Adaptations in gastrointestinal satiety during pregnancy in mice

A thesis submitted by

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For the degree of

Doctor of Philosophy

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Table of Contents

SUMMARY

Background: Increased food intake during pregnancy is associated with reduced central satiety, the development of leptin resistance and changes in sex hormones. Meal termination occurs partly via gastrointestinal vagal afferents (VAs) which sense food-related mechanical stimuli, including distension of the stomach and intestine and through nutrient-induced release of satiety hormones from intestinal enteroendocrine cells (EECs). These pathways both signal to the central nervous system to stop eating. Currently it is unknown how gastrointestinal (GI) satiety signalling adapts to permit increased food intake during pregnancy. This PhD project explored pregnancy-related adaptations in gastric VA (GVA) signalling in standard laboratory diet (SLD) fed and western diet fed-mice, intestinal expression of nutrient chemosensors and satiety hormones and the circadian timing of behaviour.

Aims:

1) a. To determine the mechanosensitivity of GVAs and food intake behaviours at different pregnancy stages compared to non-pregnant mice.
   b. To determine the effects of pregnancy-related hormones on GVA tension receptors in non-pregnant mice, as potential mechanisms underlying pregnancy related adaptations.
2) To investigate the expression of protein, fatty acid and carbohydrate nutrient receptors and satiety hormones in the duodenum, jejunum and ileum in different stages of pregnancy compared to non-pregnant mice.
3) To assess food intake and mechanosensitivity of GVAs throughout pregnancy in mice fed a SLD or western high-fat high-sugar diet (HFHSD).
4) To determine effects of pregnancy on circadian rhythms of food and water intake, sleep and activity behaviour.

Methods and results: The study in **Chapter 2** characterised the response of GVAs to stretch and changes in food intake parameters in early-, mid- and late-pregnant mice compared to non-pregnant mice. This work showed that the mechanosensitivity of GVAs was attenuated during mid- and late-pregnancy. Furthermore, addition of growth hormone (GH) to the *in vitro* organ bath decreased GVA responses to stretch in non-pregnant mice. **Chapter 3** focussed on the intestinal nutrient-sensing repertoire during pregnancy. Fatty acid (*GPR84, FFAR1,2,3,4*), protein (*GPR93, CaSR, mGLUR4, T1R1*), carbohydrate (*TRPM5, T1R2, T1R3*) receptors and gut hormones (*GCG, CCK*) were characterised in the small intestine of early-, mid- and late-pregnant mice compared to non-pregnant mice. In addition, immunofluorescence experiments were used to determine the number of FFAR4, GPR93, CCK and GLP-1 positive cells within the duodenum and jejunum of late-pregnant compared to non-pregnant mice. There were selective changes in nutrient-sensor mRNA expression during pregnancy. *FFAR4* expression was lower in late-compared to non-pregnant mice in all regions, but jejunal FFAR4 positive cells were more abundant in late- than non-pregnant mice. Duodenal *GPR93* expression was lower in late- than non-pregnant mice. In the ileum, *FFAR1* expression was greater in mid- than early- and late-pregnant mice and *FFAR2* expression was greater in mid- than early-pregnant mice. *GCG* and *CCK* expression at the transcript level and numbers of GPR93, CCK and GLP-1 immunopositive cells were unaffected by pregnancy. **Chapter 4** aimed to determine how maternal HFHSD feeding impacts adaptations in GVA function and food intake behaviours during pregnancy. The response of tension sensitive GVAs to stretch was attenuated in pregnancy within SLD-fed mice, consistent with results of **Chapter 2**, and was lower in HFHSD than SLD-fed non-pregnant mice. However, GVA responses to stretch were

similar in HFHSD-fed pregnant and non-pregnant mice. Light-phase food intake (g) and meal size (g) within each study day was higher in SLD-fed mice than HFHSD-fed mice and was greater in pregnant mice than non-pregnant mice from d 8.5 onwards. In addition, pregnant HFHSD-fed mice ate larger light-phase meals on d 14.5-16.5 than non-pregnant HFHSD-fed mice, but this was not preserved on the final study day before electrophysiology recording. Lastly, the study reported in **Chapter 5** determined the rhythms of food and water intake, activity and wakefulness across weeks 1, 2 and 3 of pregnancy compared to age-matched non-pregnant mice. From week 1, pregnant mice moved and were awake significantly less than non-pregnant mice during the dark-phase. Furthermore, the timing of peak food intake and activity late in the light-phase (time period of interest I: ZT8-ZT12) was delayed and the pregnant group ate more during the same time period in week 3 compared to the non-pregnant group. Food intake was also increased early in the dark-phase (time period of interest II: ZT12-ZT15) from pregnancy week 2.

Conclusion: The mechanosensitivity of GVAs are attenuated during pregnancy and associated with increased food intake. These GVA adaptations are likely to support increases in food intake to meet the energy demands of the growing fetus, and may be driven by increases in circulating levels of GH, but this is yet to be determined. Within the intestine, there were specific alterations in nutrient sensor *FFAR1, 2* and *4* and *GPR93*. Future research should be directed at understanding whether pregnant mice are less sensitive to luminal nutrients and whether nutrient-induced secretion of GI tract hormones changes during pregnancy. Energy balance was also altered through behaviour, where pregnant mice increased food consumption during the inactive phase and decreased movement during the active phase, when food intake was the highest. Lastly,

both pregnancy and HFHSD feeding attenuated the mechanosensitivity of GVA, however, pregnancy did not further reduce GVA mechanosensitivity in HFHSD-fed mice. Further studies are required to increase understanding of food intake regulation across pregnancy to inform strategies to improve pregnancy outcomes.

DECLARATION OF ORIGINALITY

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Georgia Sheridan Clarke

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I would like to sincerely thank my supervisors, Professor Amanda Page, Associate Professor Kathryn Gatford and Dr Sharon Ladyman for enabling me to complete a PhD in all the fields I am passionate about; pregnancy, nutrition and neuroscience. None of my work would have been accomplished without your constant support and beliefs in my abilities. I am very fortunate to be under the guidance of three influential women who have made a large reputation for themselves within their field of research and academia. I would particularly like to acknowledge Professor Page who has been my supervisor since my third year laboratory placement of my Bachelor's degree, you continuously encourage me to flourish as an individual and provide me with countless opportunities to engage within academia and create my own strong track record, thank you.

I would also like to thank all past and present members of the Vagal Afferent Research Group. Dr Hui Li, thank you for guiding me and giving constant support within the laboratory and willingness to answer all my questions which has enabled me to finish my PhD. Special thanks to the other members, Dr Maria Nunez-Salces, Dr Stewart Ramsay, Dr Rebecca O'Rielly, Dr Yoko Wang, Elaheh Hestmasti and Sebastian Overduin.

Thank you to all members of Intestinal Nutrient Sensing Group. To Associate Professor Richard Young, thank you all for your involvement in experimental discussions, your advice and feedback was countless. Thank you to Nektaria Pezos for all your technical support and friendly work environment created by Nektaria, Dr Denise Kreuch and Braden Rose.

I would like to again acknowledge Dr Ladyman for her hospitality during my visit to the Centre of Endocrinology at the University of Otago, Dunedin, New Zealand.

I appreciated the time spent within your laboratory group and the friendships I was able to make whilst in New Zealand.

Finally, I would like to express my deepest gratitude to my family. A huge thank you to my mum, Michelle, for supporting me during my PhD, listening to all my presentation practises and for believing in/motivating me when I was in doubt. I appreciate everything you do for me. To my younger sister, Paige, thank you for being my best friend and making life brighter and fun. To my partner Bradley, thank you for always listening to me and giving constant support and love. I cannot forget to acknowledge the unconditional love from my pets, Crystal (past) and Pippa.

To all my other family and all my friends, know that I appreciate you all. Especially my friends who have patiently waited for me to finish experiments before organising catch-ups.

I would also like to acknowledge my own personal growth. A younger version of myself would not have foreseen a PhD, leading committees, teaching and doing public speaking.

“You have brains in your head. You have feet in your shoes. You can steer yourself any direction you choose. You're on your own. And you know what you know. And YOU are the one who'll decide where to go.”

Dr. Seuss

PUBLICATIONS ARISING FROM THESIS

Clarke GS, Gatford KL, Young RL, Grattan DR, Ladyman SR, & Page AJ. (2021). Maternal adaptions to food intake across pregnancy: central and peripheral mechanism. Obesity 29(11) (2021) 1813-1824. Impact factor: 3.74; quartile (Q) 1 journal in the field (SCImago)

Li H*, **Clarke GS***, Christie S, Ladyman SR, Kentish SJ, Young RL, Gatford KL & Page AJ. (2021). Pregnancy-related plasticity of gastric vagal afferent signals in mice. *Am J Physiol Gastrointest Liver Physiol* 320(2) 183-192. Impact factor: 3.72; Q1.

This paper was selected by the American Physiology Society (APS select) as an article of "outstanding scientific discovery". In addition, both co-first authors (*) were invited to create a video abstract, accessible on the American Journal of Physiology website and YouTube, to highlight the publication.

Website: *https://journals.physiology.org/doi/full/10.1152/ajpgi.00357.2020*

YouTube: https://www.youtube.com/watch?app=desktop&v=qa_ha7VnICE

ACCEPTED MANUSCRIPT

Clarke GS, Li H, Ladyman SR, Young RL, Gatford KL & Page AJ. Effect of pregnancy on the expression of nutrient-sensors and satiety hormones in mice. *Peptides* (2023) Published ahead of preprint. Impact factor: 3.87; Q1

SUBMITTED MANUSCRIPT

Clarke GS, Vincent AD, Ladyman SR, Gatford KL & Page AJ. Circadian patterns of behaviour change during pregnancy in mice**.** Submitted to *The Journal of Physiology – Special call for Circadian Timing of Behaviour and Biology August 2023.* Impact factor: 6.23; Q1

PAPER IN MANUSCRIPT FORMAT

Clarke GS, Li H, Nicholas LM, Ladyman SR, Gatford KL & Page AJ. Pregnancy and a high-fat high-sugar diet each attenuate mechanosensitivity of murine gastric vagal afferents, with no additive effects. Will be submitted to *Acta Physiologica.* Impact factor: 7.52; Q1

PRESENTATIONS

Invited presentations:

Clarke GS, Li H, O'Hara SE, Gembus KM, Nicholas LM, Ladyman SR, Gatford KL & Page AJ. (2023) The impact of a high-fat high-sugar diet on gastric vagal afferent mechanosensitivity in pregnant dams and their offspring. Invited presentation – 2023. Australian and New Zealand Obesity Society (ANZOS) Conference. Adelaide, South Australia.

Clarke GS, Li H, Nicholas LM, Ladyman SR, Gatford KL & Page AJ. (2023) Plasticity of murine gastric vagal afferents during pregnancy and the impact of a western high-fat high-sugar diet. Invited presentation – 2023. Australasian Neuroscience Society (ANS) seminar series. Australian and New Zealand Audience, via zoom.

Clarke GS, Ladyman SR, Gatford KL & Page AJ. (2022) Adaptations in gastrointestinal satiety during pregnancy. Invited presentation – 2022. Centre for Neuroendocrinology and Department of Anatomy seminar series, Dunedin, New Zealand.

Clarke GS, Ladyman SR, Gatford KL & Page AJ. (2021) Adaptations in gastrointestinal satiety during pregnancy. Invited presentation – 2021. Third Thursday Seminar series, Robinson Research Institute, The University of Adelaide.

Conference abstracts:

Oral presentations:

Clarke GS, Vincent AD, Ladyman SR, Gatford KL & Page AJ (2023). Circadian patterns of behaviour change during pregnancy in mice. Oral presentation –

2023. Australasian Chronobiology Society (ACS) Conference, Adelaide, South Australia.

The above oral presentation received the Best Oral Presentation Award. This Award is presented annually for the most outstanding presentation at the Annual Meeting.

