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Nocturnal whey protein ingestion impairs postprandial glucose tolerance at breakfast

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Short title: Nocturnal protein impairs glycaemic control.

Key words: amino acids, carbohydrate, second-meal effect, sleep

1 Abstract

2 Poor postprandial glucose control is a risk factor for multiple health conditions. The second-meal
3 effect refers to the progressively improved glycaemic control with repeated feedings, an effect which
4 is achievable with protein ingestion at the initial eating occasion. The most pronounced glycaemic
5 response each day therefore typically occurs following breakfast, so this study investigated whether
6 ingesting protein during the night could improve glucose control at the first meal of the day. In a
7 randomised cross-over design, fifteen adults (7 males, 8 females; age, 22 ± 3 years; BMI, 24.0 ± 2.8
8 $\text{kg}\cdot\text{m}^{-2}$; fasting blood glucose, $4.9 \pm 0.5 \text{ mmol}\cdot\text{L}^{-1}$) woke at $0400 \pm 1 \text{ h}$ to ingest 300 ml water with or
9 without 63 g whey protein. Participants then completed a mixed-macronutrient meal tolerance test (1
10 g carbohydrate $\cdot\text{kg}$ body mass $^{-1}$, $563 \pm 104 \text{ kcal}$), 5 h 39 min following the nocturnal feeding.
11 Nocturnal protein ingestion *increased* the glycaemic response (incremental area under curve) to
12 breakfast by $43.5 \pm 55.5 \text{ mmol}\cdot 120 \text{ min}\cdot\text{L}^{-1}$ ($p=0.009$, $d=0.94$). Consistent with this effect, individual
13 peak blood glucose concentrations were $0.6 \pm 1.0 \text{ mmol}\cdot\text{L}^{-1}$ higher following breakfast when protein
14 had been ingested ($p=0.049$, $d=0.50$). Immediately prior to breakfast, rates of lipid oxidation were
15 $0.02 \pm 0.03 \text{ g}\cdot\text{min}^{-1}$ higher ($p=0.045$) in the protein condition, followed by an elevated postprandial
16 energy expenditure ($0.09 \pm 0.12 \text{ kcal}\cdot\text{min}^{-1}$, $p=0.018$). Postprandial appetite and energy intake were
17 similar between conditions. This study reveals a paradoxical second-meal phenomenon whereby
18 nocturnal whey protein feeding *impaired* subsequent glucose tolerance, whilst increasing
19 postprandial energy expenditure.

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31 INTRODUCTION

32 The postprandial glycaemic response to a fixed carbohydrate load can be used to assess the
33 proficiency of glucose control⁽¹⁾. Repetitive and/or prolonged hyperglycaemic episodes are linked to
34 a heightened risk of multiple health conditions including: type 2 diabetes mellitus, cardiovascular
35 disease and ultimately premature mortality⁽²⁾. Ineffective postprandial glucose tolerance also impairs
36 satiety perception and is thus linked to obesity, a key risk factor for type 2 diabetes^(3; 4). Even amongst
37 normoglycemic individuals⁽⁵⁾, those with poorer postprandial glycaemic control are at increased risk
38 of developing type 2 diabetes and cardiovascular disease⁽⁶⁾. The prevalence of these morbidities is
39 increasing and presents a large personal and socio-economic burden^(3; 7). Several clinical markers are
40 available to diagnose defective glucose control; incremental area under the blood glucose
41 concentration curve (iAUC) reflects the response to a glucose challenge and provides a more valid
42 indication of glucose tolerance than basal/fasted measures alone^(2; 8). This is entirely understandable
43 given that the time spent hyperglycaemic is a primary factor in the aetiology of diabetic
44 complications^(9; 10).

