concentrations of glucose. At the tissue level, skeletal muscle *GLUT4* and
457 *PPARGC1a* RNA were rhythmic, with peak levels occurring at ~1500 and ~1600 h,
458 respectively (i.e., when insulin was rising), with the lowest levels at ~0400 h (i.e., when

459 insulin was lowest). Peak *HK2* RNA occurred at ~1830 h, shortly after the rhythmic
460 peak in plasma insulin and therefore in line with the regulatory effects of insulin on
461 hexokinase activity (78,79). The observation of rhythms in the skeletal muscle
462 expression of *GLUT4* and *HK2* is contrary to previous work in mice whereby no
463 significant oscillations in these genes (80,81). Collectively, the broad alignment of the
464 rhythms of these genes with rhythmic plasma insulin reflects their involvement in
465 skeletal muscle glucose uptake and their potential to influence diurnal glucose
466 metabolism (82,83).

467

468 The diurnal profiles of NEFA and glycerol were also broadly anti-phasic to the 24-h
469 profile of insulin (**Figure 5**). Circulating NEFA and glycerol were generally supressed
470 during waking hours, before rising to peak at ~0400-0500 h, consistent with the
471 nocturnal rise reported in previous literature (84-86). Plasma triglycerides were also
472 rhythmic under controlled diurnal conditions, whereby systemic concentrations were
473 low during the morning before rising to a peak at ~2330 h (**Figure 2D**). The rhythmic
474 profile of these circulating lipids is consistent with the regulatory effects of insulin on
475 adipose tissue lipolysis (87-89) and circulating triglyceride levels (90). The anti-phasic
476 relationship between insulin with NEFA and glycerol alongside the aligned rhythms in
477 insulin and triglycerides could be reflective of feeding status and the subsequent
478 changes in adipose tissue lipolysis in the overnight fasted state (45,46,49). Circulating
479 melatonin is speculated to in part contribute towards the regulation of lipid metabolism
480 (91,92), this may be reflected in the temporal similarity in acrophase among systemic
481 melatonin NEFA and glycerol (Figure 5), however further work is required to better
482 understand the effects of melatonin on lipid metabolism.

483

484 Peak expression of skeletal muscle *PDK4* RNA (~0530 h) occurred proximally to the
485 peak in systemic NEFA (**Figure 5**). This is consistent with previous work
486 demonstrating an association between diurnal variation in *PDK4* and NEFA, which
487 may be explained by the role of this gene in stimulating fatty acid utilisation in response
488 to a rise in NEFA availability (93-97). This temporal pattern may be driven the diurnal
489 feeding pattern present in both the current and previous work (97). However, following
490 peak RNA levels, *PDK4* declined at ~0800 h, despite the continual fasted state and

491 resultant elevated NEFA availability, suggesting observed effects may not be solely
492 due to the imposed feeding pattern. The profile of genes involved in the regulation of
493 solubility, mobility, and transport of fatty acids (e.g., *CPT1B* and *FABP3*) did not align
494 with systemic concentrations of NEFA (98,99), but broadly mirrored the rhythm in
495 insulin. Furthermore, alignment between *UCP3* expression with the profile of systemic
496 NEFA is consistent with the involvement of this gene in mitochondrial fatty acid
497 oxidation (100,101).

498

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

504

505 Numerous metabolic and endocrine responses relevant to tissue turnover show
506 diurnal rhythms under semi-constant routine. Cortisol displayed the expected rhythm,
507 peaking at (~1100 h) before falling to its lowest value in the evening, approximately
508 coinciding with melatonin onset (73). Peak expression of skeletal muscle *FBXO32*
509 occurred during the morning period while cortisol was rising; consistent with the related
510 action of this gene and hormone in catabolic processes, which may be driven by the
511 diurnal overnight fast (104-108). Following muscle breakdown, autophagy is a vital
512 process to stimulate muscle regeneration (39). Expression of *FOXO3*, which promotes
513 expression of downstream targeted autophagy-related proteins, also peaked in the
514 morning when cortisol is rising, which may reflect the proposed regulatory effects of
515 cortisol in stimulating increased autophagic flux in skeletal muscle (109,110).
516 Collectively the temporal patterns of these skeletal muscle genes hint at diurnal
517 fluctuations in tissue turnover, which has previously been observed in non-human
518 models (108,111). However, serum testosterone did not display diurnal rhythmicity.
519 Previous studies have demonstrated rhythmicity in systemic testosterone, with highest
520 values early in the morning (~0800 h) and corresponding lowest values ~12 h later
521 (18,112-114). This typical rhythm was not observed in the current study, which could
522 be explained by several mechanisms, including daytime hourly nutrition (115,116),

