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

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Abstract

Nutrition-related studies avoid the participation of pre-menopausal women due to the potential effect of the menstrual cycle (MC) on their appetite regulation. It is generally accepted that women increase their energy intake during the luteal phase (LPh) compared to the follicular (FPh), however what happens in the menstrual phase (MPh) and how this might be regulated remains uncertain. Although some research indicates changes in the gastric emptying (GE) velocity, whether PYY is affected by the MC phase, remains unknown. The aim of this study was to assess whether eating the same breakfast in each of the three MC phases would change the GE time, the PYY response and post-prandial satiety such that they might affect subsequent food intake. Furthermore, the aim was to associate any potential differences to the fluctuations in estradiol (E_2) and progesterone (P_4) within a MC. Nine naturally cycling women attended to the laboratory to consume a standardised breakfast on three occasions, each of them representing one of the MC phases. Breath samples to measure GE time, plasma samples to quantify PYY levels and hunger scores were collected for a total of 4 hours after which food intake was assessed by an *ad-libitum* buffet lunch. GE and PYY levels changed significantly across the phases of the MC ($p < 0.05$). GE was correlated to P_4 and E_2-P_4 ratio ($r = -0.5$ and 0.4, respectively). To conclude, the appetite regulators PYY and GE time change depending upon the MC phases with GE time associated with the ovarian hormone levels which suggests the necessity of controlling the MC phase in studies looking at the appetite response.

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

Abbreviations

AUC	Area under the curve
E_2	Estradiol
EI	Energy intake
FPh	Follicular phase
GE	Gastric emptying
LPh	Luteal phase
MC	Menstrual cycle
MPh	Menstrual phase
P_4	Progesterone
T_{asc}	Ascension time
T_{half}	Half time
T_{lag}	Lag phase
T_{lat}	Latency time;
VAS	Visual Analogue Scale

Introduction

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

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

In fact, Brennan et al. (2009), who assessed food intake from a buffet 90 min after providing a glucose load to nine healthy women on three days of the MC (two in the FPh and one in the LPh), found that food and energy intake (EI) during LPh was significantly higher compared to FPh (~50 g and ~700 kJ difference, respectively). This was related to a faster emptying of the stomach, the time needed for emptying 50% of the gastric glucose during LPh was 15 min less than during the FPh. In addition, there was a higher post-meal release of GLP-1, blood glucose and plasma insulin levels in the LPh, thus the glycaemia response was improved when P_4 was low in the FPh. Finally, CCK response showed no changes despite the differences in hunger and EI between phases. Nevertheless this was not entirely unexpected as CCK secretion seems to be more affected by fat and protein

intake rather than glucose (Liddle, Goldfine, Rosen, Taplitz, & Williams, 1985). Whether modifications in the appetite responses are maintained with a full breakfast and whether there would be any differences during the menstrual phase (MPh) has not been previously studied. The latter seems of importance as both ovarian hormones, estradiol (E_2) and progesterone (P_4), are found at very low concentrations, in contrast to the other two phases. Having a better understanding of women's appetite physiology seems imperative in light of the global higher obesity prevalence in women than men (WHO, 2015).

The objective of the present study was to assess whether eating the same breakfast in each of the three MC phases would change the GE time, PYY response and satiety feelings of the meal to ultimately have an impact on the food intake of a buffet lunch served four hours later. Furthermore, the aim was to associate any potential differences to the naturally occurring fluctuations in E_2 and P_4 of the MC. We finally aimed to investigate whether food intake recorded during three days for each MC phase changed significantly.

Material and methods

Participants

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

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

of >10 and a DEBQ >2.5 were considered restrictive eaters and were excluded from participating in the study.

