

Changes in PYY and gastric emptying across the phases of the menstrual cycle and the influence of the ovarian hormones

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1 **Title:** Changes in PYY and gastric emptying across the phases of the menstrual cycle and the
2 influence of the ovarian hormones

3

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17 **Abstract**

18 Nutrition-related studies avoid the participation of pre-menopausal women due to the potential
19 effect of the menstrual cycle (MC) on their appetite regulation. It is generally accepted that women
20 increase their energy intake during the luteal phase (LPh) compared to the follicular (FPh), however
21 what happens in the menstrual phase (MPh) and how this might be regulated remains uncertain.
22 Although some research indicates changes in the gastric emptying (GE) velocity, whether PYY is
23 affected by the MC phase, remains unknown. The aim of this study was to assess whether eating the
24 same breakfast in each of the three MC phases would change the GE time, the PYY response and post-
25 prandial satiety such that they might affect subsequent food intake. Furthermore, the aim was to
26 associate any potential differences to the fluctuations in estradiol (E₂) and progesterone (P₄) within a
27 MC. Nine naturally cycling women attended to the laboratory to consume a standardised breakfast on
28 three occasions, each of them representing one of the MC phases. Breath samples to measure GE
29 time, plasma samples to quantify PYY levels and hunger scores were collected for a total of 4 hours
30 after which food intake was assessed by an *ad-libitum* buffet lunch. GE and PYY levels changed
31 significantly across the phases of the MC (p <0.05). GE was correlated to P₄ and E₂-P₄ ratio (r = -0.5 and
32 0.4, respectively). To conclude, the appetite regulators PYY and GE time change depending upon the
33 MC phases with GE time associated with the ovarian hormone levels which suggests the necessity of
34 controlling the MC phase in studies looking at the appetite response.

35

36 **Keywords:** menstrual cycle, PYY, gastric emptying, ovarian hormones.

37

38 **Abbreviations**

| | | | |
|----------------|----------------------|-------------------|-----------------------|
| AUC | Area under the curve | T _{asc} | Ascension time |
| E ₂ | Estradiol | T _{half} | Half time |
| EI | Energy intake | T _{lag} | Lag phase |
| FPh | Follicular phase | T _{lat} | Latency time; |
| GE | Gastric emptying | VAS | Visual Analogue Scale |
| LPh | Luteal phase | | |
| MC | Menstrual cycle | | |
| MPh | Menstrual phase | | |
| P ₄ | Progesterone | | |

39

40 **Introduction**

41 It is well known that the process of digesting food involves numerous actions by different organs
42 in order to prepare food for its absorption in the intestine. This action is regulated by different gastric
43 and intestinal hormones (e.g. gastrin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1)) that will
44 ensure the availability of the intestine to continue the digestive and absorptive process (Smolin &
45 Grosvenor, 1994). Peptide tyrosine-tyrosine (PYY) is one of the multiple regulators of the digestion
46 process and its main role is to mediate the ileal brake, i.e. the delay in the transit of the chyme through
47 the gastrointestinal tract (Onaga, Zabielski, & Kato, 2002), that results in an increase in satiety.
48 Furthermore, its satiating action is also known to originate in the central nervous system as PYY can
49 cross the blood-brain barrier and target areas known to regulate the homeostatic e.g. hypothalamus
50 and the hedonic e.g. caudolateral orbital frontal cortex, circuits (Batterham et al., 2007). PYY's
51 secretion in the distal intestine is stimulated post-prandially and this is related to the caloric and
52 macronutrient content of the meal (Adrian et al., 1985; Batterham et al., 2003).

53 Multiple studies have shown how changes in gastric emptying (GE) speed and PYY response to a
54 meal-test can have an impact on appetite sensations and subsequent food intake (Clegg & Shafat,
55 2010; Stoeckel, Weller, Giddings, & Cox, 2008). Nevertheless, many of the studies conducted in this
56 area avoid the participation of women or control their protocol by testing women at a specific phase
57 of the menstrual cycle (MC), as it is generally accepted that women can experience changes in their
58 habitual food intake upon the phase of their MC (Buffenstein, Poppitt, McDevitt, & Prentice, 1995;
59 McNeil & Doucet, 2012). These changes seem to result from a bigger meal size (rather than from an
60 increased number of meals) in the luteal phase (LPh) than the follicular (FPh) (Asarian & Geary, 2013).
61 Therefore, it could be suggested that women may experience changes in their food intake due to
62 fluctuations experienced primarily in their satiation (the process of finishing meal), rather than their
63 satiety (the process inhibiting the start of a meal), throughout the MC.

64 In fact, Brennan et al. (2009), who assessed food intake from a buffet 90 min after providing a
65 glucose load to nine healthy women on three days of the MC (two in the FPh and one in the LPh),
66 found that food and energy intake (EI) during LPh was significantly higher compared to FPh (~50 g and
67 ~700 kJ difference, respectively). This was related to a faster emptying of the stomach, the time
68 needed for emptying 50% of the gastric glucose during LPh was 15 min less than during the FPh. In
69 addition, there was a higher post-meal release of GLP-1, blood glucose and plasma insulin levels in the
70 LPh, thus the glycaemia response was improved when P₄ was low in the FPh. Finally, CCK response
71 showed no changes despite the differences in hunger and EI between phases. Nevertheless this was
72 not entirely unexpected as CCK secretion seems to be more affected by fat and protein intake rather

73 than glucose (Liddle, Goldfine, Rosen, Taplitz, & Williams, 1985). Whether modifications in the
74 appetite responses are maintained with a full breakfast and whether there would be any differences
75 during the menstrual phase (MPH) has not been previously studied. The latter seems of importance
76 as both ovarian hormones, estradiol (E₂) and progesterone (P₄), are found at very low concentrations,
77 in contrast to the other two phases. Having a better understanding of women's appetite physiology
78 seems imperative in light of the global higher obesity prevalence in women than men (WHO, 2015).

79 The objective of the present study was to assess whether eating the same breakfast in each of
80 the three MC phases would change the GE time, PYY response and satiety feelings of the meal to
81 ultimately have an impact on the food intake of a buffet lunch served four hours later. Furthermore,
82 the aim was to associate any potential differences to the naturally occurring fluctuations in E₂ and P₄
83 of the MC. We finally aimed to investigate whether food intake recorded during three days for each
84 MC phase changed significantly.

85

86 **Material and methods**

87 *Participants*

88 Participants were recruited by posters placed in Oxford Brookes University facilities e.g. library,
89 sport centre, student accommodation, and also in local libraries or gyms, as well as on social media.
90 Moreover, the study was advertised in the Oxford Brookes University Research Activity Group, on the
91 Functional Food Centre website and in the volunteers section of a local website.

92 The inclusion criteria comprised of women between 18-40 y with regular MC for the last three
93 months that lasted between 25 and 35 days and excluded those who were taking hormonal
94 contraceptives, were pregnant, lactating or had any metabolic/genetic diseases or taking any
95 medications known to interfere with their metabolism. In addition, participants who had an
96 allergy/intolerance to any of the foods given in the study, did not consume breakfast and lunch
97 habitually or were attempting to lose weight were also excluded. Finally, smokers and participants
98 with a disease (e.g. Gilbert's syndrome) or taking medication known to interfere with appetite (e.g.
99 codeine) or those who showed to be restrictive eaters were also excluded. The latter was assessed by
100 the combination of two adapted restrictive eating questionnaires: the Dutch Eating Behaviour
101 Questionnaire (DEBQ) (van Strien, Frijters, Bergers, & Defares, 1986) and the Three-factor eating
102 questionnaire – restraint eating (TFEQ FI) (Stunkard & Messick, 1985). Participants with a TFEQ score
103 of >10 and a DEBQ >2.5 were considered restrictive eaters and were excluded from participating in
104 the study.

