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Journal Item

Gastrointestinal capacity, gut hormones and appetite change during rat pregnancy and lactation

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Short title: Gut changes during rat pregnancy and lactation

Abstract

Pregnancy and lactation increase maternal appetite and adiposity, which in humans can lead to long-term body mass retention. Previous rat reproduction studies suggest that appetite-inhibiting gut hormone, peptide-YY (PYY), is elevated, despite hyperphagia; also that gastrointestinal size increases. The present study characterised changes in orexigenic (appetite-stimulating) ghrelin and anorexigenic (appetite-inhibiting) PYY and glucagon-like peptide-1 (GLP-1), and gastrointestinal architecture during pregnancy and lactation, in matched fed and fasted plasma and gut tissue samples taken during the dark phase. Enteroendocrine cells were immunolabelled, and gut masses and lengths measured. Fasted plasma ghrelin reduced during pregnancy: it was lowest by day 18, recovered to control values at parturition, then increased by the end of lactation. Ghrelin-immunoreactive stomach cells and stomach ghrelin concentrations were highest at birth, prior to the onset of lactation-associated hyperphagia. Plasma fed GLP-1 concentrations were elevated during pregnancy; and together with higher colon concentrations of PYY and GLP-1 during early lactation, they were associated with gastrointestinal tissue expansion, not satiety. Body mass increased during lactation, whereas white adipose tissue depots depleted. Extensive gut remodelling coincided with elevated colon concentrations of PYY and GLP-1. Modifications included: stomach and caecum expansion, and duodenal, ascending and descending colon circumference increases, all peaking by day 10 of lactation; increased intestinal masses and lengths peaking at lactation day 10 for small intestine and lactation day 25 for large intestine. If these physical tissue increases persist post-partum, they could

accelerate future nutrient assimilation and storage in dams, and may contribute to increased obesity risk.

Introduction

Pregnancy and lactation involve extensive maternal adaptation for foetal and neonatal growth, and to build and replace maternal energy reserves. In rats, dam body mass increases start soon after conception (Cripps & Williams, 1975), partly by adipose tissue accumulation (Lopez-Luna *et al.,* 1991, Pujol *et al.,* 2006). Post-partum, body mass increases again, to above that of non-pregnant controls, despite ~60% loss of adiposity by day 16 lactation (Naismith *et al.,* 1982). Thus maternal body composition changes, although has not been extensively studied.

Hyperphagia during rodent early pregnancy increases food intake by 20% (Crean & Rumsey, 1971, Trujillo *et al.,* 2011), with peaks of 50-60% (Cripps & Williams, 1975) compared to nulliparous controls. Maternal intake during lactation peaks during week 3 lactation to ~250-300% of controls (Crean & Rumsey, 1971, Cripps & Williams, 1975, Denis *et al.,* 2004). Hyperphagia during lactation is supported by reduced leptin and decreased adiposity (Woodside *et al.,* 2000) and although appetite increases occur, studies of changes in gut appetite-regulating hormones have so far produced conflicting results.

Ghrelin increases with fasting, elevating prior to feeding (Nakazato *et al.,* 2001) and is suppressed by increased leptin in males (Ueno *et al.,* 2004). In *ad lib* fed pregnant rats, Taylor *et al.* (2009) found total ghrelin in plasma and some gut tissues was not suppressed, despite increased leptin mid-pregnancy, whereas another study found ghrelin decreased (Shibata *et al.,* 2004). During lactation in rats, Taylor *et al.* (2009) reported no difference in either plasma or tissue total ghrelin, whereas Shibata *et al.*

(2004) found lower ghrelin and hypothalamic mRNA during lactation than late pregnancy, suggesting possible systemic reductions after birth. Inconsistencies may be due to non-standardised and/or physiologically inappropriate sampling times, such as during light periods for feeding studies, as rodents consume most food during the active dark phase. This study addressed these issues with dark phase sampling and looking closely at ghrelin-secreting cell location and abundance, to address previously conflicting findings.

Satiety hormones PYY and GLP-1 have received less research interest than ghrelin. GLP-1 may have differential abilities to regulate food intake based on nutritional status, with limited effects when fasted (Ronveaux *et al*., 2014). Also, GLP-1 treatment during proestrus was detrimental to early pregnancy events (Outeirino-Iglesias *et al.,* 2015). During rat pregnancy, plasma total PYY increased (Tovar *et al.,* 2004, Taylor *et al.,* 2009) and gradual increases were documented in descending colon and rectum (Taylor *et al.,* 2009). Taylor *et al.* (2009) reported increased plasma PYY at day 5 lactation in rats, paralleled by increases in DC and rectum; also hypothalamic PYY mRNA (days 5 + 15, Suzuki *et al.,* 2014). It appears plausible that elevated PYY has an important role in rat lactation. Why a purported satiety hormone was elevated, despite pregnancy and lactation-associated hyperphagia, remains to be established. No studies have explored circulating or gut tissue GLP-1 during lactation, hence the current focus.

Maternal adaptation during pregnancy and lactation in many animals involves GI tract structural changes (Speakman, 2008, Reiff *et al.,* 2015) to accommodate large food intake increases, although the mechanisms involved are poorly understood. Stomach tissue mass has been documented to increase during pregnancy and peak by late lactation in rats (Cripps & Williams, 1975, Taylor *et al.,* 2009) and mice (Campbell &

Fell, 1964). The small intestine is most extensively studied (Cripps & Williams, 1975, Burdett & Reek, 1979; Datta *et al.* 1995) with documented increases of: 27% length by late lactation, 140-150% mucosal epithelium mass; also surface area (Boyne *et al.,* 1966, Penzes & Regius, 1985). Colon length increases with pregnancy, and mass with lactation (Cripps and Williams, 1975; Taylor *et al.,* 2009). PYY, GLP-1 and GLP-2 are co-secreted from gut L-cells (Mojsov *et al.,* 1986) and have been linked with gut growth (Drucker *et al.,* 1996) and increased capacity for nutrient absorption (Brubaker *et al.,* 1997; Ghatei *et al.,* 2001), although not yet in reproduction. In adult female mice, PYY stimulated growth of both the small and large intestines in a dose-dependent manner; whereas colon size only increased at higher doses (Gomez *et al.,* 1995).

The aim of this study was therefore to clarify and elucidate roles of ghrelin, PYY and GLP-1 during maternal adaptation. Samples of fed and fasted plasma were obtained from the same animals, taken in the dark phase; also from matched gut tissue, with PYY and GLP-1 concentrations measured for the first time during different reproductive stages. Comprehensive measurements have explored gut capacity and expansion in detail, thus this study advances and updates the current knowledge of gut growth during pregnancy and lactation and allows investigation into potential relationships between gut peptides and intestinal remodelling.