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

Protocol

Once the participant agreed to participate in the study, she was given a fertility monitor (Clear Blue Advanced Fertility Monitor, Clearblue) to assist in the scheduling of visits to the laboratory based on the three different MC phases i.e. MPh, FPh and LPh. The three chosen days were aimed to display a very distinguishable profile in the ovarian hormones: MPh, E_2 and P_4 at low concentrations; FPh, E_2 at high concentrations while P_4 remains low; and LPh, E_2 and P_4 at high concentrations. The MPh visit was scheduled as soon as the participant notified the start of a new MC (i.e. day 1) and this was performed within 4 days of starting the MC. From day 6 of the MC, participants tested their morning urine using the fertility monitor to measure their oestrone-3-glucuronide (E3G) and luteinising hormone (LH) levels. When participants obtained the 'high' reading (i.e. E3G levels were increased) they notified the researcher who scheduled the next testing session based on the cycle day of the high reading, the MC length history of the participant and the fact that it usually takes approximately five days to reach to 'peak' after a 'high' reading (Howards et al., 2009), in order to test the participant at very high levels of E_2. Once the 'peak' reading (i.e. LH levels were high) appeared, the last session was scheduled to test when P_4 was at its highest values (in the mid-luteal phase) based on the peak day and the usual MC length of the participant. When participants did not reach 'peak' they were asked to postpone their LPh testing session until the next cycle to ensure that the P_4 levels were high enough to produce any potential effects on the parameters studied (i.e. PYY response, GE time, appetite feelings and food intake).