Clarke GS, Vincent AD, Ladyman SR, Gatford KL & Page AJ (2022). Energy metabolism and its circadian timing during pregnancy. Oral presentation – 2022. Australian Society for Medical Research (ASMR) SA meeting, Adelaide, South Australia.

The above oral presentation received the Ross Wishart Award. This Award is presented annually for the most outstanding presentation by a young researcher at the ASMR SA Annual Scientific Meeting.

Clarke GS, Li H, Ladyman SR, Young RL, Gatford KL & Page AJ. (2021) Nutrient-sensing components of the mouse intestine during pregnancy. Oral presentation – 2021. Australian Society for Medical Research (ASMR) SA meeting, Adelaide, South Australia.

Poster presentations:

Clarke GS, Li H, Nicholas LM, Ladyman SR, Gatford KL & Page AJ. (2023) A high-fat high-sugar diet prevents adaptations in gastric vagal afferent satiety signalling during pregnancy. Poster presentation – 2023. Postgraduate Florey Conference, The University of Adelaide.

Clarke GS, Li H, Nicholas LM, Ladyman SR, Gatford KL & Page AJ. (2022) Plasticity in gastric satiety signals across pregnancy and the impact of diet-induced obesity. Poster presentation – 2022. Endocrine Society of Australia/

Society for Reproductive Biology (ESA/SRB) Conference, Christchurch, New Zealand.

Clarke GS, Vincent AD, Ladyman SR, Gatford KL & Page AJ (2022). Behaviour and its circadian timing during pregnancy in mice**.** Poster presentation – 2022. International Congress on Obesity (ICO), International Conference, Melbourne, Australia.

Clarke GS, Vincent AD, Ladyman SR, Gatford KL & Page AJ (2022). Circadian timing in food intake during pregnancy. Poster presentation – 2022. Society for Ingestive Behaviour (SSIBs) Conference, via zoom.

Clarke GS, Li H, Ladyman SR, Young RL, Gatford KL & Page AJ. (2021) Nutrient-sensing components of the mouse intestine during pregnancy. Poster presentation – 2021. Australian Society for Medical Research (ASMR) National Conference, Melbourne, Australia, via zoom.

The above poster presentation received the award for the Best Student Poster Presentation Award.

Clarke GS, Li H, Ladyman SR, Young RL, Gatford KL & Page AJ. (2021) Nutrient-sensing components of the mouse intestine during pregnancy. Poster presentation – 2021. Endocrine Society of Australia/ Society for Reproductive Biology (ESA/SRB) Conference**,** via zoom.

Clarke GS, Li H, Ladyman SR, Young RL, Gatford KL & Page AJ. (2021) Nutrient-sensing components of the mouse intestine during pregnancy. Poster presentation – 2021. Society for Ingestive Behaviour (SSIBs) Conference, via zoom.

Li H*, **Clarke GS*,** Christie S, Ladyman SR, Kentish SJ, Young RL, Gatford KL & Page AJ. (2021). Plasticity of gastric satiety signals during pregnancy and in

response to pregnancy hormones**.** Poster presentation – 2021. Federation of Neurogastroenterology & Motility (FNM) Conference, national conference, Adelaide, South Australia.

Li H*, **Clarke GS*,** Christie S, Ladyman SR, Kentish SJ, Young RL, Gatford KL & Page AJ. (2020). Plasticity of gastric satiety signals during pregnancy and in response to pregnancy hormones**.** Poster presentation – 2020. Endocrine Society of Australia/ Society for Reproductive Biology (ESA/SRB) conference, via zoom.

The above poster presentation was placed into the Best of the Best Poster Session.

Li H*, **Clarke GS*,** Christie S, Ladyman SR, Kentish SJ, Young RL, Gatford KL & Page AJ. (2020). Plasticity of gastric satiety signals during pregnancy and in response to pregnancy hormones**.** Poster presentation – 2020. Postgraduate Florey Conference, The University of Adelaide, via zoom.

The above poster presentation won the Faculty of Health and Medical Sciences Executive Dean's Award for the overall highest score for poster and presentation.

ADDITIONAL CO-AUTHORED PUBLICATIONS AND CONFERENCE ABSTRACTS:

The following publications and conference abstracts resulted from collaborations and were produced during the tenure of this PhD. However, they are not part of this PhD thesis.

Publication:

H Kaur, BS Muhlhausler, PS-L Sim, AJ Page, H Li, M Nunez, **Clarke GS**, L Huang, RL Wilson, JD Veldhuis, C Chen, CT Roberts, KL Gatford 2020 Pregnancy, but not dietary octanoic acid supplementation, stimulates the ghrelin-pituitary growth hormone axis in mice. *Journal of Endocrinology* 245(2):327-342 https://doi.org/10.1530/JOE-20-0072

Conference abstracts:

Overduin TS, **Clarke GS**, Li H, Page, AJ, Young RL, Gatford KL. (2023) Differential changes in anatomical, molecular and functional determinants of intestinal glucose absorption during murine pregnancy – 2023. Australian Society for Medical Research (ASMR) SA meeting, Adelaide, South Australia

Overduin TS, **Clarke GS**, Li H, Page, AJ, Young RL, Gatford KL. (2021) Determinants of nutrient absorption across pregnancy in mice. Poster presentation – 2021. Australian Society for Medical Research (ASMR) SA meeting, Adelaide, South Australia

Overduin TS, **Clarke GS**, Li H, Page, AJ, Young RL, Gatford KL. (2021) Determinants of nutrient absorption across pregnancy in mice. Poster presentation – 2021. South Australian Health and Medical Research Institute (SAHMRI) Research Showcase, Adelaide, South Australia

Overduin TS, **Clarke GS**, Li H, Page, AJ, Young RL, Gatford KL. (2021) Changes in anatomical and functional determinants of nutrient absorption during pregnancy in mice. Oral Presentation – 2021. Barossa Valley Conference, South Australia

The following thesis and conference abstracts resulted from training undergraduate students.

Thesis:

Ward JP, **Clarke GS**, Nicholas LM, Gatford KL, Page, AJ, Li H. (2022) The impact of high-fat high-sugar diet on nutrient absorption during pregnancy. *Honours Thesis, Bachelor of Health & Medical Sciences Honours, submitted to The University of Adelaide, Awarded First Class Honours.*

Conference abstracts:

Stradwick L, **Clarke GS**, Page AJ. Gastrointestinal adaptations in intestinal L-cells during pregnancy and obesity (2023). The University of Adelaide Undergraduate Research Conference, Adelaide, Australia

Short AL, **Clarke GS**, Page AJ. Gastrointestinal adaptations in intestinal L-cells during pregnancy and obesity (2023). The University of Adelaide Undergraduate Research Conference, Adelaide, Australia

Ward JP, **Clarke GS**, Nicholas LM, Gatford KL, Page, AJ, Li H. (2022) The impact of high-fat high-sugar diet on nutrient absorption during pregnancy. The University of Adelaide Undergraduate Research Conference, Adelaide, Australia.

Roberts E, Pharm J, Ryals J, Klakhaeng P, Razaiat S, **Clarke GS**. Impact of high-fat and high-sugar diet on energy homeostasis in pregnant mice (2021). The University of Adelaide Undergraduate Research Conference, Adelaide, Australia

Graziano A, Sood D, Herana K, Katembwe D, Iliev M, Muradi N, **Clarke GS**, Page AJ. Adaptations in energy homeostasis during pregnancy (2020). The University of Adelaide Undergraduate Research Conference, Adelaide, Australia

LIST OF FIGURES:

LIST OF TABLES:

LIST OF ABBREVIATIONS

Acc	Acclimatisation
AgRP	Agouti-related peptide
alpha-MSH	alpha-melanocyte-stimulating hormone
ANOVA	Analysis of variance
ARC	Hypothalamic arcuate nucleus
B2M	β2 microglobulin
BMI	Body mass index
CART	Cocaine and amphetamine-regulated transcript
CaSR	Calcium sensing receptor
CCK	Cholecystokinin
CNS	Central nervous system
CrI	Credible interval
DMN	Doral medial nucleus
EEC	Enteroendocrine cell
FFAR1	Free fatty acid receptor 1
FFAR1	Free fatty acid receptor 1
FFAR2	Free fatty acid receptor 2
FFAR3	Free fatty acid receptor 3
FFAR4	Free fatty acid receptor 4
GCG	Proglucagon
GH	Growth hormone
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide-2
GPR84	G protein coupled receptor 84
GPR93	G protein coupled receptor 93
GPRC6A	G protein-coupled receptor 6A
GVA	Gastric vagal afferents
HFD	High fat diet
HFHSD	High-fat high-sugar diet
HPA	Hypothalamo-pituitary-adreanal
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
IOM	Institute of Medicine
LHA	Lateral hypothalamic area
MCR4	Melanocortin 4 receptor
mGLUR4	Metabotropic glutamate receptor 4
mRNA	Messenger ribonucleic acid
NP-HFHSD	Non-pregnant high-fat high-sugar diet
NP-SLD	Non-pregnant standard laboratory diet
NTS	Nucleus tractus solitarius
NYP	Neuropeptide Y
PBS-TX	Phosphate buffered saline containing 0.2% Triton
P-HFHSD	Pregnant high-fat high-sugar diet
POMC	Proopiomelanocortin

PPIA	Peptidyl-prolyl isomerase A
P-SLD	Pregnant standard laboratory diet
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
SAHMRI	South Australian Health and Medical Research Institute
SI	Small intestine
SLD	Standard laboratory diet
T1R1	Taste 1 receptor membrane 2
T1R2	Taste 1 receptor membrane 2
T1R3	Taste 1 receptor membrane 3
TRPM5	Transient receptor potential cation channel, subfamily
VA	Vagal afferents
VMN	Ventral medial nucleus
ZT	Zeitgeber

CHAPTER 1: Introduction

Maternal adaptions to food intake across pregnancy: central and peripheral mechanisms.

Clarke GS[1,2,3], Gatford KL[2,3], Young RL[3,4,5], Grattan DR[6], Ladyman SR[2,3] & Page AJ[1,3,5]

[1]Vagal Afferent Research group, Translating Nutritional Science to Good Health, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia;

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[5]Centre of Research Excellence, Translating Nutritional Science to Good Health, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia;

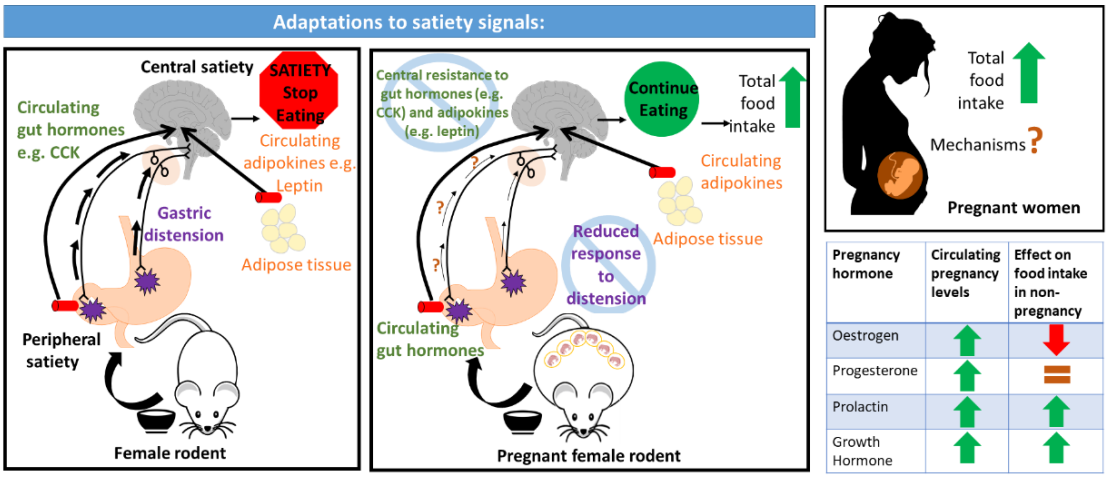
and [6]Centre of Neuroendocrinology, Department of Anatomy, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand.