Materials and Methods

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 chosen for this study to obtain an adequate volume of blood for matched fed and fasted circulating hormone analysis, with matched tissue peptide comparisons. Female

Wistar rats (Harlan, Bicester, UK; n=49) were housed in groups of three or four, with free access to standard rodent breeding diet (801730, Special Diets Service Essex, UK), water and bedding material. The animals were adjusted to 12-hour reverse lighting conditions (lights off between 11:00 and 23:00 hr) for a minimum of two weeks before study start, then permanently housed under these conditions. All procedures were carried out during the dark phase, in contrast to most prior studies, so that samples were obtained when most physiologically relevant for natural feeding behaviour. There were seven experimental groups: days 4, 12 and 18 of pregnancy and days 0, 5, 10 and 25 of lactation (n=7 per group). The time points used for sample groups were optimised based on the findings of Taylor *et al.* (2009).

The oestrus dance (Feder, 1981) was used to accurately time-mate females for pregnancy time-points, and for some lactation time-points when appropriate females presented with the dance (Stramek *et al*., 2018). Dams for the lactation time-points had their litters standardised to 8 ± 1 each by day two postpartum. Pups remained with the dams throughout the study and had free access to the cage diet when they were able to reach it, from approximately 16 days of age. Dams used on the day of birth had their litters standardised shortly after birth, when a nest had been established, and were sacrificed approximately 4-5 hours after birth. Pregnant dams for lactation time-points were separated into their own cage with nesting material between days 18 and 20 of pregnancy.

Pregnant rats had an age range of 24-38 weeks and lactating rats 18-37 weeks by the end of the study. Data from a group of 6 proestrus nulliparous females (aged 34-37 weeks) were included as a reference point for the study but the focus of this study was on the differences between the groups progressing through pregnancy and through lactation.

Body mass and food intake

Live body mass was recorded prior to tail bleed (below) and once again after the fasting period, before sacrifice. Food intake was monitored in singly housed females by weighing their food hopper in the days immediately prior to birth and into lactation before the pups started eating solid food.

Blood collection and preparation

Fed blood samples were taken from a tail vein, between 12:00 and 13:00 hr, under anaesthesia (isoflurane; IsoFlo, Abbott Laboratories, Maidenhead, UK) to minimise stress during collection, as optimised from an earlier study (Johnson *et al.,* 2016). Lubrithal (VetXX Ltd, Stoke-on-Trent, UK) was applied to the eyes of the rats whilst under anaesthetic to prevent them from drying out and a spray-on dressing (OpSite, Smith & Nephew Medical Ltd, Watford, UK) was applied to the tail tip after sample collection. Once conscious, females were immediately returned to their home cage in their established social groups (pregnancy) or with their pups (lactating).

Cage food was removed (access to water maintained) at 08:00 the following day prior to sacrifice between 12:00 and 16:00 hr. Rats were fully anaesthetised and decapitated, and a fasted blood sample was obtained from trunk blood. All blood was collected into EDTA coated tubes with additional protease inhibitor (aprotinin; Trasylol, Bayer plc, Reading, UK). All fed and 1 ml of fasted 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 (Stengel *et al.,* 2009).

Gastrointestinal tissue measurements

The gastrointestinal (GI) tract was measured and sampled in several locations: stomach, small intestine (SI), caecum, large intestine (LI; ascending and descending colon). Measurements of gastrointestinal length were made by emptying the gut of contents and free-floating the tissue in PBS, taking care not to stretch the tissue. Masses were recorded after emptying the gut and blotting the tissue dry on tissue paper. Due to differences in early sample collection, small intestine wet weight has been excluded from analysis for the day 25 lactating dam group and the proestrus controls.

Stomachs were removed and mass was recorded after opening along the greater curvature and rinsing in PBS. Once weighed, the stomach incision was extended to cut the stomach in half; one half was snap frozen for peptide extraction (see below) and the body of the other half was fixed in 4% paraformaldehyde overnight, then rinsed in 3 x 10 minutes PBS and placed in 30% sucrose at 4°C for a minimum of 48 hours until frozen in OCT for cryosectioning transversely at 10 µm. The small and large intestines were removed whole, emptied, and wet weight and length recorded. The caecum was removed and treated similarly to the stomach (see above). Approximately 1 cm of mid duodenum, proximal ascending colon and proximal descending colon was removed for circumference measurements. Circumference measurements were standardised by measurement after 20 minute incubation in PBS containing 10^{-6} M nicardipine hydrochloride to maximally relax the smooth muscle. Nicardipine could not be used to standardise gut length measurements due to the possibility of it interfering with other methodologies (e.g. peptide extraction) that the tissue subsequently underwent; circumference measurements were taken

184 from one small piece of tissue. In addition to gut measurements, all of the white
185 adipose tissue (WAT) in the abdominal cavity was carefully removed and weighed.

186 ## Gastrointestinal tissue preparation for radioimmunoassay

187 Half of each stomach, and ~2 cm portion of mid-duodenum, mid-ascending colon
188 and mid-descending colon were collected and immediately frozen on dry ice. Tissue
189 samples were extracted in 1 ml of 0.5 M glacial acetic acid per 100 g of tissue
190 collected, and boiled in a 100 °C water bath for 20 minutes. The liquid portion of the
191 boiled samples was stored at -20 °C until assayed.

192 ## Radioimmunoassay

193 As all fed plasma collected was acidified (thereby diluted), this was analysed for each
194 peptide. Fasted acidified plasma was used in ghrelin assays as recommended to
195 stabilise the acyl peptide form, and for PYY and GLP-1 assays, neat un-
196 acidified/undiluted fasted plasma was used because concentrations of both of these
197 peptides were expected to be lowest in the fasted state. All samples were analysed in
198 duplicate and according to the manufacturer's protocol for total ghrelin, total PYY and
199 total GLP-1 concentration using radioimmunoassay kits (Millipore, Watford, UK). An
200 Excel spreadsheet was used to calculate sample concentrations from the standard
201 curve, adjusted for sample dilution, with internal controls (provided with the kit)
202 confirming optimal assay performance. All samples were added to assays based on
203 sample type, not by pregnancy or lactation stage.

204 ## Total ghrelin

205 Preliminary testing measured high concentrations of ghrelin in all samples, so they
206 were diluted ten times by a reduction in sample volume in the assay tubes. Due to
207 cost, fed plasma ghrelin was not measured as a previous study (Johnson *et al.,* 2016)

found no difference between fed and fasted concentrations. Stomach tissue extracts underwent an additional 1:250 dilution for pregnant and 1:400 for lactating dams. The mean intra-assay variation was 4.4% for plasma and 10.1% for tissue and the mean inter-assay variation was 2.07% for plasma and 9.95% for tissue.

