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Pregnancy, but not dietary octanoic acid supplementation, stimulates the ghrelin-pituitary growth hormone axis in mice

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Abstract

Circulating growth hormone (GH) concentrations increase during pregnancy in mice and remain pituitary-derived. Whether abundance or activation of the GH secretagogue ghrelin increase during pregnancy, or in response to dietary octanoic acid supplementation, is unclear. We therefore measured circulating GH profiles in late pregnant C57BL/6J mice and in aged-matched non-pregnant females fed with standard laboratory chow supplemented with 5% octanoic or palmitic (control) acid ($n = 4\text{--}13/\text{group}$). Serum total and acyl-ghrelin concentrations, stomach and placenta ghrelin mRNA and protein expression, *Pcsk1* (encoding prohormone convertase 1/3) and *Mboat4* (membrane bound O-acyl transferase 4) mRNA were determined at zeitgeber (ZT) 13 and ZT23. Total and basal GH secretion were higher in late pregnant than non-pregnant mice ($P < 0.001$), regardless of diet. At ZT13, serum concentrations of total ghrelin ($P = 0.004$), but not acyl-ghrelin, and the density of ghrelin-positive cells in the gastric antrum ($P = 0.019$) were higher, and gastric *Mboat4* and *Pcsk1* mRNA expression were lower in pregnant than non-pregnant mice at ZT23. In the placenta, ghrelin protein was localised mostly to labyrinthine trophoblast cells. Serum acyl-, but not total, ghrelin was lower at mid-pregnancy than in non-pregnant mice, but not different at early or late pregnancy. In conclusion, dietary supplementation with 5% octanoic acid did not increase activation of ghrelin in female mice. Our results further suggest that increases in maternal GH secretion throughout murine pregnancy are not due to circulating acyl-ghrelin acting at the pituitary. Nevertheless, time-dependent increased circulating total ghrelin could potentially increase ghrelin action in tissues that express the acylating enzyme and receptor.

Key Words

- ▶ mouse
- ▶ pregnancy
- ▶ octanoic acid
- ▶ ghrelin
- ▶ growth hormone

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Introduction

Successful pregnancy requires substantial maternal physiological adaptations, including increases in food intake, to maintain maternal health and allow optimal growth of the fetoplacental unit to term. Growth hormone (GH) is a key endocrine regulator of growth and metabolism in a range of mammalian species and its secretion changes markedly during pregnancy. In humans, placental synthesis of variant GH in the syncytiotrophoblast increases circulating GH concentrations and progressively suppresses pulsatile pituitary secretion from mid-pregnancy onwards (Eriksson *et al.* 1989). Although the placentas of most other mammalian species, including rodents, do not produce GH (Papper *et al.* 2009), circulating GH does increase during pregnancy in the majority of species investigated to date. In contrast to humans, circulating GH profiles remain pulsatile throughout pregnancy in mice and rats (Saunders *et al.* 1976, El-Kasti *et al.* 2008, Gatford *et al.* 2017). In these species, the increase in average circulating GH concentration and secretion reflect elevated basal GH secretion, while the number and amplitude of GH pulses remains unchanged (Saunders *et al.* 1976, El-Kasti *et al.* 2008, Gatford *et al.* 2017). In addition to driving maternal metabolic adaptation to pregnancy, including insulin resistance (Catalano *et al.* 1991, Newbern & Freemark 2011, Lager & Powell 2012, Liao *et al.* 2016, Teixeira *et al.* 2019), GH likely promotes fetal growth through other mechanisms. Provided maternal nutrition is adequate, intermittent maternal treatment with exogenous GH increases fetal growth in non-human species including rats, pigs and sheep (Spencer *et al.* 1994, Sterle *et al.* 1995, Harding *et al.* 1997, Gatford *et al.* 2000, 2004, de Boo *et al.* 2008, Tung *et al.* 2012), likely through enhanced placental growth and/or function (Spencer *et al.* 1994, Sterle *et al.* 1995, Harding *et al.* 1997, Gatford *et al.* 2000, Tung *et al.* 2012). Despite the importance of GH during pregnancy in many mammals, the mechanism driving increased maternal GH in non-human species remains less well-known.

The increases in circulating GH concentration and basal GH secretion in pregnant mice (Gatford *et al.* 2017) coincide with the formation of the chorioallantoic placenta and initiation of maternal blood flow through the placenta (Pringle & Roberts 2007). This suggests that placentally-secreted factors are likely to be responsible, either indirectly or directly, for driving the increased pituitary GH secretion during rodent pregnancy.

Ghrelin, a gastric peptide hormone that acts in conjunction with hypothalamic growth hormone-releasing hormone (GHRH) and somatostatin to generate pulsatile GH secretion from the anterior pituitary, is one possible candidate. In mouse stomach, the endoproteolytic processing of the ghrelin molecule from its precursor (proghrelin) into mature ghrelin is facilitated by the enzyme prohormone convertase 1/3 (PC1/3, encoded by proprotein convertase, *Pcks1*) (Zhu *et al.* 2006). Ghrelin is secreted in its des-acyl form and requires activation by acylation of the peptide's serine 3 residue with an 8-carbon octanoyl group (Kojima *et al.* 1999). This modification is mediated by the enzyme membrane bound O-acyl transferase (Mboat4, also known as GOAT) and allows the activated acyl-ghrelin to bind to the GH-secretagogue receptor (GHSR, Kojima *et al.* 1999, Gutierrez *et al.* 2008), stimulating GH secretion. Intravenous administration of exogenous acyl-ghrelin induces GH pulses in humans and rodents (Kojima *et al.* 1999, Arvat *et al.* 2001), while mice deficient in ghrelin or Mboat4 have lower amplitude GH pulses than littermate controls (Hassouna *et al.* 2014, Xie *et al.* 2015). Acyl-ghrelin, but not des-acyl ghrelin, also acts centrally to stimulate food intake (Nakazato *et al.* 2001, Neary *et al.* 2006), and acyl-ghrelin plays an important role in metabolic adaptations to maintain circulating glucose in response to calorie restriction, particularly in pregnancy (Trivedi *et al.* 2017). Although ghrelin and Mboat4 are predominantly expressed in the stomach (Kojima *et al.* 1999), ghrelin, Mboat4 and GHSR expression are also detected in the human (Gnanapavan *et al.* 2002, González-Domínguez *et al.* 2016) and rodent (Gualillo *et al.* 2001, Nonoshita *et al.* 2010) placenta. Furthermore, maternal administration of exogenous acyl-ghrelin promotes fetal growth in rats (Nakahara *et al.* 2006). This raises the possibility that ghrelin could be synthesised by, and act directly on, the placenta, as well as through central actions, to stimulate maternal GH secretion, food intake and fetal growth.