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

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

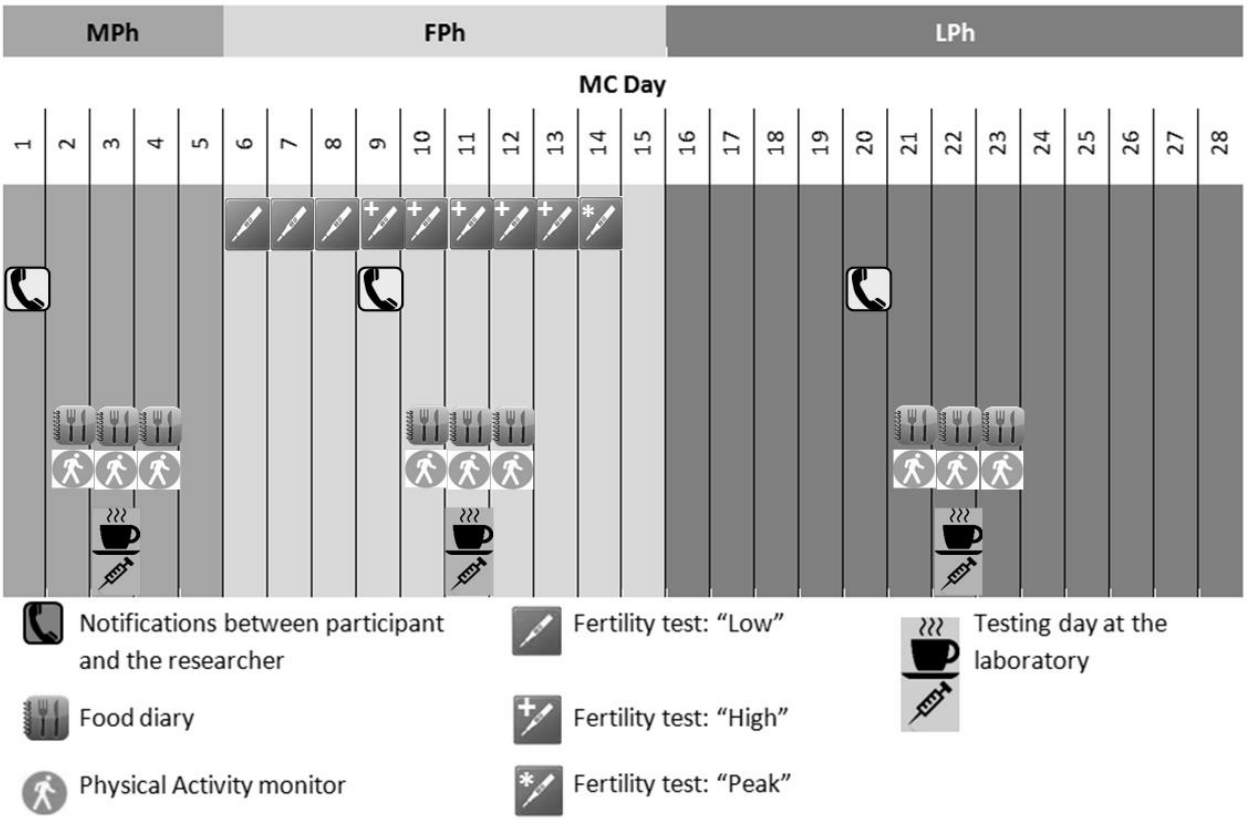


Fig 1. Example experiment timeline during a MC.

During the visits to the laboratory, participants were requested to arrive between 7:00-9:30h to have their body composition assessed by electrical bioimpedance, Tanita Body Composition Analyzer BC-418MA (Tanita Ltd, West Drayton, UK) and then a cannula (BD Venflon Pro Safety 20GA, Becton Dickinson Induction Therapy, Singapore) was inserted into a vein of the anti-cubital fosse of the arm to obtain the baseline blood sample (t = 0). The cannula was kept patent by flushing 0.9% sodium chloride into the system with a needle-free syringe after collecting each sample. Immediately after, participants filled in the visual analogue scale (VAS) for appetite sensations and the first breath sample for the measurement of GE was collected. Then the participant consumed the standardised breakfast labelled with ^{13}C octanoic acid; the breakfast consisted of scrambled eggs on toast, pineapple and a drink of their choice (water, coffee or tea with/out milk and sugar). The breakfast was standardised amongst participants and provided 375-395 kcal of which 35%, 38% and 23% were in the form of fat, carbohydrate and protein, respectively. The energy provided by the breakfast

accounted for 17-18 % of the total daily energy requirements for an average woman (19-44 years) with median physical activity level of 1.63 i.e. 2103-2175 kcal/d (SACN 2011). Participants were asked to finish their breakfast within 15min. As soon as they finished their breakfast, the first post-ingestion breath sample, blood sample and satiety scores were collected. Subsequent breath samples were taken every 15 min until 240 min. Subsequent blood samples and satiety scores were taken every 15 min until t = 60 min thereafter every 30 min until t = 240 min (Fig 2).

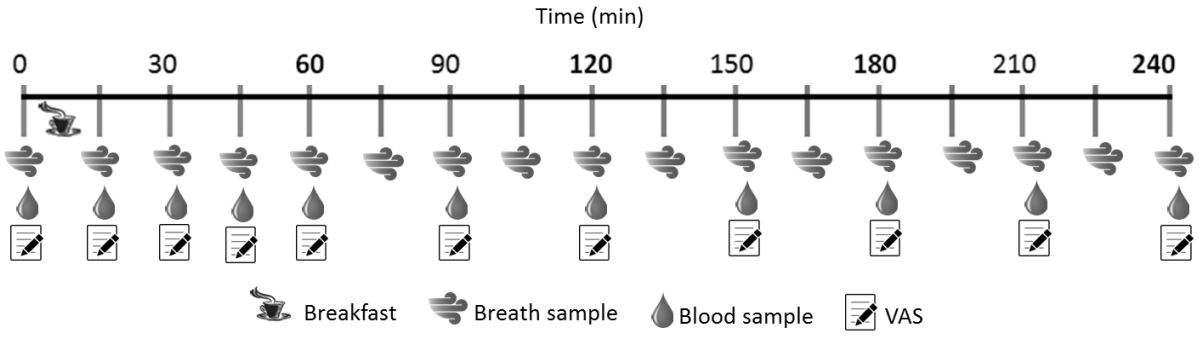


Fig 2. Timeline of events during each of the testing days in the laboratory.