Total PYY

Fasted plasma samples were added to the kits as suggested by the manufacturers' guidelines. Although fed plasma samples were analysed, the dilution effect of acidifying the samples caused peptide recovery issues so these data are not presented – see Discussion for why the interpretation of fasted PYY sample concentrations are as equally valid as fed samples during pregnancy. For both pregnant and lactating dams, ascending and descending colon extracts were diluted 1:80 prior to addition to the assays. The mean intra-assay variation was 4.4% for plasma and 6.2% for tissue and the mean inter-assay variation was 2.9% for plasma and 7.71% for tissue.

Total GLP-1

Fasted non-acidified plasma samples were extracted and added to each kit as outlined in the assay protocol. Double the volume of fed acidified plasma was required to undergo the kit extraction protocol in order to bring these samples onto the linear part of the standard curve. Ascending colon samples were diluted 1:80 and descending colon samples were diluted 1:70. The mean intra-assay variation was 4.5% for plasma and 6% for tissue and the mean inter-assay variation was 9.4% for plasma and 3.4% for tissue.

Immunofluorescence of stomach tissue

Stomach sections were stained for total ghrelin peptide using a standard immunofluorescence protocol, with incubations carried out at room temperature. Briefly, slides were incubated with normal horse serum (10%) for 90 minutes, washed in PBS, and incubated overnight with 1:800 goat anti-ghrelin antibody (Santa Cruz Biotechnology, Dallas, USA). After 3 x 10 minute washes in PBS, a biotinylated horse anti-goat IgG (Vector) at 6 µg/ml was applied for 120 minutes, followed by a further wash step. The slides were then incubated with streptavidin fluorescein (4 µg/ml) for 120 minutes before a final wash step, and were then cover-slipped using Citifluor (Agar Scientific, Stanstead, UK). Negative controls were: antibody-dilution solution only, primary antibody only, secondary antibody only and streptavidin fluorescein only.

Quantification of immunolabelled cell numbers

Images were obtained using an Olympus BX fluorescence microscope. In order to perform a manual cell count of each immunoreactive (IR) cell, serial images were taken of the entirety of each section of tissue stained using a x10 objective lens and all IR cells were counted from these images. The programme ImageJ was used to aid manual cell counting, using the cell counter plugin to mark each IR cell in each image. In order to count these images blind, an online list randomiser (http://www.random.org/lists/) was used to assign a random number to each animal number. Each image was then renamed using this random number and cell counting was completed before counts were un-blinded for statistical analysis.

Statistical analysis

Values represent mean ± S.E.M. Statistical analysis was initially carried out using a one-way ANOVA with a Tukey post-hoc test on normally distributed data (shown on figures with asterisks e.g. * $P<0.05$). Data not normally distributed were normalised by log transformation (e.g. GLP-1 AC, DC; caecum and large intestine wet masses). When data were not normally distributed and could not be normalised, a Kruskal-Wallis test was used, with subsequent pairwise comparisons (Mann-Whitney), with Bonferroni correction. A paired-samples *t*-test was used to compare plasma peptides in the fed and the fasted states, and to compare peptide concentrations in different areas/regions of the colon. As the experimental design included multiple factors including fed and fasted status, different regions of the gut and different time-points during pregnancy and lactation, as well as a proestrus control, GLM univariate analysis was used to test for any interactions. Tukey post-hoc tests were used to investigate the sources of interaction and are shown on Figures 1, 4, 5, 8, 9, 10 with e.g. a>b, $P<0.001$ etc to indicate significance between different pregnancy and lactation charts. Italicised *a>b* are also used to differentiate from other significant interactions. All statistical tests were performed using IBM SPSS Statistics 24. $P<0.05$ was considered statistically significant.

Results

Ghrelin concentrations were suppressed during pregnancy and elevated by the end of lactation

All stomach tissue samples had higher total ghrelin concentrations than plasma samples ($P<0.001$; Figure 1C > 1A; Figure 1D > 1B). GLM analysis found significant

main (direct) effects of sample type (plasma/stomach; $F(7,112)=2.470$, $P=0.023$) and stage (proestrus, pregnancy or lactation time-points; $F(1,112)=643.676$, $P=0.000$) and a significant interaction (joint effect) of sample type with reproductive stage ($F(7,112)=2.269$, $P=0.035$).

Plasma

Fasted plasma total ghrelin during pregnancy remained consistently reduced ($F(7, 49)=27.751$, $P=0.001$; Figure 1A) compared with proestrus controls and all stages of lactation (Figure 1B). During pregnancy, ghrelin was significantly ($F(2, 18)=3.767$, $P=0.043$) less concentrated in day 18 pregnant (d18P) dams compared with day 4 pregnant dams (d4P). By the day of birth (d0L), fasted plasma ghrelin concentrations had increased back to control amounts, and by day 25 of lactation (d25L) had significantly increased ($F(3, 24)=4.546$, $P=0.012$) further compared with d0L ($P=0.023$) and day 5 lactating (d5L; $P=0.017$) dams (Figure 1B).

Stomach tissue

During pregnancy, the amounts of ghrelin in stomach tissue varied within each dam group, but were reduced in comparison with proestrus controls ($P<0.014$; Figure 1C), in d12P ($P=0.035$). Stomach tissue ghrelin concentration was significantly (Kruskal-Wallis, $\chi2=10.057$, 3 df, $P=0.018$) increased by d0L compared with d12P ($P=0.001$) and d18P ($P=0.036$) dams, with a later, significant decrease by d25L ($P=0.025$) (Figure 1D). Although d0L dams had the highest concentration of ghrelin in their stomach tissue out of all of the lactating and pregnant dams, this concentration was similar to that found in the proestrus controls.

Figure 1. Concentrations of ghrelin in fasted plasma and stomach tissue throughout pregnancy and lactation

Ghrelin-immunoreactive (IR) cells in stomach tissue were found throughout the mucosa, predominantly so towards the mucosal-submucosal border, in all groups quantified: proestrus, day 12 pregnant (d12P) and d0L (Figure 2A, B, C). There was no difference in the mucosal area of the stomach, nor in the mean maximum thickness of the mucosa of muscle layers. However, the ghrelin-IR cell density was significantly different between the sample groups ($F(2, 17)=29.735$, $P<0.001$) and increased significantly from the proestrus controls to d12P, and dams in the transition stage of parturition (d0L) had a significantly higher ghrelin cell density than in both the other sample groups (Figure 2D).

