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Is the Timing of Caloric Intake Associated with Variation in Diet-Induced Thermogenesis and in the Metabolic Pattern? A Randomized Cross-Over Study.

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Abstract

Background/Objectives: food-induced thermogenesis is generally reported to be higher in the morning although contrasting results exist due to differences in experimental settings related to the preceding fasting, exercise, sleeping, and dieting. In order to definitively answer to this issue, we compared the calorimetric and metabolic responses to identical meals consumed at 8:00 am and at 8:00 pm by healthy volunteers, after standardized diet, physical activity, duration of fast and resting. **Subjects/Methods:** 20 subjects (age range 20-35 years, BMI 19-26 kg/m^2) were enrolled to a randomized cross-over trial. They randomly received the same standard meal in the morning, and 7 days after, in the evening, or vice versa. A 30-min basal calorimetry was performed; a further 60-min calorimetry was done 120-min after the beginning of the meal. Blood samples were drawn every 30-min for 180-min. General linear models (GLMs), adjusted for period and carry-over, were used to evaluate the "morning effect", i.e. the difference of morning delta (after-meal minus fasting values) minus evening delta (after-meal minus fasting values) of the variables. **Results:** fasting Resting-Metabolic-Rate (RMR) did not change from morning to evening; after-meal RMR values were significantly higher after the morning meal (1916; 95%CI 1792,2041 *vs* 1756; 1648,1863 kcal; p<0.001). RMR was significantly increased after the morning meal (90.5; 95%CI 40.4,140.6 kcal; p<0.001), while differences in areas-under-the-curve for glucose (-1800; -2564,-1036 mg/dl×h, p<0.001), log-insulin (-0.19; -0.30,-0.07 µU/ml×h; p=0.001) and fatty free acid concentrations (-16.1;-30.0,-2.09 mmol/l×h; p=0.024) were significantly lower. Delayed and larger increases in glucose and insulin concentrations were found after the evening meals.

Conclusions: the same meal consumed in the evening determined a lower RMR, and increased glycemic/insulinemic responses, suggesting circadian variations in the energy expenditure and metabolic pattern of healthy individuals. The timing of meals should probably be considered when nutritional recommendations are given.

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Introduction

An increasing number of studies have shown that the timing of food intake influences energy regulation, the risk of weight gain and obesity, independent from total daily caloric intake, dietary composition and estimated energy expenditure [1].

Early insulin secretion after a meal ingestion is significantly higher in the morning than in the evening, leading to a more rapid glucose clearance from blood [2], whereas insulin sensitivity decreases later during the day [3-4]. Circadian variations in the concentrations of hormones and peptides regulating satiety and appetite, as well as genetic patterns of the circadian clock genes have been implicated in the daily modification of specific metabolic pathways [1]. Additionally, gastric emptying seems to be more rapid at morning than in the evening [5], and an increased efficacy of dietary carbohydrates absorption has been demonstrated under late suppertime conditions [6].

A few studies evaluated the circadian variation of the energy balance with contrasting results [7-12]. The thermic effect of food, also called diet-induced thermogenesis (DIT), is defined as the increase in resting metabolic rate (RMR) after the ingestion of a meal. This component accounts for a small proportion of the total energy expenditure (about 10%), but it has been reported to be implicated in the development and persistence of obesity [13]. DIT is significantly higher after the consumption of a snack in the morning than after the same snack at night [7], and a reduced evening thermic response may be due to nocturnal insulin resistance [8]. Furthermore, habitual nighttime eating or snacking have been associated with reduced fat oxidation, potentially promoting weight gain [14].

However, data in the literature often show contrasting results [9-12] with differences in the exercise level, hours of sleeping, antecedent diets, variation in the duration of the fasting state, presence of comorbidity, and low number of the subjects enrolled likely acting as confounding factors [15]. Indeed, the ideal setting to study diurnal variation in energy expenditure is to measure post-absorptive RMR under the same conditions at different times of the day. The aim of our study was therefore to compare the calorimetric and metabolic

91 responses to identical meals (a high-protein, low-carbohydrates meal) consumed in the morning (8:00 am) and

92 in the evening (8:00 pm) by healthy volunteers, after standardizing diet, physical activity level, duration of fast

93 and resting.

94

95 **Subjects and Methods**

96 *Recruitment of participants*

97 Twenty healthy volunteers (ten males and ten females) were enrolled among students and graduates attending

98 the Department of Medical Sciences of Turin (Italy).

99 Inclusion criteria were: age 20-35 years, body mass index (BMI) 19-26 kg/m^2, habitual moderate exercise level,

100 smoking <10 cigarettes/day. Exclusion criteria were: any acute or chronic diseases, menopause, any drugs or

101 supplementations, any alimentary restrictions or specific diets, being a shift or night workers, unable to give a

102 written informed consent.

103 *Design*

104 This was a randomized cross-over trial.

105 *Outcomes*

106 The primary *outcome* was evaluating changes in RMR after the morning meal consumption compared to

107 changes in RMR after the evening meal. The secondary outcomes were analyzing changes in circulating

108 concentrations of glucose, insulin, free fatty acids (FFA) and triglycerides after the morning and evening meal

109 consumption.

110 *Intervention*

111 Participants were randomly allocated to receive a *standard meal* at 8:00 am and the week after the same

112 *standard meal* at 8:00 pm, or vice versa. Eight hours before the meal (respectively at 12:00 pm or at 12:00 am),

113 participants received the same *standard meal* (without protein supplementation) at their home, and then were

114 asked to spend the following 6-h in bed. Drinking coffee, alcohol or other beverages, and smoking were not

115   permitted. The week preceding each test, participants were instructed not to change their usual diet and to

116   refrain from heavy physical activity. A 24-h urine collection was collected the day before each test in order to

117   determine total urinary nitrogen excretion.