The potential role of ghrelin in pregnancy is supported by evidence that there are changes in maternal circulating ghrelin during pregnancy in a number of mammalian species. However, the reported changes in circulating total and acyl-ghrelin during pregnancy vary between studies. Total ghrelin concentration has been reported as lower compared to non-pregnant levels in humans and as lower or unchanged in pregnant rats (Gualillo *et al.* 2001, Shibata *et al.* 2004, Fuglsang *et al.* 2005, Tham *et al.* 2009, Johnson *et al.* 2019). Conflicting evidence is likewise available regarding changes in

acyl-ghrelin, with either elevated or lower concentrations during pregnancy in women (Palik *et al.* 2007, Tham *et al.* 2009) and elevated during late pregnancy in a single study in rats (Szczepankiewicz *et al.* 2010). Although responses to exogenous ghrelin demonstrate its functional activity during pregnancy, the role of endogenous ghrelin during pregnancy remains unclear. Pregnant mice lacking the *Mboat4* gene, which consequently cannot convert ghrelin to acyl-ghrelin (Trivedi *et al.* 2015), still undergo a similar increase in GH during late pregnancy (Trivedi *et al.* 2015), suggesting that acyl-ghrelin is not required to increase GH during pregnancy. However, in this study, GH was only measured in maternal blood collected at post-mortem and, therefore, it is unknown whether the lack of acyl-ghrelin altered the circulating patterns of GH during pregnancy (Trivedi *et al.* 2015). These pulsatile patterns of circulating GH during pregnancy are important since intermittent, but not continuous, GH administration improves placental function and increases fetal growth in a number of animal species (Clendinnen & Eayrs 1961, Gargosky *et al.* 1991, Jørgensen *et al.* 1991, Spencer *et al.* 1994).

The finding that increased acylation of ghrelin can be achieved by a nutritional approach (Nishi *et al.* 2005, Gutierrez *et al.* 2008, Kirchner *et al.* 2009) offers great translational potential as an approach to increase circulating GH. Oral supplementation with the C8 medium chain-fatty acid, octanoic acid or with the triglyceride containing this fatty acid, glyceryl trioctanoate, induced octanoylation of ghrelin in the stomach and increased abundance of circulating acyl-ghrelin in mice (Nishi *et al.* 2005, Gutierrez *et al.* 2008, Kirchner *et al.* 2009). Similarly, enteral feeding with octanoic-acid enriched formula increased circulating acyl-ghrelin in humans (Ashitani *et al.* 2009). Further, dietary supplementation with a medium chain triglyceride mixture containing 65–75% octanoic acid increased circulating levels of acyl-ghrelin in pigs (Miller *et al.* 2016).

Therefore, the aims of the present study were: (1) to characterise and compare the GH-ghrelin axis in non-pregnant and pregnant mice and (2) to determine the effect of dietary octanoic acid supplementation on this axis in both pregnant and non-pregnant animals. We hypothesised that the GH-ghrelin axis would be upregulated during pregnancy and that dietary octanoic acid supplementation would increase circulating acyl-ghrelin and GH, and pulsatile GH secretion, in both non-pregnant and pregnant mice.

Materials and methods

Ethical approval

Experimental procedures were approved by The University of Adelaide Animal Ethics Committee (studies one and two, M-2016-186) or South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee (study three, SAM395.19) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council of Australia 2013).

Animals and experimental design

Study one

Virgin female C57Bl/6J 10-week-old female mice were obtained from the Animal Resource Centre, Perth, and CBAF1 males were obtained from Laboratory Animal Services, The University of Adelaide. All animals were acclimatised to the facility for at least 7 days prior to commencement of cycle tracking, with estrus cycles tracked for at least 8 days prior to mating. All mice were housed at ~23°C with 12 h light:12 h darkness cycle (lights on at 06:00 h or zeitgeber (ZT0), with *ad libitum* access to water and meat-free rat and mouse diet (14.0 MJ/kg, 20% protein, Speciality Feeds, Glen Forrest, Australia), except when fed experimental diets as detailed subsequently. Female mice were weighed daily throughout the experiment. Estrus stage in females was classified daily by observation of cell types collected by gently flushing the vagina with 10 µL of saline (Caligioni 2009). To generate timed pregnancies, a male was placed in the females' cage overnight when one or both females were in diestrus-to-proestrus transition or proestrus. The presence of a vaginal plug on the following morning was taken as confirmation of successful mating and designated as 0.5 day of pregnancy (GD0.5). Pregnancy was subsequently confirmed by weight gain and at post-mortem.

The experimental diets were based on prior observations that adding 5 mg/mL octanoic acid to drinking water or feeding a diet supplemented with 5% glyceryl trioctanoate increased stomach acyl-ghrelin content in mice, while dietary tripalmitate did not alter ghrelin activation (Nishi *et al.* 2005). The experimental diets were therefore generated by adding 5% (wt/wt) of either octanoic acid or palmitic acid to ground meat-free rat and mouse diet (Speciality Feeds), which was then re-formed into pellets and baked for 4 h at 60°C prior to feeding. Experimental diets were fed *ad libitum* throughout

the remainder of the experiment, commencing at GD0.5 in mated groups and from the same day in age-matched controls, including the day of sampling. To confirm the fatty acid content, samples of each diet were ground, extracted into chloroform and analysed for fatty acid composition as described.

Samples were collected from pregnant mice ($n=8$ palmitic acid diet, $n=7$ octanoic acid diet) at 17.5 days after mating (term is ~ 19.3 days after mating in this strain (Murray *et al.* 2010) and from non-pregnant mice ($n=9$ palmitic acid diet, $n=10$ octanoic acid diet) when mice were in diestrus and between 15.5 and 19.5 days after commencing experimental diets. Additional non-pregnant mice ($n=2$ palmitic acid diet, $n=3$ octanoic acid diet) did not enter diestrus between 15.5 and 19.5 days after commencing experimental diets and were thus not sampled for GH, since secretion patterns change throughout the estrus cycle (Chen Chen, personal communication). All other samples were collected from these animals. To minimise stress, all females were handled daily for ~ 5 min per mouse for ≥ 14 days before sampling. GH samples were collected from each animal over a continuous 6-h period to allow patterns of GH in the circulation and of GH secretion to be determined. From 13:00 h, ZT7 (lights on at 06:00 h, lights off at 18:00 h, ZT12), 36 sequential tail-tip blood samples (each 2 μ L) were collected at 10-min intervals from each mouse, processed and stored at -80°C for later analysis as previously described (Steyn *et al.* 2011, Gatford *et al.* 2017). Shortly after collection of the final sample (19:00 h to 19:30 h), mice were terminally anaesthetised by i.p. injection of Avertin (2,2,2-tribromoethanol and tert-amyl alcohol; Sigma-Aldrich) and 0.5 to 1.0 mL of venous blood was collected via retro-orbital puncture. Whole blood (30 μ L) from each individual mouse was spotted onto customised PUFacoatTM collection cards and air dried for later analysis of free fatty acids (Liu *et al.* 2014). The remaining blood was collected into Eppendorf tubes containing 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF; at a final concentration of 2 mg AEBSF per mL whole blood), allowed to clot at room temperature for 30 min before centrifugation and plasma acidified to a final concentration of 0.05 M HCl to minimise breakdown of acyl-ghrelin (Delhanty *et al.* 2015). Following blood collection, mice were humanely killed by cervical dislocation. The uterus was removed from pregnant mice and the stomach was rapidly collected for processing as described. The stomach was opened along the greater curvature and rinsed in saline prior to dissection, along the lesser curvature, into two pieces. Mucosal scrapings