Figure 2. Representative images of stomach tissue (A-C) showing the distribution of ghrelin immunoreactive staining and (D) quantification of stomach tissue ghrelin cell density at proestrus, d12P and day of birth (d0L)

Peptide-YY (PYY) concentrations were increased during lactation

All colon tissue samples had higher total PYY concentrations than plasma samples ($P<0.001$; Figure 3C, D, E, F > Figure 3A, B) and GLM analysis of colon region (ascending/descending) found significant main (direct) effects on colon tissue PYY of stage (proestrus, pregnancy or lactation time-points; $F(7,101)=4.701$, $P<0.001$; Figure 3C, D, E, F with peak PYY concentrations at day 5 of lactation for ascending colon ($P=0.021$; Figure 3D) and descending ($P=0.024$; Figure 3F) colon compared with proestrus DC concentrations (Figure 3F).

Plasma

Both the pregnant and lactating dams had similar fasted plasma total PYY concentrations, although they were elevated compared with proestrus controls in d10L dams ($F(4,35)=4.683$, $P=0.004$; Figure 3A, B), with a tendency ($P=0.067$, n.s.) in d12P dams.

Colon tissue

Ascending and descending colon PYY concentrations varied considerably within each pregnancy group and were not significantly different from each other, although there was a numerical increase, especially in ascending colon, and PYY in the d18P dams in descending colon was two times higher than the proestrus controls (no ascending colon data), suggesting that PYY gradually elevated during pregnancy.

PYY concentrations in the ascending colon were significantly ($F(6, 46)=3.215$, $P=0.011$) highest in day 5 lactating (d5L) dams - more than double - compared with the start of pregnancy (d4P, $P=0.025$) and with the end of lactation (d25L, $P=0.021$). Descending colon PYY concentrations were over three times higher in d5L dams than the proestrus controls (Kruskal-Wallis, $\chi 2=16.955$, 7 df, $P=0.018$).

Figure 3. Concentrations of PYY in fasted plasma, ascending and descending colon tissues throughout pregnancy and lactation

Glucagon-like peptide-1 peptide concentrations were suppressed in pregnancy when fasted and elevated when fed in pregnancy and lactation

All colon tissue samples had higher total GLP-1 concentrations than plasma samples (P<0.001; Figure 5 *cf* Figure 4). GLM analysis found significant main (direct) effects on plasma GLP-1 of fed/fasted status $F(7,109)=5.208$, $P=0.000$) and stage (proestrus, pregnancy or lactation time-points; $F(1,109)=110.679$, $P=0.000$) and a significant interaction (joint effect) of fed/fasted status with reproductive stage ($F(7,109)=5.648$, $P=0.000$).

Plasma

There was a large magnitude of GLP-1 differences between fed and fasted, as well as between pregnant, lactating and proestrus controls. Fed proestrus rats had twice the amount of circulating GLP-1 than fasted proestrus animals, but fed values were highly variable (56 ± 25.7 *cf* 28 ± 6.5 pg/ml; $P=0.368$, n.s.). During pregnancy, GLP-1 concentrations were significantly - approximately 28 times - higher ($t(20)=7.463$, P<0.001) in fed plasma (165 ± 21.4 pg/ml) than in paired fasted plasma (6 ± 1.1 pg/ml) (Figure 4A *cf* 4C). For the whole lactating group, GLP-1 was 5 fold more concentrated ($t(24)=5.502$, P<0.001) in the fed state (249 ± 25.8 pg/ml) than in the fasted state (52 ± 17.8 pg/ml) (Figure 4B *cf* 4D).

Fed plasma GLP-1 in pregnant dams did not significantly differ ($P=0.30$, n.s) between groups, but was elevated 2 to 4 times during pregnancy by d12 ($P=0.024$) compared to proestrus fed nulliparous controls $F(3,23)=3.24$, $P=0.041$; Figure 4A). In contrast, fasted plasma GLP-1 concentrations, were significantly decreased at each stage of pregnancy ($F(3, 25)=8.613$, P<0.001) compared with proestrus fasted

controls, starting at approximately three times lower in early pregnancy at d4P until values were seven times lower by d18P (Figure 4C) towards the end of pregnancy. Fasted plasma GLP-1 was significantly decreased during pregnancy ($F(2,18)=3.664$, $P=0.046$), with a trend ($P=0.057$, n.s.) for d18P dams to have less fasted plasma GLP-1 than d4P dams.

Dams at the beginning of lactation had even higher ($F(4,26)=9.532$, $P<0.001$) fed plasma GLP-1 than during pregnancy, that was approximately six fold more than proestrus controls (Figure 4B). Early lactation dams (d0L and d5L) had higher fed GLP-1 values than in all the fasted and fed pregnant (except d12P fed, the third highest time point) and other lactating dams (Figure 4A, C, D). There were no significant differences in fasted plasma GLP-1 during lactation and amounts were closer to proestrus controls (Figure 4D).

Figure 4. Concentrations of GLP-1 in fed and fasted plasma throughout pregnancy and lactation.

Colon tissue

GLM analysis found significant main (direct) effects on GLP-1 concentrations of colon region (ascending/descending) $F(1,104)=43.446$, $P=0.000$) and stage (proestrus, pregnancy or lactation time-points; $F(7,104)=7.551$, $P=0.000$) and a significant interaction (joint effect) of colon region with reproductive stage ($F(7,104)=2.637$, $P=0.016$).

A comparison between different areas of the colon found that ascending colon concentrations of GLP-1 were significantly higher than in descending colon tissue during all pregnancy time points (Figure 5A, 5C; d4P $t(6)=5.224$, $P=0.002$; d12P