45 Beyond the internal validity of postprandial glycaemia as a measure of glucose control, the
46 external validity of fasted (i.e. post-absorptive) measures is also more limited given that typical eating
47 patterns in most societies mean that humans spend most or all waking hours in a fed-state (i.e. post-
48 prandial)^(9; 11). Of even greater practical relevance is the response to sequential meals, as most meals
49 are therefore consumed in an already postprandial state. The ‘second-meal effect’ describes how
50 initial glucose ingestion improves glucose tolerance at a second eating occasion⁽¹²⁾. Whilst this
51 phenomenon was first discovered with sequential oral glucose tolerance tests (OGTT), a more
52 ecologically valid method considers the effect of the mixed-macronutrient meals. This has been most
53 commonly examined in the literature as the magnitude of metabolic responses to lunch when
54 breakfast is consumed, comparative to no breakfast⁽⁸⁾. Notably, this effect also promotes a more even
55 glycaemic stability across the day, thus eliciting a potentially beneficial effect on satiety
56 perception⁽¹³⁾. The second-meal effect is hypothesised to involve several interacting mechanisms,
57 such as enhanced hepatic and peripheral insulin sensitivity, potentiated insulin secretion, and slowed
58 gastric emptying following ingestion of a second meal⁽¹⁴⁾.

59 Whereas the influence of carbohydrate (CHO) and fat on second-meal effects are relatively
60 well understood (i.e. high-CHO, low-glycaemic index (GI) and low-fat breakfasts are effective in
61 optimising the insulin secretion and glycaemic response to lunch^(15; 16; 17; 18)), the efficacy of protein
62 has received less research attention. Several studies have demonstrated that protein, specifically
63 whey, consumed as a preload can elicit the second-meal effect^(19; 20; 21). This has been attributed to
64 augmented insulin secretion and insulin priming of the muscle and liver, alongside delayed gastric

65 emptying of the second load^(20; 21). Protein ingestion has also been linked to appetite suppression and
66 a reduction in subsequent energy intake^(4; 20; 22), although other studies report no such effects^(19; 23).
67 In addition, recent evidence suggests that a high-protein breakfast may be a more potent stimulator
68 of the second-meal effect than a high-CHO breakfast^(24; 25), although this possibility requires
69 confirmation in young, normoglycaemic populations. Moreover, previous studies have only
70 examined the carry-over effects between the established daily sequence of eating occasions (i.e.
71 breakfast-lunch-dinner), yet a novel strategy would be to attenuate the profound first glycaemic
72 response after waking using a model wherein breakfast serves as the second meal of the day.

73 The purpose of this study, therefore, was to examine the effect of nocturnal whey protein
74 ingestion on the glucose response to breakfast, alongside any secondary effects on subsequent satiety
75 and energy intake later in the day. Based on extant literature examining the effects of prior feeding
76 during the morning on metabolic responses to lunch, we hypothesised that protein ingestion will
77 attenuate the glycaemic response to breakfast and reduce subsequent appetite and energy intake.

78

79 **EXPERIMENTAL METHODS**

80 *Approach to the research question*

81 The innovative nature of this investigation stems from the unique combination of protein
82 ingestion with nocturnal feeding. Whey protein was chosen in preference to alternative protein
83 sources due to a shorter gastric emptying time, resulting in a more rapid development and greater
84 magnitude of hyperaminoacidaemia and therefore a more rapid insulin release. This is consistent with
85 the rationale for our stated hypothesis above, that an increased morning insulin requirement is due to
86 a lack of residual insulin from a preceding meal⁽²⁶⁾. As such, this novel strategy may reduce the insulin
87 requirement to breakfast, consequently improving glucose control.

88 Whey also reduces subsequent food intake and appetite to a greater extent than casein, egg or
89 soy^(20; 26; 27). A whey protein dose between 20-40 g can be effective in reducing appetite, while as
90 little as 10 g is reported to stimulate the second-meal effect⁽²⁰⁾. A large, but palatable dose of 63 g
91 was used in this novel protocol to conclusively elucidate any effects on sequential glycaemic response
92 and satiety (i.e. if effects are observed then that provides proof-of-principle and could warrant further
93 examination of lower doses). This protein solution was made up with 300 ml of water instead of milk
94 to avoid confounding effects of fat ingestion and to allow any effects of protein to be isolated.

95 The time of protein feeding was 0400 ± 1 h to ensure a post-absorptive state following dinner
96 and to allow sufficient time before breakfast for the acute initial metabolic response to the ingested

97 protein to subside. The time of feeding resulted in, on average, a 5 h 39 min period between protein
98 ingestion and breakfast, which aligns with the majority of studies investigating the second-meal effect
99 using 3-6 h between sequential meals^(13; 18; 28; 29). Additionally, participants were instructed to be in
100 bed, lights out, at 23:00, which would therefore result in a minimum of 5 h separating dinner and the
101 nocturnal protein feed. A mixed macronutrient tolerance test was deemed more appropriate than an
102 OGTT as this study focuses on the response to sequential meals to provide practical results applicable
103 to daily living. A porridge breakfast aligns with this aim as a common breakfast meal, providing
104 information on the ability to process a realistic glucose and fat challenge.