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

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

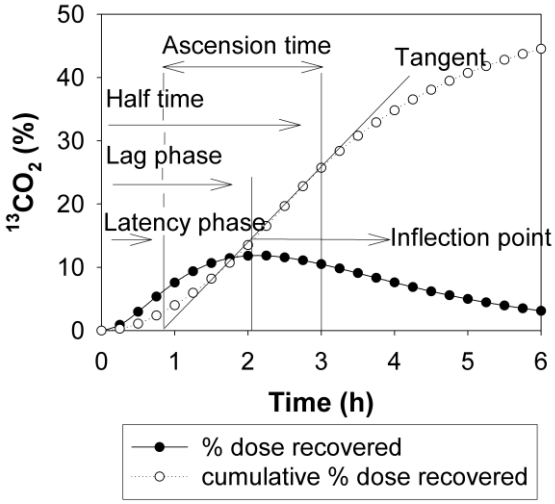
Food	Serving		Energy		Fat	Carbs	Fibre	Prot	Salt
	units	g	(kJ)	(kcal)	(g)	(g)	(g)	(g)	(g)
Hummus		50	664	161	14	4	2	4	1
Apples Gala	1	135	304	71	0	16	2	1	0
Banana	1	158	636	150	0	36	4	2	0
Clementines	2	226	398	95	0	20	3	2	0
Carrots		70	123	30	0	6	2	0	0
Celery sticks		80	32	8	0	1	1	0	0
Tomatoes	18	142	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 ϒ		215	1288	310	13	26	14	15	2
Soya Fruit Yoghurt ϒ	1	129	396	94	3	12	2	5	0
Vegetable Spring Rolls ϒ	6	115	1173	281	14	33	3	6	0
Peanut Butter Sandwich ϒ		113	1477	355	20	23	7	17	0
Beetroot, Mint hummus Sandwich ϒ		109	755	180	5	23	6	7	1
TOTAL (vegan buffet)		2914	11413	2721	95	361	62	78	6

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

Measurements

Gastric emptying Breath samples were collected by blowing into a small glass tube (Labco Exetainer, Labco Limited, UK) through a straw while having the nose blocked with a nose-clip. Participants blew into the tube while removing the straw to immediately cap the tube which was then stored at room temperature for analysis. Breath samples were analysed using an isotope ratio mass spectrometer (ABCA, Sercon Ltd, Chesire UK) to quantify the excess amount of labelled oxidised octanoic acid (i.e. $^{13}CO_2$) above baseline for each time point, as previously described elsewhere (Clegg & Shafat, 2010). This was expressed as the percentage of dose recovered per hour and this was fitted into a non-linear regression model (Ghoos et al., 1993). From this model several parameters were measured. Lag phase (T_{lag}) and half time (T_{half}) were calculated using the formulae derived by Ghoos et al. (1993). T_{lag} is the time taken to maximal rate of $^{13}CO_2$ excretion (Jackson, Bluck, & Coward, 2004) and is equivalent to the time of the inflection point (Schommartz, Ziegler, & Schadewaldt, 1998). T_{half} is the time it takes 50% of the 13C dose to be excreted (Jackson et al., 2004). Latency phase (T_{lat}) (Schommartz et al., 1998) is the point of intersection of the tangent at the inflection point of the $^{13}CO_2$-excretion curve representing an initial delay in the excretion curve. Ascension time (T_{asc}) (Schommartz et al., 1998) is the time course between the T_{lat} and T_{half}, representing a period of high $^{13}CO_2$-excretion rates (Fig. 3).