Obesity 29(11) (2021) 1813-1824.Impact factor: 3.74; quartile (Q) 1 journal in the field (SCImago).

DOI: 10.1002/oby.23224

1.1. Graphical Abstract:

Maternal adaptions to food intake across pregnancy: central and peripheral mechanisms



Pregnancy hormone	Circulating pregnancy levels	Effect on food intake in non-pregnancy
Oestrogen	↑	↓
Progesterone	↑	=
Prolactin	↑	↑
Growth Hormone	↑	↑

1.2. Overview

Sections 1.4.-1.13 are taken directly from a published invited review that I was first author on and therefore my thesis introduction is reproduced exactly as published. Due to the timing of this review's publication, it does contain some experimental work mentioned in **Chapter 2**. The rationale of this review was to identify gaps in gastrointestinal satiety signals during pregnancy and link this with central satiety mechanisms and food intake behaviour. From this review it was clear that there was very limited data on adaptations within the stomach and intestine during pregnancy. This paper has been published as:

Clarke GS, Gatford KL, Young RL, Grattan DR, Ladyman SR, & Page AJ. (2021). Maternal adaptions to food intake across pregnancy: central and peripheral mechanism. Obesity 29(11) (2021) 1813-1824.

1.3. Authorship Document

Statement of Authorship

Title of Paper	Maternal adaptations to food intake across pregnancy: Central and peripheral mechanisms.
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Manuscript DOI: 10.1002/oby.23224 This was an invited review from the Obesity Journal. Clarke GS, Gatford KL, Young RL, Grattan DR, Ladyman SR, Page AJ. Maternal adaptations to food intake across pregnancy: Central and peripheral mechanisms. Obesity. 2021;29(11):1813-24.

Principal Author

Name of Principal Author (Candidate)	Miss Georgia Clarke		
Contribution to the Paper	Wrote the manuscript. Prepared figures. Edited and drafted revised manuscript. Approved final version of manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/07/23

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);
II. permission is granted for the candidate in include the publication in the thesis; and
III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	A/Prof Kathryn Gatford		
Contribution to the Paper	Edited and drafted revised manuscript. Approved final version of manuscript.		
Signature		Date	14/7/23

Name of Co-Author	A/Prof Richard Young		
Contribution to the Paper	Edited and drafted revised manuscript. Approved final version of manuscript.		
Signature		Date	7/7/23

Name of Co-Author	Prof David Grattan		
Contribution to the Paper	Edited and drafted revised manuscript. Approved final version of manuscript.		
Signature		Date	7/7/23

Name of Co-Author	Dr Sharon Ladyman		
Contribution to the Paper	Edited and drafted revised manuscript. Approved final version of manuscript.		
Signature		Date	7/7/23

Name of Co-Author	Prof Amanda Page		
Contribution to the Paper	Prepared figures. Edited and drafted revised manuscript. Approved final version of manuscript.		
Signature		Date	7/7/23

1.4. Study importance questions

1.4.1. What major reviews have already been published

It is widely known that pregnancy is associated with increased maternal food intake and reduced energy expenditure, which is important for creating enough energy to support the mother and the growing fetus(es) and placenta. In human and non-human species, reviews strongly support that both under- and over-nutrition impact fetal and mother health outcomes in the short and long-term, which signifies the importance of maintaining healthy pregnancy weight gain and intervening when not achieved. At the foundation level, the regulation of food intake and energy homeostasis in a healthy state has been extensively studied, however, during pregnancy the mechanisms permitting increased food intake are mostly unknown. Experts in central satiety, Prof David Grattan and Dr Sharon Ladyman, have published the majority of the current research on central satiety and the role of prolactin on food intake during pregnancy. However, there is very little known about peripheral or gastrointestinal satiety during pregnancy. Prior studies in gastrointestinal changes during pregnancy, largely focus on anatomical changes at a gross (weight and length) and microscopic scale (microstructure e.g. villi) or change in function (e.g. intestinal transit or gastric emptying). To the best of our knowledge, this is the first review combining adaptions in central and peripheral satiety and hypothesising potential physiological mechanisms. This review is the basis of a PhD project, with one research article recently published on adaptations in gastric vagal afferents during pregnancy from our group.

1.4.2. What does your study add?

This review presents an overview of the current literature on known changes in central and peripheral mechanism of satiety during pregnancy. We also detail how the pregnancy hormones could play a role in these adaptions. Furthermore, this review highlights the need for extensive research in gastrointestinal satiety signalling during pregnancy. Future research highlighted includes:

1. Establishing whether nutrient sensing mechanisms within the intestine are attenuated during pregnancy
2. Establishing whether nutrient induced gastrointestinal hormone release within the intestine are attenuated during pregnancy
3. Establishing whether gastrointestinal vagal afferent innervation and response to food related stimuli is altered during pregnancy
4. Determine changes in nutrient induced satiety across pregnancy
5. Determine the role of pregnancy hormones mediating adaptations in satiety signalling during pregnancy

1.5. Abstract

A sufficient and balanced maternal diet is critical to meet the nutritional demands of the developing fetus and to facilitate deposition of fat reserves for lactation. Multiple adaptations occur to meet these energy requirements, including reductions in energy expenditure and increases in maternal food intake. The central nervous system plays a vital role in the regulation of food intake and energy homeostasis, and responds to multiple metabolic and nutrient cues, including those arising from the gastrointestinal tract. This review describes the nutrient requirements of pregnancy and the impact of over- and under-nutrition on the risk of pregnancy complications and adult disease in progeny. The central and peripheral regulation of food intake is then discussed, with particular emphasis on the adaptations that occur during pregnancy and the mechanisms that drive these changes, including the possible role of the pregnancy-associated hormones progesterone, oestrogen, prolactin and growth hormone. We identify the need for deeper mechanistic understanding of maternal adaptations, in particular, changes in gut-brain axis satiety signalling. Improved understanding of food intake regulation during pregnancy will provide a basis to inform strategies that prevent maternal under- or over-nutrition, improve fetal health and reduce the long-term health and economic burden for mothers and offspring.

1.6. Introduction

Effective regulation of food intake and energy expenditure is essential for the optimal functioning of an organism and is achieved through complex integration of appetite control regions of the central nervous system (CNS) with peripheral metabolic and nutrient cues. In very basic terms, peripheral feedback to the CNS is composed of two components, post-ingestive signals arising from the gastrointestinal (GI) tract which contribute to meal termination, and long term adiposity signals that signal the level of body energy storage.

Although energy homeostasis is tightly controlled, the underlying processes show a high level of plasticity in response to environmental or physiological demand. This is perhaps best exemplified during pregnancy, where the mother experiences additional metabolic demands due to fetal development and maternal physiological changes, and this demand is met by maternal adaptions in energy expenditure, food intake and nutrient absorption [1,2]. These adaptations, and appropriate changes in maternal nutrient intake, are essential, as over- or under-nutrition are now well established to negatively impact normal fetal development and long-term health of offspring [3,4]. A clearer understanding of the mechanisms underlying appetite regulation in pregnancy has the potential to lead to improved strategies that secure a healthy gestational weight gain. This review will examine current knowledge on food intake and its regulation during pregnancy, and the gaps in knowledge that require further attention.

1.7. Energy requirements during pregnancy

1.7.1 Energy homeostasis during pregnancy

During pregnancy, healthy women gain on average 10-15% of their pre-pregnancy weight and rats and mice gain ~50%, largely due to growth of the conceptuses and uterus, but also due to deposition of fat in preparation for lactation (**Figure 1.1**). This demands increased provision of energy, which is made available through a shift in energy homeostatic components. It is now well understood that the metabolic rate increases during pregnancy and physical activity reduces, or remains unchanged, in pregnant women reviewed in [5] as occurs during pregnancy in mice and rats [1,6]. Despite this, increased energy is also made available through increased maternal food intake along with an increased capacity for maternal nutrient absorption [2].

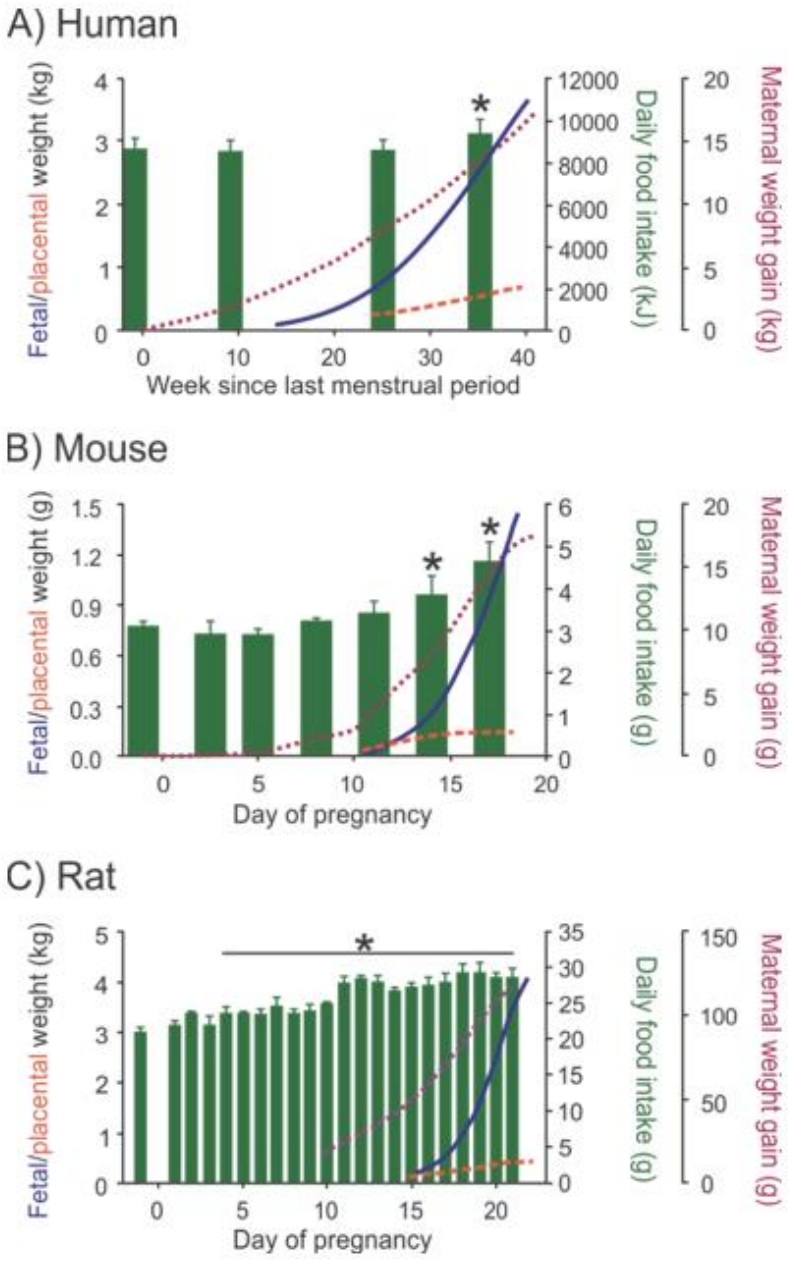


Figure 1.1. Human (A), mouse (B) and rat (C) maternal weight gain, fetal/placental weight and food intake across pregnancy.