523 sleep fragmentation (117), and the potential acute effect of muscle biopsies on
524 systemic cortisol (118). The lack of rhythmicity could also be due to the sensitivity of
525 measurement through the use of commercial enzyme-based immuno-assays rather
526 than gold standard measurement by liquid chromatography mass spectrometry
527 (119,120). Equally, neither free testosterone nor sex hormone-binding globulin were
528 assessed as part of these analyses, both of which have been reported to display clear
529 daily rhythms (114,121). Finally, *MYOD1*, an important myogenic regulatory factor,
530 displayed a similar peak and nadir to insulin. This is in line with the proposed effects
531 of insulin on muscle protein turnover, hinting at diurnal patterns in skeletal muscle
532 turnover, which are plausibly driven by patterns of feeding and fasting (78,122).

533

534 Plasma CTX was lowest during the day in the fed state and peaked during the
535 biological night in the fasted state (~0500 h) in a remarkably similar rhythm and
536 amplitude to previous literature (123-125). Feeding reliably suppresses bone
537 resorption, and acute fasting dampens typical rhythmicity (124). The current data
538 therefore highlight the influence of diurnal feeding-fasting cycles on the typical 24 h
539 patterns of systemic CTX (126,127). However, plasma CTX was higher at the end of
540 the measurement period than the beginning, suggesting that other factors, such as
541 sleep and wake cycles, may also impact bone resorption and future work should seek
542 to establish the contribution of sleep on bone resorption independent of nutritional
543 status (128,129).

544

545 Despite the novelty of simultaneously collected plasma and muscle samples under
546 controlled diurnal conditions in a 24 h period, the current data must be interpreted in
547 light of several factors. Participants were fed relative to individualised requirements,
548 to account for the role of resting metabolic rate as a driver of energy intake and
549 appetite (53,54). However, 24-h bed rest eliminates the influence of physical activity
550 on circadian clocks, glucose, lipid, and protein metabolism in skeletal muscle as well
551 as bone turnover (42,130-132). This is especially pertinent given that muscle samples
552 were collected from the legs, which typically sustain greater load bearing than upper
553 limbs, so bed rest may elicit greater metabolic perturbation (133). The potential for
554 multiple tissue biopsies on localised inflammation must also be acknowledged.

555 However, biopsies were taken from alternating limbs with each following biopsy on the
556 same limb being taken 3 cm proximally to the initial incision. This is in line with Van
557 Thienin and colleagues (134), who reported inflammatory markers were upregulated
558 at the distal, but not at the proximal site when taking sequential samples from the same
559 limb. Equally, it is a limitation of this study that sleep duration and quality were not
560 objectively measured, so it is not possible to comment on the impact of nocturnal
561 sampling on those outcomes or their potential influence on the primary outcomes. It
562 should also be considered that the bright light in the laboratory may have delayed the
563 melatonin onset time and therefore suppressed the release of melatonin in the first
564 part of the night (135).

565

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

573

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

581

582 In summary, this was the first study to measure diurnal rhythms in human skeletal
583 muscle gene expression alongside systemic metabolites and hormones under
584 controlled diurnal conditions. The diurnal pattern in genes relating to carbohydrate and
585 lipid metabolism tended to reflect the pattern of insulin across 24 hours, which may in

586 part be driven by the diurnal influence of cyclic feeding and fasting. This study provides
587 novel context for metabolic regulation at both the tissue and systemic level.

588

589 **Contributions** Conceptualisation and Methodology; H.A.S., J.D.J., J-P.W., and J.A.B.
590 Data Collection, Analysis, Visualisation and Interpretation; H.A.S., M.D., J-P.W., I.T.,
591 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
592 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.
593 All authors read and approved the final manuscript.