105

106

[Figure 1]

107

108 ***Participants***

109 Fifteen individuals who self-identified as healthy volunteered to participate (7 males, 8
110 females; age, 22 ± 3 years; BMI, 24.0 ± 2.8 kg·m⁻²; resting heart rate, 69 ± 15 bpm; resting mean
111 arterial pressure, 72 ± 6 mmHg; fasting blood glucose, 4.9 ± 0.5 mmol·L⁻¹; sleep chronotype, 54 ± 7
112 (intermediate)). Participants were informed of the study objectives, requirements and any potential
113 risks before written consent was obtained. Ethical approval was received from the University of Bath
114 research ethics committee (SESHES-18R1-004). Exclusion criteria included allergy or intolerance to
115 any of the breakfast constituents or any metabolic conditions which may have posed undue personal
116 risk to the participant or introduced bias into the experiment.

117

118 ***Study design***

119 This study employed a randomised crossover design, comprising two conditions; nocturnal
120 ingestion of either a protein solution (PRO) or water (CON). The following morning participants
121 visited the laboratory to complete a mixed-macronutrient meal tolerance test. Participants were not
122 blinded to the condition as it would have been immediately apparent upon ingestion and to analyse
123 subsequent food choices participants needed to be aware of their consumption as they would in free-
124 living conditions. Trials were completed within a month for males, or within 4-7 days for females to
125 ensure the phase of the menstrual cycle was consistent between trials in order to avoid large
126 systematic differences in glucose control due to menstrual cycle phase⁽³⁰⁾. All procedures were
127 performed in accordance with the declaration of Helsinki.

128 *Experimental procedures*

129 Participants refrained from vigorous physical activity and replicated their diet the day prior to
130 both laboratory visits. Compliance was confirmed verbally upon laboratory arrival. A schematic for
131 the study protocol is shown in **Figure 2**. Prior to data collection, participants completed the Horne &
132 Östberg Morningness-Eveningness Questionnaire⁽³¹⁾, to assess sleep chronotype and establish
133 habitual morning or evening preference. Participants were instructed to be in bed, lights out at 23:00,
134 awaking at 07:00 and arriving in the laboratory the following morning at 09:00. Participants awoke
135 at 04:00 to consume the relevant solution. Compliance was confirmed with a text message to a
136 researcher at this time. Based on the questionnaire results, these time components were adjusted \pm 1
137 h for morning or evening preference. This aimed to minimise disturbance to habitual sleep patterns
138 due to the confounding effect on glucose tolerance⁽³²⁾.

139 Upon laboratory arrival the following morning, anthropometric (height and mass) and pre-
140 breakfast resting measures were collected. Fingertip blood glucose concentration was recorded using
141 an automatic glucose analyser (coefficient of variation 5.03%) (FreeStyle Optium, Abbott
142 Laboratories Ltd, Berkshire, UK). Three 5 min expired gas samples were collected using Douglas
143 bags (Hans Rudolph, MO, USA), which were analysed using a Servomex 1440 Gas Analyser
144 (Servomex Group Ltd., UK) and the volume of expired air determined by evacuating the Douglas bag
145 with a dry gas meter (Harvard Apparatus, Kent, UK). Heart rate and blood pressure (Omron M2
146 Compact blood pressure monitor, Omron Healthcare Co., Netherlands) were collected, alongside 100
147 mm visual analogue scales.

148 A standardised porridge breakfast was then consumed, followed by a 2 h resting period in the
149 seated position with water consumption permitted (ad libitum). Blood glucose measurements were
150 collected every 15 min for the first 60 min and then every 30 min until 120 min. Five min expired air
151 samples were collected at 20, 40, 75 and 100 min. At 60 and 120 min appetite VAS were completed.
152 Upon completion of the tolerance test participants were free to leave the laboratory and completed a
153 food diary for the remainder of the day under free-living conditions.