Maternal weight (red dotted line) and food intake (green bars) increases in the third trimester in women and in late pregnancy in mice and rats, during rapid fetal (orange dashed line) and placental growth (purple dotted line).

1.7.2 Nutrition in pregnancy influences long term health of progeny

Fetal weight in rats, mice and humans, increases throughout pregnancy, most rapidly in the later stages of pregnancy (**Figure 1.1**). The Institute of Medicine (IOM) provide guidelines for optimal weight gain during pregnancy in a BMI-dependent manner, such that higher pre-pregnancy BMI reduces the total recommended gestational weight gain [4]. The effects of a maternal diet that differs from these guidelines has been extensively reviewed, and it is widely accepted that maternal undernutrition and excessive gestational weight gain increase the risk of adverse short- and long-term health consequences for the fetus and mother. For example, pregnant women who gain less weight than recommended guidelines are at increased risk of pre-term birth and small for gestational age infants (reviewed in [4]). Maternal nutrient restriction during pregnancy can also lead to increased rates of obesity, coronary heart disease and elevated circulating cholesterol levels in later life for the fetus [3]. In contrast, excessive weight gain during pregnancy increases the risk of large for gestational age infants (macrosomia, birthweight >4000g) and Caesarean delivery [4], which also increases the risk of obesity and type 2 diabetes in the progeny later in life [3].

The macronutrient composition of the diet during pregnancy is as important as the caloric intake. The IOM recommends daily dietary reference values for grams of carbohydrate, protein, fibre and fatty acids per day for a healthy weight gain, to maintain fetal development, and for healthy long term outcomes in progeny (reviewed in [7]). For example, IOM recommends women consume ~60-70 g/day of protein during the first trimester, which increases by ~25 g/day during the second and third trimester [7]. This is important, as a diet with a lower or higher ratio of protein is associated with restricted fetal growth, and with insulin resistance and higher blood pressure in progeny in adult life [7]. Similar findings

have been shown with varying ratios of carbohydrates and lipids [7], highlighting the importance of maintaining a balanced macronutrient profile during pregnancy. Overall, the extent of metabolic reprogramming at a cellular, tissue or functional level depends on the specific dietary exposure, its duration and the stage of fetal development when exposure occurs [3]. In the current environment, excessive weight gain during pregnancy is common, with over 50% of women exceeding the IOM guidelines for optimal weight gain [4], which signifies the need for a weight management intervention.

1.8. Regulation of food intake

1.8.1 Hypothalamic regulation of food intake

Many different central brain regions and neuronal networks are involved in the regulation of food intake, such as the hypothalamo-pituitary-adrenal (HPA) axis, oxytocin system and various orexigenic and anorectic neurons populations with the medial basal region of the hypothalamus. For the most part, these systems have been under-investigated in the context of food intake regulation during pregnancy. For example, within the HPA axis, cortisol is known to increase food intake in non-pregnant rats and humans, however, it is unknown whether increases in plasma cortisol in the late stages of pregnancy in humans [8], mice [9] and rats [10] alter food intake. In addition, oxytocin levels rise during pregnancy, to peak during week forty in women [11] and between day eighteen and twenty one in rats [12]. However, intracerebroventricular injection of oxytocin fails to suppress food intake in pregnant rats (d 16) in contrast to non-pregnant rats [13] adding support for the development of progressive oxytocin resistance during pregnancy, however, this requires more investigation. Despite this, the hypothalamus provides the most evidence for adaptations in food intake

regulation, mostly because many of the hormones that normally act in this region to regulate food intake, increase their secretion (e.g. leptin, insulin) during pregnancy. Due to this knowledge, this review will focus on pregnancy-related changes within the hypothalamus as a result of leptin and insulin.

The control of appetite regulation in the brain involves a complex neuronal network that includes both orexigenic and anorectic factors. Normally, these factors are constantly modulated through stimulation or inhibition by many stimuli to maintain appetite at appropriate levels for current energy expenditure. The hypothalamus plays a pivotal role in the regulation of food intake, via integrating signals from peripheral and central sources to coordinate food intake and achieve energy homeostasis [14,15]. The hypothalamic arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventromedial hypothalamic nucleus (VMN) and dorsomedial nucleus (DMN) are critical nuclei involved in this central circuitry [15]. These regions, and others in the brain, are responsive to satiety signals from the periphery, such as the adipose-derived hormone leptin and insulin from the pancreas. Both of these hormones are too big to diffuse into the brain and are actively transported across the blood-brain-barrier by, as yet, undefined mechanisms [1]. Various regions are also responsive to ghrelin, which is orexigenic and secreted by the stomach [16].

In particular the ARC, located in the medial basal hypothalamus, is a key area for appetite regulation. It is located in close proximity to the fenestrated capillaries of the median eminence allowing its rapid exposure to circulating metabolic hormones, such as leptin, insulin and ghrelin. The ARC contains two neuronal populations whose role in energy homeostasis is well-characterised, the pro-opiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART) neurones and the neuropeptide Y (NPY) and agouti-related peptide

(AgRP) neurones [15]. The POMC/CART neurones are anorectic and express the POMC precursor polypeptide, which undergoes cleavage processing to yield multiple neuropeptides including alpha- melanocyte-stimulating hormone (α-MSH). This is the peptide predominantly responsible for the effects on appetite, demonstrated by studies showing that administration of α-MSH can prevent the hyperphagia otherwise exhibited by POMC-deficient mice (reviewed in [16]). The anorectic actions of α-MSH are principally mediated by the melanocortin 4 receptor (MC4R) in the PVN, although MC4R are widely expressed in the hypothalamus and actions at other sites certainly contribute to its action [16]. Projections from the PVN to the lateral parabrachial nucleus form the downstream circuitry that regulate this feeding pathway. The orexigenic NPY/AgRP neurones also project to multiple brain regions. These neurones are GABAergic and GABA released from NPY/AgRP neurones is critical to promote food intake [16]. The peptides NPY and AgRP are also highly orexigenic and their release via projections to other brain areas also regulates food intake. NPY acts through its own cognate receptors, Y1, Y2 and Y5, within the ARC, PVN and VMN reviewed in [17], whereas, AgRP is an endogenous antagonist to the melanocortin 4 receptor and inhibits the anorectic actions of the melanocortin system, particularly in the PVN [16]. Thus, these NPY/AgRP neurones regulate appetite via multiple pathways, contributing to the complexity of this system. NPY/AgRP and POMC neurones within the ARC express leptin receptors [18], insulin receptors [19] and ghrelin receptors [20]. Binding of leptin or insulin at its receptor augments the activity of POMC neurones, but attenuates the activity of NPY/AgRP neurones [16] decreasing food intake and increasing energy expenditure.

Alongside the ARC, many other areas have been implicated in appetite regulation. As mentioned above, the PVN forms a critical nexus receiving inputs

from the ARC while the brainstem, amygdala, LHA, VMH and DMH also each contain neurone populations that are involved in appetite regulation. For example, the LHA receives input from the ARC and contains neuropeptides such as melanin concentrating hormone, orexin and neurotensin which can all influence food intake [21].

1.8.2 Gastrointestinal regulation of food intake

Satiation signals arise from multiple regions of the GI tract. The presence of food and nutrients in the stomach and intestine is communicated to the brainstem and hypothalamus via the gut-brain axis, comprising of neural signals from vagal afferent (VA) neurones and endocrine signals from gut hormones [14]. Vagal afferents innervating the GI tract respond to mechanical and chemical stimuli to detect the arrival, amount and nutrient composition of a meal [14]. Gastrointestinal VAs project centrally within the nucleus tractus solitarius (NTS) whereupon information is integrated with brainstem, limbic and hypothalamic signals to coordinate reflex control of GI motility and gastric emptying, along with behavioural responses and sensations, such as fullness and satiety [14].

1.8.2.1 Gastric and intestinal responses to mechanical distension

Gastric mechanosensitive VAs are classified into two functional classes; mucosal and tension receptors [14]. Mucosal receptors respond to fine tactile stimuli, such as the movement of food over the receptive field, which may discriminate food participle size, and in turn, regulate gastric emptying [14]. Tension receptors respond to mechanical stretch or distension of the gastric wall, and trigger vago-vagal reflexes that control GI function and generate sensations of satiety and fullness, leading to meal termination [14]. It is well established that VAs display plasticity, which is important for matching food intake to energy demands. For

example, the mechanosensitivity of gastric VAs are modulated by nutritional status, with the sensitivity of both subtypes reduced after short-term fasting, to facilitate increased food intake [22]. Gastric VAs also exhibit a diurnal rhythm, with a nadir in mechanosensitivity during the dark phase, when nocturnal mice are active and require energy [23]. Similar to the stomach, the small intestine is innervated by VAs, with the highest density in the proximal intestine, i.e. duodenum [14]. A recent study has shown that the tension-sensitive duodenal VAs have a major role in the inhibition of food intake [24].

1.8.2.2 Intestinal enteroendocrine response

Satiety signals are also generated by interactions between nutrients and mucosal enteroendocrine cells, distributed along the entire length of the small intestine [14]. These cells express a wide range of nutrient receptors which are tuned to detect macronutrient breakdown products: amino acids (G protein-coupled receptor 93, G protein-coupled receptor family C group 6 member A, calcium-sensing receptor, umami taste receptor T1R1-T1R3, metabotropic glutamate receptor 4), monosaccharides (sweet taste receptor T1R2-T1R3, sodium-glucose co-transporter 1) and fatty acids (free fatty acid receptor FFAR1, 2, 3 and 4, G-protein coupled receptor 84) [14]. Activation of these nutrient receptors initiates an intracellular signalling cascade which culminates in the release of peptide/hormones, such as cholecystokinin (CCK) or glucagon-like peptide 1 (GLP-1), which can enter the circulation to access the brain and activate their cognate receptors CCK-A and GLP-1R in the hypothalamus [14], however, these central effects are not normally seen under normal physiology, since these peptides have a relatively short half-life in the circulation and do not readily cross the blood brain barrier [25]. These gut peptides also have paracrine actions at CCK-A [26] and GLP-1R [14] receptors on VAs, activating these nerves once

released from the enteroendocrine cell (**Figure 1.2**). These paracrine actions are best evidenced in *in vivo* experiments, where CCK [27] and GLP-1 [28] levels in plasma were elevated after oral administration of nutrients, in association with reduced food intake in both rats and men. Consistent with these pathways, in male rats a nutrient gavage activated NTS neurones, assessed via c-fos expression [28], while a vagotomy abolishes the satiating effects of CCK (unilateral abdominal vagotomy [26]) and GLP-1 at high concentrations (subdiphragmatic vagotomy [29]), suggesting the satiating effects were mediated via VAs.

Enteroendocrine cells can also make direct synaptic connections with VAs via a basal specialisation, known as a neuropod [30]. Stimulation of a neuropod results in the release of small and large vesicles packaged with satiety peptides and neurotransmitters, e.g. glutamate to activate VA nerves [30].