118   The *standard meal* consisted of: 100g white bread, 100g ham, 50g cheese, 125g yogurt, 200ml fruit juice, plus

119   25g protein supplement (Resource Instant Protein, Nestlè, Switzerland). The nutritional composition of the

120   meal was: 30% protein, 31% fat, 39% carbohydrates; total kcal 1168. The participants had to consume each

121   meal in 25-30-min. Participants were taken by car to the laboratory. At 7:00 am (or pm, according to the

122   randomization), the participants underwent to anthropometric measurements and to the insertion of a 16-G

123   indwelling catheter into an antecubital vein of the forearm, subsequently kept patent by the slow infusion of

124   500 ml of saline solution until the end of the testing session. In order to avoid blood drawing related stress, all

125   the blood samples have been withdrawn from an extension line tubing.

126   A 30-min basal calorimetric exam was performed (**Figure 1**). Participants remained in a supine position but

127   awake and motionless on a hospital bed for the whole period, except during the meal, when they could sit to

128   eat and were allowed to void. At 8:00 am (or pm), the participants consumed the meal, and then rested in a

129   supine position for 90-min, followed by a second 60-min calorimetric evaluation. In order to obtain a better

130   compliance to the experiment, the second calorimetric evaluation lasted 60-min (from 120 to 180 min from the

131   beginning of the meal), since we have previously performed a pilot study in six volunteers to evaluate the

132   tolerance to the calorimetric exam, and have found that maintaining immobility while awaking was difficult for

133   more than 1 hour consecutive.

134   Blood samples were drawn every 30-min from the first calorimetric exam (baseline) until the end of the second

135   (post-prandial) (Figure 1). Time 0 was after the first calorimetry and before the meal. Times 30, 60, 90, 120, 150

136   and 180 were referred to the time intervals in minutes from the beginning of the meal. The same time

137   schedule was adopted in the case of the morning meal (at 8:00 am) and the evening meal (at 8:00 pm). The

138   second test was carried out after 7 days from the first.

139   *Sample size*

140   A sample size of 20 subjects (10 in the "morning-first" group and 10 in the "evening-first" group) was required

141   to test a 0.66 effect size with a power equal to 80% and a two-tailed α-value=0.05.

142   *Randomization*

143   The random sequence (morning/evening, evening/morning meal) was computer-generated, using blocks of

144   different lengths in random order.

145   *Measurements*

146   The Minnesota Leisure Time Physical Activity questionnaire [16], previously validated in an European cohort

147   [17], was completed by all the participants together with a 3-day food record, which consisted of a detailed

148   written food diary [18]. Subjects were instructed to record everything they ate or drank during 2 consecutive

149   week days and 1 week-end day. The 3-day food record data were loaded on the Win Food Pro 3 software

150   (Medimatica, Colonnella, Teramo, Italy), and the mean nutritional values for the 3 days were reported.

151   Body weight and height were measured with subjects wearing light clothes using a mechanical column scale

152   (SECA 711, Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm, respectively. Waist circumference was

153   measured by a plastic tape meter at the top of the iliac crest, after a normal expiration. Fat and fat-free mass

154   were determined by single frequency bioelectrical impedance using manufacturer equations (BIA 101, Akern,

155   Italy). Indirect calorimetry by Deltatrac II (DATEX, Division of Instruments Corp. Helsinki, Finland) was used to

156   measure the rate of energy expenditure, by measuring the inspired and expired concentrations of oxygen (VO_2)

157   and carbon dioxide (VCO_2), which reflect nutrient metabolism. Deltatrac II has become one of the reference

158   tools for reliable measurements, validated in vitro and in vivo, and its accuracy has been detailed in several

159   studies [19]. Before measurements, the instrument was warmed-up for 30-min. The canopy was placed over

160   the subjects' face and carefully checked to prevent air leakage. After 5-min of initial calibration, gas sample

161   were continuously analyzed by a paramagnetic and infrared gas chamber for sensing O_2 and CO_2 respectively.

162   During the calorimetric exam, participants were not allowed to talk. The exams were performed in a quiet

163   room with a temperature kept at 23-25° C. The energy expenditure was calculated within 3-h from the

164   beginning of the meal, because it has been reported that the DIT response to meal can be assessed with

165   sufficient precision within 3-h and prevent the movements occurring with more prolonged periods of

166   immobility [15].

167   Blood samples were immediately centrifuged and aliquots of plasma were stored separately at -80° until

168   analysis. The following determinations were performed: glucose, insulin, FFA, triglycerides. Serum glucose was

169   measured by enzymatic colorimetric assay (Menarini Diagnostics, Florence, Italy); serum insulin was

170   determined by immunoradiometric assay (Beckman Coulter, Immunotech, Prague, Czech Republic; intra-assay

171   coefficients of variation ≤3.99% and inter-assay coefficients of variation ≤4.8%). FFA concentrations were

172   measured by a fluorometric assay (Sigma-Aldrich, St. Louis, MO, USA). Plasma triglycerides were assayed by

173   enzymatic colorimetric method (Hitachi, Roche Diagnostics, Mannheim, Germany). Total urinary nitrogen

174   excretion was determined on the 24-h urine collection of the day before each test. Total nitrogen excretion

175   was assessed by a kinetic assay (Hitachi, Roche Diagnostics, Mannheim, Germany).

176   *Definitions*

177   The physical activity level was calculated as the product of the duration and frequency of each activity (in

178   hours/week), weighted by an estimate of the metabolic equivalent of the activity (METS) and summed for the

179   activities performed.

180   Both basal and after-meal RMR were calculated according to the formula of Weir [20]. RMR was calculated also

181   in relation to fat free mass (FFM) determined by bio-impedance as: RMR/FFM and expressed as kcal/kg.

182   DIT was considered as the difference between average after-meal RMR and the basal RMR (after-meal RMR −

183   basal RMR).

184   The Respiratory Quotient (RQ) was the ratio between VCO_2 and VO_2 (VCO_2/VO_2).

185   Glucose and fat oxidation were calculated according to the Ferranini formulas [21]:

186   Carbohydrate (CHO) oxidation (g/min) = 4.55 VCO_2 (l/min) − 3.21 VO_2 (l/min) − 2.87 N (g/min)

187   Fat oxidation (g/min) = 1.67 VO_2 (l/min) – 1.67 VCO_2 (l/min) -1.92 N (g/min)

188   VO_2=oxygen consumption; VCO_2=carbon dioxide production; N= urinary nitrogen excretion.

189   Rate of N, an index of protein oxidation, was assumed to be constant during the calorimetry.

190   Area-under-the curve (AUC) values for glucose, insulin, FFA and triglycerides were calculated according to the

191   trapezoidal model [22].