from the corpus and antrum, the glandular regions of the stomach, were collected from one half of the stomach, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For the immunohistochemistry experiments, the other half of the stomach was pinned out flat in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PFA-PB) and rocked at room temperature for 3 h before cryoprotection in 30% sucrose-PB solution overnight. The stomachs were then embedded in optimal cutting temperature compound (Tissue-Tek, ProSciTech, Queensland, Australia), frozen and stored at -80°C until sectioning (Li *et al.* 2018). The numbers of implantations, fetuses and resorption sites were counted and each fetus and placenta was dissected and weighed. Placentas were alternately snap-frozen in liquid nitrogen or fixed for histological analysis as described. Complete suppression of GH secretion was observed in two pregnant mice, who had normal litter size and fetal weights at post-mortem. Data for these animals were excluded from all analyses, because a loss of GH secretion occurs acutely in stressed mice (Steyn *et al.* 2012) and these two animals were therefore not considered to reflect the normal physiology of pregnancy.

Study two

In study one, plasma samples were collected from animals at the completion of GH sampling, when most animals had not eaten. Upon analysis, we found no difference in the circulating plasma acyl-ghrelin concentrations between groups. Given the rapid turnover of acyl-ghrelin in circulation (Delhanty *et al.* 2015), we considered it possible that any effects of diet on ghrelin production and release may only have been evident close to the time of feeding. Therefore, in order to determine whether there was an increase in basal circulating plasma acyl-ghrelin levels in mice that had undisturbed access to food overnight, we conducted a second study, with the same interventions, but with samples collected late in the active feeding period (darkness phase) for mice (Bake *et al.* 2014) and without serial sampling for GH, to minimise disruption to feeding. Animal management, breeding and diets were as described for study one. Animals were humanely killed at 07:30 h to 08:00 h (lights on at 09:00 h, lights off at 21:00 h), and blood and tissue samples were collected as described previously.

Study three

Since circulating ghrelin was not consistently different between non- and late-pregnant mice in the first two studies, we collected plasma from a third cohort of mice

to assess changes in ghrelin abundance throughout pregnancy. Mice were sampled shortly after lights on, at a time when they have recently eaten (Kentish *et al.* 2013). Female C57Bl/6J 10–12 week-old female mice were obtained from SAHMRI Bioresources. All mice were housed at ~22°C with 12 h light:12 h darkness cycle (lights on at 07:00 h, with *ad libitum* access to water and standard rodent diet (18.6% protein, 6.2% fat, 44.2% carbohydrate, Teklad standard diet, Envigo, Cambridgeshire, United Kingdom). For timed mating, female mice were pair-housed with a C57BL/6J male at 17:00 h, and pregnancy was confirmed by the presence of a vaginal plug at 07:00 h (assigned as day 0.5 of pregnancy) before being returned to individual cages. Plugged females were randomly assigned to either early (6.5 days, $n=8$), mid (12.5 days, $n=8$) or late (18.5 days, $n=8$) stage pregnancy end points. Age-matched females that were not housed with a male for mating were used as non-pregnant controls ($n=8$). Mice were anaesthetised at ZT0–ZT1 by isoflurane inhalation (5% in oxygen) and venous blood was collected from the inferior vena cava and processed as described to obtain plasma for ghrelin assays. Mice were then humanely killed via decapitation.

Analysis of fatty acids in feed and blood

The fatty acid composition of blood and feed samples was assessed as previously described (Liu *et al.* 2014, Kanakri *et al.* 2017). Briefly, whole dried blood spots from the PUFACoat™ cards and extracted feed samples were trans-esterified with 2 mL of 1% H₂SO₄ in methanol at 70°C for 3 h. After adding distilled water and heptane to the vial, the samples were shaken using a vortex. Samples were then left standing and the top heptane layer containing fatty acid methyl esters was extracted and analysed using gas chromatography (Liu *et al.* 2014). Abundance of each fatty acid is expressed as a percentage of total fatty acids in the sample.

Hormone analyses

Serum concentrations of acyl-ghrelin and total ghrelin were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (EZRGRA-90K, Rat/mouse Ghrelin (Active) ELISA and EZRGRT-91K, Rat/mouse Ghrelin (Total) ELISA, respectively; R&D Systems), in accordance with the manufacturer's instructions. A single ELISA plate was used to measure all samples from each study. Within-assay coefficients of variation were each <10% (study 1: total ghrelin: 4.1%, acyl-ghrelin: 7.4%; study 2: total ghrelin: 5.8%, acyl-ghrelin: 3.8%).

Analysis for GH was performed using an in-house mouse GH ELISA as described and validated previously (Steyn *et al.* 2011). Within- and between-assay coefficients of variation were 2.0 % and 16.8 %, respectively, for a mouse plasma QC sample containing 27.7 ng/mL ($n=16$ assays). All samples from a single mouse (serial samples for analysis of secretion pattern) were analysed on the same ELISA plate. Kinetics and secretory patterns of pulsatile GH secretion were determined by deconvolution analysis following parameters established previously for GH secretion in mouse (Steyn *et al.* 2011, 2012). The orderliness and regularity of serial GH serum concentrations were calculated by approximate entropy (ApEn) analysis as described previously; a higher absolute ApEn denotes greater irregularity and indicates a loss in stability of feedback of GH regulation (Veldhuis *et al.* 2001).