594

595 **Data Availability Statement**

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

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1066

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 ($kg{\cdot}m^{-2}$)	24.1 ± 2.7
Resting Metabolic Rate ($kcal{\cdot}day^{-1}$)	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)

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1068

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

1069

1070

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
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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1072

1073 **Figure Legends Section**

1074

1075 **Figure 1 –** Schematic representation of the study protocol.

1076

1077 **Figure 2** – 24-hour profile for melatonin onset adjusted A) plasma glucose B) plasma
1078 NEFA C) plasma glycerol D) plasma triglycerides E) plasma urea. Solid lines denote
1079 the regression that best fits the data with the horizontal dotted line representing the
1080 24-hour mean concentration used for the null comparison. The dotted vertical line
1081 denotes melatonin onset. The shaded areas represent 24-h melatonin profile.

1082

1083 **Figure 3** – 24-hour profile for melatonin onset adjusted A) plasma insulin B) plasma
1084 c-terminal telopeptide (CTX) C) serum cortisol D) serum testosterone. Solid lines
1085 denote the regression that best fits the data with the horizontal dotted line
1086 representing the 24-hour mean concentration used for the null comparison. The
1087 dotted vertical line denotes melatonin onset. The shaded areas represent 24-h
1088 melatonin profile.

1089

1090 **Figure 4 –** Relative changes in skeletal muscle RNA expression across the 24-h
1091 semi-constant routine. Diurnal rhythmicity (as determined by cosinor analysis) are
1092 denoted by a clock symbol.

1093

1094 **Figure 5 –** Peak (circles) and nadir (triangles) timings of circulating metabolites,
1095 hormones, telopeptides, and skeletal muscle genes displaying significant diurnal
1096 rhythmicity. The dark/fasted period is depicted in the shaded grey region.

1097

2-d recorded food diary

7-d physical activity monitoring, controlled sleep-wake cycles and meal timings

d1 d2 d3

-7 d

-2 d

In laboratory

1900 h

0800 h

0800 h

Enter laboratory

24-h sample collection

d1 d2 d3

Hourly Blood:

Muscle biopsy:

Isocaloric Feed:

0800 h (day 2)

1400 h (day 2)

2000 h (day 2)

0200 h (day 3)

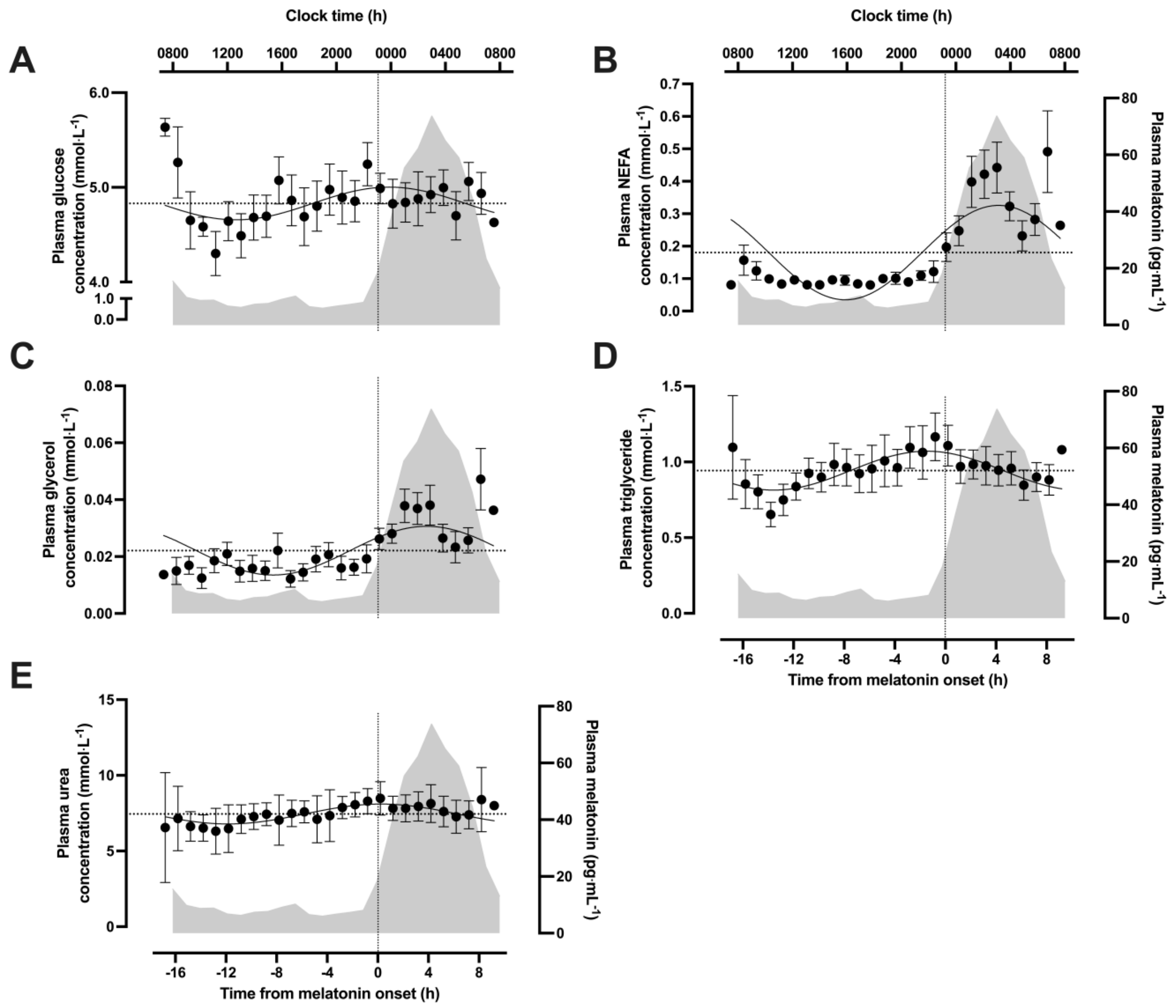
0800 h (day 3)