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

189 *PYY and E_2 and P_4 levels*. Blood samples were collected with K2E-EDTA tubes (BD Vacutainer,
190 Becton Dickinson, UK). A 4 ml blood sample was withdrawn from the cannula for every time point,
191 except for the baseline when 8 ml were collected to measure the ovarian hormones. After collection,
192 blood samples were kept in ice until they were centrifuged at 4°C for 10 minutes at 4000 rpm (MC-6,
193 Sarstedt Ltd, Leicester, UK) to extract the plasma. These were then frozen at -80°C in different
194 aliquots until analysis. E_2 and P_4 levels were measured by an ElectroChemiLuminescense
195 immunoassay (ECLIA) with a Cobas e411 semi-automated analyser (Roche diagnostics Burgess Hill,
196 UK) and total PYY concentrations were assessed with a direct sandwich enzyme-linked-
197 immunosorbent assay (ELISA) kit (EMD Millipore). Samples of the same participant in the three
198 phases of the MC were analysed within the same ELISA plate. Averaged intra-duplicates coefficient
199 of variance (CV) for the total PYY ELISA assay was 6.3 ± 1.4 %. Averaged inter-plate CV for the quality
200 controls of the PYY ELISA assay was 13.3 ± 4.1 %.

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

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

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

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

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

Calculations and statistical analyses

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

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

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

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

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

Results

Participants characteristics

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

Table 2. Participants' characteristics at baseline

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

MC characteristics and ovarian hormones

Average MC length was 29 ± 3 days. Of the nine participants included, four had a "peak" reading i.e. ovulation was confirmed by the fertility monitor, within their first MC, while three participants only ovulated on the second MC. Averaged "peak" reading happened on day 14 ± 3 of the MC. The two remaining participants were asked to attend to the laboratory when it was expected to be their mid-LPh despite not having had a peak reading in the fertility monitor. Nevertheless, plasma P_4 levels indicated that these participants had ovulated as P_4 concentrations were > 15.9 nmol/L which is considered high enough to have ovulated (Piers et al., 1995). Moreover, one of these two participants had a positive LH peak in her personal fertility monitor, thus participants were kept in the study as they seemed to have ovulated despite not having been detected by the fertility monitor used in the study.

There were significant differences in E_2 and P_4 concentrations amongst the three phases of the MC ($p < 0.001$ and <0.0001, respectively). E_2 levels were significantly increased in the FPh and LPh compared to the MPh, and P_4 levels were significantly higher in the LPh compared to the other two phases (Fig 4).

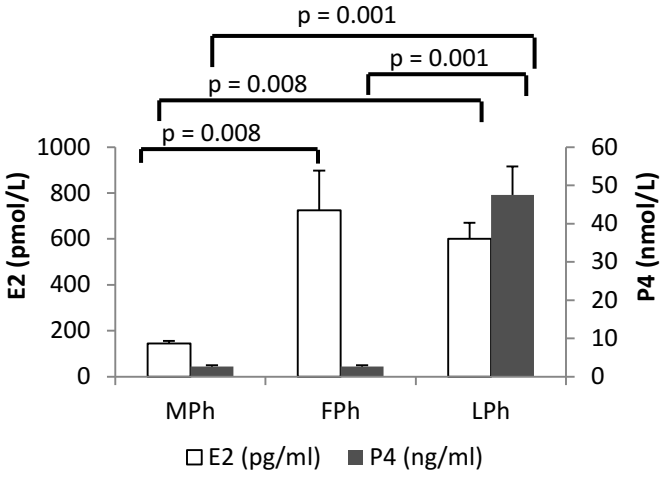


Fig 4. E_2 and P_4 concentrations in the different phases of the MC (means ± SD).

GE

There was a significant overall effect of the phase of the MC on T_{half} and T_{asc} (Table 3) but none of the specific comparisons between phases indicated a significant difference. However the effects observed seem to suggest trends that T_{half} was quicker in the LPh compared to the FPh and the MPh

(mean difference: 28 ± 31 and 13 ± 15 min, p = 0.081 and 0.092, respectively) and T_{asc} was faster in the LPh compared to the FPh (mean difference: 27 ± 29 min, p = 0.077). There was a trend towards a difference in T_{lag} across the phases of the MC (p = 0.072). No differences were found in T_{lat} across the phases of the MC.