105 Ethical approval for the study was obtained from the University Research Ethics Committee at
106 Oxford Brookes University. All participants gave written informed consent prior to commencing the
107 study.

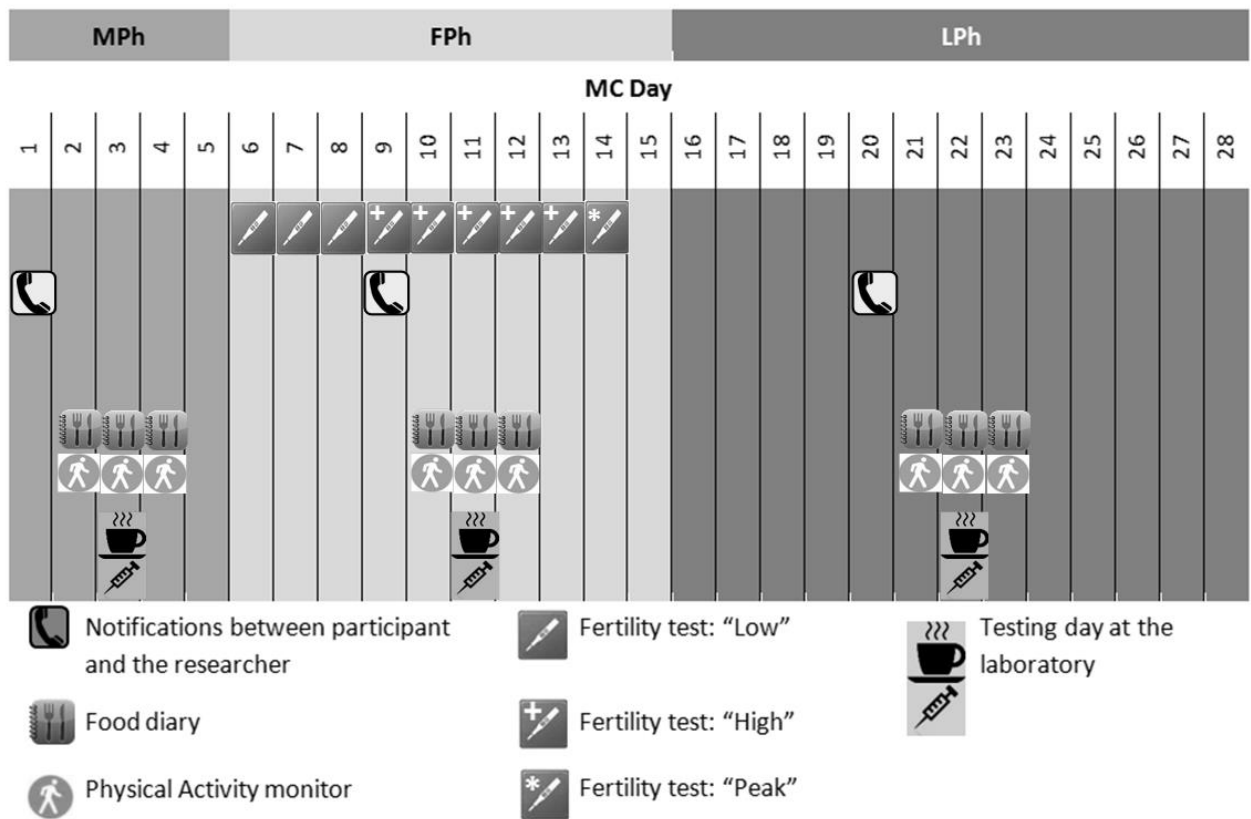
108

109 *Protocol*

110 Once the participant agreed to participate in the study, she was given a fertility monitor (Clear
111 Blue Advanced Fertility Monitor, Clearblue) to assist in the scheduling of visits to the laboratory based
112 on the three different MC phases i.e. MPh, FPh and LPh. The three chosen days were aimed to display
113 a very distinguishable profile in the ovarian hormones: MPh, E₂ and P₄ at low concentrations; FPh, E₂
114 at high concentrations while P₄ remains low; and LPh, E₂ and P₄ at high concentrations. The MPh visit
115 was scheduled as soon as the participant notified the start of a new MC (i.e. day 1) and this was
116 performed within 4 days of starting the MC. From day 6 of the MC, participants tested their morning
117 urine using the fertility monitor to measure their oestrone-3-glucuronide (E3G) and luteinising
118 hormone (LH) levels. When participants obtained the 'high' reading (i.e. E3G levels were increased)
119 they notified the researcher who scheduled the next testing session based on the cycle day of the high
120 reading, the MC length history of the participant and the fact that it usually takes approximately five
121 days to reach to 'peak' after a 'high' reading (Howards et al., 2009), in order to test the participant at
122 very high levels of E₂. Once the 'peak' reading (i.e. LH levels were high) appeared, the last session was
123 scheduled to test when P₄ was at its highest values (in the mid-luteal phase) based on the peak day
124 and the usual MC length of the participant. When participants did not reach 'peak' they were asked
125 to postpone their LPh testing session until the next cycle to ensure that the P₄ levels were high enough
126 to produce any potential effects on the parameters studied (i.e. PYY response, GE time, appetite
127 feelings and food intake).

128 Once the visit to the laboratory was scheduled, participants were also asked to record their food
129 intake for three days in each MC phase: (1) the day before coming to the laboratory, (2) the testing
130 day and (3) the day after the visit to the laboratory. An example testing timeline within a MC is given
131 in Fig 1. In addition, participants were asked to wear a body monitoring system (SenseWear®,
132 BodyMedia) to estimate their PA levels to facilitate the validation of the EI from the food diary and
133 detect any potential misreporting.

134 In the evening before each test day, participants were asked to avoid the consumption of caffeine
135 and alcohol and any strenuous exercise that they would not usually do as part of their normal daily
136 lifestyle.

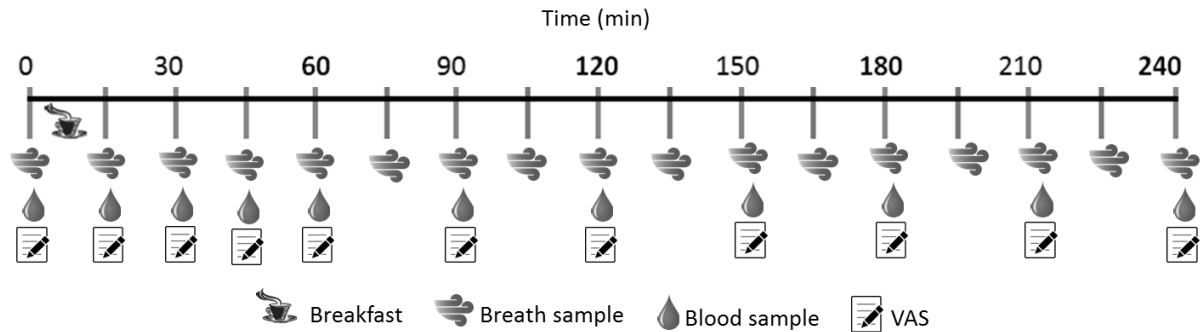


138

139 **Fig 1.** Example experiment timeline during a MC.