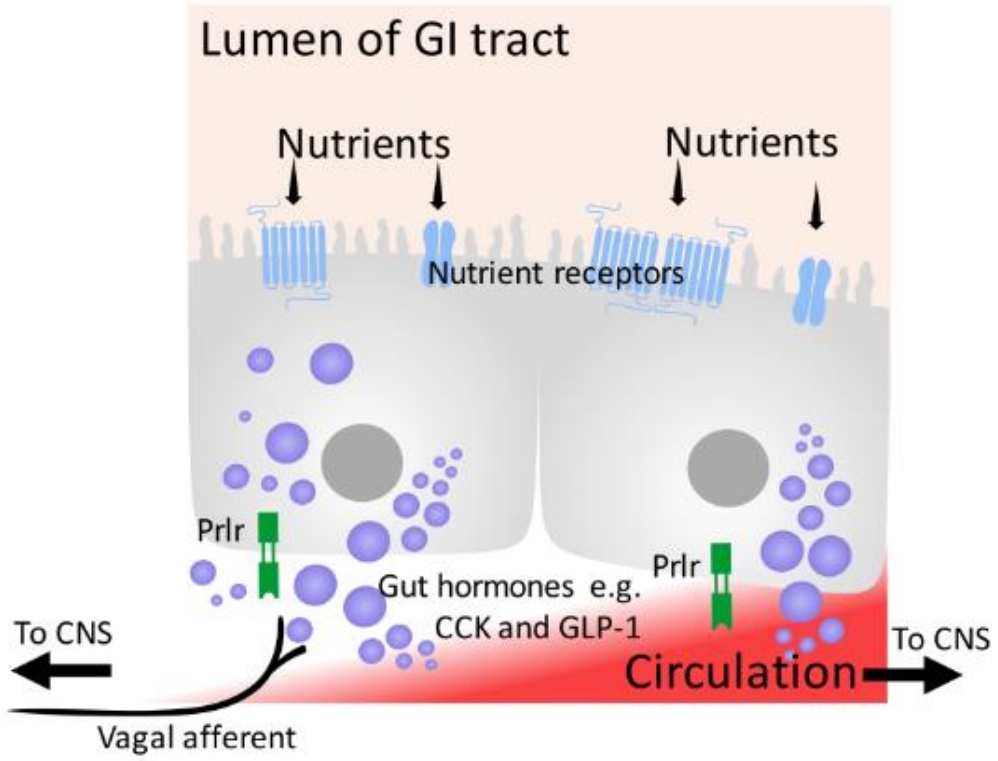


Figure 1.2. Model of satiety signals generated in the small intestine.

Nutrients in the lumen of the gastrointestinal (GI) tract activate specific nutrient receptors initiating an intracellular process that triggers gut hormone(s) release, including cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1). These signal direct to the central nervous system (CNS) via circulatory access, or upon activation of GI vagal afferent endings. How these satiety signals adapt during pregnancy is unknown. Prolactin receptor (Prlr) is highly expressed in the small intestine and, we propose, plays a major role in driving pregnancy adaptations in satiety signalling.

1.9. Changes in food intake regulation during pregnancy

1.9.1 Maternal food intake in pregnancy

Maternal food intake begins to increase from early gestation and increases progressively before peaking in late gestation (**Figure 1.1**). Maternal food intake increases ~10% in the third trimester in humans [31] and increases by ~20-30% before birth in rats and mice [6,32]. These increases in food intake proceed despite elevated fat deposition and circulating leptin in the mothers, via specific adaptations in central and peripheral pathways of food intake regulation.

1.9.2. Adapting central regulation of food intake during pregnancy

As food intake increases during pregnancy, so too do levels of the orexigenic neuropeptides NPY and AgRP [33,34] suggesting that elevations in these neuropeptides contribute to the increase in appetite during pregnancy. Two key long-term metabolic regulatory hormones, leptin and insulin, both increase during pregnancy, respectively due to increases in fat mass together with placental production of leptin in some species, and adaptations of the beta-cells required to maintain glucose homeostasis in the face of gestational insulin resistance. Both leptin and insulin usually have suppressive effects on NPY and AgRP mRNA expression [15], and, therefore, the increases seen during pregnancy indicate that there is a dissociation of the control of NPY and AgRP by these hormones. Our understanding of the maternal adaptations in the central regulation of food intake during pregnancy has been particularly focused on the attenuated sensitivity of the brain to anorexic factors, which may facilitate increased food intake during pregnancy.

There is a general consensus that leptin levels increase during pregnancy (**Figure 1.3**), apart from a small decrease seen in late pregnancy in rats [35]. Many different factors contribute to raising leptin concentrations in different species, including: 1) increased production from accumulating adipose tissue; 2) a proposed reduction in clearance due to increased binding proteins; and 3) production of leptin by the placenta (reviewed in [1]), with up to ~14% entering the fetal circulation, where it plays an important role in fetal development and organ maturation reviewed in [36], and the remainder entering the maternal circulation. Overall, increases in food intake are maintained (reviewed in [1]), despite elevated leptin. The ability of centrally administered leptin to reduce food intake is preserved until mid-pregnancy, after which central leptin resistance subsequently develops, at least in terms of the ability of leptin to induce a reduction in food intake [32].

Evidence would suggest there are multiple mechanisms contributing to leptin resistance. Firstly, it has been shown that leptin transport into the brain during mid pregnancy in the mouse is impaired, which may be due to increased binding of leptin in the blood as the placenta secretes a leptin binding protein (reviewed in [1]). While the identity of this binding protein is unknown, it is possible a circulating secretory isoform of the leptin receptor increases in mid pregnancy in rats [1]. Furthermore, mRNA levels of a proposed leptin transporter in the choroid plexus are reduced by early pregnancy in rats [37], suggesting that transport of leptin into the brain at this location may also be reduced during pregnancy.

As well as a likely reduction in leptin access to the brain due to reduced blood-brain barrier transport, some first-order neurones show impaired responses to leptin during pregnancy. An attenuated response to intracerebroventricular leptin in the VMN is evident in pregnant compared to non-pregnant rats, despite the

leptin response in the ARC being unchanged [1]. This VMN-specific attenuation of leptin-induced phosphorylation of signal transduced and activator of transcription 3 (STAT3) is likely due to a VMN-specific reduction in leptin receptors during pregnancy [37]. Indeed, fewer VMN cells responded to supraphysiological doses of intraperitoneal leptin in pregnant mice, while leptin-induced pSTAT3 in the ARC was unaffected [38], as in rats. Moreover, similar levels of endogenous pSTAT3 were reported in the ARC of pregnant and non-pregnant rats [39], despite the fact that circulating leptin levels were higher in pregnant rats [32]. This adds support for pregnancy-related leptin resistance in the ARC, possibly secondary to impaired leptin transport [40], but this requires additional research to confirm.

As part of the changes in maternal glucose regulation, insulin secretion is elevated during pregnancy. To maintain sufficient glucose in the blood to facilitate transfer across the placenta to the fetus, in the later stages of pregnancy, maternal peripheral tissues become insulin resistant, requiring higher levels of insulin for glucose uptake. To meet this demand pancreatic beta-cells undergo pregnancy-specific adaptations including a decreased threshold for glucose-stimulated insulin secretion [1]. While elevated insulin is required for peripheral actions during pregnancy, insulin actions in the brain, such as suppression of food intake, would be counterproductive at a time when the pregnant female needs to increase food intake. To prevent this, similar to leptin, insulin resistance develops in the feeding circuits of the hypothalamus during pregnancy. Insulin-induced intracellular signalling is attenuated in both the ARC and VMN during pregnancy [41] and this central insulin resistance is likely to prevent increases in plasma insulin from influencing appetite. Insulin transport into the brain is unaffected by pregnancy, yet it does appear that insulin degradation in the brain may be more rapid during pregnancy [1] which may also contribute to central insulin

insensitivity. Both insulin and leptin influence food intake by downstream pathways that engage the melanocortin system [15]. During pregnancy the satiety response to α-MSH is also absent [1], suggesting that it is not only first order leptin- and insulin- responsive neurones that become insensitive, but that reduced sensitivity also develops in the downstream pathways activated by these neurones. Thus, it would appear that at least four mechanisms act during pregnancy to prevent suppression of food intake by satiety factors, facilitating the increase in food intake during pregnancy. These are: 1. impaired transport of satiety-inducing factors into the brain; 2. Down-regulation of their receptors in some hypothalamic nuclei; 3. Loss of response to leptin in specific first-order neurones, in some cases via receptor down-regulation; and 4. loss of response to downstream mediators like αMSH.

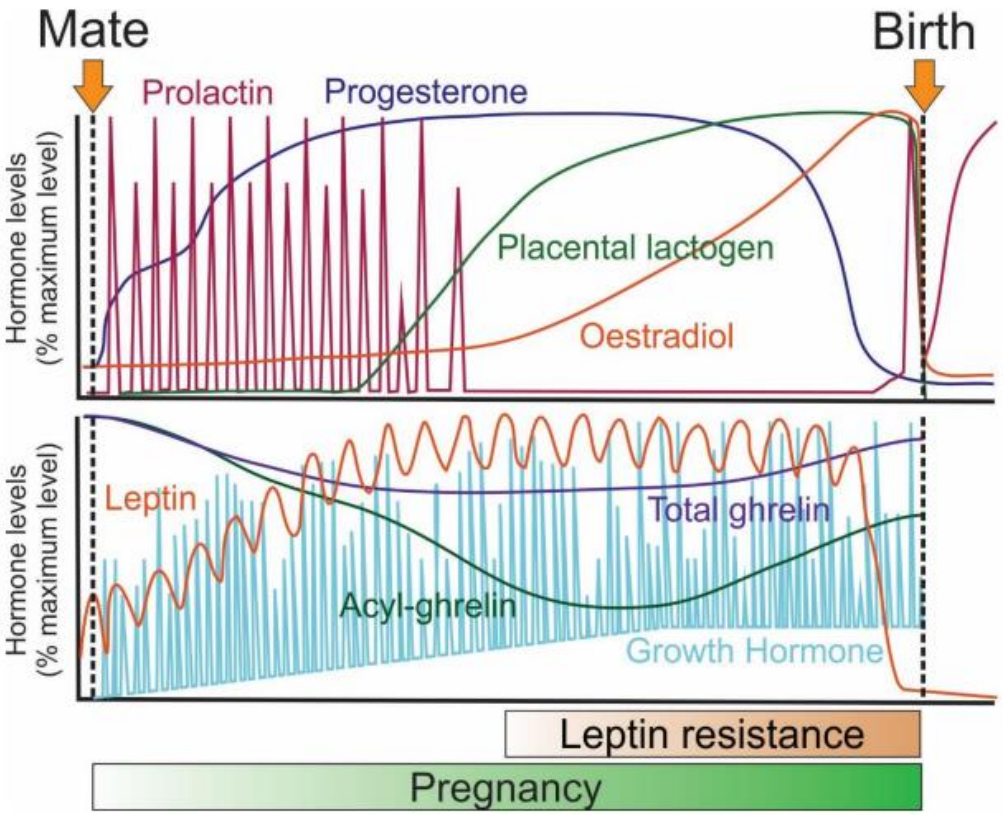


Figure 1.3. Schematic representation of hormonal changes and development of leptin resistance across pregnancy in rats and mice.

After mating, prolactin (top panel, red line) is secreted in surges up until day 10, when placental lactogen increases (top panel, green line). Circulating oestradiol concentrations (top panel, orange line) progressively increase during pregnancy and progesterone (top panel, purple line) begins to increase on days 3-4. Leptin (bottom panel, orange line) progressively increases during pregnancy, but leptin resistance develops during the second half of pregnancy. Growth hormone (bottom panel, blue line) is secreted in surges throughout pregnancy with the basal secretion and circulating concentrations increasing in the first two thirds of pregnancy. Acyl-ghrelin (bottom panel, green line) and total ghrelin concentrations (bottom panel, purple line) gradually decrease during pregnancy with a nadir on, or shortly after the pregnancy mid-point.

1.10. Changes in GI Satiety signalling during pregnancy

1.10.1 Adaptions in gastrointestinal vagal afferents

Gastrointestinal VAs are ideally placed to respond to the arrival, amount and chemical composition of a meal, and are a likely target for maternal adaptations that increase food intake during pregnancy.