154

155

[Figure 2]

156

157

158

159 *Nutritional composition*

160 Experimental solutions were prepared by researchers and provided to participants the night
161 before the trial. The protein solution contained: 75 g whey protein powder (Myprotein, Northwich,
162 UK), resulting in an intake of 63 g protein (21 g protein per 25 g serving), 300 ml water and 0 kcal
163 vanilla flavouring drops to taste (Myprotein, Northwich, UK). An amino acid profile for the whey
164 protein used is depicted in **Figure 1**. The control solution was 300 ml water. Breakfast consisted of
165 29% porridge oats (Sainsbury's, UK), 67% whole milk (3.7/100 g fat; Sainsbury's, UK) and 4%
166 granulated sugar (Sainsbury's, UK). Porridge was provided in quantities to deliver 1 g CHO·kg body
167 mass⁻¹, obtained from 80% oats and 20% sugar. This resulted in an energy intake of 563 ± 104 kcal,
168 containing 18.0 ± 3.3 g protein and 72.4 ± 13.9 g CHO. The quantity was replicated for the second
169 trial, regardless of any minor change in body mass. Participants were instructed to consume the
170 porridge within 15 min to standardise effects of eating rate upon appetite hormones⁽³³⁾.

171

172 *Statistical analysis*

173 All data are presented as mean ± standard deviation. Statistical analyses were performed using
174 the Statistical Package for the Social Sciences (IBM SPSS Statistics 25), with statistical significance
175 accepted at an alpha level of $p \leq 0.05$. A minimum sample size of 11 was calculated in order to detect
176 an effect size of 0.95 with 80% power (G*Power 3.1.9.4), based on the effect size in similar studies
177 in normoglycaemic individuals^(24;34). Data from one female participant who volunteered for the study
178 were not included in the analysis because she experienced very poor sleep quantity in the control
179 condition (>2 SD below the mean), given the established negative effect of sleep deprivation on
180 glucose tolerance⁽³²⁾. This is illustrated by her almost doubled blood glucose iAUC in the CON
181 condition (396 *versus* 195 mmol·120min·L⁻¹).

182 Blood glucose iAUC was calculated using the trapezoid method⁽³⁵⁾. Rates of oxygen
183 utilisation and carbon dioxide production were used to calculate respiratory exchange ratio (RER)
184 and energy expenditure from expired gas samples⁽³⁶⁾. Substrate oxidation rates were calculated in
185 accordance with the stoichiometric equations outlined by Frayn⁽³⁷⁾ assuming negligible protein
186 oxidation. Of the three resting gas samples, the average of those within a 100 kcal·day⁻¹ agreement
187 in energy expenditure was taken to calculate pre-breakfast values of substrate utilisation, RER and
188 energy expenditure. If none of the three bags met this criterion, the lowest of the three samples was
189 considered the most reflective measure of resting metabolic rate (this was the case for 5 out of 27
190 bags). Scores from the appetite VAS were combined to give an average appetite score at each time
191 point according to the equation outlined by Gonzalez and Stevenson⁽⁸⁾. Regarding sleep VAS, a

192 difference >10 mm is deemed of clinical relevance and more meaningful than statistical
193 significance⁽³⁸⁾, and thus this criterion was used when examining intraindividual differences in VAS
194 scores between conditions.

195 A Shapiro-Wilk test was used to assess the normality of intraindividual differences between
196 conditions. Consequently, a paired *t*-test was applied to analyse normally distributed parametric data
197 and a Wilcoxon test used for non-parametric data. Order effects were examined with a paired *t* test
198 for iAUC between trial 1 and 2 alongside a two-way mixed-models ANOVA (treatment x sequence);
199 there was a 2.2% decrease in iAUC from trial 1 to 2 with no significant order effect ($p=0.875$) and no
200 treatment x sequence interaction ($p=0.509$, $F=0.463$). Pearson correlation coefficients were calculated
201 for the difference in iAUC between conditions and both body mass and sleep quality (calmness and
202 wakefulness). A two-way repeated measures ANOVA (treatment x time) was used to examine
203 differences in blood glucose response and appetite over time. The Greenhouse-Geiser correction was
204 applied for epsilon values <0.75 and the Huynh-Feldt correction applied for less severe asphericity.
205 Effect size was calculated using pooled standard deviation, with Cohen's thresholds applied⁽³⁹⁾.