Time of Day

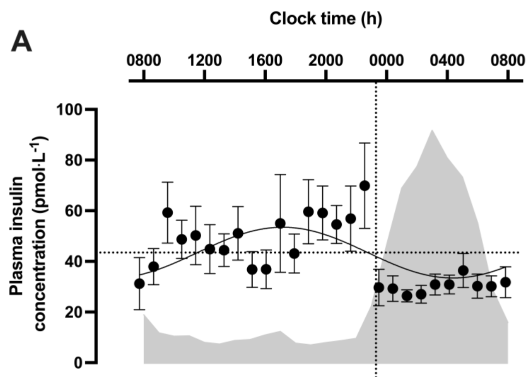
Wake/Light

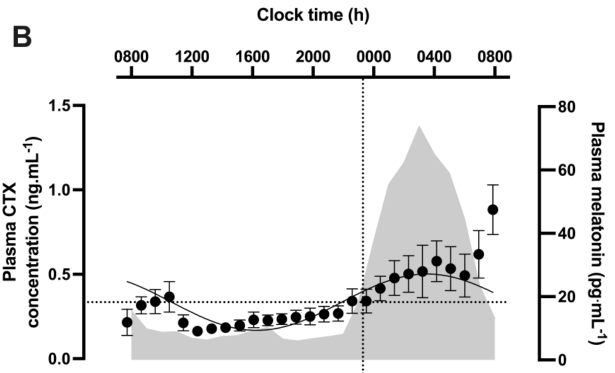
Sleep/Dark

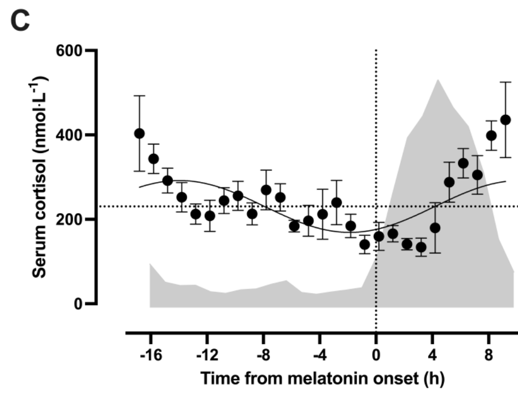