1.10.2. Meal Patterns and GVA activity during pregnancy

In mice, the increase in daily food intake from mid-pregnancy onwards occurs predominantly due to an increase in meal size and meal duration in the light phase, without a change in meal number [6,42]. Meal duration also increases in pregnant rats, without a change in meal frequency [43]. These eating patterns are regulated in part by gastric VA signals [23]. Indeed, the mechanosensitivity of gastric VAs is attenuated during pregnancy in mice [42], which would permit a larger quantity of food to be consumed prior to initiating signalling for meal termination. It is likely that intestinal VAs act in a similar manner during pregnancy, as they also respond to mechanical distension [24], however, this requires future investigation. Meal frequency patterns in pregnant women vary between 17 and 29 weeks of pregnancy For example, some pregnant women consume a “snack-dominant” diet (more snacks than meals, [44]) at 28 weeks of pregnancy, while others consume a regular “main meal” diet with three meals with two or more snacks [45]. The former “snack-dominant” pattern has been linked to increased total energy intake whereas the regular “main meal” pattern has not; these data, however, are limited to women in their second and early third trimester [44,45]. In another small study of pregnant women, total daily food intake increased by 775 kilojoules in the third trimester compared to pre-pregnancy levels [31], however, meal patterns were not reported. Overall, however, pregnant women consume

around four meals per day [44,45], which is similar in pattern to the non-pregnant US population [46]. A more detailed analysis of meal intake patterns and the individual meal size (both volume and caloric content) in pregnant women will provide further insight into precise meal adaptations.

1.10.3 Gastric emptying and GVA activity

When the rate of gastric emptying slows, a larger volume of food remains in the stomach, which in turn, increases signals from mechanosensitive gastric VAs that induce satiety [47]. Evidence of changes to the rate of gastric emptying in pregnant women is equivocal, with studies reporting no change [48], or decreased emptying rates [49], which is consistent in rats and mice [50,51].

1.11. Intestinal nutrient sensing and circulating GI hormones

1.11.1. Nutrient sensing during pregnancy

Altered GI sensing of nutrients has the potential to exert a major influence on food intake during pregnancy. To the best of our knowledge, there is no evidence of changes in intestinal nutrient receptor expression during pregnancy, nutrient-evoked gut hormone release, or any effect of these on food intake. However, nutrient sensors in other organs, including the tongue, show altered expression during pregnancy, and have been hypothesised to influence food intake or metabolism [52]. For example, expression of the lipid sensor FFAR2 in pancreatic islets was higher on day 15 of pregnancy, compared to non-pregnant mice [53]. Furthermore, transcripts for the lingual nutrient sensor for ‘umami’ the taste receptor subunit, T1R1, were elevated in the tongue at mid-pregnancy in mice, whereas there were no changes in sweet taste subunits T1R2 or T1R3 across pregnancy stages [54]. Altered expression of T1R1 may be important for altered

taste perception and promotion of specific nutrient intake, such as protein [14], as well as overall food intake. Given evidence of pregnancy-related changes in nutrient sensors during pregnancy, it is likely that nutrient sensors in the small intestine also adapt during pregnancy, contributing to regulation of food intake.

1.11.2. Circulating GI hormones and Pregnancy

The role of maternal gut hormones in GI adaptations to normal pregnancy is under investigated, and few studies have explored the capacity of these hormones to alter GI mucosal structure and affect satiety [55,56]. Current evidence on plasma acyl-ghrelin (the active form of ghrelin) concentrations during pregnancy is equivocal, with reports of an increase [57] or decrease [58] in women, an increase in rats [59] and a decrease in mice [60]. Furthermore, the effects of ghrelin on food intake during pregnancy is mostly unknown. One study in mid-stage pregnant rats, showed that acyl-ghrelin, administered via a subcutaneous osmotic mini-pump, increased food intake compared to saline infusion in pregnant rats [61]. However, since this was exogenous acyl-ghrelin and was not compared with non-pregnant controls, more investigations are required to determine the precise effects of ghrelin on food intake during pregnancy.

The satiety effects of gut-derived CCK and GLP-1 are largely mediated through VA signalling as stated earlier. Fasting plasma levels of CCK are reported to be increased or unchanged in pregnant women, compared to menstruating women [62,63]. Fasting levels of circulating CCK also rise in canine pregnancy [64], however, CCK levels have not been reported during pregnancy in rats and mice. Fasting levels of active GLP-1 increase in pregnant women, especially between the second and third trimesters [56]. However, fasted plasma total GLP-1 levels were reported to be reduced by day 4 of pregnancy in rats compared to proestrus

controls [55]. While it is likely that these differences relate to species variation, the profile of change in active and total GLP-1 during pregnancy requires further investigation.

Together, the balance of evidence supports an increase in intestinal satiety hormones during pregnancy, which is counterintuitive to increased food intake. This may reflect changes in sensitivity to these hormones during pregnancy, as pregnant rats have been shown to be resistant to the short-term satiating effect of peripherally administered CCK [39]. During pregnancy, reduced responses to CCK may be mediated by attenuated expression of CCK receptor transcript or protein in key brain regions controlling food intake, such as the ARC [65]. Moreover, both central and peripheral leptin treatment modulate CCK sensitivity, such that when leptin levels are low the effect of CCK on food intake is reduced in rats [66]. CCK also increases leptin transport into the brain and pSTAT3 in the hypothalamus, in a synergistic effect [67]. Since pregnancy is associated with leptin resistance, it is possible that attenuated central leptin signalling reduces CCK sensitivity, and facilitates increased food intake. Less is understood about how food intake regulation by GLP-1 changes during pregnancy, largely because GLP-1 treatment in pregnancy significantly reduces pup birth weight [68]. GLP-1 appears to have little effect on food intake in pregnant women, as maternal body weight was shown to be negatively associated with fasting active GLP-1 plasma concentrations [56], but more in-depth research is required.

The intestine significantly increases in length, weight and surface area during pregnancy [2], which is likely to augment nutrient-dependent gut hormone release. The density of GLP-1 containing L-cells is higher in pregnant compared to non-pregnant mice, a change absent in GLP-1R knockout pregnant mice, suggesting GLP-1 action at GLP-1R drives these changes [69]. Such changes may parallel

contributions of GLP-1 to metabolic adaptations in pregnancy. Although not a focus of this review, these satiety hormones may also act in other ways during pregnancy. For example, CCK stimulates post-meal induction of sleep via the vagal nerve [70]. Increased CCK may contribute to the increase in day-time sleepiness reported by women during pregnancy [71] and the increase in sleep or rest periods observed in pregnant mice [6]. Such actions of CCK could promote a positive energy balance by reserving energy previously utilised for activity. CCK and GLP-1 also increase pancreatic, specifically β-cell, expansion and regulate insulin secretion [56,63].

1.12. What is driving increase food intake in pregnancy?

Pregnancy is associated with marked changes in the hormonal milieu, characterised by higher circulating levels of growth hormone (GH), prolactin, progesterone, and loss of oestradiol cycling (**Figure 1.3**) all of which are important in pregnancy recognition, placental and fetal growth and development of lactation reserves. These hormones can also interact with central nuclei associated with the regulation of metabolism, several of which express receptors for prolactin, GH, oestrogen and progesterone and respond to oestrogen [72], GH [73] and prolactin [74]. These central satiety responses are described in detail below. Pregnancy hormones can also interact within the GI tract, where the intestinal mucosa expresses receptors for oestrogen (cytoplasm of lamina propria stroma cells [75]), prolactin (enterocyte cells [76]), GH (enterocytes and enteroendocrine cells [77]), and progesterone (external intestinal smooth muscle cells in the duodenum and jejunum [78]). However, the functional role of these receptors and their response in the intestine is not well understood.

1.12.1 Oestrogen and progesterone

Ovariectomy in rats results in hyperphagia and weight gain, while oestrogen treatment restores normal food intake [72]. Food intake-suppressing effects of oestrogen involve both central and peripheral mechanisms. Centrally, oestradiol implants into the PVN and VMH reduced food intake in ovariectomised rats [79], and these effects were dependent on oestrogen receptor located in these regions [72]. Moreover, oestradiol treatment in rats also stimulates POMC [80] and inhibits AgRP/NPY neurone activity in mice [81]. Oestradiol may also modulate leptin signalling, with indications that exogenous oestradiol increases hypothalamic leptin receptor expression [82].

Oestradiol has recently been shown to act in the GI tract to enhance the mechanosensitivity of gastric VAs to stretch [42]. This is likely a direct effect, as oestradiol receptors are expressed on VAs [83] and oestradiol treatment restores the excitability of a subpopulation of VAs in ovariectomised female rats [84]. Furthermore, oestradiol potentiates the satiating effects of the intestinal hormone CCK [85] and attenuates the appetite promoting effects of gastric ghrelin [86]. However, since oestrogen increases during pregnancy, it seems unlikely that leptin resistance or the increased food intake, observed during pregnancy, are driven by oestrogen. Further investigation is required to elucidate the effects of oestrogen in pregnancy.

In contrast to oestrogen, progesterone does not influence food intake alone, unless administered at high pharmacological doses [72,87], however, progesterone can increase food intake by blocking the effects of oestrogen when co-administered [87]. Although multiple ARC subnuclei, including neurones expressing AgRP, NPY and α-MSH, express the progesterone receptor (reviewed in [88]), the

effect of progesterone on these neurones remains to be investigated. Furthermore, progesterone had no effect on the mechanosensitivity of gastric VAs to stretch in non-pregnant female mice [42], consistent with the lack of effect of systemic progesterone administration on food intake. Although progesterone appears not to influence food intake directly, it may nevertheless act indirectly by altering release of satiety hormones in response to nutrients or the satiating potency of these GI hormones. For example, oral administration of progesterone increased plasma GLP-1 concentrations in male mice compared to vehicle controls [89]. Within the same study, progesterone increased proglucagon gene expression and GLP-1 secretion in intestinal GLUTag cell lines [89], a cell line which expresses the progesterone receptor. Therefore, elevated plasma GLP-1 levels during pregnancy may be partly mediated via progesterone actions on intestinal L-cells, however, this requires further investigation. It is also important to recognise that during pregnancy, both progesterone and oestrogen are elevated. The net effect of these hormones is likely the sum of the satiating effect of oestrogen diminished in the presence of progesterone [87]. Further studies are required to determine the effect of these hormones in combination.

1.12.2. Prolactin

Prolactin acts centrally to increase food intake [74] and can induce a leptin resistant state [1]. In rats and mice, daily prolactin surges during early pregnancy are replaced, later in pregnancy, by secretion of placental lactogens, which act at the same prolactin receptors (**Figure 1.3**). There is strong evidence to suggest that prolactin action is critical for neurophysiological adaptations during pregnancy [90]. For example, central prolactin administration reduces hypothalamic leptin sensitivity [1]. This suggests that surges in prolactin and elevated circulating

placental lactogens induce central leptin resistance, to drive pregnancy-induced hyperphagia.

In contrast to the evidence for prolactin-mediated attenuation of central satiety, there is currently no evidence that prolactin modulates peripheral satiety pathways, since exogenous prolactin had no effect on the *in vitro* mechanosensitivity of gastric VAs to stretch in non-pregnant mice [42]. While it is currently unknown whether prolactin receptors are expressed on GI VAs, they are expressed in intestinal mucosal cells [76]. Accordingly, indirect actions of prolactin within the mucosa that regulate peripheral satiety cannot be excluded. Indeed, prolactin actions on the mucosa in pregnancy may be similar to those of progesterone, and drive intestinotrophic changes that increase nutrient-dependent gut hormone release. In support of this concept, increases in intestinal length are associated with elevated plasma prolactin concentrations in lactating rats [91]. In addition, acute and chronic hyperprolactinemic elevate plasma CCK concentrations in male rats [92] and prolactin interacts with CCK to influence GI motility and gastric emptying [92], however whether these responses are replicable in females is yet to be determined.