206

207

208 **RESULTS**

209 ***Blood glucose concentration***

210 Plasma glucose concentrations increased more rapidly in PRO than in CON, reaching a 7.5%
211 higher peak (7.9 ± 1.4 versus 7.3 ± 0.9 mmol•L⁻¹, $p=0.049$, $d=0.50$, moderate effect; **Figure 3A**) and
212 then remaining higher than the CON trial until the final blood sample, such that there was a main
213 effect of treatment ($p=0.037$, $F=5.392$) but no treatment x time interaction ($p=0.308$, $F=1.241$; **Figure**
214 **4**). Accordingly, there was a 28.1% mean increase in postprandial blood glucose iAUC in the PRO
215 condition (161.8 ± 55.7 v 116.3 ± 39.2 mmol•120 min•L⁻¹ ($p=0.009$, $d=0.94$; **Figure 3B**). Time spent
216 above the clinical threshold of 7.5 mmol•L⁻¹ blood glucose concentration was longer in the PRO trial
217 for 9 out of 14 participants by an average of 8.2 ± 13.4 min (15.9 ± 17 v 7.7 ± 11.6 min, $p=0.039$,
218 $d=0.56$, moderate effect). Although not significant ($p=0.206$, $d=0.48$, small effect), on average there
219 was a 6.4 ± 18.3 min delay in time to peak glucose with PRO (37.5 ± 16.4 min) compared to CON
220 (31.1 ± 9.2 min).

221

222

[Figure 3]

223 [Figure 4]

224

225 ***Substrate utilisation, respiratory exchange ratio and energy expenditure***

226 Lipid oxidation was 30.8% greater at pre-breakfast (following intervention but prior to the
227 test meal) in the PRO trial compared to CON ($p=0.045$) (**Table 1**). There were no significant
228 differences between conditions at pre-breakfast for RER, CHO oxidation, or energy expenditure.
229 Mean responses over the 120 min postprandial period did not differ between conditions for RER,
230 CHO oxidation, or lipid oxidation. Postprandial energy expenditure was greater in the PRO trial
231 ($p=0.018$).

232

233 [Table 1]

234

235 ***Subjective appetite and energy intake***

236 There were no significant differences in any appetite ratings or combined appetite score at
237 pre-breakfast (all $p>0.05$, **Table 2**). There was a main effect of time ($p<0.001$, $F=11.411$), but not
238 treatment ($p=0.674$, $F=0.185$) nor treatment x time interaction ($p=0.681$, $F=0.222$) for postprandial
239 combined appetite score. Further, there was a main effect of time ($p<0.03$), but not treatment nor
240 treatment x time interaction (all $p>0.05$), for hunger, fullness, satisfaction and prospective
241 consumption during the postprandial period. There was no difference in volitional energy intake
242 ($p=0.736$) in CON (1728 ± 681 kcal) compared to PRO (1666 ± 766 kcal).

243

244 [Table 2]

245

246 ***Sleep quantity and quality***

247 There was no difference in self-reported sleep quantity between conditions (450 ± 52 v $444 \pm$
248 52 min, $p=0.788$). Reductions >10 mm were observed in the sleep quality measures of wakefulness
249 (15 mm) and calmness (12 mm) in the PRO versus CON condition. However, there was no correlation
250 between the difference in iAUC between conditions and the change in sleep wakefulness ($r=0.017$,
251 $p=0.953$) or calmness ($r=0.176$, $p=0.546$) between trials.

252 **DISCUSSION**

253 This is the first study to investigate nocturnal protein ingestion in relation to the second-meal
254 effect at breakfast. Contrary to our hypothesis, the primary findings demonstrate impaired
255 postprandial glucose tolerance at breakfast after nocturnal protein ingestion, evidenced by the
256 increased glycaemic response (iAUC) and peak blood glucose concentrations. Pre-breakfast (fasted)
257 lipid oxidation was also elevated following protein ingestion, whilst postprandial after breakfast
258 energy expenditure was higher. There was no difference in appetite or volitional energy intake
259 between conditions. These findings illustrate that protein elicited a response opposite to the second-
260 meal effect, impairing sequential glucose tolerance, with no effect on appetite or energy intake. This
261 is the opposite response to that reported in the available literature regarding daytime meals during
262 waking hours^(15; 16; 18; 20).