192   We defined as Delta the difference between each variable at fasting and after the meal:

193   Delta = variable value after the meal – variable value at fasting

194   In the case of the calorimetric variables, the values at fasting corresponded to the results of the basal

195   calorimetry; delta RMR therefore coincided with DIT. In the case of the laboratory variables, the values at

196   fasting corresponded to the values at time 0.

197   *Blinding*

198   Due to the nature of the intervention, blinding participants and health professionals was not feasible. The

199   laboratory personnel who performed the biochemical analyses was blinded to the group assignment.

200   *Ethics*

201   The study was approved by the ethics committee of "Città della Salute e della Scienza" of Turin; all the

202   procedures conformed to the principles of the Helsinki Declaration. All participants provided written informed

203   consent to take part to the study.

204   *Statistical methods*

205   Clinical and laboratory variables were presented as mean and standard deviation (SD). Triglyceride and insulin

206   values were logarithmically transformed in order to approximate normal distribution. The Student's *t*-test for

207   paired data was applied to investigate within-subject changes of the variables at morning and at evening.

208   Within-subject changes in the variables after the morning and the evening meal consumption were further

209   analyzed to estimate the "morning effect", i.e. the difference between the morning delta and the evening

210   delta:

211   "Morning effect" = morning delta – evening delta

212   In the case of the AUCs, the "morning effect" was the difference between morning AUC for the variable minus

213   evening AUC for the variable.

214   General linear models (GLM) with patients as random effects were performed to assess and adjust for the

215   possible period and carry-over effects and to estimate crude and adjusted "morning effects" and 95%

216   confidence intervals (95% CI). In order to make easier the interpretation of log-transformed variables, the

217   adjusted estimates of triglycerides and insulin AUCs were expressed both as differences and as ratios.

218   In an explorative analysis we analyzed by GLM the "morning effect" on glucose, insulin, FFA and triglyceride

219   values at all time-points.

220   The repeated measures from 0 to 180 minutes of glucose, insulin, FFA and triglycerides were graphically

221   reported as means and as means and 95%CIs of variations from time 0.

222   Statistical analyses were performed using STATA 13.1 (StataCorp LP, College Station, Texas).

223

224   **Results**

225   Mean age, weight, height, body mass index (BMI), and waist circumference of the participants were 27.6±3.4

226   years, 67.3±12.5 kg, 1.70±0.01 m, 23.4±3.2 kg/m^2, and 81.8±8.0 cm, respectively. Fat mass and fat-free mass

227   determined by bioelectrical impedance analyses were 14.5±6.0 kg and 53.5±11.3 kg.

228   Participants were moderately active: their median METs h/week were 46.3, and usually consumed a high-lipid

229   low-fiber diet (total kcal 1989.9±523.0; fat 39.9±15.7 % total kcal; saturated fatty acids 11.1±2.6 % total kcal;

230   monounsaturated fatty acids 14.9±4.2 % total kcal; carbohydrates 46.6±6.6 % total kcal; fiber 11.9±3.5 g/day).

231   There were no meaningful differences between the two groups of randomization (morning-first *versus* evening-

232   first) for anthropometric and fasting RMR values (**Table 1SI, Supplementary Information**).

233   In **Table 1**, the morning and evening calorimetric variables at fasting and after-meal in the 20 participants are

234   reported. Fasting RMR was slightly lower at evening. After-meal RMR and DIT values were significantly higher

235   after the morning meal. Fasting and after-meal RQs at morning were significantly higher than the

236   corresponding RQs at evening. Both fasting and after-meal CHO oxidation values were significantly higher and

237   fasting and after-meal fat oxidation significantly lower in the morning in comparison with the evening values.

238   Period and carry-over effects were tested by using GLM and resulted not statistically significant for all

239   variables. The crude and un-adjusted effects of the "morning effects" did not differ; therefore, only the

240   adjusted effects were reported. The difference in delta RMR values, i.e. the difference in DIT, indicated a higher

241   DIT increase after the morning than after the evening meal (**Table 2**). On the other hand, the difference in delta

242   RQ values, and the differences between morning and evening AUCs for glucose, insulin and FFA concentrations

243   were negative, indicating a significantly higher effect of the evening meal on these variables than the morning

244   meal.

245   Adjusted estimates of the "morning effects" tested by GLM showed significantly lower values of glucose at

246   time 60, 90, 120, 150; lower values of insulin at time 90, 120, 150; lower values of FFA at time 120, 150, 180,

247   and lower values of triglycerides at time 60 **(Table 2SI, Supplementary Information)**.

248   Basal values of glucose, insulin, FFA and triglycerides did not differ from morning to evening. In **Figures 2-5**

249   (panels A) the mean values according to the different time points of glucose, insulin, FFA and triglycerides were

250   reported. In the panels B of the Figures 2-5, the mean and 95%CI variations from time 0 of the same variables

251   were presented.

252

253   **Discussion**

254   The results of the present paper indicate that the time of food intake per se affects both the thermogenic and

255   the metabolic responses to meals. This could have practical implications since the time to eat should probably

256   be considered when planning a healthy diet.

257

258   *Energy expenditure and metabolic responses to meals*

259   Our data showed that the meal-induced DIT increase after the morning meal was higher than that observed at

260   dinner, while basal RMRs were only slightly lower at evening, consistent with data available in the literature

261   [7,15]. Furthermore, glucose, insulin and FFA AUCs values resulted significantly higher after the evening meal

262   rather than at morning.