140 During the visits to the laboratory, participants were requested to arrive between 7:00-9:30h to
 141 have their body composition assessed by electrical bioimpedance, Tanita Body Composition Analyzer
 142 BC-418MA (Tanita Ltd, West Drayton, UK) and then a cannula (BD Venflon Pro Safety 20GA, Becton
 143 Dickinson Induction Therapy, Singapore) was inserted into a vein of the anti-cubital fosse of the arm
 144 to obtain the baseline blood sample (t = 0). The cannula was kept patent by flushing 0.9% sodium
 145 chloride into the system with a needle-free syringe after collecting each sample. Immediately after,
 146 participants filled in the visual analogue scale (VAS) for appetite sensations and the first breath sample
 147 for the measurement of GE was collected. Then the participant consumed the standardised breakfast
 148 labelled with ¹³C octanoic acid; the breakfast consisted of scrambled eggs on toast, pineapple and a
 149 drink of their choice (water, coffee or tea with/out milk and sugar). The breakfast was standardised
 150 amongst participants and provided 375-395 kcal of which 35%, 38% and 23% were in the form of fat,
 151 carbohydrate and protein, respectively. The energy provided by the breakfast accounted for 17-18 %
 152 of the total daily energy requirements for an average woman (19-44 years) with median physical
 153 activity level of 1.63 i.e. 2103-2175 kcal/d (SACN 2011). Participants were asked to finish their
 154 breakfast within 15min. As soon as they finished their breakfast, the first post-ingestion breath

155 sample, blood sample and satiety scores were collected. Subsequent breath samples were taken every
 156 15 min until 240 min. Subsequent blood samples and satiety scores were taken every 15 min until t =
 157 60 min thereafter every 30 min until t = 240 min (Fig 2).



158

159 **Fig 2.** Timeline of events during each of the testing days in the laboratory.

160 Immediately after the last blood sample, the cannula was removed and the participant was
 161 offered an *ad-libitum* lunch buffet composed by a variety of dishes/foods. The selected foods were
 162 chosen with the aim to satisfy all tastes and possible conditions (e.g. lactose intolerance, vegetarian
 163 diets, etc.) thus food intake was not restrained by choice or quantity (Table 1). Participants were
 164 invited to eat until comfortably full within 30 min.

165 **Table 1.** Foods available in buffet lunch with nutritional composition per portion provided.

| Food | Serving | | Energy | | Fat (g) | Carbs (g) | Fibre (g) | Prot (g) | Salt (g) |
|------------------------|---------|-----|--------|--------|------------|--------------|--------------|-------------|-------------|
| | units | g | (kJ) | (kcal) | | | | | |
| Hummus | | 50 | 664 | 161 | 14 | 4 | 2 | 4 | 1 |
| Apples Gala | 1 | 135 | 304 | 71 | 0 | 16 | 2 | 1 | 0 |
| Banana | 1 | 158 | 636 | 150 | 0 | 36 | 4 | 2 | 0 |
| Clementines | 2 | 226 | 398 | 95 | 0 | 20 | 3 | 2 | 0 |
| Carrots | | 70 | 123 | 30 | 0 | 6 | 2 | 0 | 0 |
| Celery sticks | | 80 | 32 | 8 | 0 | 1 | 1 | 0 | 0 |
| Tomatoes | 18 | 142 | 119 | 28 | 0 | 4 | 1 | 1 | 0 |
| Potato Salad * | | 270 | 1858 | 448 | 35 | 28 | 3 | 4 | 1 |
| Tuna & Sweetcorn Pasta | | 295 | 2295 | 549 | 25 | 59 | 3 | 20 | 1 |
| Moroccan Couscous | | 245 | 2112 | 502 | 16 | 75 | 11 | 9 | 0 |
| Bright Salad | | 83 | 93 | 22 | 0 | 3 | 2 | 1 | 0 |
| Cheese, Babybel | 4 | 95 | 1207 | 291 | 23 | 0 | 0 | 21 | 2 |
| Low-fat Yoghurt | 1 | 120 | 406 | 96 | 1 | 19 | 0 | 3 | 0 |
| Sausages | 8 | 66 | 779 | 187 | 14 | 7 | 1 | 9 | 1 |
| Chicken Nuggets | 5 | 77 | 770 | 185 | 10 | 14 | 1 | 10 | 0 |
| Cheese & Tomato Pizza | 1 | 160 | 1934 | 459 | 12 | 68 | 2 | 18 | 1 |
| Bread sticks | | 19 | 331 | 78 | 1 | 14 | 1 | 2 | 0 |
| Crisps, ready salted | 1 bag | 24 | 527 | 126 | 8 | 12 | 1 | 1 | 0 |

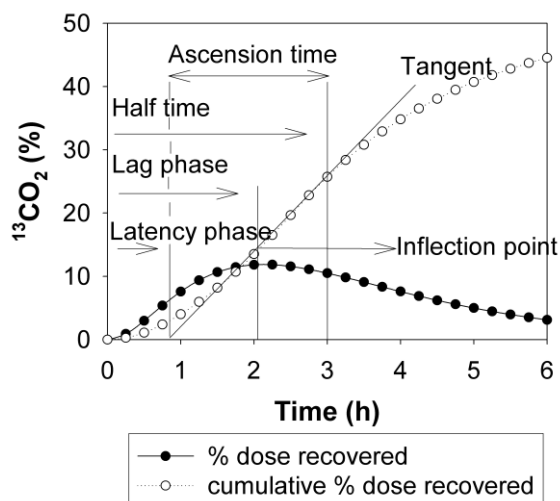
| | | | | | | | | | |
|----------------------------------|-----------|------|-------|------|-----|-----|----|-----|----|
| KitKat | 4 fingers | 45 | 958 | 229 | 11 | 29 | 1 | 3 | 0 |
| Orange Juice | | 500 | 985 | 230 | 0 | 54 | 0 | 5 | 1 |
| Water | | 500 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Egg Mayonnaise Sandwich * | | 139 | 1202 | 287 | 13 | 26 | 5 | 14 | 1 |
| Chicken Sweetcorn Sandwich* | | 139 | 1199 | 287 | 12 | 29 | 5 | 14 | 1 |
| TOTAL (non-vegan buffet) | | 3594 | 17974 | 4291 | 184 | 494 | 51 | 142 | 11 |
| Bean & Mint Salad Y | | 215 | 1288 | 310 | 13 | 26 | 14 | 15 | 2 |
| Soya Fruit Yoghurt Y | 1 | 129 | 396 | 94 | 3 | 12 | 2 | 5 | 0 |
| Vegetable Spring Rolls Y | 6 | 115 | 1173 | 281 | 14 | 33 | 3 | 6 | 0 |
| Peanut Butter Sandwich Y | | 113 | 1477 | 355 | 20 | 23 | 7 | 17 | 0 |
| Beetroot, Mint hummus Sandwich Y | | 109 | 755 | 180 | 5 | 23 | 6 | 7 | 1 |
| TOTAL (vegan buffet) | | 2914 | 11413 | 2721 | 95 | 361 | 62 | 78 | 6 |

166 Nutritional composition of the foods was based on manufacturer's information. Carbs, carbohydrates; Prot,
 167 protein. * Item removed in the vegan buffet. Y Item included in the vegan buffet only.

