Glucagon-like peptide-1 (GLP-1) increases in plasma and colon tissue prior to estrus and circulating levels change with increasing age in reproductively competent Wistar rats

How to cite:

For guidance on citations see FAQs.

© 2017 Elsevier Inc.
https://creativecommons.org/licenses/by-nc-nd/4.0/

Version: Accepted Manuscript

Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.1016/j.peptides.2017.02.010

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online’s data policy on reuse of materials please consult the policies page.
Title: Glucagon-like peptide-1 (GLP-1) increases in plasma and colon tissue prior to estrus and circulating levels change with increasing age in reproductively competent Wistar rats

Authors: Michelle L. Johnson, M. Jill Saffrey, Victoria J. Taylor

PII: S0196-9781(17)30051-7
DOI: http://dx.doi.org/doi:10.1016/j.peptides.2017.02.010
Reference: PEP 69749

To appear in: Peptides

Received date: 27-10-2016
Revised date: 30-1-2017
Accepted date: 21-2-2017

Please cite this article as: Johnson Michelle L, Saffrey M Jill, Taylor Victoria J. Glucagon-like peptide-1 (GLP-1) increases in plasma and colon tissue prior to estrus and circulating levels change with increasing age in reproductively competent Wistar rats. Peptides http://dx.doi.org/10.1016/j.peptides.2017.02.010

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Glucagon-like peptide-1 (GLP-1) increases in plasma and colon tissue prior to estrus and circulating levels change with increasing age in reproductively competent Wistar rats

Michelle L. Johnson, M. Jill Saffrey, Victoria J. Taylor

School of Life, Health and Chemical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

*Corresponding author: vicky.taylor@open.ac.uk

Michelle L. Johnson: medmjoh@leeds.ac.uk

M. Jill Saffrey: jill.saffrey@open.ac.uk

1 Present address: Women’s Health Research Group, Section of Cancer & Pathology, Leeds Institute of Cancer & Pathology, St James’s University Hospital, Beckett Street, Leeds LS9 7TF, UK
highlights

- Increased GLP-1 levels were found in plasma and colon tissue during proestrus.
- Reduced stomach contents were recorded on the morning of estrus.
- High GLP-1 at proestrus may indicate increased satiety leading into estrus.
- Plasma and colon PYY unchanged during estrous cycle; possible differential PYY/GLP-1 secretion.
- Increasing age affects GLP-1 regulatory control in reproductively competent females.
Abstract:
There is a well-documented association between cyclic changes to food intake and the changing ovarian hormone levels of the reproductive cycle in female mammals. Limited research on appetite-controlling gastrointestinal peptides has taken place in females, simply because regular reproductive changes in steroid hormones present additional experimental factors to account for. This study focussed directly on the roles that gastrointestinal-secreted peptides may have in these reported, naturally occurring, changes to food intake during the rodent estrous cycle and aimed to determine whether peripheral changes occurred in the anorexigenic (appetite-reducing) hormones peptide-YY (PYY) and glucagon-like peptide-1 (GLP-1) in female Wistar rats (32-44 weeks of age). Total forms of each peptide were measured in matched fed and fasted plasma and descending colon tissue samples for each animal during the dark (feeding) phase. PYY concentrations did not significantly change between defined cycle stages, in either plasma or tissue samples. GLP-1 concentrations in fed plasma and descending colon tissue were significantly increased during proestrus, just prior to a significant reduction in fasted stomach contents at estrus, suggesting increased satiety and reduced food intake at this stage of the cycle. Increased proestrus GLP-1 concentrations could contribute to the reported reduction in food intake during estrus and may also have biological importance in providing the optimal nutritional and metabolic environment for gametes at the potential point of conception. Additional analysis of the findings demonstrated significant interactions of ovarian cycle stage and fed/fasted status with age on GLP-1, but not PYY plasma concentrations. Slightly older females had reduced fed plasma GLP-1 suggesting that a relaxation of regulatory control of this incretin hormone may also take place with increasing age in reproductively competent females.