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

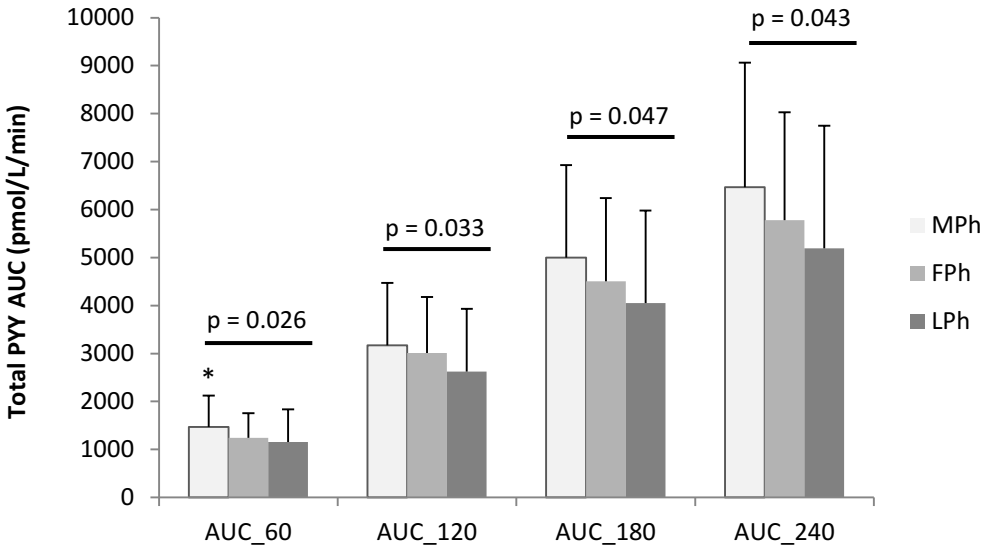
T_{half}, half time; T_{lag}, lag phase; T_{lat}, latency time; T_{asc}, ascension time. Mean ± SD

Total PYY

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

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

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



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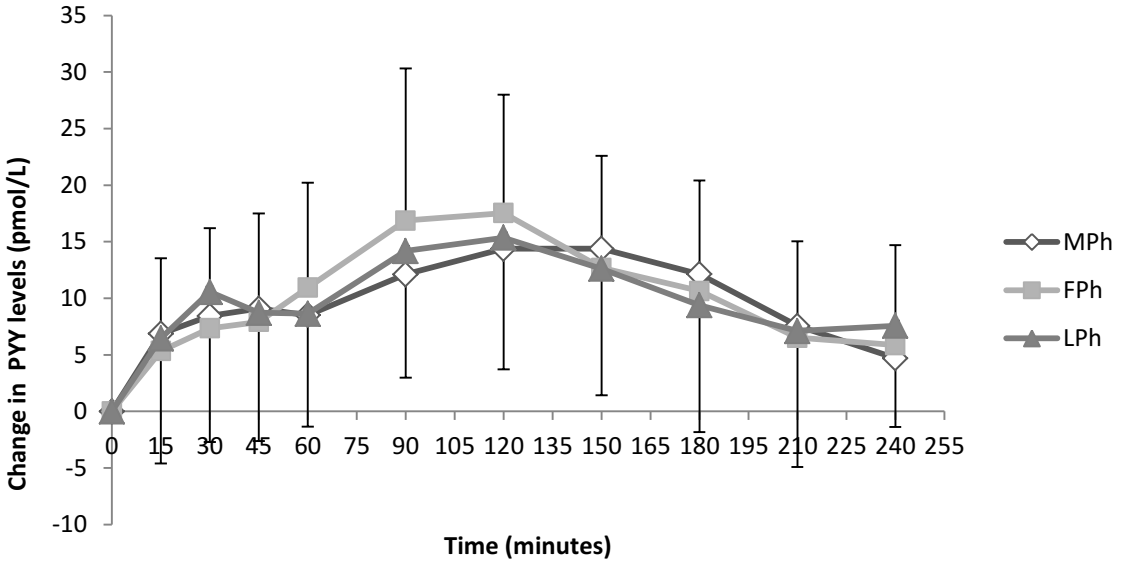
307 **Fig 5**. Total PYY AUCs at t = 60, 120, 180 and 240 min in the different phases of the MC (means ±
308 SD). *Significantly different to the LPh within the same time AUC.