1098

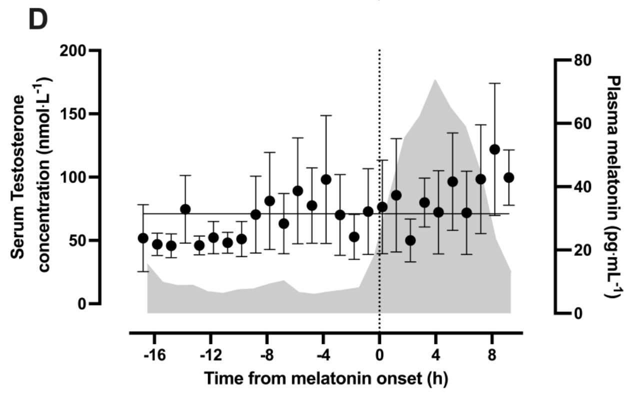


1099









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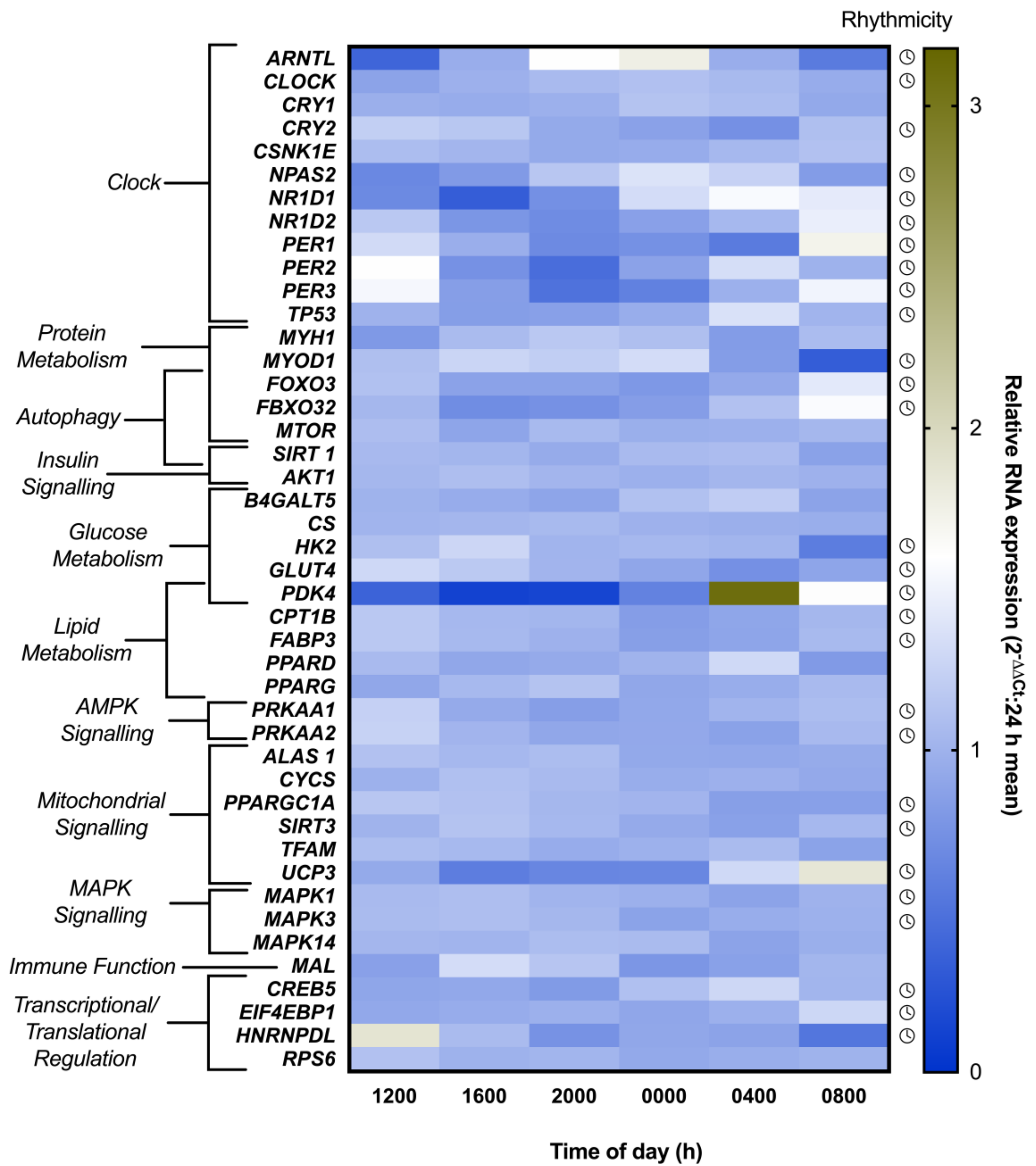