Keywords: estrous cycle; ovarian cycle; appetite; gut peptides; GLP-1; PYY; satiety
1 Introduction:
Reduced food intake in reproductively cycling females has been associated with high levels of estradiol in the circulation; rats consume less during estrus, following the earlier peak in ovarian estradiol secretion during proestrus [1-3]. Eckel et al. (2000) found a significant reduction in food intake at estrus to be accompanied by a reduction in water consumption and also reported a significant decrease in body mass in ad libitum fed rats. Another study in ovariectomised (OVX) female rats further demonstrated that cyclic administration of near physiological estradiol levels allowed maintenance of feeding patterns and body mass, both of which were lost in non-treated OVX control rats [4]. Despite previously observed cyclic changes to food intake and body mass during the reproductive cycle, the optimal nutritional and metabolic environment to ensure healthy gametes at the time of ovulation and potential conception, prior to early development events, is not well characterised. Likewise, the possible roles of appetite-regulating gut hormones at this time have not been explored in detail.

Appetite-influencing hormone concentrations in the peripheral circulation, including ghrelin, PYY and GLP-1, are generally suppressed in conditions of obesity and in type II diabetes [5-9]. Gastrointestinal bypass surgery results in reduced food intake and body mass gain, and rapid improvements in glucose homeostasis in people with type II diabetes. It is also associated with marked changes to gut hormone secretion, with postprandial levels of satiety-inducing gut hormones being significantly increased [10-13]. With the globally increased prevalence of obesity in younger human populations, new drugs and endogenous hormone combinations are being tested as alternatives to risky gastric surgery treatments that are effective in altering metabolic/gut hormone profiles towards weight loss. In this context, it will be important to establish what effects altering endogenous appetite hormone concentrations may have on females of reproductive age. Before such work is performed, however, it is necessary to determine how appetite hormone levels change during the ovarian cycle, which is the aim of the present study.

PYY and GLP-1 are anorexigenic (appetite-inhibiting) gut peptides that are co-secreted predominantly from the population of L cells in the distal colon in response to intake of specific nutrients in food [reviewed by 14, 15], although recent studies
using perfused male rat guts *ex vivo* have demonstrated the presence and variable secretion of both peptides from proximal and distal areas of small intestine [16]. PYY and GLP-1 are released in a biphasic manner and plasma levels increase from approximately 15 minutes after the start of food consumption, proportional to meal size, to signal satiety [14, 17]. Both peptides additionally have physical effects on the gastrointestinal tract, such as delaying gastric emptying via an ileal brake mechanism, and decreasing gastric acid and intestinal secretions [18, 19]. Additionally, GLP-1 acts as an incretin, signalling between the gut and pancreatic beta cells to increase glucose-dependent insulin secretion [15]. The incretin properties of GLP-1 have been utilised to treat type-II diabetes and to achieve modest body mass reductions [20]. Little is known about the potential influence of the reproductive cycle hormones’ interaction with PYY and GLP-1, but it may be anticipated that during estrus, when food intake is lowest, PYY and GLP-1 may contribute to the anorexigenic tone.

The main objectives of this study were to establish if any changes in circulating and tissue concentrations of the anorexigenic hormones PYY and GLP-1 occurred in relation to previously documented natural food intake changes during the rodent estrous cycle. It is the first study to consider co-secreted PYY and GLP-1 concentrations in the same matched fed and fasted plasma and in corresponding (fasted) colon tissue samples in female rats at each stage of the estrous cycle. This work also took into account the nocturnal feeding patterns of rodents, with quantification of hormone concentrations during the dark phase at each estrous cycle stage. Secondary aims included checking for any age effects, as data analysis of total ghrelin fed and fasted concentrations from the same samples found decreased amounts in slightly older females [21].

2. Materials and methods

2.1 Animals
This work was licensed under the Home Office Animals (Scientific Procedures) Act 1986 and had approval from The Open University Ethics Committee; rats were specifically chosen for this study to obtain enough blood for fed and fasted comparison. Nulliparous female Wistar rats (Harlan, UK, n=43) were housed in groups of four and maintained under a 12 hour reverse light cycle (lights off between
11.00 and 23.00) with free access to standard breeding diet and water. All procedures were carried out during the dark phase so that samples were obtained when most physiologically relevant for natural feeding behaviour. Females were kept near a cage of male rats to keep the females cycling normally. Daily estrous monitoring was undertaken at 24-hourly intervals between 11.00 (lights off) and 13.00 to determine cycle stages by vaginal lavage, as described by Becker et al. [22]. Rats were between 32-44 weeks of age at the end of the study. Rats were monitored for a minimum of 2 complete cycles (often up to 4 cycles) to obtain sample groups at each stage of the estrous cycle.