389 $t(6)=4.466$, $P=0.004$; d18P $t(6)=4.234$, $P=0.005$). During lactation, ascending colon
390 GLP-1 was higher than descending colon (Figure 5C, 5D) at d0L ($t(6)=3.651$,
391 $P=0.01$) and d10L ($t(6)=4.369$, $P=0.005$), therefore GLP-1 concentrations in different
392 regions of the colon were only similar at d5L, due to a possible transient rise in
393 descending colon; and they were also equivalent at d25L when GLP-1 in both tissue
394 regions had reduced to close to control values.
395 Within ascending colon, GLP-1 concentrations were significantly different between
396 the pregnant dams ($F(2, 18)=3.919$, $P=0.039$; Figure 5A) and d18P dams had twice
397 the concentration of ascending colon GLP-1 than day 12 pregnant (d12P) dams,
398 although this did not reach significance with ANOVA post hoc tests ($P=0.051$, n.s.).
399 Descending colon tissue concentrations of GLP-1 were not significantly different with
400 pregnancy stage, but were approximately two to four times higher than in proestrus
401 controls (Figure 5C).
402 Ascending colon GLP-1 concentrations decreased as lactation progressed and were
403 significantly ($F(3, 22)=4.164$, $P=0.018$) higher in d0L dams than in d25L dams
404 ($P=0.016$; Figure 5B). GLP-1 concentrations in descending colon tissue were
405 significantly different ($F(3, 22)=4.493$, $P=0.013$) between the dam groups, and were
406 highest at d0L and d5L before a sharp decrease by d10L ($P=0.020$), with a
407 significant decrease also found between d0L and d10L ($P=0.030$) dams (Figure 5D).
408 Descending colon GLP-1 concentrations were higher at d0L and d5L ($P=0.001$;
409 $P=0.036$) than in the proestrus controls.
410 GLM analysis confirmed that GLP-1 concentrations in late pregnancy (d18P)
411 ascending colon (Figure 5A) were higher than all other time-points in both regions of
412 colon ($P=0.027$-0.000; Figure 5B, C, D) and d10L DC concentrations (Figure 5D)

were lower than all the pregnant AC concentrations (*P*=0.047-0.000; Figure 5A), as well as d0 and d5L AC (*P*=0.016-0.000; Figure 5B).

Figure 5. Concentrations of GLP-1 in ascending and descending colon throughout pregnancy and lactation

Peripartum food intake

The four dams that could be monitored continuously for the most consecutive days around birth, halved their food intake from day 3 to day 1 prior to birth (Figure 6). Food intake rapidly increased into the lactation period, doubling from d1L to d2L. Food intake continued to increase and was highest at d8L, when monitoring stopped. The d8L dams consumed approximately 250% more than the mean daily food intake of all of the normally cycling nulliparous females (15.5 ± 0.09 g, n=43) used in a prior study (Johnson *et al.,* 2016).

Figure 6. Food intake during the late peri- and early postpartum period

Changes in body and gastrointestinal size during pregnancy and lactation

As expected, body mass significantly increased ($F(2, 18)=12.565$, $P<0.001$) with the advancing stages of pregnancy until birth (Figure 7A). Body mass gain continued during lactation ($F(2, 18)=12.942$, $P<0.001$; analysis excluding d0L as fed mass included gravid uterus; Figure 7B) and body mass exceeded that of proestrus control rats ($F(7,47)=16.208$, $P<0.001$) by d10L ($P=0.038$). Mass of abdominal cavity white

adipose tissue (WAT) was significantly largest (F(2, 18)=6.248, P=0.009; Figure 7C) by d18P, and showed a significant decline after d0L (F(3, 24)=13.899, P<0.001; Figure 7D) until the end of lactation. Day 18 pregnant dams had higher abdominal WAT values than proestrus nulliparous controls, while control rats had up to three times more WAT (F(7,49)=10.495, P<0.001) than rats at day 10 and day 25 of lactation (P<0.009; P<0.001; Figure 7C and D).

Figure 7. Changes in body mass and abdominal white adipose tissue mass throughout pregnancy and lactation

Stomach and caecum masses only increased during lactation, peaking d10L

Stomach tissue wet mass did not change throughout pregnancy and remained similar to the controls (Figure 8A). During lactation, stomach mass significantly increased (Kruskal Wallis, $\chi 2$=15.015, 3 df, P=0.002), becoming heavier than early pregnancy time points and control values by d10L, and d25L dams had significantly heavier stomachs than d0L (P=0.018) and d5L (P=0.002) dams (Figure 8b) and all pregnant groups (F(7,48)=12.648, P<0.001).

Similarly, caecum wet mass did not change significantly during pregnancy (Figure 8C), but was significantly (F(3, 24)=40.888, P<0.001) heavier in both the d10L and d25L dams than in the d0L (P<0.001) and d5L (P<0.001) dams (Figure 8D) and all pregnant groups (F(7,48)=12.648, P<0.001). Additionally, d5L dam caecum mass was heavier than in d4P animals (P=0.013).

Figure 8. Stomach mass and caecum mass throughout pregnancy and lactation

Small intestine mass and length increased during pregnancy and lactation, peaking by d10L

Late pregnant, d18P dams had significantly heavier (F(2, 18)=4.782, P=0.022; Figure 9A) and longer (F(2, 18)=5.365, P=0.015; Figure 9C) small intestines than d4P dams. Further growth continued during lactation: d5L dams had significantly heavier small intestines than d0L (P=0.022) dams, and in mid-lactation, d10L dams had heavier (F(2,18)=39.220, P<0.001) small intestines than both d0L (P<0.001) and d5L (P<0.001) dams (Figure 9B); there were no data for d25L (see methods). The small intestine also significantly increased in length in later lactation (F(3, 24)=17.944, P<0.001; Figure 9D), being significantly longer in both d10L dams (where peak growth had been reached) and d25L dams, than in d0L (P<0.001) and d5L (P<0.001) dams. Day 10 and 25 lactation dams had approximately 48% longer small intestines than proestrus controls, d0L and d5L dams and all pregnant groups (F(7,49)=20.546, P<0.001).

Large intestine mass increased during pregnancy and lactation, with length only increased by late lactation, peaking d25L

Large intestines of d18P dams were significantly heavier (F(2, 18)=7.931, P=0.003; Figure 9E) than the d4P (P=0.003) and d12P (P=0.046) groups, although they were significantly (F(2, 18)=5.506, P=0.014) shorter in d12P dams than in d4P (P=0.044) and d18P (P=0.017) dams (Figure 9G). Dams at the end of the lactation period (d25L) had significantly (F(3, 19)=19.322, P<0.001) heavier large intestine tissue than d0L

(*P*<0.001), d5L (*P*<0.001) and d10L (*P*=0.001) dams (Figure 9F) and all pregnant dam groups (*F*(7,47)=11.104, *P*<0.001). Dams also had significantly shorter (*F*(3, 24)=15.519, *P*<0.001) large intestines at d0L (*P*<0.001) and d5L (*P*=0.001) compared to d25L dams, with d0L dams also having shorter large intestines than d10L (*P*=0.004) dams (Figure 9H). Both the pregnant dams and d5L dams had similar large intestine lengths to the proestrus controls (Figure 9G, H).