263   A circadian pattern in the thermic and metabolic responses to nutrients could be therefore hypothesized.

264   DIT consists of two components: an obligatory component, linked to the energy expenditure by digestion,

265   absorption and metabolism of nutrients, and an additional facultative component, likely mediated by the

266   sympathetic nervous system [23-24]. A circadian rhythmicity of circulating norepinephrine and epinephrine has

267   been found, with increased values in the morning [25]. Epinephrine increases metabolic rate, RQ and glucose

268   oxidation [26]. Other explanations for the reduced evening post-prandial thermic and metabolic answers may

269   be the slower evening gastric emptying, with increased efficacy of dietary carbohydrates absorption [5-6], and

270   the reported decrement in insulin sensitivity as evening progresses [3]. Possible contributors to the daily

271   variations in insulin secretion, with higher morning beta-cell function and action, could be the circadian

272   fluctuation in the concentrations of cortisol, ACTH, glucagon-like peptide 1, glucose-dependent insulinotropic

273   polypeptide, as well as in the meal-induced glucagon responses, showing a delayed peak after evening meals

274   [2-3,27-29]. Insulin resistance may determine a decreased thermic effect of glucose because of the diminished

275   insulin-mediated glucose uptake and metabolism by skeletal muscle that results in diminished glucose-induced

276   thermogenesis [8,30]. Furthermore, sympathetically mediated thermogenesis is decreased in the presence of

277   hyperinsulinemia or insulin resistance [26,30]. Therefore, both the circadian variation in catecholamine

278   concentrations and in insulin sensitivity might contribute to the evening reduction of DIT. Consistent with the

279   reported reduced insulin sensitivity at evening, we found lower glucose/insulin AUCs in the morning and

280   delayed and larger increases in the concentrations of glucose and insulin in the evening (Figures 2-3, Table 2SI,

281   Supplementary Information).

282   A circadian control of FFA metabolism has been described, with highest levels around noon [31] or in the

283   afternoon [2], and an increased activity of lipoprotein lipase during the daytime [27]. The circadian activity of

284   clock target genes in adipocytes may impact on lipid breakdown and adipokine function, by regulating FFA

285   mobilization from adipose tissue, thus acting on energy homeostasis and metabolic processes [32-33]. It is

286   difficult to establish with our data if the higher increase in FFA AUC after the evening meal was the

287   consequence of insulin resistance or rather the cause of the impaired insulin sensitivity, because of the known

288   effects of FFA on the reduction in glucose uptake and phosphorylation in skeletal muscles and on the

289   impairment in insulin signaling and action [34-35].

290

291   Not all the authors found a lower DIT after the evening meal [9-12]. Westrate failed to find a significant

292   difference between morning and afternoon DIT in 10 normal weight young men, but the fasting period was

293   shorter in the afternoon than in the morning and the metabolic conditions of the participants to this study

294   were not the same [9]. Nevertheless, in the morning, DIT was slightly higher, energy balance (the difference

295   between energy intake and expenditure) lower, and early post-meal values of RQs and CHO oxidation

296   significantly increased, in line with our results [9]. Another study found a higher energy expenditure at night

297   during enteral nutrition, but it involved older patients hospitalized in a Neurologic unit on artificial nutrition,

298   therefore these results are difficult to be compared with those in our healthy individuals [11]. Sato found no

299   differences in 24-h energy expenditure in a room-size respiratory chamber between normal (7:00 pm) or late

300   (10:30 pm) evening meals in 10 young Japanese, but the measure of DIT was not available, the times of the two

301   experimental meals were not so different (approximately 3-h), and, consistent with our results, postprandial

302   glucose AUC values were significantly increased after the late meal [10]. Similarly, delaying the time of an

303   identical meal of about 3-h for a week did not change postprandial energy expenditure, but decreased CHO

304   oxidation and glucose tolerance [12].

305 In our study protocol, we used extreme conditions (meals at 12-h distance) and a high-protein content, which is

306 known to exert a greater effect on energy expenditure [36-37]. It is possible that with a mixed meal we could

307 have found lower values of DIT and lower differences between the morning and evening tests.

308 Nevertheless, the circadian variation in DIT might explain at least in part the previously found large intra-

309 individual variability in DIT and CHO and fat oxidation, which could not be accounted by variation in the

310 antecedent diets or in the method of measurement [7,15]. Intriguingly, the significant difference in morning-

311 evening DIT in our sample persisted even when energy expenditure was calculated in relation to the FFM

312 (Table 2).

313

314 *Respiratory Quotients*

315 RQ values generally range within 0.70-1.00, with 100% lipid oxidation at 0.70, and 100% carbohydrate

316 oxidation at 1.00 [19]. We found reduced CHO oxidation and RQ values and increased fat oxidation (Table 1) in

317 the evening, suggesting a modification of the metabolic pathways toward a higher utilization of lipid substrates

318 in the evening, supported by the faster reduction of FFA levels after the evening meal (Figure 3, panel B),

319 although starting from higher FFA values (Figure 3, panel A).

320 After the experimental infusion of glucose/insulin [23] as well as after a meal [38], both an increase in CHO

321 oxidation and a decreased lipid oxidation have been reported, in line with our data. Other studies have found a

322 higher RQ in the morning than in the evening [7,9,12,15,39]. A significantly higher lipid oxidation and a lower

323 CHO oxidation were described with meals at 6:00 pm when compared with meals at 10:00 am in obese

324 subjects both after a short (3 days) and a long (18 days) term protocol, and the morning has been considered

325 the time of the day in which anabolic functions take place [40], in line with the circadian rhythm of the

326 glycogen synthesis [41]. Even if delta RQs were slightly higher after the evening meal (Table 2), the increased

327 evening fat oxidation and FFA AUCs in our sample suggested, in line with the higher insulin resistance at that

328 clock time, a preferential use of lipid substrates in our sample.

329

330 *Clinical perspectives*

331 Human studies have shown that both in adolescents and in adults, consuming more of the daily energy intake

332 at evening is associated with an increased risk of obesity, hyperglycemia, lipid abnormalities, metabolic

333 syndrome, non-alcoholic fatty liver diseases, and cardiovascular diseases [1,4,12,14,28,42-45]. Circadian

334 misalignment has adverse metabolic and cardiovascular consequences [4,46]. Examples of chronic conditions

335 of this phenomenon are shift work and sleep deprivation, both conditions being associated with an increased

336 risk of obesity, metabolic syndrome and cardiovascular diseases [27,31,33].