2.2 Blood and tissue collection and preparation
Fed blood samples from the tail vein were taken between 13.00 and 15.00, after completion of estrous monitoring, the day before the cycle stage to be studied. Some animals did not progress as predicted with their estrous cycle, so there is a mismatch between the number of animals in the fasted state cycle stages (proestrus n=12; estrus n=11; metestrus n=9, diestrus n=11) and the numbers obtained for the fed cycle stages the day before (proestrus n=14; estrus n=7; metestrus n=6, diestrus n=16).

Rats were fasted from 08.00, before the beginning of the dark period (when maximum food consumption occurs), 4 – 8 hours prior to culling. Cage food was removed at 08.00 the day after tail vein fed sample collection, and animals were then sacrificed between 12.00 and 16.00. Fed blood samples were immediately acidified by dilution at 1:10 in buffer (0.1 M ammonium acetate, 0.5 M NaCl, pH 3.6) as recommended for optimal peptide preservation and recovery [23]. Rats were anaesthetised and decapitated, and a fasted blood sample was obtained from trunk blood. All blood was collected into EDTA coated tubes with a protease inhibitor. Stomachs were removed and masses were recorded both before and after opening along the greater curvature and rinsing in PBS, enabling the mass of remaining stomach contents to be calculated. Samples of descending colon were removed and immediately frozen for later peptide extraction and assay.

2.3 Radioimmunoassays
Samples of fed and fasted plasma and fasted colon tissue extract were analysed according to the manufacturer’s protocol for total PYY and GLP-1 concentrations using radioimmunoassay kits of the same batch number per peptide (Millipore, UK). All tissue samples were diluted prior to addition to RIAs using distilled water. Fasted plasma samples for GLP-1 measurement were extracted and added to each kit as outlined in the assay protocol. Samples were added to assays based on sample type, not cycle stage. PYY: intra-assay %CV: 2.97 ± 1.617 (n=3), inter-assay %CV: 2.85 ± 3.172 (n=3). GLP-1: intra-assay %CV: 3.34 ± 1.279 (n=3), inter-assay %CV: 6.28 ± 1.551 (n=3).

2.4 Statistics
Values represent mean ± S.E.M. Statistical analysis was carried out using a one-way ANOVA with a Tukey post-hoc test on normally distributed data. When data were not normally distributed, and could not be transformed (e.g. by log transformation) to normality, a Kruskal-Wallis test was used, with follow-up pairwise comparisons (Mann-Whitney), with Bonferroni correction. A paired-samples t-test was used to compare hormones in the fed and the fasted state. Further statistical testing used a univariate general linear model (GLM) with PYY or GLP-1 concentration as the primary dependent variable and fed/fasted status and stage in cycle as independent variables, with age in weeks as a covariate. To evaluate our secondary aims, Pearson correlations were used to determine if either hormone concentrations were correlated in fed and fasted states and with age. All statistical tests were performed using IBM SPSS Statistics 21. P<0.05 was considered statistically significant.

3. Results
At culling, the mean age of the rats was 38 ± 0.49 weeks (32 – 44 weeks) and mean body mass was 269.2 ± 2.59 g (239.6 – 303.9 g). There were no significant differences in age (P=0.180) or body mass (P=0.673) between the different cycle stage groups using ANOVA. There was no difference in body mass with age (P=0.555).

3.1 Fasted stomach contents indicated least food consumed leading up to estrus
Stomach contents data were analysed against the cycle stage of the preceding day, as the rats were fasted from the beginning of the cull day (from 08.00; before lights off at 11.00), therefore analysis of remaining stomach contents provided an indication of food consumption during the previous day/cycle stage. When fasted from estrus and dissected at metestrus, rats had significantly ($F(3, 39)=3.187$, $P=0.034$) more stomach contents (0.96 g) than those fasted from proestrus and dissected at estrus (0.44 g; $P=0.028$) [21].