Figure 9. Changes in small and large intestine sizes throughout pregnancy and lactation

Gut circumferences were greater in pregnancy and lactation, peaking by d10L

Gut circumference measurements for duodenum and ascending colon were greater in the pregnant dams, significantly at d4P (*F*(7,49)=4.108; 5.906, *P*<0.001) compared with proestrus controls, with only descending colon values similar (Figure 10A). Between the different pregnant groups, neither the duodenum nor descending colon tissue circumferences differed, however, ascending colon circumference was significantly wider (*F*(2, 18)=3.953, *P*=0.038) in early pregnant d4P dams compared with d12P dams (*P*=0.033), which coincided with reduced large intestine length in d12P rats. d4P and d18P rats also had wider AC values than d25L rats (Figure 10 A, B).

Lactating dams at d10 had larger duodenum circumferences than proestrus controls (Figure 10B), at the same time as the small and large intestine growth increases at

d10L (Figure 9). By late lactation/weaning duodenum circumference (F(3, 24)=3.052, P=0.048) had significantly reduced by d25L from the earlier peak at d10L (P=0.046). Ascending colon circumference (F(3, 24)=6.506, P=0.002) was also significantly increased in early, compared to late lactation: d0L (P=0.045), d5L (P=0.035), and peaked in d10L (P=0.001) dams compared to d25L dams and proestrus controls (Figure 10B). The reduction in late lactation coincided with peak large intestine increases (Figure 9).

Circumference of descending colon (F(3, 24)=4.346, P=0.014) was also significantly smaller in d25L than in d10L (P=0.012) dams. Both ascending and descending circumferences at d25L were reduced compared to earlier in lactation and were also smaller than proestrus controls – this coincided with the large intestine peak mass and length increases at d25L.

Figure 10. Changes in gut circumference throughout pregnancy and lactation

Discussion

This is the first study to analyse total orexigenic ghrelin, anorexigenic PYY and GLP-1, in matched fed and fasted plasma and gut tissues, with samples taken during the nocturnal, active phase. We provide new and updated information about organ remodelling in dams, including changes to GI capacity and tissue sizes, matched with peripherally circulating and tissue concentrations of ghrelin, PYY and GLP-1.

Ghrelin stimulates appetite, thus is expected to increase during pregnancy. However, fasted plasma ghrelin was decreased between d4P and d18P, consistent with animal (Shibata *et al.,* 2004) and human studies (Fuglsang *et al.,* 2005, Tham *et al.,* 2009). Suppression of this appetite-stimulating signal occurred throughout pregnancy for

reasons unknown, and despite ghrelin-secreting cell increases by d12P compared with proestrus controls that continued until the onset of lactation. Unknown mechanisms underlie peripheral ghrelin suppression during early pregnancy, although increasing leptin may be involved later. What initiates and supports early pregnancy hyperphagia if the only peripheral orexigenic hormone is not involved? These observations caution against endogenous appetite hormone use as body mass control therapies during pregnancy until established that alterations are not harmful to developing embryos.

Reduction in food intake before birth followed low systemic ghrelin at d18P, and the 100% increase post-partum occurred after peak ghrelin-IR cells and the highest stomach ghrelin measured. Fasted plasma ghrelin increased from early to d25 lactation; dams had 20% higher than proestrus controls. Shibata *et al.* (2004) reported no changes to fed plasma ghrelin in rats between d5L and d15L, and Suzuki *et al.* (2014) likewise found no difference in fed acyl ghrelin. The plasma differences we report are novel and arguably more reliable, as samples were both fasted and taken in the more physiologically-relevant dark phase. Late lactation ghrelin increases could be explained by decreased WAT/reduced leptin (Woodside *et al.,* 2000, Taylor *et al.,* 2009), which in turn could unsuppress circulating ghrelin (Ueno *et al.,* 2004). Dams still need to eat more to replenish energy reserves following weaning (very low WAT masses d25L), despite body mass increases (see later discussion). Dams had larger stomach masses by lactation end, with potentially more ghrelin-secretory cells following the measured increase at parturition, contributing to higher circulating ghrelin by d25L.

PYY was not altered during pregnancy, agreeing with Valsamakis *et al.* (2010), although values were higher than non-pregnant. Fasted plasma PYY was 63%

556 higher than controls at d10L, demonstrating elevated peripheral concentrations,
557 following earlier peaks in d5L colon tissue. Other studies report plasma PYY fed and
558 fasted d5L peaks (Tovar *et al.,* 2004, Taylor *et al.,* 2009; Suzuki *et al.,* 2014).
559 Together, these studies indicate that fed/fasting status of *ad lib* fed dams does not
560 diminish observed PYY peaks. During lactation, AC and DC PYY peaked in d5L
561 dams, similar to d5L plasma peaks (Taylor *et al.,* 2009; Suzuki *et al.,* 2014). Despite
562 gut region/timing differences between studies, increases were consistent. It remains
563 to be established why a purported satiety hormone elevates during physiological
564 states of hyperphagia; some explanations relating to GI remodelling are explored
565 below.
566 GLP-1 is a satiety hormone and decreases in insulin-resistant states (Toft-Nielsen *et*
567 *al.,* 2001, Muscelli *et al.,* 2008, Lim *et al.,* 2009). Pregnancy-associated insulin
568 resistance could explain decreases in fasted plasma GLP-1 throughout pregnancy,
569 with lowest values by d18P. As with PYY, there were unexpected increases, as fed
570 GLP-1 was greatly elevated (25x) in pregnancy and highest in d18P AC tissue.
571 Likewise, fed GLP-1 was also very high with d0 and d5 peaks in lactation; the main
572 source of circulating GLP-1 was likely AC, as DC was very low after d5L. In contrast,
573 human studies found no GLP-1 lactational changes (Larson-Meyer *et al.,* 2016).
574 The current study found both PYY and GLP-1, so-called ‘satiety’ hormones, to be
575 increased, with high values persistent in lactation despite food intake increasing 199%
576 by d5L. High sustained PYY and GLP-1 may initiate the ileal brake mechanism (Lin *et*
577 *al*., 1996; Maljaars *et al.*, 2008), against a low ghrelin background and reduced gastric
578 emptying (Levin *et al*., 2006), thus slowing gut transit times to allow digestion and
579 nutrient extraction from increased feed. The currently accepted role of PYY and GLP-
580 1 is to rise in response to intake and signal fullness, causing a compensatory appetite