168

169 *Measurements*

170 Gastric emptying Breath samples were collected by blowing into a small glass tube (Labco
 171 Exetainer, Labco Limited, UK) through a straw while having the nose blocked with a nose-clip.
 172 Participants blew into the tube while removing the straw to immediately cap the tube which was then
 173 stored at room temperature for analysis. Breath samples were analysed using an isotope ratio mass
 174 spectrometer (ABCA, Sercon Ltd, Chesire UK) to quantify the excess amount of labelled oxidised
 175 octanoic acid (i.e. $^{13}\text{CO}_2$) above baseline for each time point, as previously described elsewhere (Clegg
 176 & Shafat, 2010). This was expressed as the percentage of dose recovered per hour and this was fitted
 177 into a non-linear regression model (Ghoos et al., 1993). From this model several parameters were
 178 measured. Lag phase (T_{lag}) and half time (T_{half}) were calculated using the formulae derived by Ghoos
 179 et al. (1993). T_{lag} is the time taken to maximal rate of $^{13}\text{CO}_2$ excretion (Jackson, Bluck, & Coward, 2004)
 180 and is equivalent to the time of the inflection point (Schommartz, Ziegler, & Schadewaldt, 1998). T_{half}
 181 is the time it takes 50% of the ^{13}C dose to be excreted (Jackson et al., 2004). Latency phase (T_{lat})
 182 (Schommartz et al., 1998) is the point of intersection of the tangent at the inflection point of the $^{13}\text{CO}_2$ -
 183 excretion curve representing an initial delay in the excretion curve. Ascension time (T_{asc}) (Schommartz
 184 et al., 1998) is the time course between the T_{lat} and T_{half} , representing a period of high $^{13}\text{CO}_2$ -excretion
 185 rates (Fig. 3).



186

187 **Fig 3.** GE time points (Clegg & Shafat, 2010)

188 PYY and E₂ and P₄ levels. Blood samples were collected with K2E-EDTA tubes (BD Vacutainer,
 189 Becton Dickinson, UK). A 4 ml blood sample was withdrawn from the cannula for every time point,
 190 except for the baseline when 8 ml were collected to measure the ovarian hormones. After collection,
 191 blood samples were kept in ice until they were centrifuged at 4°C for 10 minutes at 4000 rpm (MC-6,
 192 Sarstedt Ltd, Leicester, UK) to extract the plasma. These were then frozen at -80°C in different aliquots
 193 until analysis. E₂ and P₄ levels were measured by an ElectroChemiLuminescence immunoassay (ECLIA)
 194 with a Cobas e411 semi-automated analyser (Roche diagnostics Burgess Hill, UK) and total PYY
 195 concentrations were assessed with a direct sandwich enzyme-linked-immunosorbent assay (ELISA) kit
 196 (EMD Millipore). Samples of the same participant in the three phases of the MC were analysed within
 197 the same ELISA plate. Averaged intra-duplicates coefficient of variance (CV) for the total PYY ELISA
 198 assay was 6.3 ± 1.4 %. Averaged inter-plate CV for the quality controls of the PYY ELISA assay was 13.3
 199 ± 4.1 %.

200 Appetite sensations. Feelings of satiety were assessed by four questions (1) ‘How hungry do you
 201 feel?’, (2) ‘How full do you feel?’, (3) ‘How strong is your desire to eat?’ and (4) ‘How much food do
 202 you think you can eat?’ in which participants had to rate their appetite sensations with the VAS,
 203 namely, by putting a mark in a 100 mm line per each question, where 0 = (1) ‘not hungry at all’, (2)
 204 ‘extremely full’; (3) ‘not strong at all’ and (4) ‘nothing at all’ and 100 = (1) ‘extremely hungry’, (2) ‘not
 205 at all full’; (3) ‘extremely strong’ and (4) ‘a large amount’. The distance between the origin (score = 0)
 206 and the mark was used to measure the participant’s score.

207 Ad libitum food intake. The researcher weighed out all the foods before and after the participant
 208 had lunch and then food intake was analysed using an excel spreadsheet designed from the

209 manufacture's food information provided in the food label. Ad-libitum food intake assessment
210 included the measurement of energy, carbohydrate, protein, sugar, fat, saturated fat, fibre and
211 sodium.

212 Food intake from food diaries. For three days of each MC phase participants were asked to weigh
213 out and record all the foods and beverages consumed with as much detail as possible (e.g. brand,
214 cooking process). If participants could not weigh out a meal, they were asked to provide portion sizes
215 by using household measures (e.g. cups) and/or by taking pictures of the foods eaten. The selected
216 days of each phase included one of the visits to the laboratory (on day 2 of the 3-days), therefore
217 participants had to only record anything consumed after leaving the testing facilities on the test day.
218 Food intake recorded was measured by the use of a nutrition analyses software program (Nutritics
219 V3.74 Professional Edition) and intakes of energy, carbohydrate, sugar, protein, fat, saturated fat, fibre
220 and sodium were determined per day and per phase of the MC for each participant.

221 Physical activity. Participants were requested to wear the body monitoring system on the upper
222 right arm (triceps muscle) throughout the day (24 hours) except during activities in which the skin is
223 in contact with water (e.g. showering) as the equipment instructions advise (Body Media, 2006). Data
224 was downloaded and analysed as total daily energy expenditure (kcal/d) using the BodyMedia
225 software once individual characteristics (i.e. date of birth, height, weight, sex) were entered into the
226 system. Averaged daily energy expenditure across the same nine days as the food diaries were
227 recorded. This was then compared to the energy intake estimated from the food diaries.

228

229 *Calculations and statistical analyses*

230 PYY peak was defined as the highest PYY concentrations achieved post-baseline. Concentrations
231 of PYY were used to calculate the total area under the curve (AUC) using the trapezoidal method at
232 min 60, 120, 180, 210 and 240 from baseline (before breakfast).

233 Each appetite sensation question was analysed separately by calculating the derived AUC from
234 the scores of all the time points. AUC was calculated using the trapezoidal method at min 60, 120, 180,
235 210 and 240 from baseline. The employment of VAS has been validated in many studies and the use
236 of total AUCs with baseline levels as covariates has been recommended over individual time scores or
237 incremental AUCs within participants (Blundell et al., 2010).

238 One-way repeated measures ANOVA or Friedman test was used to test differences across the
239 phases of the MC for PYY AUCs, ovarian hormone levels, GE parameters and food intake across the

240 phases of the MC. When significant differences were found, a Bonferroni post-hoc pairwise
241 comparison or a Wilcoxon signed-rank test was performed, according to the normality of the data. A
242 2-way repeated measures ANOVA with time and MC phase as factors was used to analyse the change
243 in PYY levels from baseline within subjects as an assessment of the post-prandial changes across the
244 MC. AUC for VAS was analysed with another 2-way repeated measures ANOVA that included the
245 baseline scores as covariates in the analyses.

246 Associations between EI and PYY, GE and appetite feelings as well as between ovarian hormones
247 and the appetite markers (i.e. EI and PYY, GE and appetite feelings) were analysed by Pearson's or
248 Spearman's correlation, according to the normality of the data.

249 A sample size of nine women was based on the only other study that has looked at appetite
250 hormones responses in the MC (Brennan et al., 2009).