### 3.2 GLP-1

GLP-1 concentrations in fed plasma (101.6 ± 13.15 pg/ml) were significantly ($t(41)=5.266$, $P<0.001$) higher (approximately three times) than in fasted plasma (28.4 ± 15.17 pg/ml) in the whole group. Fed plasma had significantly (Kruskal-Wallis, $\chi^2=7.871$, 3 df, $P=0.049$) more GLP-1 during proestrus than in diestrus ($P=0.034$; Figure 1 i). Fasted plasma concentrations of GLP-1 were similar at each cycle stage (Figure 1 ii). Higher large intestine tissue concentrations were also found at proestrus: in descending colon tissue (Figure 1 iii), GLP-1 was significantly more concentrated ($F(3, 39)=3.921$, $P=0.015$) at proestrus than during estrus ($P=0.045$) and diestrus ($P=0.029$).

As the experimental design included multiple factors including fed and fasted status and stage of ovarian cycle, GLM univariate analysis was used to test for any interactions. Although rats used for the study were within a limited age range, hormone concentrations may vary with age, so cull age was added into the linear model as a covariate. There were significant main (direct) effects of fed/fasted status ($F(1,76)=36.851$, $P=0.001$), ovarian cycle stage ($F(3,76)=3.024$, $P=0.035$) and age ($F(1,76)=5.729$, $P=0.019$) on total GLP-1 plasma concentrations and a significant interaction (joint effect) of fed/fasted status with ovarian cycle stage ($F(3,76)=2.766$, $P=0.048$) as fixed factors, with age included in the model as a covariate. There was no effect of body mass ($P=0.531$) or empty stomach mass ($P=0.124$).

Tukey post hoc tests were used to investigate the sources of interaction, with significant differences between fed and fasted cycle stage groups shown on Figure 1(i) and (ii). Fed proestrus rats had the highest GLP-1 plasma concentrations and higher amounts than all fasted cycle stages. Fed proestrus GLP-1 concentrations
were also higher than fed diestrus rats ($P<0.001$, Fig 1 i). Fed estrus rats also had higher GLP-1 than all fasted cycle stages: proestrus, metestrus ($P<0.05$; with trends for estrus $P=0.056$, ns and diestrus $P=0.073$, ns).

**Figure 1. GLP-1 concentrations during the rat estrous cycle**

Fed plasma (i) and descending colon (iii) GLP-1 were significantly increased at proestrus compared to diestrus, and additionally at metestrus in colon tissue. No changes were seen in fasted plasma GLP-1 levels (ii) throughout the cycle (*) ($P<0.05$). General linear modelling univariate analysis differences are shown between cycle stages in fed samples (i) and between fed (i) and fasted (ii) figures (a>b, $P<0.001$, c>d, $P<0.05$).

### 3.3 Plasma, but not colon tissue GLP-1 concentrations were different with age

General linear modelling (GLM) of GLP-1 data analysis also revealed a significant main (direct) effect of age on plasma concentrations of GLP-1, and furthermore, scatterplots of the data (Figure 2) show fed and fasted plasma GLP-1 concentrations correlated with age: fed negatively ($r=-0.534$, $P<0.001$, $n=42$, Figure 2 i) and fasted positively ($r=0.445$, $P=0.003$, $n=43$, Figure 2 ii). Younger rats had higher fed GLP-1 concentrations, whereas they had lower fasted concentrations, in comparison with
slightly older animals. GLM also showed that colon tissue levels were not affected by age (Figure 2 iii).

**Figure 2 Correlation of plasma GLP-1 concentrations and rat age in weeks**
(i) fed GLP-1 ($r=-0.534$, $P<0.001$, $n=42$); (ii) fasted GLP-1 ($r=0.445$, $P=0.003$, $n=43$); (iii) descending colon GLP-1 ($r=0.221$, $P=0.154$, ns). Note y axes scales and units differ (proestrus, ●; estrus, ○; metestrus, ♦; diestrus, ◊).

### 3.4 PYY

Matched fed and fasted plasma sample analysis for the whole group showed that PYY concentrations were significantly ($t(41)=13.397$, $P<0.001$) higher (approximately twice) in the fed state ($453.6 \pm 20.05$ pg/ml) than the fasted state ($177.6 \pm 7.18$ pg/ml). The consistent decreases in both PYY and GLP-1 in the circulation from fed to fasted states confirmed an adequate fasting time in these animals.
There were no significant differences in either fed or fasted plasma concentrations of PYY in the different cycle stage groups (Figure 3 i, ii). Concentrations of PYY in the descending colon (Figure 3 iii) did not differ significantly between the cycle stages. GLM univariate analysis did not reveal any differences in total PYY plasma concentrations in fed or fasted states, between ovarian cycle stages or with age in the same animals/samples that did have GLP-1 differences. There were no significant correlations between fed or fasted PYY concentrations with age.