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



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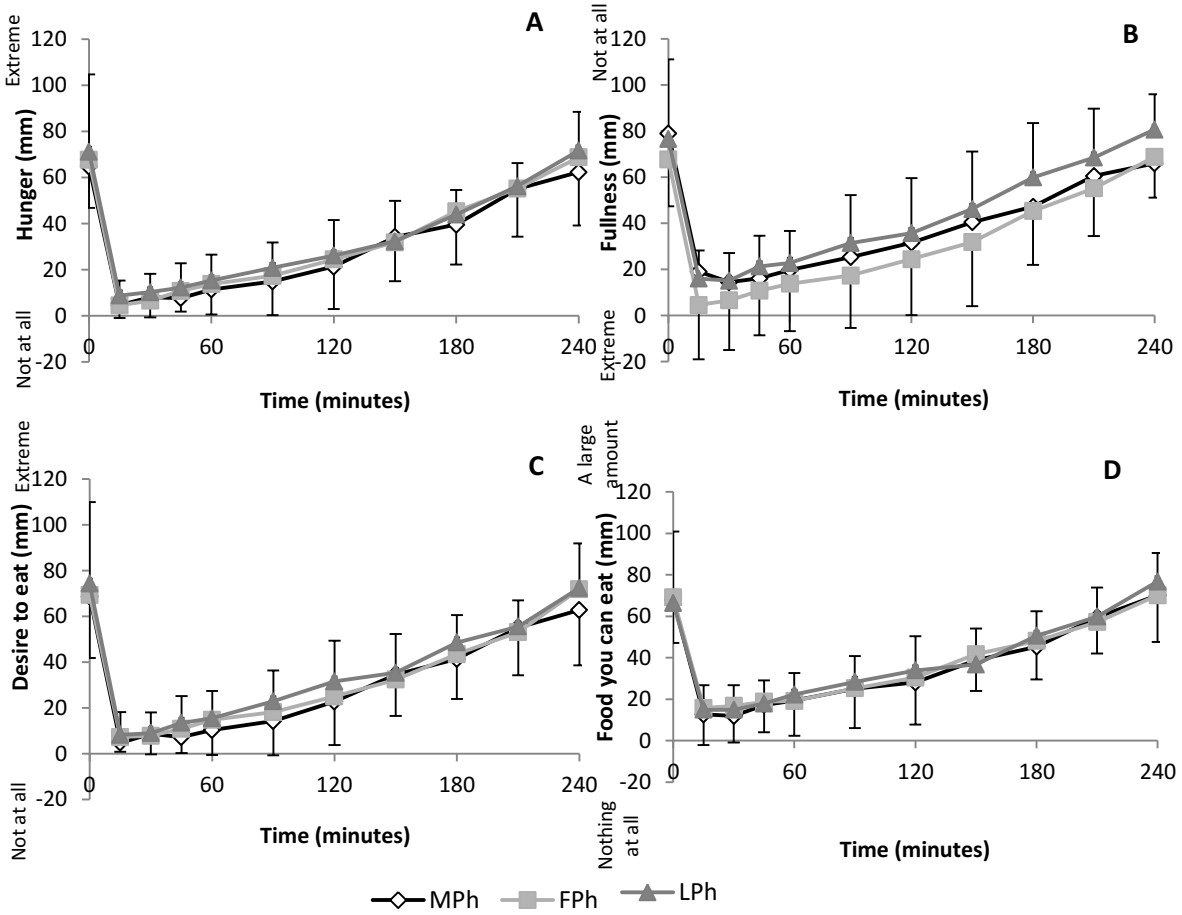
313 **Fig 6**. Change in PYY levels from baseline to each time point in the different phases of the MC
314 (Means ± SD).