263 The differences in glucose tolerance observed in the present study may be due to the 63 g dose
264 of protein, as the majority of studies demonstrating a beneficial effect of a protein preload have used
265 doses in the range of 10-55 g^(19; 20; 21). Further, previous studies have most commonly utilised a protein
266 preload 30 min prior to the second meal^(19; 20; 21), which differs from 5 h 39 min separating meals in
267 the present study. Although both Meng *et al.*⁽²⁴⁾ and Park *et al.*⁽²⁵⁾ demonstrated that protein
268 stimulates the second-meal effect with 4 h separating meals, both studies used high-protein breakfast
269 foods, rather than the addition of whey protein used in the aforementioned preload studies^(19; 20; 21).
270 Moreover, these studies were conducted in adults with type 2 diabetes⁽²⁵⁾ and postmenopausal
271 women⁽²⁴⁾. Regarding appetite, our results support the findings of Allerton *et al.*⁽²⁸⁾, which also
272 reported no attenuation in appetite when whey protein was added to breakfast⁽²⁸⁾ ingested with a
273 longer duration (i.e. 3 h) between meals than used in other preload studies^(19; 20; 21).

274 Protein ingestion in excess of physiological needs can stimulate ureagenesis and the use of
275 glucogenic amino acids in gluconeogenesis⁽⁴⁰⁾. Boden and Tappy⁽⁴¹⁾ demonstrated the
276 hyperaminoacidemia following protein ingestion stimulated endogenous glucose production despite
277 hyperinsulinemia. Studies employing stable isotope methodologies have demonstrated ~17-19% of
278 protein ingested is converted to glucose^(42; 43). Therefore, assuming similar rates in the current study,
279 it could be hypothesised that 17-19% of the 63 g protein ingested in the current study resulted in
280 glucose production, producing 10.7 – 12.0 g glucose. This is supported by the findings of Ang *et al.*
281⁽⁴⁴⁾, who demonstrated that a 75 g of whey protein dose resulted in 11 g endogenous glucose
282 production. Fromentin *et al.*⁽⁴²⁾ state that the percentage of dietary amino acids converted to glucose
283 was mainly related to the provision of glucogenic amino acids. The whey protein utilised in this study
284 comprised 79.8/100 g glucogenic or mixed amino acids (**Figure 1**), providing a high availability of
285 gluconeogenic precursors. In addition, the relative lack of other sources of gluconeogenic precursors

286 (glycerol, pyruvate, lactate) in our whey protein solution, comparative to egg⁽⁴²⁾ or cottage cheese⁽⁴³⁾,
287 may have further increased the contribution of amino acids to gluconeogenesis. Hence, glucose
288 production may have been at the higher end of this estimate.

289 There are multiple underlying mechanisms through which the above-reasoned endogenous
290 glucose production could alter glucose control following breakfast. Firstly, it may be that there was
291 still some residual endogenous glucose production from the nocturnal protein by the time breakfast
292 was ingested. Indeed, Ang *et al.* ⁽⁴⁴⁾ reported that endogenous glucose production was still elevated
293 at 4 h following whey protein ingestion, which could therefore supplement the carbohydrate directly
294 ingested at breakfast and thus explain the elevated postprandial glycaemia in the PRO condition. It
295 should be noted, however, that the breakfast in the present study was provided almost 6 h following
296 the protein dose, so the contribution of the ingested protein towards endogenous glucose production
297 may have subsided by the time of the second delivery of ingested nutrients.

298 Secondly, any glucose generated from the ingested amino acids even prior to breakfast could
299 still contribute to a more positive carbohydrate balance (i.e. increased glycogen availability) and thus
300 potentially limit the capacity for further non-oxidative carbohydrate disposal at breakfast. Certainly
301 the 10-12 g of glucose that would theoretically arise via gluconeogenesis from the ingested protein
302 could make a meaningful contribution towards the total capacity for hepatic glycogen stores. The
303 current study employed a somewhat unusual protocol in that a large provision of energy was delivered
304 at an atypical time of day when neither required nor expected by the body. Overnight fasting depletes
305 hepatic glycogen stores and stimulates gluconeogenesis^(45; 46), hence the liver may have been more
306 sensitive to the large protein dose ingested during the night. It is unlikely that any large proportion of
307 glucose produced would be oxidised in this rested and fasted state and therefore is more likely to be
308 stored, increasing hepatic glycogen stores. An increase in glycogen stores may reduce the liver's
309 capacity for first pass extraction following breakfast, reducing hepatic glucose uptake and resulting
310 in more glucose remaining in circulation⁽⁴⁷⁾. In the present study, the mean time between protein
311 ingestion and breakfast was 5 h 39 min. Peak liver glycogen concentration is achieved around 5 h
312 postprandially⁽⁴⁶⁾, therefore it is possible this peak may have coincided with breakfast, reducing the
313 capacity for first pass extraction. However, rather than liver glycogen storage, the primary driver of
314 postprandial glucose tolerance is thought to be insulin-stimulated glucose uptake into peripheral
315 tissues and the synthesis of skeletal muscle glycogen^(48; 49), yet 10-12 g of newly synthesised glucose
316 would represent a much smaller contribution to whole-body muscle glycogen reserves and thus less
317 likely to impair skeletal muscle glucose uptake at breakfast. The present results regarding glycaemic
318 control therefore warrant future studies to examine such mechanisms by measuring glucose
319 synthesis/disposal, along with muscle and liver glycogen concentrations.