251

252 **Results**

253 *Participants characteristics*

254 Fifteen women signed the consent form of which three had to be excluded because of violating
255 the inclusion criteria (i.e. irregular MC and suspicion of suffering Gilbert's syndrome). Of the twelve
256 women who started the study, two withdrew due to personal reasons and another who completed
257 the study had to be excluded because of unconfirmed ovulation and unavailability to reschedule the
258 LPh testing day. Thus the following results are based on a population of nine NC women (Table 2).

259 **Table 2.** Participants' characteristics at baseline

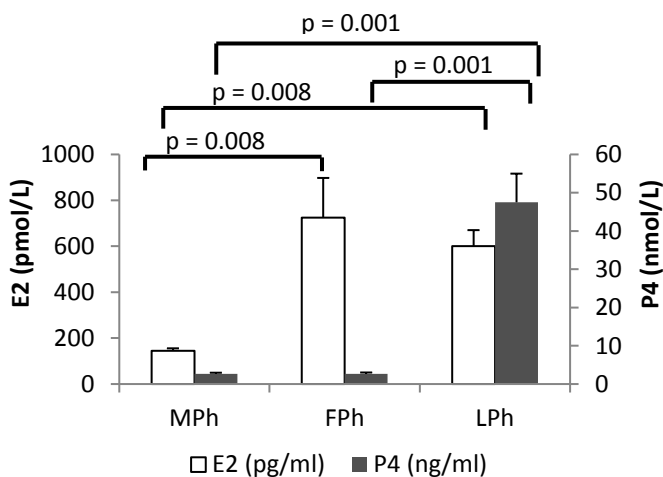
| | Mean \pm SD |
|--------------------------|-----------------|
| Age (years) | 31 \pm 6 |
| Height (m) | 1.67 \pm 0.09 |
| Body weight (kg) | 63.4 \pm 12.8 |
| BMI (kg/m ²) | 22.6 \pm 2.7 |
| Fat Mass Percentage (%) | 29.0 \pm 7.4 |
| Fat Mass (kg) | 19.1 \pm 7.8 |
| Fat Free Mass (kg) | 44.4 \pm 6.3 |
| Waist-to-hip ratio | 0.77 \pm 0.07 |

260

261 *MC characteristics and ovarian hormones*

262 Average MC length was 29 ± 3 days. Of the nine participants included, four had a “peak” reading
 263 i.e. ovulation was confirmed by the fertility monitor, within their first MC, while three participants
 264 only ovulated on the second MC. Averaged “peak” reading happened on day 14 ± 3 of the MC. The
 265 two remaining participants were asked to attend to the laboratory when it was expected to be their
 266 mid-LPh despite not having had a peak reading in the fertility monitor. Nevertheless, plasma P₄ levels
 267 indicated that these participants had ovulated as P₄ concentrations were > 15.9 nmol/L which is
 268 considered high enough to have ovulated (Piers et al., 1995). Moreover, one of these two participants
 269 had a positive LH peak in her personal fertility monitor, thus participants were kept in the study as
 270 they seemed to have ovulated despite not having been detected by the fertility monitor used in the
 271 study.

272 There were significant differences in E₂ and P₄ concentrations amongst the three phases of the
 273 MC ($p < 0.001$ and < 0.0001 , respectively). E₂ levels were significantly increased in the FPh and LPh
 274 compared to the MPh, and P₄ levels were significantly higher in the LPh compared to the other two
 275 phases (Fig 4).



276
 277 **Fig 4.** E₂ and P₄ concentrations in the different phases of the MC (means \pm SD).

278
 279 *GE*

280 There was a significant overall effect of the phase of the MC on T_{half} and T_{asc} (Table 3) but none of
 281 the specific comparisons between phases indicated a significant difference. However the effects
 282 observed seem to suggest trends that T_{half} was quicker in the LPh compared to the FPh and the MPh
 283 (mean difference: 28 ± 31 and 13 ± 15 min, $p = 0.081$ and 0.092 , respectively) and T_{asc} was faster in
 284 the LPh compared to the FPh (mean difference: 27 ± 29 min, $p = 0.077$). There was a trend towards a

285 difference in T_{lag} across the phases of the MC ($p = 0.072$). No differences were found in T_{lat} across the
 286 phases of the MC.

287 **Table 3.** GE parameters shown in minutes for MPh, FPh and LPh.

| GE parameter (min) | MPh | FPh | LPh | p |
|--------------------|----------|----------|----------|-------|
| T_{half} | 101 ± 23 | 116 ± 46 | 88 ± 22 | 0.015 |
| T_{lag} | 48 ± 8 | 51 ± 14 | 43 ± 12 | 0.072 |
| T_{lat} | 52 ± 7 | 53 ± 12 | 48 ± 13 | 0.264 |
| T_{asc} | 128 ± 23 | 143 ± 41 | 116 ± 13 | 0.011 |

288 T_{half} , half time; T_{lag} , lag phase; T_{lat} , latency time; T_{asc} , ascension time. Mean ± SD

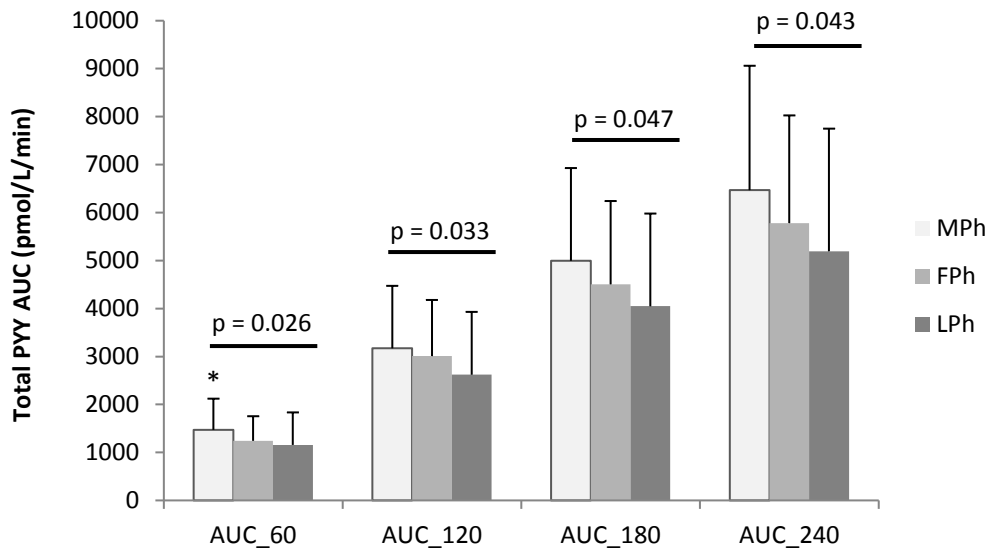
289

290 *Total PYY*

291 Due to blood collection issues, a total of four samples (1%) could not be obtained. These were
 292 the 150-240 min samples of one participant's FPh, therefore, comparisons from min 150 onwards are
 293 only from 8 participants.

294 PYY levels were significantly different at baseline across the phases of the MC ($p = 0.004$), being
 295 significantly lower in the LPh compared to the MPh (14.97 ± 10.11 vs 22.81 ± 11.89 pmol/L) ($p = 0.008$),
 296 but not to the FPh (16.22 ± 7.08 pmol/L, $p = 0.079$). PYY peak was lower in the LPh compared to the
 297 MPh and FPh (29.20 ± 12.38 vs 33.35 ± 11.83 and 32.94 ± 12.00 pmol/L, respectively) but it was not
 298 significantly different ($p = 0.264$).