337 The timing of meals also influences the success of weight loss strategies: late-lunch eaters lose less weight than

338 early eaters [47]; overweight/obese women lose significantly more weight after a low-calorie dinner weight-

339 loss program than after an isocaloric high-calorie dinner program [48]. Therefore, dietary recommendations

340 should probably include indications on the time-of-day for food consumption, besides advice on food quality

341 and quantity.

342

343 *Limitations*

344 First of all, caution is needed when trying to link results from short-term studies to long-term effects.

345 We did not evaluate the energy expenditure longer than 3-h after the beginning of the meal, according to some

346 [7], but not other authors who recommended >5-h measurements [13]. However, our experiment was

347 consistent with studies showing that energy expenditure is lower after 3-h and that DIT response to meals can

348 be assessed with sufficient accuracy for the comparison across subjects within this time interval [7,15,49].

349 Furthermore, most of the differences in the metabolic patterns we found were present in the first 2-h after

350 meal. We used the glucose equation to calculate CHO oxidation [21], even if after an overnight fast most of

351 plasma glucose turnover is derived from liver glycogenolysis, but comparisons were performed within-

352 individuals under the same conditions and, as Ferrarini stated [21], assuming exclusive glucose oxidation does

353 not introduce a major error.

354

355 *Conclusions*

356 Consuming a high-protein, low-carbohydrates meal at evening seems energetically and metabolically

357 unfavorable with respect to the consumption of the same meal at morning. Energy expenditure and

358 metabolism may be tightly linked to circadian rhythms; gaining further insights into these processes may be

359 useful to curb the current increasing rate of metabolic disorders.

360

361 **Acknowledgments**

365

366 **Conflicts of interest:** The authors report no conflict of interest.

367

368 Supplementary information is available at journal's website.

369

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Figure Legends

Figure 1

The arrows (↓) indicate the time when the blood samples were drawn. During the visit, participants were submitted to anthropometric measures and a venous catheter was peripherally inserted. "Basal" corresponded to the time before the first calorimetric examination was performed. The time 0 was after the first calorimetry and before the meal. The times 30, 60, 90, 120, 150 and 180 were referred to the time interval in minutes from the **beginning** of the meal.

The same time schedule was adopted in the case of the morning meal (at 8:00 am) and the evening meal (at 8:00 pm).

Figure 2

Mean glucose values at the different time points (panel A). Variation of glucose from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 3

Mean insulin values at the different time points (panel A). Variation of insulin from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).
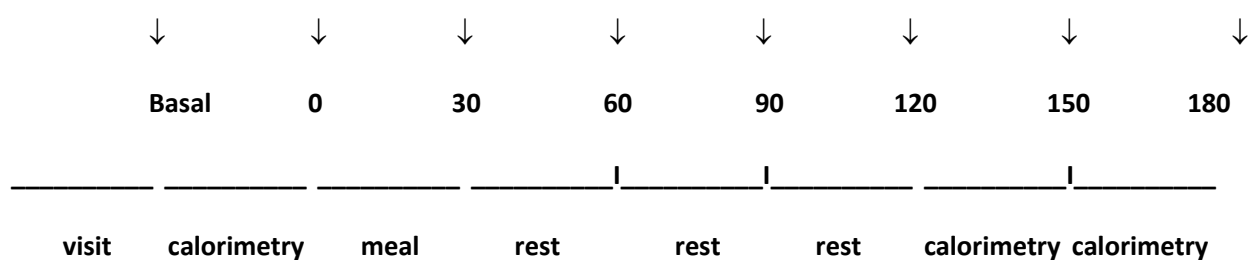
Figure 4

Mean FFA values at the different time points (panel A). Variation of FFA from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 5

Mean triglyceride values at the different time points (panel A). Variation of triglycerides from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 1. Time schedule of the study

↓	↓	↓	↓	↓	↓	↓	↓
Basal	0	30	60	90	120	150	180

_____ _____ _____ _____ | _____ | _____ _____ | _____

| visit | calorimetry | meal | rest | rest | rest | calorimetry | calorimetry |

The arrows (↓) indicate the time when the blood samples were drawn. During the visit, participants were submitted to anthropometric measures and a venous catheter was peripherally inserted. "Basal" corresponded to the time before the first calorimetric examination was performed. The time 0 was after the first calorimetry and before the meal. The times 30, 60, 90, 120, 150 and 180 were referred to the time interval in minutes from the **beginning** of the meal.

The same time schedule was adopted in the case of the morning meal (at 8:00 am) and the evening meal (at 8:00 pm).