Ghrelin and *Mboat4* gene expression in stomach and placenta

Total RNA from snap-frozen placenta and gastric mucosal scrapings was extracted using PureLink RNA Mini Kit (12183018A; Invitrogen) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Quantitative real-time PCR reactions (qPCR) were performed using a 7500 Fast Real-time PCR System (Life Technologies) and Express One-Step SuperScript qRT-PCR Kit (11781-200, Invitrogen). Predesigned Taqman gene expression assays (4331182, Life Technologies) that target *Ghrelin* (Mm00445450_m1), *Mboat4* (Mm01200389_m1) and *Pcsk1* (encodes for PC 1/3; Mm00479023_m1) were used. *B2m* (Mm00437762_m1), *Hprt* (Mm01545399_m1) and *Ppia* (Mm02342429_g1) with an expression stability value of 0.035 were used as reference genes for the gastric mucosa, while *Polr2a* (Mm00839502_m1) and *Ubc* (Mm01198158_m1) were identified as suitable reference genes in the mouse placenta (Solano *et al.* 2016). qPCR reactions were carried out under the following conditions: RT, 50°C for 15 min; initial PCR activation, 95°C for 20 s; PCR cycles 95°C for 3 s, 60°C for 30 s repeated for 40 cycles. Each assay was run in triplicate. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the delta CT method as described previously (Li *et al.* 2018).

Immunohistochemistry and histology

Stomach ghrelin localisation

Stomach tissue was sectioned (10 µm), air dried at RT before being rinsed in PBS+0.2% Triton X-100

(PBS-TX; Sigma-Aldrich) and blocked with 10% donkey serum at room temperature. To detect ghrelin immunoreactivity, sections were then incubated with rabbit-anti-ghrelin primary antibody (1 in 800 dilution in PBS-TX; Abcam Cat# ab129383, RRID:AB_11159267ab129383, Abcam) for 20 h at 4°C and donkey-anti-rabbit 488 secondary antibody (1 in 200 in PBS-TX, Cat# A-21206, RRID:AB_2535792 Thermo Fisher Scientific) for 1 h at RT. The slides were mounted using ProLong Antifade (Life Technologies). Slides where primary antibody was omitted showed no labeling and served as negative controls. Slide sections were visualised using an epifluorescence microscope (BX-51, Olympus) equipped with filters for Alexa Fluor® 488, with images acquired using a CoolSnapfx monochrome digital camera (Roper Scientific, Tuscon, AZ, USA). Ghrelin-positive cells were counted manually on images taken at 10× magnification by two people blind to treatment. Ten stomach tissue sections were randomly chosen from each mouse. In each section, ghrelin-positive cells were counted in an area of 400×400 µm in both proximal corpus and distal antrum. The number of ghrelin-positive cells in corpus or antrum from each mouse was averaged from the ten selected sections.

Placental morphology

Histological analyses were performed on two placentae from each dam (palmitic acid $n=14$ placentas, octanoic acid $n=12$ placentas). One dam from the palmitic acid group was excluded from this analysis due to a small litter size (a single fetus); however, the placenta was collected and frozen for gene expression analysis. Bisected placentae were fixed in 4% paraformaldehyde (PFA), washed in 1 X PBS over 48 h and stored in 70% ethanol prior to being paraffin embedded. Full-faced placental sections cut to 5 µm thickness were stained with Masson's Trichrome following standard protocols (Roberts *et al.* 2003). Areas of junctional zone and placental labyrinth for each placental section were visualised and measured with NDP.view 2 software (Hamamatsu Photonics, Shizuoka, Japan). Total cross-sectional area and the proportion of junctional zone to placental labyrinth were calculated.

Placental ghrelin localisation

Full-faced sections cut to 5 µm thickness were deparaffinised and rehydrated, using the same placentas as mentioned previously (palmitic acid $n=14$ placentas, octanoic acid $n=12$ placentas). Antigen retrieval was performed by microwaving slides in citrate buffer (0.2% citric acid in distilled water) for 10 min at 150 W.

In order to quench endogenous peroxidase, sections were incubated in 3% H₂O₂ in 1× PBS for 10 min. Non-specific binding was blocked by incubation with 10% goat serum in 1× PBS for 30 min before addition of rabbit anti-ghrelin antibody (1 in 250 dilution, Abcam Cat# ab129383, RRID:AB_11159267ab129383) and incubated overnight at RT. Bound antibody was detected using biotinylated goat anti-rabbit IgG (1 in 200 dilution, Agilent Cat# E0432, RRID:AB_2313609, DAKO) for 1 h, followed by streptavidin-conjugated horseradish peroxidase (1 in 500 dilution, Cat# P0397, DAKO) for 1 h, both at RT. Immunolabelling was visualised by incubating sections with diaminobenzidine (Cat# K346811-2, DAKO) and by counterstaining with hematoxylin before mounting in DPX (Sigma-Aldrich). Areas of ghrelin staining were visualised with NDP.view 2 software (Hamamatsu Photonics).

Statistical analyses

For studies one and two, effects of diet (palmitic acid supplemented cf. octanoic acid supplemented) and pregnancy status (non-pregnant cf. pregnant) on maternal outcomes were analysed by two-way ANOVA. For study three, effects of pregnancy stage (non-, early-, mid- and late-pregnant) were analysed by one-way ANOVA. Where outcomes differed between pregnancy stages, post-hoc analyses were performed using the Bonferroni correction. Effects of diet on fetal and placental outcomes was analysed using repeated measures ANOVA, treating each fetus or placenta as a repeated measure on the dam. GH secretion data were natural log-transformed to achieve equal variances before analysis. Data are presented as mean ± S.E.M. and $P<0.05$ was considered statistically significant.

Results

Pregnancy outcomes were unaffected by maternal diet

Maternal weight gain during the study, body and liver weights at post-mortem (Table 1) and absolute and relative organ weights (liver, kidneys and spleen, data not shown) were higher in pregnant than non-pregnant mice and unaffected by diet. Pregnancy outcomes including litter size, fetal weight and placental gross structure were also unaffected by maternal diet (Table 1).

Circulating fatty acid profiles were altered by diet and pregnancy

As expected, the content of C16:0 (palmitic acid) was higher in the palmitic acid-supplemented feed

Table 1 Maternal and pregnancy characteristics (study one ZT13).

	Non-pregnant		Pregnant		P value		
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy	D*P
Number of dams	11	13	8	6			
Final body weight (g)	21.3 ± 0.1	21.9 ± 0.3	34.5 ± 1.3	34.0 ± 1.5	0.953	<0.001	0.542
Weight gain (g/d)	0.05 ± 0.01	0.05 ± 0.02	0.83 ± 0.07	0.78 ± 0.10	0.488	<0.001	0.593
Liver (g)	0.75 ± 0.03	0.76 ± 0.02	1.30 ± 0.08	1.20 ± 0.16	0.550	<0.001	0.431
Liver (% of body weight)	2.5 ± 0.5	3.2 ± 0.3	3.8 ± 0.2	3.6 ± 0.6	0.563	0.073	0.306
Litter size			6.4 ± 0.8	7.3 ± 0.8	0.432		
Average fetal weight (g)			1.2 ± 0.1	1.1 ± 0.1	0.626		
Average placental weight (g)			0.1 ± 0.1	0.1 ± 0.1	0.276		
Average junctional zone area (mm ²)			2.5 ± 0.2	2.5 ± 0.1	0.938		
Average labyrinth zone area (mm ²)			5.2 ± 0.2	4.7 ± 0.2	0.158		

Maternal data were analysed using two-way ANOVA, and data for fetal outcomes were analysed using one-way ANOVA. Data are presented as mean ± s.e.m. D*P, diet*pregnancy interaction; FA, fatty acid.