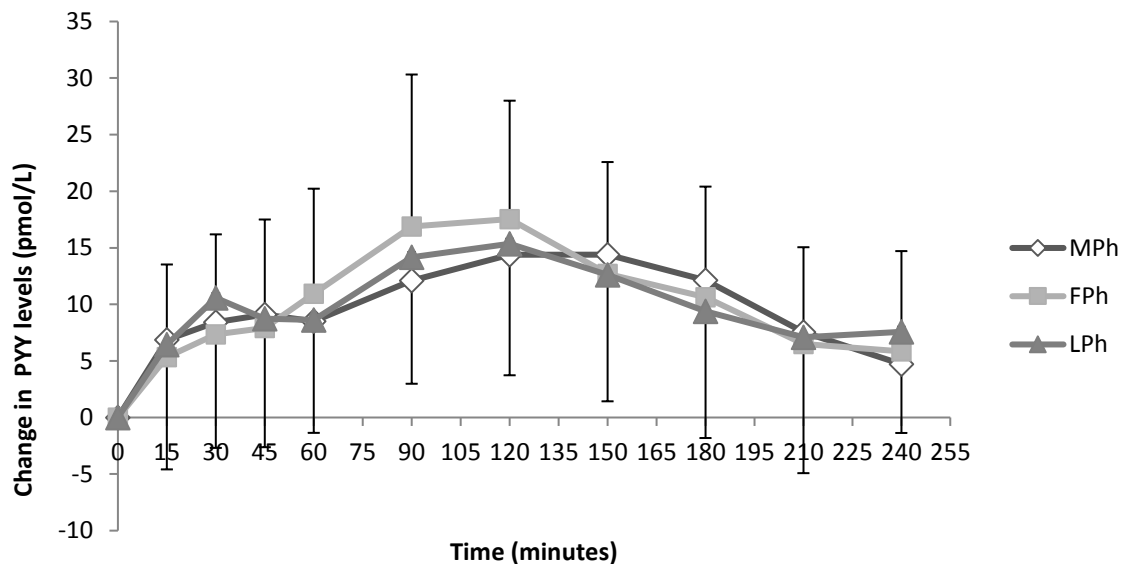
299 There was a significant overall effect on PYY AUC at $t = 60, 120, 180$ and 240 min, but only at $t =$
 300 60 min there was a significant difference between specific phases, i.e. LPh vs. MPh (1157 ± 678 vs. 1471
 301 ± 650 pmol/ml/min) ($p = 0.021$) (Fig 5). However the effects observed seem to mainly reflect that the
 302 PYY AUCs at $t = 120, 180$ and 240 min were smaller in the LPh compared to the MPh ($p = 0.066, 0.129$
 303 and 0.113 , respectively).



304

305 **Fig 5.** Total PYY AUCs at t = 60, 120, 180 and 240 min in the different phases of the MC (means ±
 306 SD). * Significantly different to the LPh within the same time AUC.

307 The 2-way ANOVA analyses looking at the change in PYY levels from baseline to every time point
 308 showed that only *time* had a significant effect ($p < 0.001$), whereas *phase* or *phase x time* interaction
 309 had no statistical effect on PYY change ($p = 0.846$ and 0.213 , respectively) (Fig 6).



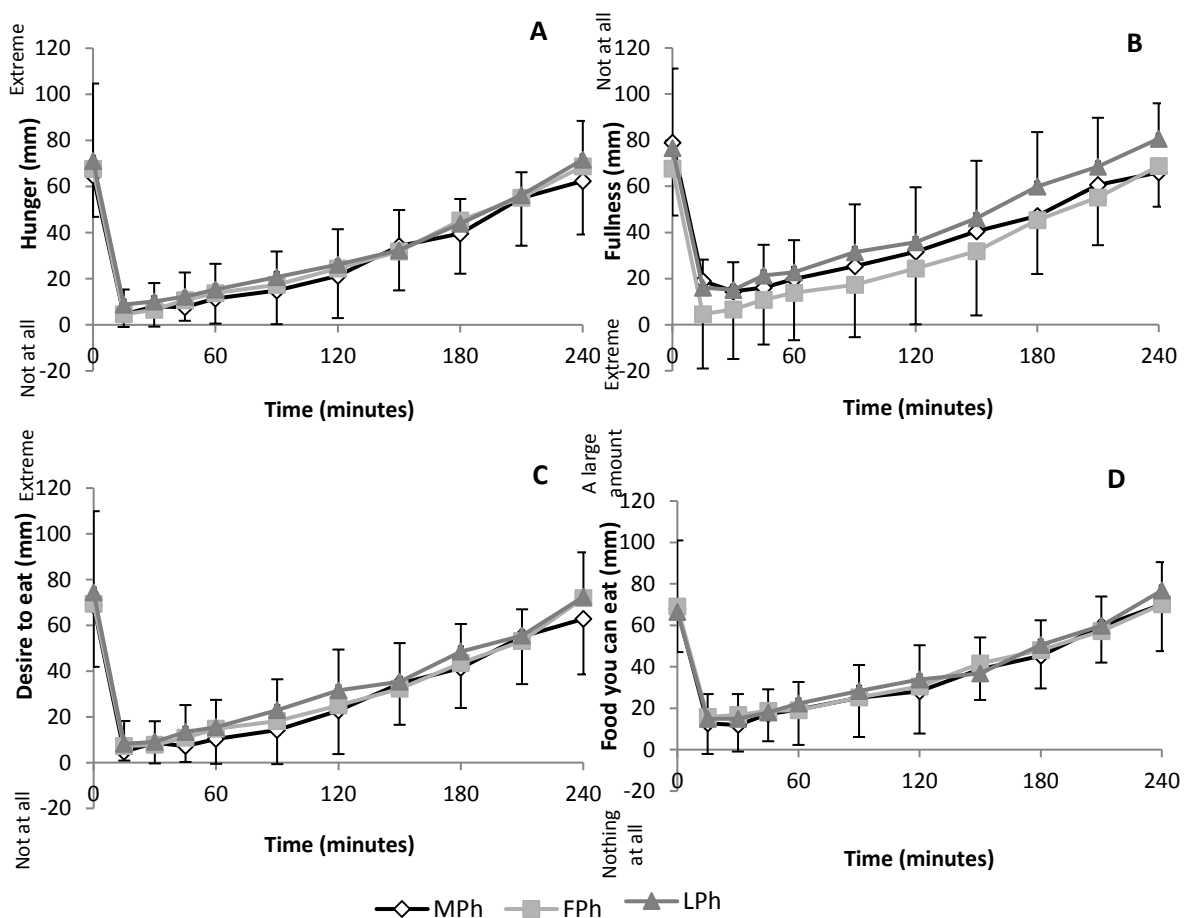
310

311 **Fig 6.** Change in PYY levels from baseline to each time point in the different phases of the MC
 312 (Means ± SD).

313

314 *Satiety ratings*

315 There were no significant differences in AUC for any of the four satiety questions when analysing
 316 them in a two-way-ANOVA (*time x phase*) with the baseline measurements as covariates. AUC at the
 317 end of the 4 hours test for “*how hungry do you feel?*”; “*how full do you feel?*” “*how strong is your desire*
 318 *to eat?*” and “*how much food do you think you can eat?*” were 6932 ± 2961 , 7404 ± 2010 and $7802 \pm$
 319 2080 mm/min; 8924 ± 3153 , 10358 ± 4261 and 10340 ± 3638 mm/min; 7039 ± 3147 , 7479 ± 2108 and
 320 8268 ± 2388 mm/min for MPh, FPh and LPh, respectively (Fig 7).