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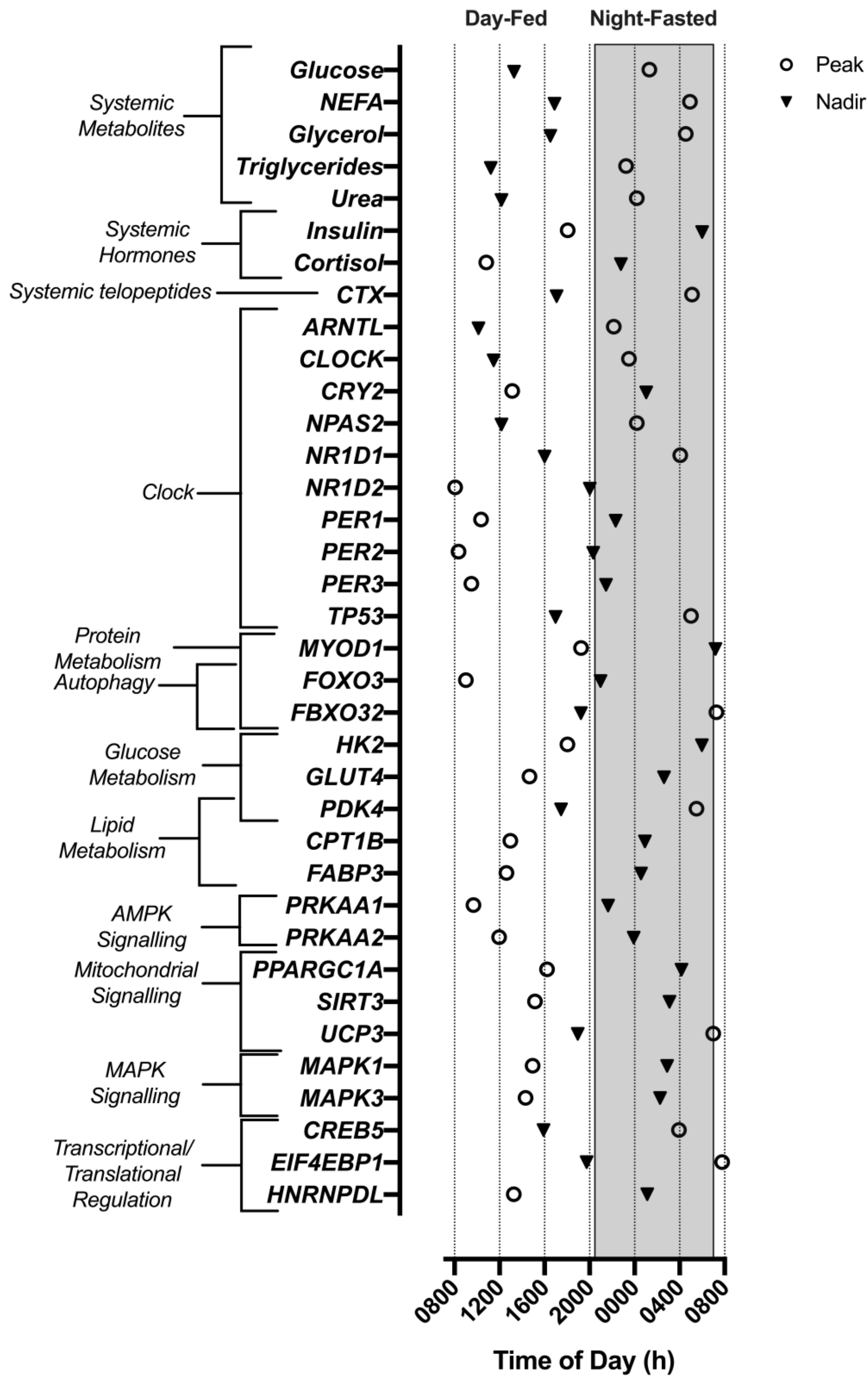

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