1.12.3. GH

GH levels increase throughout human pregnancy, with pituitary-derived GH predominant in early pregnancy, while the placental GH variant predominates from mid to late pregnancy (**Figure 1.3**, [93]). Rats and mice do not possess the placental variant and instead pituitary-derived GH levels increase during pregnancy, likely in response to placental signals [60,94]. Systemic infusion of human placental GH into pregnant mice reduced maternal insulin sensitivity without affecting food intake or body weight [95]. This data is consistent with

elevated GH contributing to the development of insulin resistance during pregnancy [95]. The lack of effect of GH on appetite and growth in this study may reflect different receptor affinities of human and murine GH, or between the pituitary and placental variants, or be due to stimulation of food intake by increasing endogenous pituitary GH during pregnancy. Despite the findings of this study, there is strong evidence supporting a role of GH in food intake regulation during pregnancy. Intracerebroventricular administration of GH, or selective cerebral overexpression of GH, increased food intake in mice [96,97] and inactivation of whole brain specific GH receptors improved insulin sensitivity and decreased food intake and body adiposity in pregnant mice [98]. It is possible these effects are mediated through modulation of appetite hormone signalling, since depletion of GH receptors in AgRP expressing neurones, in leptin receptor expressing cells increases the sensitivity of VMN neurones to leptin [98]. The human placental GH variant also binds the prolactin receptor, but with far lower affinity than prolactin, whilst placentally produced human placental lactogen has high affinity for the PRL receptor, and therefore a greater potential to increase food intake [99]. In addition, an intracerebroventricular central injection of GH increased hypothalamic expression of AgRP and NPY mRNA, while incubating hypothalamic sections *in vitro* with GH activated 25% of AgRP/NPY neurones [96]. Finally, the orexigenic effect of the gastric hormone ghrelin occurs via its action at ARC AgRP/NPY neurones [100]. Together, these findings support central actions of GH in contributing to the pregnancy-related development of insulin resistance, increased food intake and adiposity.

In addition to central mechanisms, we have shown that GH reduces the *in vitro* mechanosensitivity of gastric VAs to stretch in non-pregnant mice [42], a potential link between increases in GH and food intake during pregnancy. However, further

studies are required to confirm these findings in pregnant mice and to elucidate whether the GH receptors are expressed on these VAs and, therefore, whether these are likely to be direct or indirect effects. In terms of gut hormone secretion, there is evidence suggesting that elevated plasma GLP-1 levels during pregnancy are not mediated via GH. In fact, evidence in non-pregnant fasted rats suggests that GH suppresses circulating GLP-1, which was reduced in hypophysectomised animals and restored to control levels after an infusion of GH [101].

Overall, it is clear that there is limited knowledge on how pregnancy hormones interact with the GI tract to modulate GI satiety signals during pregnancy, especially considering the elevated levels of the satiety hormones CCK and GLP-1 [55,63]. The existing evidence suggests that increases in food intake during pregnancy likely reflect down-regulation of both central and peripheral satiety pathways, potentially mediated by pregnancy hormones, but many of these pathways have not been directly assessed to date.

1.13. Conclusion

This review covers an underdeveloped area of research on maternal adaptations that support a positive energy balance during pregnancy, and that facilitate the healthy growth of the fetus and deposition of lactation reserves. It details adaptations in central hypothalamic satiety mechanisms and potential decreases in GI satiety signalling during pregnancy. It is clear that hypothalamic satiety is downregulated during pregnancy due to the development of central leptin resistance, and likely that many brain regions are involved in regulating food intake during pregnancy. Future research should include investigations in other areas of the brain involved in food intake regulation, including the caudal brain,

involved in the processing of VA signals, and other hypothalamic pathways including the hypothalamo-pituitary-adrenal (HPA) axis and oxytocin system, since cortisol and oxytocin secretion increases during pregnancy. Furthermore, while there is limited data on changes in GI satiety signals during pregnancy, a recent report of attenuated mechanosensitivity in gastric VAs in pregnant mice, and strong correlation between VA mechanosensitivity and meal size during pregnancy [42], provides the first support of such adaptations during pregnancy. Whether intestinal VAs adapt in a similar way is an avenue of future research. Even less is known of the potential for changes in intestinal nutrient sensing during pregnancy, although higher circulating levels of gut-derived satiety hormones [55,56,63] in tandem with increased food intake during pregnancy [6,42], imply that resistance to GI satiety hormone signals may occur. Adaptations in central and peripheral satiety pathways during pregnancy are likely to be driven by changes in levels of pregnancy hormones such as prolactin, oestrogen, progesterone and growth hormone, since increases in food intake parallel the timing of pregnancy-related changes in the circulating abundance of these hormones. As the primary function of the GI tract is to receive, digest and absorb food, a deeper understanding of pregnancy-related changes in GI nutrient sensing and satiety signalling offers potential new avenues to develop dietary and nutritional strategies that safeguard maternal nutrient intake, and which optimise maternal and fetal health.

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Author contribution: G.C. wrote the manuscript. K.G., R.Y., D.G., S.L. and A.P contributed to the editing of the manuscript.

CHAPTER 2: Pregnancy-related plasticity of gastric vagal afferent signals in mice.

Li H[1,2]*, **Clarke GS[1,2,3]*,** Christie S[1,2], Ladyman SR[4], Kentish SJ[1,2], Young RL[1,2], Gatford KL[1,2,3] & Page AJ[1,2]

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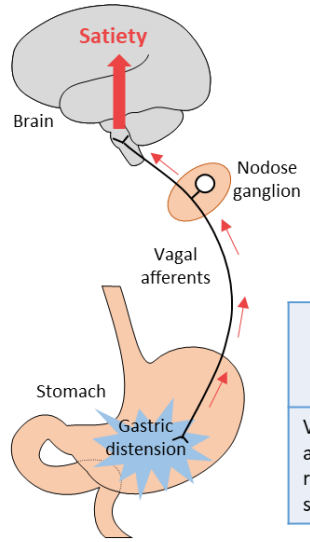
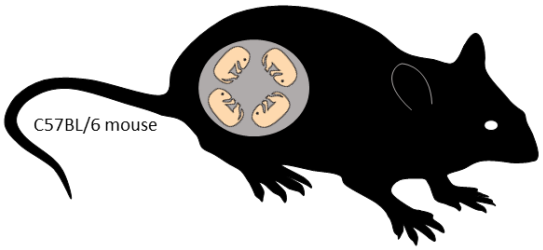
DOI: 10.1152/ajpgi.00357.2020

2.1. Graphical abstract:





	During Pregnancy	Effect of pregnancy associated hormones			
		Growth hormone	Oestradiol	Progesterone	Prolactin
Vagal afferent response to stretch	↓	↓	↑	↔	↔

2.2. Overview:

Chapters 2, 3, 5 describe outcomes from the same cohort of early-, mid-, late-pregnant and non-pregnant mice. Within this broader animal study, I led the animal management and timed-matings and the post-mortems including taking all intestinal issue and processing it. More specifically, my contribution to generating outcomes in **Chapters 2, 3 and 5** were as followed. **Chapter 2**: Analysed all the metabolic cage data and prepared figures, ran all the statistics needed for the paper and contributed to the writing of the manuscript. **Chapter 2** is also the first paper to describe this experimental cohort and is published. **Chapter 3:** Completed all the RNA extraction and PCR experiments across each pregnancy stage and region, analysed PCR data, worked up antibodies, completed the immunohistochemistry and counts, ran the statistics, created the figures and wrote the manuscript. **Chapter 5:** Extracted the raw data from the metabolic cage system, created the figures and wrote the manuscript. **Chapter 3** has been accepted for publication and **Chapter 5** is under review.

Sections 2.4.-2.9. are reproduced exactly as published. I am joint co-first author on this paper and it was published as:

Li H*, **Clarke GS*,** Christie S, Ladyman SR, Kentish SJ, Young RL, Gatford KL & Page AJ. (2021). Pregnancy-related plasticity of gastric vagal afferent signals in mice. *Am J Physiol Gastrointest Liver Physiol* 320(2) 183-192. Impact factor: 3.72; Q1.

This paper was selected by the American Physiology Society (APS select) as an article of "outstanding scientific discovery". In addition, both co-first authors (*) were invited to create a video abstract, accessible on the American Journal of Physiology website and YouTube, to highlight the publication.

Website: *https://journals.physiology.org/doi/full/10.1152/ajpgi.00357.2020*

YouTube: https://www.youtube.com/watch?app=desktop&v=qa_ha7VnICE

2.3 Authorship Document

Statement of Authorship

Title of Paper	Pregnancy-related plasticity of gastric vagal afferent signals in mice
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript DOI: 10.1152/ajpgi.00357.2020 Published in the American Journal of Physiology, this paper was selected by the American Physiology Society (APS select) as an article of "outstanding scientific discovery". In addition, both co-first authors were invited to create a video abstract, accessible on the American Journal of Physiology website and YouTube, for highlighting the publication. Li H, Clarke GS, Christie S, Ladyman SR, Kentish SJ, Young RL, et al. Pregnancy-related plasticity of gastric vagal afferent signals in mice. Am J Physiol Gastrointest Liver Physiol. 2021;320(2):G183-g92.

Principal Author

Name of Principal Author (Candidate)	Miss Georgia Clarke
Contribution to the Paper	Conception and design of research, performed experiments and analysed data. Interpreted results of experiments. Prepared figures. Edited and revised manuscript. Accepted final version of manuscript.
Overall percentage (%)	35%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 07/07/23

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Equal first author: Dr Hui Li
Contribution to the Paper	Performed experiments and analysed data. Prepared figures. Drafted manuscript. Edited and revised manuscript. Accepted final version of manuscript.
Signature	Date: 7/7/23

Name of Co-Author	Dr Stewart Christie
Contribution to the Paper	Performed experiments and analysed data. Edited and revised manuscripts. Accepted final version of manuscript.
Signature	Date: 7/7/2023

Name of Co-Author	Dr Sharon Ladyman		
Contribution to the Paper	Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	7/7/23

Name of Co-Author	Dr Stephen Kentish		
Contribution to the Paper	Performed experiments and analysed data. Edited and revised manuscripts. Accepted final version of manuscript.		
Signature		Date	13/7/23

Name of Co-Author	A/Prof Richard Young		
Contribution to the Paper	Conception and design of research. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	7/7/23

Name of Co-Author	A/Prof Kathryn Gatford		
Contribution to the Paper	Conception and design of research. Performed experiments and analysed data. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	14/7/23

Name of Co-Author	Prof Amanda Page		
Contribution to the Paper	Conception and design of research, performed experiments and analysed data. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	7/7/23

2.4. Abstract

Gastric vagal afferents (GVAs) sense food related mechanical stimuli and signal to the central nervous system, to integrate control of meal termination. Pregnancy is characterised by increased maternal food intake, which is essential for normal fetal growth and to maximise progeny survival and health. However, it is unknown whether GVA function is altered during pregnancy to promote food intake. This study aimed to determine the mechanosensitivity of GVAs and food intake during early-, mid-, and late- stages of pregnancy in mice. Pregnant mice consumed more food compared to non-pregnant mice, notably in the light phase during mid- and late pregnancy. The increased food intake was predominantly due to light phase increases in meal size across all stages of pregnancy. The sensitivity of GVA tension receptors to gastric distension was significantly attenuated in mid- and late pregnancy, while the sensitivity of GVA mucosal receptors to mucosal stroking was unchanged during pregnancy. To determine whether pregnancy associated hormonal changes drive these adaptations, the effects of oestradiol, progesterone, prolactin and growth hormone, on GVA tension receptor mechanosensitivity were determined in non-pregnant female mice. The sensitivity of GVA tension receptors to gastric distension was augmented by oestradiol, attenuated by growth hormone and unaffected by progesterone or prolactin. Together, the data indicate that the sensitivity of GVA tension receptors to tension is reduced during pregnancy, which may attenuate the perception of gastric fullness and explain increased food intake. Further, these adaptations may be driven by increases in maternal circulating growth hormone levels during pregnancy.