321 **Fig 7.** Appetite sensations scores (mm) before and after breakfast in the different phases of the
 322 MC: (1) ‘*How hungry do you feel?*’ (A), (2) ‘*How full do you feel?*’ (B), (3) ‘*How strong is your desire*
 323 *to eat?*’ (C) and (4) ‘*How much food do you think you can eat?*’ (D). (Means \pm SD)

324

325

326 *Ad-libitum, post-lunch and averaged food intake*

327 For this section of the results, a participant’s data was excluded as her eating behaviour and food
 328 diary analyses showed a strong indication that she was restricting her EI during the *ad-libitum* buffet-
 329 lunch as well as underreporting her food intake in the food diary.

330 During the buffet lunch there were no significant differences in EI, carbohydrate, protein or fat
 331 intake between phases of the MC (Table 4). Similarly no differences were observed in food intake once
 332 participants left the laboratory. In addition, as an average of the three days in each MC phase, non-
 333 significant differences were found in food intake. Finally, food intake as an average of the day before
 334 and after the laboratory visit, i.e. food intake under free-living conditions, did not change significantly
 335 for energy, carbohydrate, fat or protein intake across the MC.

336 **Table 4.** Food intake during and after the *ad libitum* lunch and as an average of the three
 337 measured days in each MC phase.

| | MPh | FPh | LPh |
|--------------------------------------|------------|------------|------------|
| <i>Ad libitum</i> lunch | | | |
| Energy (kcal) | 931 ± 193 | 984 ± 178 | 956 ± 194 |
| Carbohydrate (g) | 113 ± 20 | 119 ± 20 | 116 ± 27 |
| Fat (g) | 38 ± 11 | 41 ± 10 | 39 ± 8 |
| Protein (g) | 29 ± 6 | 30 ± 6 | 30 ± 7 |
| <i>After Ad libitum</i> lunch | | | |
| Energy (kcal) | 1131 ± 339 | 1308 ± 660 | 1192 ± 485 |
| Carbohydrate (g) | 134 ± 47 | 156 ± 93 | 141 ± 54 |
| Fat (g) | 42 ± 14 | 52 ± 25 | 56 ± 31 |
| Protein (g) | 43 ± 21 | 39 ± 16 | 34 ± 18 |
| Average of 3 days | | | |
| Energy (kcal) | 2352 ± 358 | 2368 ± 604 | 2443 ± 412 |
| Carbohydrate (g) | 271 ± 41 | 274 ± 70 | 279 ± 54 |
| Fat (g) | 101 ± 21 | 97 ± 25 | 106 ± 22 |
| Protein (g) | 84 ± 17 | 77 ± 10 | 85 ± 15 |
| Free-living conditions | | | |
| Energy (kcal) | 2292 ± 146 | 2203 ± 598 | 2386 ± 520 |
| Carbohydrate (g) | 264 ± 52 | 255 ± 64 | 270 ± 67 |
| Fat (g) | 103 ± 26 | 91 ± 26 | 103 ± 27 |
| Protein (g) | 79 ± 19 | 70 ± 13 | 84 ± 20 |

338 Means ± SD

339

340 *Relationships between PYY, GE, appetite feelings and EI*

341 There was a significant moderate correlation between peak PYY and T_{half} and T_{asc} ($r = 0.396$ and
342 0.410 , $p = 0.041$ and 0.034 , respectively). Moreover, there was a trend for a moderate correlation of
343 PYY AUC at time 180 and 240 with T_{half} and T_{asc} ($r = 0.4$ for all, $p = 0.08$ and 0.07 for T_{half} and T_{asc}
344 correlations, respectively).

345 No significant correlations were found between EI and PYY, GE or appetite feelings.

346

347 *Relationships between appetite markers and the ovarian hormones*

348 There was a moderate negative correlation between T_{half} and T_{asc} and P_4 levels ($r = -0.490$ and -
349 0.426 , $p = 0.010$ and 0.027 , respectively). Moreover, T_{half} and T_{asc} were positively correlated to $E_2:P_4$
350 ratio ($r = 0.437$ and 0.407 , $p = 0.023$ and 0.035).

351 There were no correlations between PYY AUCs or peak PYY and the ovarian hormones. Similarly,
352 no correlations between appetite sensations or food intake and the ovarian hormones were found.

353

354 **Discussion**

355 The aim of this study was to investigate whether appetite responses vary after consuming the
356 same breakfast in the different phases of the MC. This research is of importance in order to extend
357 the current knowledge in appetite regulation in a subset of the adult population who seems to be at
358 a higher risk of developing obesity than men (WHO, 2015).

359 Our results showed that the time to empty half of the breakfast from the stomach to the
360 duodenum (T_{half}) was significantly different across the phases of MC, being on average 28 and 13
361 minutes quicker in the LPh compared to the FPh and MPh, respectively. It could be suggested that
362 the reduction in the GE time (represented by T_{half}) was because of a significantly shorter T_{asc} and,
363 potentially, a faster T_{lag} in the LPh compared to the other phases. In the LPh, high GE rates might have
364 been reached quicker and once attained, these were sustained for a shorter period which resulted in
365 a reduction of the time required to empty the same amount of food from the stomach when compared
366 to the other two phases of the MC. Because T_{asc} was maintained for less time in the LPh, GE rates
367 achieved during that period had to be of a higher velocity to achieve a shorter T_{half} .

368 Faster GE during the LPh in comparison to the FPh has previously been described (Brennan et al.,
369 2009), however, others have found opposite results (Gill, Murphy, Hooper, Bowes, & Kingma, 1987)
370 or no differences (Horowitz et al., 1985; Monés et al., 1993) between these two phases, thus a definite
371 position in this matter cannot be made with the available evidence. Discrepancies amongst studies
372 might be due to different test meals in terms of calories and nutrient composition (Horowitz et al.,
373 1985) and the fact that some women might not have ovulated as this was not tested in all studies
374 (Monés et al., 1993). Furthermore, attention is warranted as our outcome does not only support those
375 who found differences between the LPh and the FPh (Brennan et al., 2009), but also suggests that the
376 GE effect seen in the LPh is large enough to be compared to the MPh, as well. As far as we know, this
377 is the first study to add the MPh as another time point to investigate GE within the MC and our findings
378 suggest that this should be included in future investigations.

379 To our knowledge, this is the first investigation to indicate that fasting and post-prandial PYY
380 levels significantly change amongst the phases of the MC. The results indicate that when participants
381 are fasted in the LPh there are lower PYY levels compared to the MPh. Moreover, the results suggest
382 that PYY response is smaller after the consumption of the same breakfast in the LPh compared to the
383 other MC phases. Nevertheless, the MC effect on the PYY response seems to partly result from the
384 significant differences found at baseline (when fasted) as the statistical significance was lost when
385 looking at the change in PYY levels from baseline.