(4.1 g/100 g feed) than in the octanoic acid-supplemented feed (0.5 g/100 g feed). Similarly, the content of C8:0 (octanoic acid) was higher in the octanoic acid-supplemented feed (1.5 g/100 g feed) than in the palmitic acid-supplemented feed (0.07 g/100 g feed).

Study one

In mice sampled at ZT13, total saturated fatty acid levels were higher in mice fed the palmitic acid than the octanoic acid diet ($P=0.014$) and higher in pregnant than non-pregnant mice ($P<0.001$, Table 2). Conversely, total monounsaturated fatty acid levels were higher in the blood of mice fed the octanoic acid diet ($P=0.006$) and in pregnant compared to non-pregnant mice ($P=0.046$, Table 2). Octanoic acid was undetectable in blood, regardless of diet or pregnancy status (data not shown). Circulating palmitic acid (C16:0) comprised a higher percentage of total lipids in mice fed the palmitic acid diet, compared to those fed an octanoic acid diet ($P<0.001$), and were also higher in pregnant than non-pregnant mice overall ($P<0.001$, Table 2).

Study two

In mice sampled at ZT23, effects of diet on total saturated fatty acids differed between non-pregnant and pregnant groups (interaction $P=0.022$, Table 2). Thus, in mice fed the palmitic acid-supplemented diet, the concentration of total saturated fatty acids was similar between non-pregnant and pregnant mice. However, within mice fed the octanoic acid-supplemented diet, the concentration of total saturated fatty acids was 7% higher in pregnant than non-pregnant mice. Circulating total saturated fatty acid concentrations did not differ between diets within either non-pregnant or pregnant groups. Total trans-esterified and monounsaturated

fatty acid concentrations were similar between groups ($P>0.05$, Table 2).

Circulating GH was higher in pregnant than non-pregnant mice but was unaffected by diet

Circulating GH was pulsatile in all mice and mean plasma GH concentrations increased during pregnancy (study one, Fig. 1 and Table 3). Total and basal GH secretion rates were significantly higher in pregnant mice ($P<0.001$, Table 3) compared to non-pregnant mice, irrespective of diet. The irregularity of GH pulses (approximate entropy; $P=0.033$) and mode of secretion ($P=0.001$) were also higher in pregnant mice compared to non-pregnant mice and unaffected by diet (Table 3). Pulse frequency, mass of GH released per burst and pulsatile GH secretion rate were similar between dietary groups and between pregnant and non-pregnant animals (Table 3).

Total but not acyl-ghrelin is higher in pregnant than non-pregnant mice, depending on time of day

In mice sampled at ZT13 (study one), serum total ghrelin levels were significantly higher in pregnant than non-pregnant mice ($P=0.004$, Fig. 2) and were unaffected by diet, while serum acyl-ghrelin concentrations were similar irrespective of diet or pregnancy status. In mice sampled at ZT23 (study two), total and acyl-ghrelin levels in mice were similar between dietary groups and between pregnant and non-pregnant animals (Fig. 2).

When assessing changes in ghrelin abundance across pregnancy (study three), serum total ghrelin did not differ between pregnancy stages ($P=0.059$, Fig. 3A). In contrast, serum acyl-ghrelin concentrations changed with pregnancy stage ($P=0.008$, Fig. 3B).

Table 2 Circulating maternal fatty acid concentrations.

	Non-pregnant		Pregnant		P value		
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy	D*P
Study one							
<i>n</i> animals	11	13	8	6			
Total saturated FA (% of total lipids)	42.0 ± 0.2	40.6 ± 0.4	44.1 ± 0.5	43.3 ± 0.5	0.014	<0.001	0.466
16:0 (% of total lipids)	24.7 ± 0.3	22.1 ± 0.2	26.4 ± 0.3	24.3 ± 0.3	<0.001	<0.001	0.307
Total trans-esterified FA (% of total lipids)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.674	0.978	0.593
Total monosaturated FA (% of total lipids)	16.2 ± 0.3	17.8 ± 0.2	17.5 ± 0.5	17.9 ± 0.3	0.006	0.046	0.092
Study two							
<i>n</i> animals	13	9	5	6			
Total saturated FA (% of total lipids)	44.3 ± 0.3	43.4 ± 0.5	45.1 ± 0.5	46.5 ± 0.5	0.680	0.000	0.022
16:0 (% of total lipids)	28.2 ± 0.2	25.6 ± 0.4	28.3 ± 0.2	28.4 ± 0.5	0.004	0.001	0.002
Total trans-esterified FA (% of total lipids)	0.3 ± 0.02	0.2 ± 0.02	0.3 ± 0.03	0.3 ± 0.02	0.949	0.191	0.485
Total monosaturated FA (% of total lipids)	14.9 ± 0.6	15.1 ± 0.2	14.4 ± 0.4	15.9 ± 0.4	0.141	0.830	0.279

Fatty acids were measured by GCMS in blood collected from non-pregnant and pregnant mice, fed chow supplemented with either 5% palmitic acid or 5% octanoic acid. Mice in study one were sampled between 1 and 2 h after lights off, and mice in study two were sampled between 1 and 0 h before lights on, at the end of the natural feeding period. Data were analysed by two-way ANOVA and are presented as mean ± S.E.M.

D*P, diet*pregnancy interaction; FA, fatty acids.

Acyl-ghrelin concentrations were lower in mid-pregnant compared to non-pregnant mice ($P=0.007$) and did not differ between other pregnancy stages (Fig. 3B).

Stomach and placental ghrelin expression may explain higher total but not acyl-ghrelin in pregnancy

Gastric *ghrelin*, *Mboat4* and *Pcsk1* mRNA levels were similar in all groups at ZT13 (study one, Table 4). Gastric *ghrelin* mRNA expression was unaffected by pregnancy or diet at ZT23 (study two, Table 4). However, the gastric mRNA expression of *Mboat4* and *Pcsk1* was lower in pregnant compared to non-pregnant mice at ZT23 (Table 4).