Figure 2. Mean glucose values at the different time points (panel A) and variation of glucose from time 0 (panel B)
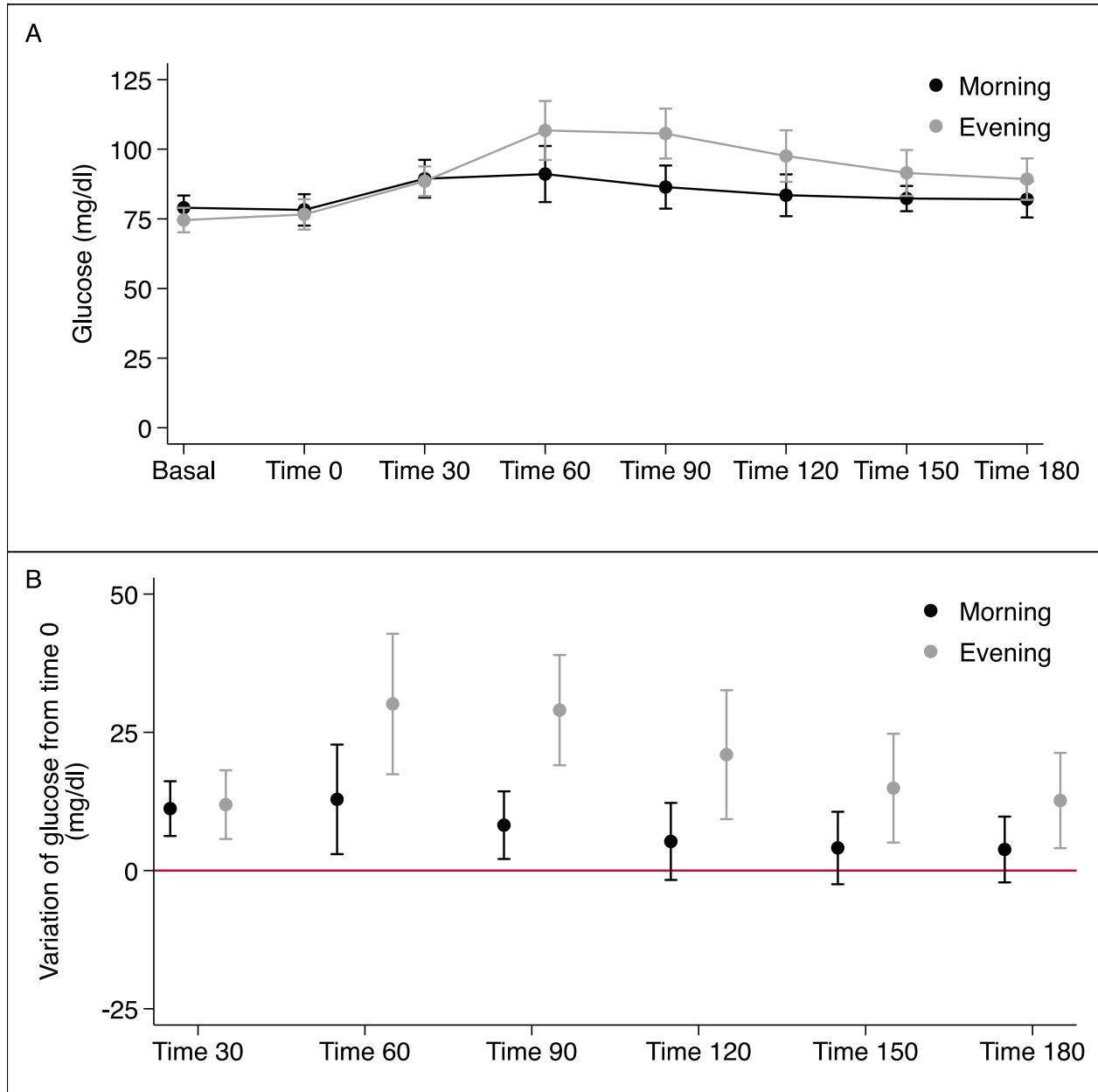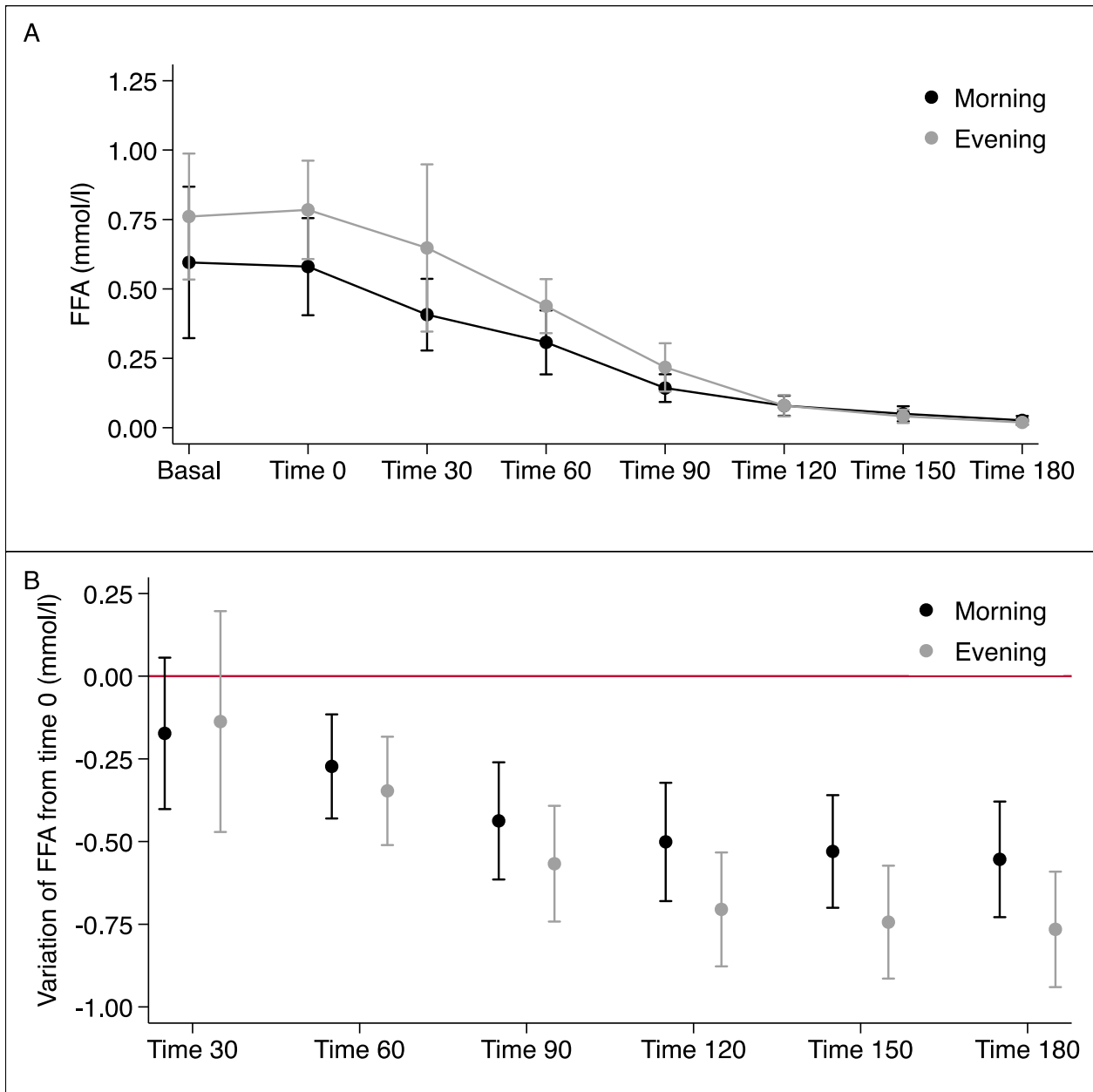