320 The time of feeding in the present study may also have impacted gluconeogenesis and insulin
321 sensitivity. Protein was fed at a time when the relative concentrations of both glucagon and cortisol
322 were increasing. Elevated glucagon concentration stimulates hepatic gluconeogenesis and amino acid
323 uptake^(50; 51), while cortisol also stimulates hepatic gluconeogenesis, alongside reducing insulin
324 sensitivity⁽⁵⁰⁾. Betts *et al.* ⁽⁵²⁾ demonstrated that the overnight cortisol response is elevated following
325 the co-ingestion of protein with CHO before bed. Thus, the protein ingestion in the present study may
326 have augmented the natural circadian elevation in cortisol thus reducing insulin sensitivity. There is
327 also evidence for circadian rhythmicity in circulating amino acids, with lowest concentrations
328 between 0400 and 0800 h^(53; 54). Feigin *et al.* ⁽⁵⁴⁾ demonstrated that the ingestion of a large protein
329 bolus at 0800 h resulted in an additional increase in blood amino acid concentrations, exceeding that
330 anticipated from typical circadian periodicity, an interference not replicated with an identical protein
331 load consumed at 2000 h. This further supports the idea that the body may not be entrained to deal
332 with a large bolus protein dose at the time of feeding in the present study. Finally, whey protein
333 ingestion and an increase in plasma branched-chain amino acids (BCAA), particularly leucine, have
334 also been demonstrated to impair insulin sensitivity directly, especially in high doses ^(55; 56; 57). The
335 high BCAA (22.6/100 g) and specifically leucine content (10.6/ 100 g) of the whey protein (**Figure**
336 **1**) may therefore have impaired glucose uptake via that mechanism. As such, any priming effect of
337 insulin secretion following protein ingestion may have been insufficient to overcome this reduced
338 insulin sensitivity at breakfast. Therefore, the arrival of a large dose of amino acids at an atypical time
339 of day might, via the hypothesised influence on hepatic gluconeogenesis and insulin sensitivity, result
340 in impaired glucose control upon waking in a manner specific to this time-frame. This further
341 highlights the novel nature of the current study employing an atypical feeding time. Additional study
342 is warranted to investigate if the glucose tolerance to a second meal is impaired following protein
343 ingestion prior to other daily meals in a more 'conventional' feeding pattern.

344 Pre-breakfast lipid oxidation was elevated in the PRO trial and post-prandial energy
345 expenditure was also then higher. It should be noted, however, that these calculations were not
346 adjusted based on 24-h nitrogen excretion and so assume negligible protein oxidation, which may not
347 be a valid assumption in this experiment⁽³⁷⁾. Witard *et al.* ⁽⁵⁸⁾ reported that ingesting 40 g whey protein
348 isolate stimulates phenylalanine oxidation for 4 h, so the 63 g ingested in the present study is likely
349 to have stimulated a similar response, although this may have reduced by the time measurements
350 were made 6 h later at breakfast. Nonetheless, if protein undergoes gluconeogenesis and is oxidised,
351 then the respiratory quotient for that process is ~0.8 (the same as for direct protein oxidation), whereas
352 the respiratory quotient if the newly synthesised glucose is stored is 0.4⁽⁵⁹⁾. Any persistent protein
353 oxidation or gluconeogenesis 6 h after the nocturnal bolus would therefore be expected to slightly

354 reduce the whole-body RER based on measured oxygen uptake and carbon dioxide production.
355 However, the fact that the RER under both conditions were very similar and in the range of 0.85-0.89
356 means any such difference is likely to be small and thus it remains a reasonable alternative explanation
357 that the ingested protein did in fact increase lipid oxidation.