**Figure 3. PYY concentrations during the rat estrous cycle**

Neither fed (i) nor fasted (ii) plasma PYY differed with cycle stage. Descending colon PYY (iii) varied with cycle stage, but not significantly.

**Figure 4 Correlation of plasma PYY concentrations and rat age in weeks**

(i) fed PYY ($r=0.143$, $P=0.368$, ns); (ii) fasted PYY ($r=0.278$, $P=0.071$, ns); (iii) descending colon PYY ($r=0.469$, $P=0.002$). Note y axes units differ (proestrus, ●; estrus, ○; metestrus, ♦; diestrus, ◊).
4. Discussion
This study has established that circulating and colon tissue concentrations of GLP-1 were altered at defined stages of the rat estrous cycle. During proestrus, GLP-1 concentrations were significantly highest in descending colon tissue as well as in fed plasma. These increases in the anorexigenic hormone GLP-1 occurred in advance of the onset of the estrus phase of the cycle, in agreement with previously reported decreased feed intakes [1-3]. Increased expression of Glp-1r has previously been demonstrated in the hypothalamus and ovaries at proestrus in Sprague-Dawley rats [24], suggesting increased responsiveness to GLP-1 at this time, when circulating levels are high. Despite being secreted from the same cell type as GLP-1, neither fed nor fasted PYY plasma or tissue concentrations differed at defined stages of the cycle, although descending colon PYY individual concentrations varied considerably within each group. Approximately 70% of colonic L cells co-secrete PYY [reviewed
by 15], so it is likely that differential secretion of GLP-1 and PYY takes place throughout the rat ovarian cycle. We measured total concentrations, so the possibility remains that the proportions of the different forms of PYY [14] could be altered at different stages, despite the absence of any increases in total levels, as were found for GLP-1; and requires further study to determine.

GLP-1 acts as an incretin, signalling between the gut and pancreatic beta cells to increase glucose-dependent insulin secretion [14], but as GLP-1 secretion starts to occur before glucose reaches the distal gut in rats, the initial release is thought to be vagally stimulated [25]. This initial increase is followed later by the direct GLP-1 stimulatory effect of nutrients arriving at the distal L cells. Diurnal variations of GLP-1 secretion in male rats in response to oral glucose administration have been demonstrated [25], and were limited to the first phase response. Therefore, if this also occurs in female rats, this earlier ‘non nutrient’ mechanism may be involved in the increased GLP-1 secretion observed prior to the estrus phase. However, it likewise remains possible that differences in other hormones, neuropeptides, or nutrients that stimulate second phase GLP-1 secretion such as insulin, gastric inhibitory polypeptide (GIP) or leptin, which activate Erk1/2 or AKT; cAMP and STAT3 signalling mechanisms in L cells, respectively, could also be involved in the differences observed in this study. Further research is needed in females to explore the secretory patterns of cells in different areas of the gastrointestinal tract (similar to work done by Svendsen et al., [16] on male rat perfused small intestine) and confirm the possibility of differential release of PYY and GLP-1 throughout the oestrous cycle.

Studies have demonstrated a link between high exogenously administered GLP-1 levels and a reduction in food intake in rats [26] and humans [27], and also a reduction in the intake of high-fat food in mice when injected centrally [28]. As the animals used in the present study were group housed, daily food intake with cycle stage could not be monitored closely, but weighing stomach contents at dissection provided an indication of food and water consumption based on what remained after the fasting period. The mass of remaining stomach contents indicated that the rats either consumed the least between proestrus and estrus and the most shortly after, or that stomach emptying may have been inhibited by higher GLP-1 concentrations.
during proestrus. Increased levels of both GLP-1 and PYY have been linked with a reduced gut transit time in rodents [29, 30] and humans [31, 32]. Although gastric motility has not been studied during the ovarian cycle, it has been reported that there is reduced food intake during the stage of proestrus [1-3, 33, 34]. These combined observations suggest that satiety was likely increased leading into estrus, further supported by the increased endogenous GLP-1 findings from this study.