At ZT13 (study one), the effects of pregnancy on the density of ghrelin-positive cells in the corpus region of the stomach differed between diets (interaction, $P=0.003$, Fig. 4C). In mice fed the palmitic acid diet, the density of ghrelin-positive cells in the stomach corpus was higher in pregnant than non-pregnant mice ($P<0.001$, Fig. 4). Pregnancy status did not affect the density of ghrelin-positive cells in the corpus of mice fed the octanoic acid diet. In pregnant mice, the density of ghrelin-positive cells in the corpus was higher in mice fed the palmitic acid compared to mice fed the octanoic acid diet ($P=0.012$, Fig. 4). There was no effect of diet on the density of ghrelin-positive cells in the corpus in non-pregnant mice. In the antrum region, the density of ghrelin-positive cells was higher in pregnant mice than non-pregnant mice ($P=0.019$), irrespective of diet (Fig. 4E).

At ZT23 (study two), the density of ghrelin-positive cells in the corpus region was higher in pregnant mice than non-pregnant mice ($P=0.023$), irrespective of diet (Fig. 4D). However, the density of ghrelin-positive cells in the antrum region of the stomach at this sampling time was similar in all groups (Fig. 4F).

Placental *ghrelin* and *Mboat4* mRNA levels were measured in pregnant animals and were unaffected by diet in both studies (Table 5). In the placenta, cytoplasmic ghrelin immunostaining was mostly localised to labyrinthine trophoblast and nuclear immunostaining was present in both labyrinth and junctional zones (Fig. 5). Localisation was unaffected by diet.

Discussion

In the present study, circulating basal and total GH, as well as total, but not acyl, ghrelin concentrations were significantly higher in pregnant compared to non-pregnant mice. Contrary to our hypothesis, dietary supplementation with octanoic acid did not increase acyl-ghrelin, circulating GH concentration or pulsatile GH secretion in either pregnant or non-pregnant mice.

The increased circulating levels of GH we observed during late pregnancy are consistent with previous studies in mice (Gatford *et al.* 2017) as well as rats (Saunders *et al.* 1976, El-Kasti *et al.* 2008). This further confirms

Figure 1

Representative circulating GH profiles in non-pregnant females fed palmitic acid diet; $n = 7$ (A) or octanoic acid diet; $n = 10$ (B), in mice at GD17.5 or age-matched diestrus controls days of eating experimental diets and in pregnant mice fed palmitic acid diet; $n = 8$ (C) or octanoic acid diet; $n = 4$ (D) at GD17.5. Shading indicates darkness period.

that maternal GH is elevated in multiple species during pregnancy (Saunders *et al.* 1976, Eriksson *et al.* 1989, El-Kasti *et al.* 2008, Gatford *et al.* 2017) despite the lack of a placentally-expressed GH variant gene in rodents and most other non-primate species (Papper *et al.* 2009). Interestingly, GH was elevated in pregnant mice despite the fact that maternal acyl-ghrelin levels were similar in late pregnant and non-pregnant mice and actually lower at mid-pregnancy than in non-pregnant females. These observations are consistent with the findings of Trivedi and colleagues, who demonstrated that circulating GH, measured in single samples at post-mortem, was similarly higher in late pregnant than non-pregnant animals in

both *Mboat4*-knockout and WT mice (Trivedi *et al.* 2015). Together, these findings imply that endogenous circulating acyl-ghrelin is not an important regulator of changes in GH during pregnancy.

Total serum ghrelin concentrations were higher in pregnant than in non-pregnant mice; however, this was only observed in mice that were sampled at the end of the light phase (study one) and not in those sampled at the end of the darkness phase (study two) or early in the light phase (study three). Ghrelin secretion undergoes a pre-prandial increase and a postprandial decline in humans, reflecting its function in stimulating hunger and initiation of meals (Cummings *et al.* 2001, Tschöp *et al.* 2001). Besides the stimulation of food intake, ghrelin decreases energy expenditure in rodents and promotes the storage of fatty acids in adipocytes (Tschöp *et al.* 2000, Mano-Otagiri *et al.* 2009). The difference in effects of pregnancy on total ghrelin abundance in the light and darkness phases might therefore reflect greater diurnal ghrelin variation in pregnancy, possible due to greater nutrient demand and appetite drive (Ladyman *et al.* 2010). Consistent with food intake patterns in non-pregnant female and male mice (Yanagihara *et al.* 2006, Kentish *et al.* 2016), the majority of food intake occurs during the darkness phase in pregnant mice (Ladyman *et al.* 2018). Lower acyl-ghrelin concentrations in fed mice at mid-pregnancy (study three) might be a consequence of increasing food intake during pregnancy (Ladyman *et al.* 2018), since postprandial suppression of plasma ghrelin is proportional to the amount of calories ingested (Callahan *et al.* 2004). Additional studies are needed to characterise the circadian rhythm in ghrelin and determine whether these change in pregnancy.

The higher circulating total ghrelin concentrations in pregnant than in non-pregnant mice in study one might be explained by greater ghrelin production in the stomach, as well as potentially by placental production. Specific cells in the stomach, particularly X/A-like enteroendocrine cells, are the main site of ghrelin production and acylation in non-pregnant animals (Date *et al.* 2000), and in male mice preproghrelin mRNA expression is substantially higher in the stomach than in other tissues (Yang *et al.* 2008). Although ghrelin mRNA expression was detected in placenta, its abundance was >1000-fold lower than in stomach, suggesting that its contribution to circulating ghrelin is relatively minor, although abundant ghrelin was evident in placentas stained for ghrelin protein. Gene expression of ghrelin in the stomach was not different in pregnant and non-pregnant mice in either study, consistent with similar stomach, hypothalamic and

Table 3 Circulating maternal growth hormone (GH) and parameters of pulsatile GH secretion following deconvolution and approximate entropy (ApEn) analysis.

	Non-pregnant		Pregnant		P value		
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy	D*P
n samples	7	10	8	4			
Mean circulating GH (ng/mL)	3.9 ± 1.0	6.1 ± 1.3	11.4 ± 2.7	18.5 ± 9.2	0.155	0.004	0.433
Total GH secretion rate (ng/mL 6 h)	169.2 ± 43.0	247.7 ± 58.3	491.6 ± 118.1	819.1 ± 409.1	0.159	<0.001	0.382
Basal GH secretion rate (ng/mL 6 h)	18.0 ± 11.3	68.6 ± 24.8	312.2 ± 85.4	404.6 ± 187.3	0.344	<0.001	0.780
Interval between bursts	19.9 ± 10.0	24.3 ± 8.0	26.0 ± 6.7	28.0 ± 10.3	0.343	0.151	0.724
Number of GH pulses/6 h	4.1 ± 0.8	4.2 ± 0.6	5.4 ± 0.7	5.8 ± 0.9	0.773	0.073	0.832
Mode of secretory bursts (min)	15.0 ± 2.0	11.3 ± 0.5	7.6 ± 1.2	8.7 ± 1.4	0.353	0.001	0.096
ApEn	0.8 ± 0.1	0.9 ± 0.04	1.0 ± 0.1	1.0 ± 0.1	0.573	0.033	0.528
Mass of GH secreted/burst (ng/mL)	34.5 ± 8.5	47.8 ± 9.9	39.3 ± 11.0	62.8 ± 31.0	0.187	0.472	0.709
Pulsatile GH secretion rate (ng/mL 6 h)	151.2 ± 44.7	179.1 ± 40.0	179.4 ± 40.5	414.5 ± 262.2	0.130	0.130	0.230