358 In conclusion, consumption of a whey protein solution during the night impaired postprandial
359 glucose control at breakfast, whilst increasing postprandial energy expenditure, with no effect on
360 satiety or energy intake. As such, this approach would not be recommended to improve postprandial
361 glucose control following breakfast. This paradoxical second-meal phenomenon may relate to an
362 influence of protein oxidation on the availability of hepatic and/or skeletal muscle glycogen and/or
363 insulin sensitivity.

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389 Authorship

390 JAB and JTG formulated the research question, ESS, EA, KC, JH, GP, JTG and JAB designed the
391 study, ESS, EA, KC, JH and GP collected the data, ESS analysed the data, ESS, HAS, JTG and JAB
392 contributed to writing the manuscript and all authors approved the final version of the manuscript.

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408 TABLES

409 **Table 1. Pre-breakfast and postprandial (120 min) values for substrate utilisation, RER and**
 410 **energy expenditure. At pre-breakfast n = 13 due to difficulties associated with expired air**
 411 **collection. *significant difference between CON v PRO.**

	CONTROL	PROTEIN	<i>p</i>
<i>Pre-breakfast</i>			
RER	0.88 ± 0.06	0.85 ± 0.05	0.102
CHO oxidation (g•min ⁻¹)	0.21 ± 0.10	0.18 ± 0.09	0.173
Lipid oxidation (g•min ⁻¹)	0.06 ± 0.02	0.07 ± 0.02	0.045*
Energy expenditure (kcal•day ⁻¹)	1901 ± 375	1952 ± 302	0.196
<i>Postprandial (120 min period)</i>			
RER	0.89 ± 0.03	0.89 ± 0.05	0.804
CHO oxidation (g•120 min ⁻¹)	28.17 ± 7.37	31.88 ± 12.29	0.084
Lipid oxidation (g•120 min ⁻¹)	6.85 ± 2.11	6.58 ± 2.75	0.694
Energy expenditure (kcal•120 min ⁻¹)	168 ± 27	179 ± 33	0.018*

412 Carbohydrate (CHO), respiratory exchange ratio (RER). Values expressed as mean ± standard
 413 deviation.

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426 **Table 2. Pre-breakfast (0 min) and postprandial (60, 120 min) subjective appetite responses.**

Variable	0 min		60 min		120 min	
	CON	PRO	CON	PRO	CON	PRO
Hunger	42 ± 29	42 ± 28	27 ± 15	28 ± 21	40 ± 23	42 ± 23
Fullness	34 ± 29	32 ± 31	60 ± 21	59 ± 23	54 ± 23	53 ± 23
Satisfaction	38 ± 27	36 ± 28	63 ± 14	54 ± 24	52 ± 24	51 ± 24
Prospective consumption	55 ± 28	55 ± 28	33 ± 16	43 ± 20	47 ± 26	55 ± 26
Combined appetite score	57 ± 26	57 ± 24	34 ± 15	40 ± 18	45 ± 23	48 ± 20

427 Control (CON), protein (PRO). All variables demonstrated a main effect of time ($p < 0.03$). Values
 428 expressed as mean ± standard deviation.

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592 **FIGURE LEGENDS**593 **Figure 1. Amino acid profile of the whey protein used.**

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595 **Figure 2. Protocol schematic. A mixed macronutrient tolerance test was conducted following**
596 **nocturnal ingestion of either protein (PRO) or water (CON).**

597

598 **Figure 3. A) Peak blood glucose concentration (conc.) and B) blood glucose iAUC. Dashed lines**
599 **denote female (n = 7) and solid lines denote male (n = 7) participants. Darker lines represent**
600 **individuals with a BMI >25 kg.m⁻² (n = 4) and lighter lines denote those with a BMI <25 kg.m⁻²**
601 **(n = 10). *significant difference between CON v PRO trials.**

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603 **Figure 4. Blood glucose concentration following breakfast ingestion at 0 min. The dashed line**
604 **represents a blood glucose concentration of 7.5 mmol·L⁻¹ as a reference threshold.**

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