386 PYY secretion occurs by direct contact depending on the presence of food in the lower intestine
387 (ileum, colon) where the L-cells are located (Fu-Cheng et al., 1995). Its secretion can also start earlier
388 via neural or hormonal mechanisms, by digestive events that occur at upper sections of the
389 gastrointestinal tract, i.e. duodenum and stomach (Fu-Cheng et al., 1995). For instance, there is
390 evidence that gastrin, which is known to stimulate the production of gastric acid, can inhibit the
391 release of PYY as seen in rats (Gomez et al., 1996). Meanwhile, the increase in gastric acid
392 concentrations will trigger the synthesis of PYY as part of the ileal brake of the digestion process, thus
393 creating a feedback loop between the upper and lower gastrointestinal tract. In some (Adamopoulos,
394 Dessypris, Xanthopoulos, & Chryssicopoulos, 1982) but not all (Frick, Bremme, Sjögren, Lindén, &
395 Uvnäs-Moberg, 1990; Uvnäs-Msoberg, Sjögren, Westlin, Anderson, & Stock, 1989) studies, gastrin
396 levels were elevated in the LPh when compared to the FPh which could partly explain the impairment
397 in the PYY release and the consequent unavailability to reduce the GE time in the LPh in our
398 participants. This was supported by the positive correlation found between T_{half} and PYY peak and the
399 tendency for a positive correlation with the PYY AUC at $t = 180$ and 240 min.

400 Another potential mechanism that could have contributed to the different PYY responses would
401 be changes in the CCK secretion. CCK release after the infusion of long-chain fatty acids in the
402 duodenum has been shown to up-regulate PYY secretion by CCK-receptor 1 (Degen et al., 2007), thus
403 if CCK secretion is inhibited in the LPh that could in turn impair PYY release. Brennan et al. (2009)
404 found that CCK secretion was maintained across the MC, although this could have been influenced by
405 the fact that participants only ingested a glucose drink and carbohydrates are known to be less
406 effective in stimulating the CCK than fats (Hildebrand et al., 1990), thus there could still be a potential
407 for CCK modulating the changes in PYY secretion across the phases of the MC.

408 One interesting finding of the current study was the significant negative correlation between P_4
409 and T_{half} . Despite being only a moderate correlation, our results agree with Brennan et al. (2009) and
410 corroborate the idea that the ovarian hormones might have an influence on GE. Furthermore, the fact
411 that the ratio between E_2 and P_4 is also significantly correlated, suggests that both hormones may
412 modulate the changes in the GE process. Although our results did not indicate a direct association
413 between PYY levels and the ovarian hormones, these may have exerted their influence by other factors
414 involved in the digestive process e.g. GE, other appetite-hormonal secretions. Considering the
415 naturally occurring changes in E_2 levels between the MPh and FPh it seemed necessary to investigate
416 three rather than two phases and this was corroborated by the outcome of the study.

417 Although increases in food intake in the *ad-libitum* lunch were expected in the LPh as seen in
418 previous literature (McNeil & Doucet, 2012), our results did not find significant fluctuations in EI or
419 macronutrient intake across the phases of the MC. This could be due to the fact that the majority of
420 the food intake of the day (while in the laboratory) was already purposely kept constant, thus leaving
421 little room for any changes. Nevertheless, food intake under the free-living conditions, which was 183
422 and 94 kcal/d higher in the LPh compared to the FPh and MPh, respectively, was not significantly
423 different throughout the MC phases, either. Despite not reaching the statistical significance,
424 fluctuations were within the spectrum of +50-100 kcal/d which are recognised to be of enough
425 magnitude to induce the progressive development of obesity (Mozaffarian, Hao, Rimm, Willett, & Hu,
426 2011).

427 The unchanged food intake during the lunch buffet may be expected since there were no
428 significant differences in the appetite sensations post-breakfast, suggesting that food intake was
429 responding to actual appetite perceptions and not to other extrinsic factors. However, direct
430 correlations were not found between food intake and appetite sensations which manifest the
431 difficulty in assessing subjective measurements.

432 Although the assessment of food intake in a controlled setting presents important advantages,
433 such as the availability to accurately quantify what is consumed, it also presents several limitations
434 that cannot be ignored. For instance, eating behaviour can be altered due to eating in a non-familiar
435 and unnatural environment, or because of the expectations the participants believe that the
436 researcher might have (Stubbs, Johnstone, O'Reilly, & Poppitt, 1998). Nevertheless, we tried to
437 minimise this effect by providing a sensible variety of foods that the participant could be familiar with.
438 On the other hand, although food diaries can avoid the limitations of the laboratory setting, they can
439 also present different drawbacks such as the misreporting displayed by one of our participants as well
440 as in other studies (de Vries, Zock, Mensink, & Katan, 1994). Therefore, both methods agreed on the
441 idea that there were no significant alterations in food intake throughout the phases of the MC.
442 Inconsistencies with other studies might rely on the limited sample size, although others have proved
443 significant differences with the same number of participants (Dalvit-McPhillips, 1983). Thus,
444 differences between the latter study and ours could partly be due to the dietary assessment
445 techniques employed i.e. dietary interview during 60 days vs. 9 days of food diaries. Finally, although
446 PYY response significantly changed throughout the MC, the magnitude of the change (mean difference
447 in total PYY AUC at min 240 in LPh: 15 and 23% compared to the MPh and FPh, respectively) might not
448 have been substantial enough to elicit modifications in appetite sensations and subsequent food
449 intake.

450 There were limitations to this study. Despite GE time and PYY response showing to be significantly
451 different across the MC, pairwise comparisons could not achieve the statistical significance and that
452 could be due to the small sample size or the inter-individual variation. In fact, if applying a t-test to
453 compare T_{half} between the LPh and FPh or MPh, significant differences would have been found;
454 however, with the ANOVA and the Bonferroni correction, the statistical significance was diminished.
455 Nevertheless, we employed the same sample size used by previous studies (Brennan et al., 2009). The
456 current study did not distinguish the two different forms of PYY, i.e. PYY₁₋₃₆ and PYY₃₋₃₆. It is well known
457 that with food intake PYY₁₋₃₆ is cleaved by the dipeptidyl peptidase IV to PYY₃₋₃₆ and that only the latter
458 form has almost exclusive and high affinity to Y-2 receptors of the ARC. This is of relevance as Y-2
459 receptors are the only subtype of Y-receptors that can induce appetite and body weight suppression
460 by stimulating the activity of the α -MSH through the inhibition of the NPY release (Ballantyne, 2006).
461 Thus, although unlikely, changes in the concentrations of total PYY could respond to alterations in the
462 proportion between the two forms of PYY. Thus it cannot be dismissed that lower PYY response in the
463 LPh was mainly relying on a diminished PYY₁₋₃₆ secretion and conversion, therefore, inducing minimal
464 changes in food intake. Future studies could improve our findings by measuring the two forms of PYY.

465

466 **Conclusion**

467 To our knowledge this is the first study to investigate GE time and PYY response after consuming
468 the same breakfast three times in the MC in which ovarian hormones, E₂ and P₄ presented very
469 distinguishable levels. Our results found significant differences in GE time and PYY response that
470 suggest the LPh as the quickest in GE time with the smallest PYY response of the all MC phases.. Finally
471 changes in the GE time could be influenced by the fluctuations in the ovarian hormones.

472 Further research needs to be done to confirm these findings and to have a better understanding
473 of the underlying mechanisms for these changes in GE time and PYY response across the MC as they
474 could potentially direct us to novel dieting strategies in women. Finally, our findings suggest that any
475 functional food studies aimed to change satiation should take into account the likely modifications in
476 the processing of food that women might experience throughout the MC by re-testing their products
477 in the different MC phases to ultimately be able to demonstrate the effects of a dietary intervention
478 in this population.

479

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483

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