581 reduction but the opposite occurred in pregnant and lactating dams, despite
582 hyperphagia (except briefly at parturition).
583 This study also investigated physical gut changes to help explain contradictory
584 'appetite' hormone observations. Following birth, dam body mass increased although
585 WAT reserves decreased, reflecting body composition changes. We explored GI tract
586 remodelling and have described a number of modifications that show how dam
587 physiology altered to accommodate lactational demands of eight growing pups.
588 Neither stomach nor caecum mass changed across pregnancy, but wet masses
589 increased by end of lactation, consistent with previous studies (Cripps & Williams.
590 1975; Taylor *et al.,* 2009). Small intestine wet weight and length increased between
591 d4P and d18P, by 20% and 15%. This increase in size and capacity could be an early,
592 rapid adaptation to increase absorption of nutrients from more food, to support the
593 production of reproductive tissues; also building reserves for lactation. Lactating dams
594 had further increased small intestine wet weight and length by d10L, 50% longer than
595 non-pregnant controls. Large intestine mass increased with pregnancy and was even
596 heavier by d25L, in agreement with Cripps & Williams (1975). Any variation in adaptive
597 changes between studies are likely due to differences in diet composition and food
598 quantities consumed. In our study, there was consistency of timing of SI and LI tissue
599 expansion with increased stomach and caecum masses, although SI increases
600 peaked earlier at d10 of lactation, whereas LI growth continued to d25L.
601 Gut circumferences provided further novel information. Although neither duodenum
602 nor DC were different between pregnancy stages, AC circumferences were. Changes
603 along the GI tract could be an additional mechanism to support pregnancy/lactation,
604 increasing surface area and gut capacity to process nutrients from more food, altering
605 transit times. Day 4P dams had largest AC circumferences, with higher values in

duodenum and DC, compared to controls. These data may reflect the earliest pregnancy adaptation to rapidly increase capacity, especially of caecum to hold more food (as microbiome composition changes: Mann *et al*., 2018), prior to gut lengthening later in pregnancy, further increasing surface area. In early pregnancy there are less competing demands for space between gravid uterus and abdominal organs, making temporary expansion possible.

In lactating dams, duodenum, AC and DC had increased circumferences leading up to d10L peaks, with decreases from d10L to d25L. Narrowing of colon circumferences coincided with maximal tissue hypertrophy – increased lengths and masses – and capacity, which may reflect final adjustments to maximal feed intakes during lactation. Thus, in addition to later gut hypertrophy, early maternal adaptations to hyperphagia included widening/dilation of specific portions of GI tract to accommodate greater volumes of food and aid nutrient acquisition. In lactating rats on restricted diets, Campbell and Fell (1964) found SI was similarly dilated, with only partial hypertrophy compared to *ad lib* fed. That study also reported that the absorptive capacity of SI did not differ between nulliparous and lactating rats with varying gut hypertrophy, suggesting changes are proportionate to requirements. Datta *et al.* (1995) also reported that food restriction prevented SI hypertrophy and Tovar *et al.* (2004) found a 30% food restriction completely suppressed pregnancy-associated PYY rises. Combined, these findings indicate both physical (food mass) and hormonal (PYY/GLP-1) stimulation are needed to initiate and maintain gut growth, as has been expounded in this study, where extensive organ remodelling, with increases in SI and LI masses and lengths, occurred during later lactation, coincident with elevations of and following PYY and GLP-1 peak concentrations that had occurred earlier in lactation.

PYY and GLP-1 are co-secreted, with GLP-2 (Mojsov *et al.,* 1986); and have previously been linked with gut growth in adult female mice (PYY: Gomez *et al.,* 1995), male mice (Drucker *et al.,* 1996) and rats (GLP-2 > GLP-1: Ghatei *et al.,* 2001). PYY and GLP-2 cause substantial intestinal hypertrophy, with smaller GLP-1 increases. Our findings of gut hypertrophy under conditions of high PYY and very high GLP-1 concentrations highlight that it is imperative to further elucidate the role of L-cells and their secretory products after gut surgeries for body mass reduction, as these techniques lead to rapidly increased concentrations of appetite hormones (Chandarana *et al*., 2011) and may be stimulating intestinal growth in attempts to regenerate remaining gut tissues (le Roux *et al.,* 2010).

This study has revealed that when maternal nutritional requirements are highest, additional and unexpected supportive changes to increased intake occur in ‘appetite’ hormones PYY and GLP-1. These hormonal alterations are likely to be homeorhetic adjustments (Bauman and Currie, 1980) to pregnancy, and especially lactation, that have a more pronounced influence on GI remodelling, than satiety. The observed structural changes contribute towards meeting dam enhanced energy requirements and maximising nutrient recovery from the increased intake of pregnancy and more extreme hyperphagia of lactation. They may aid feeding efficiency via altered metabolism and nutrient uptake, slowing food passage rate (Hammond, 1997) and facilitate caecum microbe diversity changes (Mann *et al*., 2018). Our observations show how gut adaptations continue until (and possibly persist beyond) weaning, in rat dams that raise litters of 8 pups.

In conclusion, despite hyperphagia, fasted plasma ghrelin was suppressed in pregnancy, although ghrelin-IR cells and stomach ghrelin were highest at birth, supporting onset of lactation-associated hyperphagia. Plasma fed GLP-1 was

elevated during pregnancy, and increases of colon PYY and GLP-1 during early lactation were associated with GI expansion, not satiety. All three 'appetite' hormones were altered in unexpected ways, with important implications for any surgical or pharmaceutical interventions designed to act as weight-control measures, in reproductive age females. Extensive stomach, caecum and gut expansion and remodelling coincided with PYY and GLP-1 peaks. Then increased intestinal masses, lengths and circumferences followed, with peaks at d10L for SI and d25L for LI. These modifications demonstrate how lactating rats process and assimilate more food to support eight pups to weaning. More important questions arise, including what implications may be for future maternal health if GI expansion persists, as modifications could accelerate future nutrient assimilation and storage, leading to long-term body mass retention. Also, whether the increased incidence of obesity and insulin resistance in younger human populations may additionally amplify maternal adaptations, thus influencing metabolic programming and future health of any offspring.

Declaration of interest

VJT served on the Council of Management for the Society for Reproduction and Fertility (SRF) 2013-16. MLJ was Postdoc representative on SRF Council of Management 2017-19. MLJ was the winning recipient of the SRF Post Doctoral Scientist Prize talk based on this work: Johnson ML, Saffrey MJ, Taylor VJ (2015) *Hyperphagia of pregnancy and lactation is associated with changes in appetite-regulating hormones and gastrointestinal modifications in Wistar rats.* Society for Reproduction and Fertility Annual Conference 2015, 20-22 July 2015, St Catherine's College, Oxford, UK. A repeat talk was given at the Annual Meetings of the

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Figure 1. Concentrations of ghrelin in fasted plasma and stomach tissue throughout pregnancy and lactation

Fasted plasma ghrelin during (A) pregnancy (* P<0.05; a>b, ***P<0.001) and (B) lactation (* P<0.05); GLM: (a>b, P<0.001); stomach tissue ghrelin during (C) pregnancy (c>d, P<0.05) and (D) lactation (* P<0.05); GLM: (a>b, P<0.001, c>d, P<0.05). (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of lactation, n=7; d5L, day 5 of lactation, plasma, n=7; stomach tissue, n=5; d10L, day 10 of lactation, n=7; d25L, day 25 of lactation, n=7. Dotted line represents mean of proestrus controls, n=6).