Data were analysed by two-way ANOVA and are presented as mean ± s.e.m. D*P, diet*pregnancy interaction.

pituitary ghrelin expression in pregnant and non-pregnant mice reported previously (Trivedi *et al.* 2017). However, the 1.5-fold greater density of ghrelin-positive cells in the stomach antrum of pregnant than non-pregnant mice sampled at ZT13 (study one) suggests a greater capacity to secrete ghrelin, although this was not seen at ZT23 (study two). We saw a similar pattern for the stomach corpus, with greater ghrelin-positive cell density in pregnant than non-pregnant mice at ZT23, although at ZT13 the effect of pregnancy was only significant within the subset fed the palmitic acid diet. The discrepancy between ghrelin gene expression levels and number of ghrelin-positive

cells in the stomach may be because gene expression was measured from the entire gastric mucosa, whereas ghrelin-positive cell count was carried out in specific sections of the stomach. Given the variability within groups and relatively low sample size, we suggest that this is probably

Figure 2

Circulating maternal serum total (A and C) and acyl (B and D) ghrelin in non-pregnant and pregnant mice fed palmitic acid diet (white bars) or octanoic acid diet (grey bars), sampled at ZT13 (study one, A and B) or ZT23 (study two, C and D), in mice at GD17.5 or age-matched controls. Data were analysed by two-way ANOVA and are presented as mean ± s.e.m. Animal numbers are indicated by numerals within bars.

Figure 3

Circulating maternal plasma total (A) and acyl (B) ghrelin throughout pregnancy in non-pregnant (white bars), early, mid and late pregnant (grey bars) mice sampled at ZT0-1 (study three). Data were analysed by one-way ANOVA and are presented as mean ± s.e.m. Animal numbers are indicated by numerals within bars.

Table 4 Stomach ghrelin, *Mboat4* and *Pcsk1* mRNA expression.

	Non-pregnant		Pregnant		P value	
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy
Study one (ZT13)						
n samples	5	5	5	5		
<i>Ghrelin</i>	5.4 ± 0.3	4.9 ± 0.3	4.9 ± 0.2	4.4 ± 0.2	0.090	0.070
<i>Mboat4</i>	0.0059 ± 0.0007	0.0061 ± 0.0004	0.0059 ± 0.0002	0.0060 ± 0.0004	0.716	0.927
<i>Pcsk1</i>	0.015 ± 0.001	0.017 ± 0.001	0.016 ± 0.001	0.015 ± 0.001	0.553	0.829
Study two (ZT23)						
<i>Ghrelin</i>	3.8 ± 1.0	3.2 ± 0.9	2.7 ± 0.6	2.4 ± 0.6	0.587	0.243
<i>Mboat4</i>	0.0074 ± 0.0006	0.0067 ± 0.0009	0.0052 ± 0.0004	0.0051 ± 0.0005	0.553	0.007
<i>Pcsk1</i>	0.021 ± 0.003	0.018 ± 0.002	0.015 ± 0.001	0.016 ± 0.002	0.644	0.049

Gene expression was quantified using qRT-PCR and is expressed relative to reference genes. Gene expression data were analysed by two-way ANOVA. Data are presented as mean ± S.E.M. D*P, diet*pregnancy interaction; *Mboat4*, membrane bound O-acyltransferase; *Pcsk1*, proprotein convertase.

a chance finding and not likely to reflect diet-specific outcomes. An increased density of ghrelin-expressing cells is consistent with a recent report in rats, where the density of ghrelin immunopositive cells in the stomach mucosa is increased by ~10% at mid-pregnancy compared to non-pregnant animals and increases by a further ~30% by the day of birth (Johnson *et al.* 2019). We also characterised ghrelin protein expression in the murine placenta. Cytoplasmic expression was primarily localised to the labyrinth (nutrient exchange) region, while nuclear expression was evident in both labyrinth and junctional (endocrine crosstalk) regions. This is the first report of ghrelin protein localisation in the rodent placenta, and its presence is consistent with prior reports of ghrelin mRNA expression in placenta of rats and humans (Gualillo *et al.* 2001).

Despite greater circulating total ghrelin in pregnancy, we did not detect greater circulating acyl-ghrelin in pregnant mice, with a lower acyl:total ghrelin ratio in pregnant compared to non-pregnant mice and lower circulating acyl-ghrelin at mid-pregnancy. The decreased ratio of acyl to total ghrelin we observed in mice are broadly consistent with those of Tham and co-authors in women, who reported markedly lower maternal acylated ghrelin concentrations during pregnancy than postpartum (Tham *et al.* 2009). These authors suggested that lower acyl-ghrelin during human pregnancy probably reflected decreased acylation, since they found lower rather than higher activity of the key de-acylating enzyme butylcholinesterase in the pregnant group (Tham *et al.* 2009). We observed comparatively lower gastric mRNA expression of *Mboat4* and *Pcsk1* in pregnant mice compared to non-pregnant mice at ZT23. Although the acylation of ghrelin is independent of processing by PC 1/3 (Zhu *et al.* 2006), lower gastric gene expression of *Mboat4* at ZT23 may explain the lack of subsequent increase in circulating serum acyl-ghrelin concentrations despite higher total ghrelin concentrations in pregnant compared to non-pregnant mice sampled at ZT13. Further experiments are required to determine whether the lower acyl:total ghrelin ratio reflects slower acylation or more rapid deacylation in pregnancy. Despite the lack of elevation of circulating acyl-ghrelin in pregnancy, it is possible that the higher total ghrelin seen before substantial feeding might lead to increased local ghrelin action in tissues expressing both *Mboat4* and the ghrelin-receptor, such as the pancreas, placenta and hypothalamus (Yang *et al.* 2008, Trivedi *et al.* 2017). Consistent with a potential role for elevated ghrelin during pregnancy, ghrelin receptor mRNA expression increased in hypothalamus