Mean glucose values at the different time points (panel A). Variation of glucose from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 3. Mean insulin values at the different time points (panel A) and variation of insulin from time 0 (panel B)
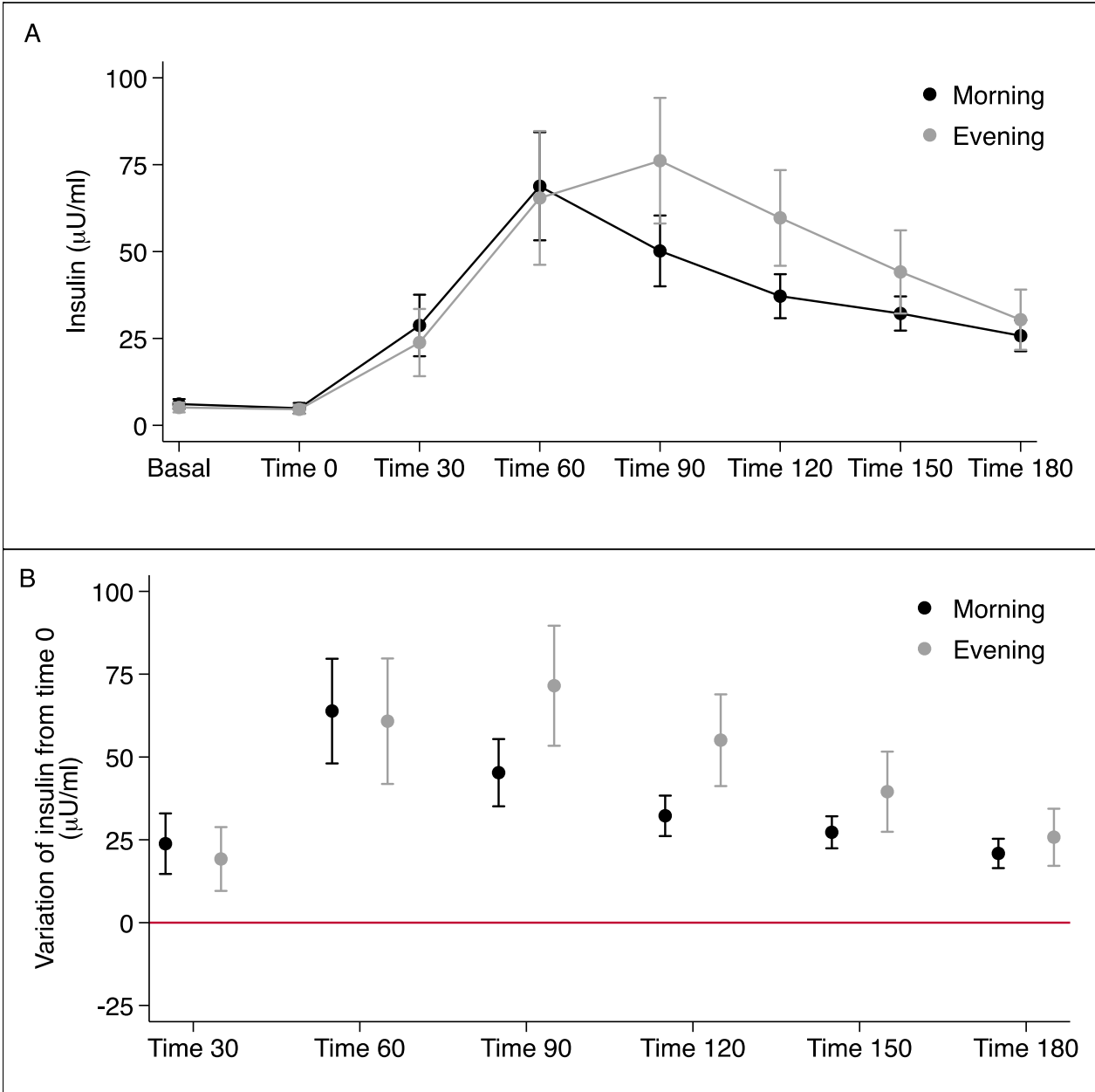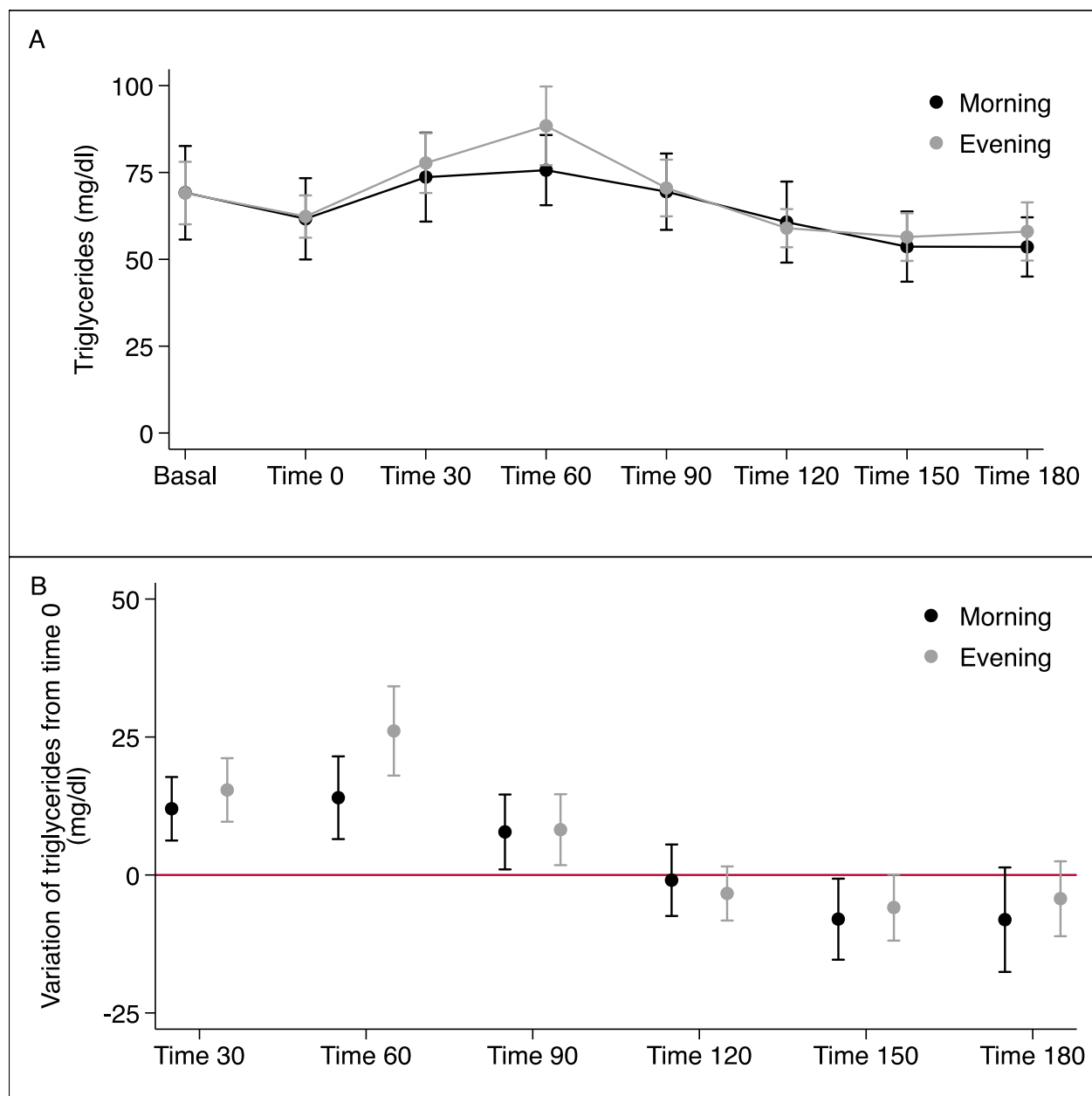
Mean insulin values at the different time points (panel A). Variation of insulin from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 4. Mean FFA values at the different time points (panel A) and variation of FFA from time 0 (panel B)