Figure 2. Representative images of stomach tissue (A-C) showing the distribution of ghrelin immunoreactive staining and (D) quantification of stomach tissue ghrelin cell density at proestrus, d12P and day of birth (d0L)

L, luminal/mucosal surface; M, mucosal-submucosal border (proestrus, n=6; d12P, day 12 of pregnancy, n=7; d0L, day 0 of lactation, n=7. ** P<0.01, *** P<0.001).

Figure 3. Concentrations of PYY in fasted plasma, ascending and descending colon tissues throughout pregnancy and lactation

Fasted plasma PYY in (A) pregnancy; and (B) lactation (a>b, P<0.005); ascending colon PYY during (C) pregnancy; and (D) lactation (*P<0.05); GLM: (c>d, P<0.025); descending colon PYY during (E) pregnancy; and (F) lactation (c>d, P<0.025). GLM: (c>d, P<0.025). (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of lactation, n=7; d5L, day 5 of lactation, n=7; d10L, day 10 of lactation, n=7; d25L, day 25 of lactation, fed plasma, n=6; fasted plasma, n=7. Dotted line represents mean of proestrus controls, n=6; no control data for AC).

Figure 4. Concentrations of GLP-1 in fed and fasted plasma throughout pregnancy and lactation.

GLP-1 in fed plasma during (A) pregnancy, (c>d, *P*<0.025); and (B) lactation (* *P*<0.05); GLM: (a>b, *P*<0.001; e>f, *P*<0.05); and GLP-1 in fasted plasma during (C) pregnancy (a>b, *P*<0.001); and (D) lactation; GLM: (a>b, *P*<0.001; c>d, *P*<0.025); note y axes differences between Figures (C) and (A), (B) and (D). (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of lactation, n=7; d5L, day 5 of lactation, n=5; d10L, day 10 of lactation, n=7; d25L, day 25 of lactation, fed plasma, n=6; fasted plasma, n=7. Dotted line represents mean of proestrus controls, n=6.)

Figure 5. Concentrations of GLP-1 in ascending and descending colon throughout pregnancy and lactation

Ascending colon GLP-1 concentrations during (A) pregnancy and (B) lactation (* *P*<0.05); descending colon GLP-1 during (C) pregnancy; (D) lactation (* *P*<0.05, e>f, *P*<0.05); *t*-tests: pregnancy (a>b, *P*<0.01); lactation (*a>b*, *P*<0.01); GLM: (c>d, *P*<0.03; e>f, *P*<0.05); (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of lactation, n=7; d5L, day 5 of lactation, n=5; d10L, day 10 of lactation, n=7; d25L, day 25 of lactation, n=7. Dotted line represents mean of proestrus controls, n=6.)

Figure 6. Food intake during the late peri- and early postpartum period

898 Dam food intake from 3 days PP until day 8 of lactation (d3PP-d1PP, days 3-1
899 peripartum; d0L-d8L, days 0 to 8 of lactation; n=4. Dotted line represents mean of
900 proestrus controls, n=6).
901
902 **Figure 7. Changes in body mass and abdominal white adipose tissue mass**
903 **throughout pregnancy and lactation**
904 Changes in fed body mass during (A) pregnancy (** P<0.01, *** P<0.001) and (B)
905 lactation (** P<0.01, *** P<0.001; e>f, P<0.05); (fed state is shown to avoid effects of
906 short-term fast and d0L fed masses not shown as they include gravid uterus) and
907 dissected WAT mass during (C) pregnancy (* P<0.05; a>b, P<0.001) and (D)
908 lactation (** P<0.01, *** P<0.001; a>b, P<0.001; c>d, P<0.01). (d4P, day 4 pregnant,
909 n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of
910 lactation, n=7; d5L, day 5 of lactation, n=7; d10L, day 10 of lactation, n=7; d25L, day
911 25 of lactation, n=7. Dotted line represents mean of proestrus controls, n=6).
912
913 **Figure 8. Stomach mass and caecum mass throughout pregnancy and**
914 **lactation**
915 Changes in empty stomach mass during (A) pregnancy and (B) lactation (** P<0.01;
916 * P<0.05; a>b, P<0.001); a>b, P<0.001, c>d, P<0.01, e>f, P<0.05; and empty
917 caecum mass during (C) pregnancy and (D) lactation (*** P<0.001); a>b, P<0.001,
918 c>d, P<0.02. (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18
919 pregnant, n=7; d0L, day 0 of lactation, n=7; d5L, day 5 of lactation, n=7; d10L, day
920 10 of lactation, n=7; d25L, day 25 of lactation, n=7. Dotted line represents mean of
921 proestrus controls, n=6; no data for caecum: (C), (D)).

Figure 9. Changes in small and large intestine sizes throughout pregnancy and lactation

The mass (A, B, E, F) and length (C, D, G, H) of the small (A, B, C, D) and large (E, F, G, H) intestines during pregnancy and lactation. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; a>b, $P<0.001$, c>d, $c>d$, $P<0.01$, e>f, $P<0.05$. There were no data for small intestine masses at d25L or proestrus controls for small and large intestine masses. (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of lactation, mass, n=4; length, n=7; d5L, day 5 of lactation, n=7; d10L, day 10 of lactation, n=7; d25L, day 25 of lactation, mass, n=5; length, n=7. Dotted line represents mean of proestrus controls, n=6).

Figure 10. Changes in gut circumference throughout pregnancy and lactation

Changes in duodenum (D), ascending colon (AC) and descending colon (DC) circumferences during (A) pregnancy and (B) lactation. * $P<0.05$, ** $P<0.01$; a>b, $a>b$, $P<0.001$, c>d, $P<0.01$, e>f, $e>f$, $P<0.05$. (d4P, day 4 pregnant, n=7; d12P, day 12 pregnant, n=7; d18P, day 18 pregnant, n=7; d0L, day 0 of lactation, n=7; d5L, day 5 of lactation, n=7; d10L, day 10 of lactation, n=7; d25L, day 25 of lactation, n=7. Dotted line represents mean of proestrus controls, n=6).