315

316 *Satiety ratings*

317 There were no significant differences in AUC for any of the four satiety questions when
318 analysing them in a two-way-ANOVA (*time x phase*) with the baseline measurements as covariates.
319 AUC at the end of the 4 hours test for *"how hungry do you feel"; "how full do you feel?" "how strong*
320 *is your desire to eat"* and *"how much food do you think you can eat?"* were 6932 ± 2961, 7404 ±
321 2010 and 7802 ± 2080 mm/min; 8924 ± 3153, 10358 ± 4261 and 10340 ± 3638 mm/min; 7039 ±
322 3147, 7479 ± 2108 and 8268 ± 2388 mm/min for MPh, FPh and LPh, respectively (Fig 7).



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

326

327

Ad-libitum, post-lunch and averaged food intake

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

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

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

	MPh	**FPh**	**LPh**
***Ad libitum* 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
After *Ad libitum* 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

Means ± SD

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

343 There was a significant moderate correlation between peak PYY and T_{half} and T_{asc} (r = 0.396 and
344 0.410, p = 0.041 and 0.034, respectively). Moreover, there was a trend for a moderate correlation of
345 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}
346 correlations, respectively).

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

348

349 *Relationships between appetite markers and the ovarian hormones*

350 There was a moderate negative correlation between T_{half} and T_{asc} and P_4 levels (r = -0.490 and -
351 0.426, p= 0.010 and 0.027, respectively). Moreover, T_{half} and T_{asc} were positively correlated to E_2:P_4
352 ratio (r = 0.437 and 0.407, p= 0.023 and 0.035).

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

356

357 **Discussion**

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

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

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

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

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

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

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

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

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

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

There were limitations to this study. Despite GE time and PYY response showing to be significantly different across the MC, pairwise comparisons could not achieve the statistical significance and that could be due to the small sample size or the inter-individual variation. In fact, if applying a t-test to compare T_{half} between the LPh and FPh or MPh, significant differences would have been found; however, with the ANOVA and the Bonferroni correction, the statistical significance was diminished. Nevertheless, we employed the same sample size used by previous studies (Brennan et al., 2009). The current study did not distinguish the two different forms of PYY, i.e. PYY_{1-36} and PYY_{3-36}. It is well known that with food intake PYY_{1-36} is cleaved by the dipeptidyl peptidase IV to PYY_{3-36} and that only the latter form has almost exclusive and high affinity to Y-2 receptors of the ARC. This is of relevance as Y-2 receptors are the only subtype of Y-receptors that can induce appetite and body weight suppression by stimulating the activity of the α-MSH through the inhibition of the NPY release (Ballantyne, 2006). Thus, although unlikely, changes in the concentrations of total PYY could respond to alterations in the proportion between the two forms of PYY. Thus it cannot be dismissed that lower PYY response in the LPh was mainly relying on a diminished PYY_{1-36} secretion and conversion, therefore, inducing minimal changes in food intake. Future studies could improve our findings by measuring the two forms of PYY.

Conclusion

To our knowledge this is the first study to investigate GE time and PYY response after consuming the same breakfast three times in the MC in which ovarian hormones, E_2 and P_4 presented very distinguishable levels. Our results found significant differences in GE time and PYY response that suggest the LPh as the quickest in GE time with the smallest PYY response of the all MC phases.. Finally changes in the GE time could be influenced by the fluctuations in the ovarian hormones.

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

Acknowledgments

The authors thank the participants for their involvement and cooperation during the study. We also thank Dr Ryan Pink for his assistance with the ELISA assays.

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