Mean FFA values at the different time points (panel A). Variation of FFA from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 5. Mean triglyceride values at the different time points (panel A) and variation of triglycerides from time 0 (panel B)

Mean triglyceride values at the different time points (panel A). Variation of triglycerides from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Table 1. Calorimetric variables before and after morning and evening meals

	Morning		Evening		p-value
Number	20		20		
Fasting RMR (kcal)	1588.5	[1464.9;1712.1]	1518.5	[1407.7;1629.3]	0.098
After-meal RMR (kcal)	1916.0	[1791.5;2040.5]	1755.5	[1648.0;1863.0]	<0.001
DIT (kcal)	327.5	[279.0;376.0]	237.0	[195.1;278.9]	0.003
Fasting RMR[1] (kcal/kg FFM)	30.2	[27.8;31.7]	29.1	[27.0;31.2]	0.180
After-meal RMR[1] (kcal/kg FFM)	36.7	[34.6;38.8]	33.7	[31.4;36.0]	<0.001
DIT[1] (kcal/kg FFM)	6.46	[5.16;7.76]	4.62	[3.55;5.70]	0.003
Fasting RQ	0.87	[0.85;0.89]	0.80	[0.78;0.82]	<0.001
After-meal RQ	0.90	[0.89;0.92	0.85	[0.82;0.88]	0.002
RQ Difference	0.03	[0.01;0.05]	0.05	[0.02;0.08]	0.055
Fasting CHO oxidation (g/min)	0.13	[0.10;0.15]	0.05	[0.02;0.07]	<0.001
After-meal CHO oxidation (g/min)	0.20	[0.18;0.22]	0.12	[0.08;0.16]	<0.001
CHO oxidation difference	0.07	[0.05;0.09]	0.08	[0.05;0.10]	0.856
Fasting fat oxidation (g/min)	0.01	[0.01;0.02]	0.04	[0.03;0.05]	<0.001
After-meal fat oxidation (g/min)	0.01	[0.00;0.02]	0.03	[0.02;0.04]	0.006
Fat oxidation difference	-0.01	[-0.01;0.00]	-0.01	[-0.03;0.00]	0.116

Mean [95%CI]; p-values calculated by the t-test for paired data

RMR = Resting Metabolic Rate; DIT = Diet Induced Thermogenesis; RQ = Respiratory Quotient; CHO =

carbohydrates

[1]energy expenditure calculated in relation to fat free mass

Table 2. Estimates of "morning effect" adjusted for period and carry over effects by General Linear Models

(GLMs)

	Effects	95% CI	p-value
RMR[1] (kcal)	90.5	[40.4,140.6]	<0.001
RMR[1,2] (kcal/kg FFM)	1.84	[0.81,2.87]	<0.001
RQ[3]	-0.02	[-0.04,-0.001]	0.035
CHO oxidation[3] (g/min)	0.00	[-0.02,0.02]	0.848
Fat oxidation[3] (g/min)	0.01	[-0.00,0.02]	0.089
Glucose AUC[4] (mg/dl×h)	-1800.1	[-2564.1,-1036.0]	<0.001
Log-insulin AUC[4,5] (μU/ml×h)	-0.19	[-0.30,-0.07]	0.001
Log-insulin AUC[4,6]	0.83	[0.74,0.93]	0.001
FFA AUC[4] (mmol/l×h)	-16.1	[-30.0,-2.09]	0.024
Log-triglycerides AUC[4,5] (mg/dl×h)	-0.08	[-0.21,0.05]	0.230
Log-triglycerides AUC[4,6]	0.92	[0.81,1.05]	0.230

RMR = Resting Metabolic Rate; RQ = Respiratory Quotient; CHO = carbohydrates; AUC = Area-under-the curve;

FFA = free fatty acids

[1]morning Diet-Induced-Thermogenesis (DIT) – evening DIT

[2]RMR calculated in relation to fat free mass

[3]Morning delta minus evening delta

[4]Morning AUC – evening AUC

[5]Estimated effects expressed as difference in log-terms

[6]Estimated effects expressed as ratio.