Due to the important timing, close to potential conception, it is possible that an increase in GLP-1 at proestrus may also be involved in setting appropriate metabolic/nutritional conditions for vital reproductive events taking place at this critical stage of the ovarian cycle. Recent studies of the orexigenic gut hormone ghrelin found that both hyper- and hypoghrelinemia had negative effects throughout fertilisation, implantation, and both embryo and fetal developmental stages in mice [35]. That study demonstrated the importance of optimal ghrelin levels from the fertilisation period and during the very early stages of pregnancy. Likewise in this study, from proestrus to estrus when the rats were sexually receptive, higher fed and tissue GLP-1 levels may have provided a permissive signal of adequate nutritional reserves to support pregnancy, enhanced insulin sensitivity and acted to delay or prolong nutrient absorption by slowing gastric emptying and intestinal transit [15]. This hypothesis is consistent with the finding that descending colon GLP-1 levels were increased despite the period of fasting, which would be expected to decrease GLP-1 production to increase appetite; also that the fed plasma samples from proestrus rats taken the day before had the highest concentrations. In support of this possibility, another recent study has demonstrated regulatory links between GLP-1 and the hypothalamic-gonadal-pituitary axis and increased litter size in female rats [24].

The increasing incidence of obesity in females of reproductive age (with suppressed GLP-1, PYY and ghrelin concentrations) across the globe [36] is of concern for the health of these women and their offspring. The evidence for an effect of parental diet and body composition on metabolic programming and epigenetic changes of offspring before [37, 38], during and after conception and implantation [39, 40] is growing. Mice with diet-induced obesity have been shown to have poor oocyte quality leading to poor blastocyst survival rates and embryos that survived showed
multiple abnormalities such as aneuploidy [41]. Human fetuses are also at risk, presenting with growth retardation and brain development abnormalities [42]. Obese women are known to have impaired reproductive function and can suffer infertility, miscarriage and further, obstetric complications [43], so it is essential that more is understood about the role of gut hormones in normal eating behaviour and fundamental reproductive functions, particularly at the time of conception.

This study has also revealed interactions between fed/fasted status and cycle stage on GLP-1 plasma concentrations with increasing age in reproductively competent rats (between 32 and 44 weeks of age), but not on co-secreted PYY. Both fed and fasted total ghrelin (appetite-enhancing) concentrations from these plasma samples [21] were likewise significantly reduced in the slightly older animals. GLP-1 and ghrelin are both involved, in opposing roles, in glucose homeostasis [39]. This current work provides further evidence that total GLP-1 concentrations are an important component of the complex dynamic physiological changes taking place in the ovarian cycle of female rats. Furthermore, if the regulatory control of appetite-influencing hormones such as GLP-1 and ghrelin are attenuated between fed and fasted states with increasing age in reproductively competent females, such changes could impact on dam body composition, reproductive success and offspring metabolic programming.

5. Conclusion

These new findings that GLP-1 concentrations are increased in fed plasma and descending colon tissue at proestrus, whilst PYY are not, and that there are interactions between fed/fasted status, ovarian cycle and age (whilst still reproductively competent), add to the existing knowledge about natural food intake reductions during the ovarian cycle in rats. Alterations in anorexigenic gut hormone concentrations and fasted stomach contents at proestrus could contribute to the reported reduction in food intake during estrus in rats, in addition to the known effects of estradiol. There is growing evidence that so-called appetite hormones act as signals to the reproductive tract of an optimal nutritional environment to support pregnancy. As GLP-1 concentrations are also implicated in glucose regulation, studying this gut hormone throughout reproductive life stages is important for
examining the links between maternal appetite and glucose regulation and how this may be implicated in the metabolic programming of offspring.

**Acknowledgements**

The authors thank Steve Walters, Karen Evans, Agata Stramek and Sophie Brooks for outstanding technical support. They are also grateful to Professor Paul Garthwaite for statistical guidance with the GLM models and data analysis. The School of Life, Health and Chemical Sciences (LHCS), The Open University, provided the funding for this work.
6. References


[34] Eckel LA, Houpt TA, Geary N. Spontaneous meal patterns in female rats with and without access to running wheels. Physiology & behavior. 2000;70:397-405. doi: 10.1016/S0031-9384(00)00278-X.