Figure 4

Immunohistochemistry for ghrelin-positive cells in the corpus of non-pregnant (A) and pregnant (B) mice. Arrows indicate ghrelin-positive cells, L and M indicate lumen and mucosal regions, respectively, scale bar = 100 μ m. Effects of pregnancy and diet on the density of ghrelin-positive cells in the corpus (C and D) and antrum (E and F) regions of the stomach in mice fed palmitic acid diet (white bars) or octanoic acid diet (grey bars). Samples were collected at ZT13 (study one, C and E) or ZT23 (study two, D and F), in mice at GD17.5 or age-matched controls. Data were analysed by two-way ANOVA and are presented as mean $\pm$ S.E.M. Animal numbers are indicated by numerals within bars. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0072>.

and pituitary during rat pregnancy (Szczepankiewicz *et al.* 2010). It is also possible that non-acylated ghrelin has a functional role in pregnancy, since growing evidence suggests that des-acyl ghrelin has GHSR1a independent roles in energy and glucose metabolism (Thompson *et al.* 2004, Zhang *et al.* 2008, Trivedi *et al.* 2017). We did not

measure des-acyl ghrelin in the present study, but post-test meal concentrations of des-acyl (but not acyl) ghrelin were higher in women with gestational diabetes during late pregnancy and postpartum compared to normal pregnant women (Tham *et al.* 2009), suggesting it either regulates or responds to maternal metabolism.

Contrary to our hypothesis, dietary supplementation with octanoic acid did not alter circulating GH or ghrelin concentrations regardless of whether the mice had a full or empty stomach. Although we were able to confirm the presence of octanoic acid in our supplemented diet, we were not able to confirm its presence in the circulation, despite the fact that abundance of other circulating fatty acids were altered in response to the experimental diets. Similarly, Lemarié and co-authors could not detect C8 fatty acids in rat plasma in response to C8-supplementation with 0, 8 or 21% of total fatty acids (Lemarie *et al.* 2015). This could possibly be due to rapid gastrointestinal hydrolysis and absorption of medium chain-fatty acids, specific transport through the portal vein and rapid beta-oxidation in the liver (Bach & Babayan 1982). The lack of increase in acyl-ghrelin was surprising given that previous studies in mice found increased stomach acyl-ghrelin in male mice using similar dose regimens used in the present study (Nishi *et al.* 2005). Furthermore, dietary supplementation with octanoic acid increased circulating acyl-ghrelin concentrations in studies conducted in humans, mice and cattle (Ashitani *et al.* 2009, Kirchner *et al.* 2009, Fukumori *et al.* 2013). However, in other studies, dietary supplementation with octanoic acid increased stomach acyl-ghrelin of mice (Nishi *et al.* 2005) and stomach octanoic acid content in rats (Lemarie *et al.* 2015) without any changes in circulating acyl-ghrelin secretion. Interestingly, dietary supplementation with medium chain triglycerides (MCT) increased circulating acyl-ghrelin in humans and pigs of both sexes and circulating growth hormone concentrations in pigs

Table 5 Placental *ghrelin* and *Mboat4* mRNA expression.

	Palmitic acid	Octanoic acid	P value
Study one (ZT13)			
n samples	5	5	
<i>Ghrelin</i>	0.0021 $\pm$ 0.0001	0.0023 $\pm$ 0.0001	0.258
<i>Mboat4</i>	0.0007 $\pm$ 0.0002	0.0011 $\pm$ 0.0003	0.292
Study two (ZT23)			
n samples	5	6	
<i>Ghrelin</i>	0.0022 $\pm$ 0.0001	0.0023 $\pm$ 0.0002	0.922
<i>Mboat4</i>	0.0021 $\pm$ 0.0005	0.0013 $\pm$ 0.0004	0.216

Gene expression was quantified using qRT-PCR and is expressed relative to reference genes. Gene expression data were analysed by one-way ANOVA. Data are presented as mean $\pm$ S.E.M.

Mboat4, membrane bound O-acyltransferase.

Figure 5

Immunohistochemistry for ghrelin localisation in GD17.5 murine placentas from dams fed palmitic acid (A) and octanoic acid (B) diet. L and J indicate labyrinthine and junctional zones, respectively. Scale bar = 100 μ m. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0072>.

(Miller *et al.* 2016, Kawai *et al.* 2017), suggesting a possible alternate dietary approach to increase circulating acyl-ghrelin abundance. In addition, we did not see any differences in circulating GH patterns in response to diet. Although the current study utilised the established method of characterising patterns of pulsatile GH secretion in mice (Steyn *et al.* 2011), it was necessary to exclude data from two pregnant animals where pulsatile GH secretion was lost, indicative of an acute response. Pulsatile GH secretion was evident in the majority of mice, indicating that the acclimatisation and sampling protocol were mostly successful in preventing stress responses (Steyn *et al.* 2012). Loss of GH secretion in two pregnant mice but no non-pregnant mice on the present study, and from one late pregnant mouse and no non-, early- or mid-pregnant mice in our previous study (Gatford *et al.* 2017), suggests that pregnancy may increase susceptibility to

handling-induced stress, despite extensive acclimatisation. Together with the need for consistency in the estrous cycle stage of non-pregnant mice, this resulted in a limited sampling size for GH measurements. Therefore, we cannot exclude the possibility of a diet effect on GH based only on the present study. Maternal and fetal weights did not differ with diet, which was not unexpected given that we did not anticipate any restriction of fetal growth in this model of normal pregnancy. Although mice ate both experimental diets readily, we cannot exclude the possibility that food intake may differ between diets and suggest this should be measured in subsequent studies. Future studies may find it beneficial to adapt a dietary approach based on dietary supplementation with medium chain triglycerides containing up to 65–75% of octanoic acid, which has been shown to increase circulating levels of acyl-ghrelin as well as average plasma GH levels in pigs (Miller *et al.* 2016).

Overall, increased total and basal GH during murine pregnancy does not appear to be regulated by maternal circulating acyl-ghrelin. Maternal total ghrelin secretion in pregnancy may follow an altered diurnal pattern, possibly due to the pregnancy-associated changes in nutrient demand and energy intake. A higher number of gastric ghrelin-expressing cells in pregnant mice may explain the time-dependent higher concentrations of circulating total ghrelin compared to non-pregnant mice. Interestingly, despite elevated total ghrelin, acyl-ghrelin concentrations were not elevated in pregnancy or by dietary supplementation with octanoic acid at the concentrations used in the present study. Our results confirm upregulation of the GH-ghrelin axis in pregnancy, but the mechanisms underlying increases in circulating GH in murine pregnancy remain to be explained.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Authors contribution statement

H K, B S M, A J P, C T R and K L G conceived and designed the experiments. H K, B S M, P S S, A J P, H L, M N S, G S C, L H, R L W, J D V, C T R and K L G

were involved in the acquisition of data. H K, B S M, A J P, H L, L H, J D V and K L G analysed and interpreted the data. H K and K L G drafted the article. H K, B S M, A J P, H L, L H, R L W, C C, C T R and K L G critically revised the article. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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