NEW & NOTEWORTHY

This study provides first evidence that gastric vagal afferent signalling is attenuated during pregnancy and inversely associated with meal size. Growth hormone attenuated mechanosensitivity of gastric vagal afferents, adding support that increases in maternal growth hormone may mediate adaptations in gastric vagal afferent signalling during pregnancy. These findings have important implications for the peripheral control of food intake during pregnancy.

KEYWORDS: Food intake, gastric vagal afferents, growth hormone, pregnancy, satiety signals

2.5. Introduction

Gastric vagal afferents (GVA) sense food related mechanical stimuli and transfer this information to the brain to modulate food intake and gastric function [102]. There are two functional classes of mechanosensitive GVAs, tension and mucosal receptors [103,104]. Tension receptors, with afferent endings distributed in the muscular layers, sense the level of gastric distension, and transfer signals to the brain to generate feelings of satiety and fullness [105]. Mucosal receptors, with afferent endings distributed in the gastric mucosal layers, are activated by movement of food particles over the receptive field in the stomach. Although there is no direct evidence, mucosal receptors are thought to be involved in the regulation of gastric motor function delaying gastric emptying [106]. This mechanism may allow large food particles to be sufficiently digested before transit to the small intestine. The subsequent accumulation of undigested food in the stomach could activate GVA tension receptors and, therefore, activation of mucosal receptors may indirectly inhibit food intake. Together, evidence indicates that both types of GVAs play distinct but complementary roles in the generation of satiety signals and control of food intake.

GVAs are highly plastic and can adapt to physiological changes to assist with the maintenance of energy homeostasis. For example, the sensitivity of GVAs to gastric distension is modulated by nutritional status in mice, with reduced mechanosensitivity observed after short term food restriction [22]. GVAs are also modulated according to circadian cues, with a nadir in mechanosensitivity during the dark phase when the majority of food is consumed [107]. In addition, the mechanosensitivity of GVAs is modulated by a wide variety of hormones, originating from within or outside the gastrointestinal tract, including the gastric hormone ghrelin [108] and adipokines, including apelin [109] and adiponectin [110]. This

allows GVAs to modulate food intake in response to local and remote hormonal information.

Maternal food intake is increased during pregnancy to support maternal metabolism and adipose deposition as well as the development of the growing fetus. In humans, energy requirements increase by 10% in late pregnancy, compared to non-pregnant women [111]. In mice, maternal food intake increases in mid-pregnancy and peaks a few days before birth with a maximum increase of ~25% compared to pre-pregnancy [6,38]. In rodents, this increase in maternal food intake is due, in part, to reduced satiety signalling in the central nervous system as a consequence of central resistance to anorectic hormones, including insulin [41], cholecystokinin (CCK) [39] and leptin [38]. It is unknown whether peripheral gastrointestinal signalling is altered during pregnancy and contributing to the increase in food intake.

Pregnancy is associated with changes in maternal hormones, which contribute to the physiological adaptations in pregnancy. The circulating levels of oestradiol, progesterone, prolactin and growth hormone are markedly elevated in pregnant women and mice [93,112-116], and these hormones are critical for pregnancy maintenance, fetal growth and/or milk production. In addition, some of these hormones are well known to regulate appetite, with oestradiol attenuating and prolactin and growth hormone augmenting food intake [72,117,118]. However, it is unknown whether these hormones can modulate GVA signalling.

Therefore, the current study aimed to determine the mechanosensitivity of mouse GVAs in early, mid- and late stage pregnancy, compared to non-pregnant age-matched mice. Further, the effect of the pregnancy associated hormones, oestradiol, progesterone, prolactin and growth hormone on GVA function was

determined in non-pregnant mice to establish the potential role for these to alter GVA signalling during pregnancy**.**

2.6. Materials and methods

2.6.1. Animals

All experimental studies were approved by the animal ethics committee of the South Australian Health and Medical Research Institute (SAHMRI) and carried out in accordance with the Australian code for the care and use of animals for scientific purposes, 8th edition 2013 and the ARRIVE guidelines [119]. C57BL/6 mice were obtained from SAHMRI Bioresources and housed at 22°C, under a 12:12 light/dark cycle, with lights on at 0700 h. Mice were provided *ad libitum* access to water and standard laboratory diet (18.6% protein, 6.2% fat, 44.2% carbohydrate; Teklad standard diet, Envigo, Cambridgeshire, United Kingdom).

2.6.2. Pregnancy experimental design

Female C57BL/6 mice (10 - 12 weeks old, 18 - 22 g) were weighed and placed into metabolic cages for a 7-day acclimatisation (Promethion Sable System, Las Vegas, USA). After this period, the female mice were pair-housed with a C57BL/6 male mouse in a home cage at 1700 h. Female mice were checked daily at 0700 h and pregnancy was confirmed by the presence of a vaginal plug (assigned as day 0.5 of pregnancy). Plugged mice were then returned to individual metabolic cages, and were randomly assigned to either early (6.5 days, $N = 10$), mid- (12.5 days, $N = 10$) or late- (17.5 days, $N = 11$) stage pregnancy end points. Control female mice ($N = 12$) were pair-housed without a male in a normal home cage and returned to metabolic cages on age-matched plugging days. At the corresponding end points, mice were anaesthetised between 0700 and 0800 h

via isoflurane inhalation (5% in oxygen) and humanely culled by decapitation. The stomach with attached vagal nerves was dissected for electrophysiology as described below.

2.6.3. Metabolic monitoring

Body weight and food intake were recorded individually in the metabolic cages, with weight was recorded each time the mouse interacted with the body mass monitor tube. The body weight on a specific day was the averaged body weight recorded on that day. Food intake was defined as the reduction in food hopper weight, measured by high precision sensors in real time, with 3 mg resolution. Meals were defined as a reduction in food hopper weight with a minimum food intake duration of 20 seconds. Meal size was defined as the reduction in food hopper weight during each meal. Meal duration was defined by the time spent interacting with the hopper during the meal [6]. Data were transformed using the Promethion data software package ExpeData version 1.9.14 (Promethion Sable System, Las Vegas, USA) using analytical macro 6 which analysed data in 12 h time periods corresponding to the light and dark periods on each day of pregnancy.

2.6.4. *In vitro* mouse gastric vagal afferent recording

The mechanosensitivity of GVAs was determined in non-pregnant mice (N = 7), and mice at early (N = 7), mid (N = 7) and late stages of pregnancy (N = 6) using the *in vitro* mouse GVA preparation, described in detail previously [103,120]; tissue was collected between 0700 and 0800 h. Briefly, the stomach was opened and placed mucosal side up in an organ bath filled with modified Krebs solution. Nifedipine (1 µM) was added to the Krebs to prevent smooth muscle contraction. The vagal nerves were placed into another chamber, filled with liquid paraffin.

The nerves were teased apart into small bundles and placed onto a platinum recording electrode for single fibre recording. Afferent impulses were amplified (DAM50, World Precision Instruments, Sarasota, FL, USA), filtered (Band-pass filter 932, CWE, Ardmore, PA, USA), and recorded.

GVA tension and mucosal receptors were identified by applying stretch stimuli and mucosal stroking to the stomach. For tension receptors, a hook with a cantilever system was attached to the stomach near the receptive field. The response of tension receptors to circular tension was determined by placing weights (0.5 - 5 g) on the cantilever system for 1 minute. For mucosal receptors, the response to mucosal stroking was determined by stroking over the receptive field with calibrated von Frey hairs (10 - 1000 mg). One to five individual GVA tension or mucosal receptors were recorded per mouse, and these responses were averaged per mouse. When there was more than one recording within a GVA type, the data were averaged to generate one set of data per mouse. Single units were discriminated by the shape, duration and amplitude of the action potentials using Spike 2 software (Cambridge Electronic Design, UK).

The effects of pregnancy-associated hormones on GVA tension receptors were determined in 8-10-week-old female C57BL/6 mice (N = 12). Mice fed *ad libitum* were humanely culled between 0700 and 0900 h via CO_2 inhalation for collection of tissue for electrophysiology. The effects of oestradiol (10, 100 and 1000 pM, Sigma, NSW, Australia), growth hormone (3, 30, 300 ng/ml; NOVNBP199601, Novus Biologicals, CO, USA), progesterone (30, 100 and 300 nM; P8737, Sigma), or prolactin (30, 100 and 300 ng/ml; SRP4688, Sigma) were assessed at circulating concentrations reported in pregnant mice [112,115,121] (N = 5/hormone). In this way, responses of GVA tension receptors to 3 g tension was determined at baseline and after hormone incubation (N = 5/hormone) with hormones at each

concentration. Oestradiol and progesterone doses, were added to the Krebs solution sequentially and superfused over the gastric tissue for 20 minutes before determining mechanosensitivity. Prolactin and growth hormone were added to a small chamber (4 x 2 cm) surrounding the tissue and incubated for 5 minutes prior to determining mechanosensitivity. The mechanosensitivity of GVAs to mechanical stimuli did not change in time control experiments, omitting the addition of hormones in superfusion or chamber application [122] .

2.6.5. Statistical analysis

Body weight and food intake parameters were consistent in all pregnant mice and were therefore combined within pregnancy stage groups. Statistical analysis was conducted using SPSS version 26 (IBM Corporation, Armonk, New York, USA). Body weight was analysed using a linear mixed model to assess the effect of pregnancy (pregnant vs non-pregnant), with day as a repeated factor and litter size included as a covariate. In preliminary analysis, litter size did not affect food intake parameters, which were therefore analysed using linear mixed models to assess effects of pregnancy (pregnant vs non-pregnant), with day as a repeated factor. Where a pregnancy * day interaction was significant, the pregnancy effect on each day was analysed by one-way ANOVA, and the effect of day in pregnant and non-pregnant groups was analysed using a linear mixed model, with day as a repeated factor. The mechanosensitivity of GVA mucosal and tension receptors was analysed using linear mixed models to assess the effect of pregnancy stage (non-, early, mid- or late- pregnancy), with mechanical stimuli intensity (von Frey hair or circular tension respectively) as a repeated factor. Where the effect of pregnancy was significant, non-pregnant and pregnancy stage groups were compared using Bonferroni pairwise comparisons. To determine if there was a

correlation between GVA sensitivity and meal size the response of GVA tension (5 g) and mucosal (200 mg) receptors, attained during the light phase, was plotted against the average meal size during the light phase and a Pearson correlation performed. The effect of each hormone on GVA tension receptors was analysed using a general linear model, with mechanical stimulus intensity as a repeated factor. When the effect of a hormone was significant, outcomes at different hormone concentrations were compared using Bonferroni pairwise comparisons. Data were expressed as mean ± SEM. $P < 0.05$ was considered significant. The raw data from this study are available from the corresponding author upon reasonable request.

2.7. Results

2.7.1 Mouse body weight increased during pregnancy

The body weight of control and pregnant mice did not differ during the acclimatisation period. Pregnant mice gained more weight than non-pregnant mice during the experiment (**Figure 2.1**) and were heavier than age-matched non-pregnant control mice from day 7 of pregnancy onwards (**Figure 2.1**). The body weight of mice at late pregnancy (day 17) was 55% higher than that of non-pregnant age-matched mice (34.7 ± 3.5 g vs 22.5 ± 1.1 g). Body weight of mice also increased with litter size. Consequently, pregnant mice with large litters (10 - 11 pups) gained more weight compared to mice with smaller litters (< 7 pups, **Supplementary Fig. 2.1**).

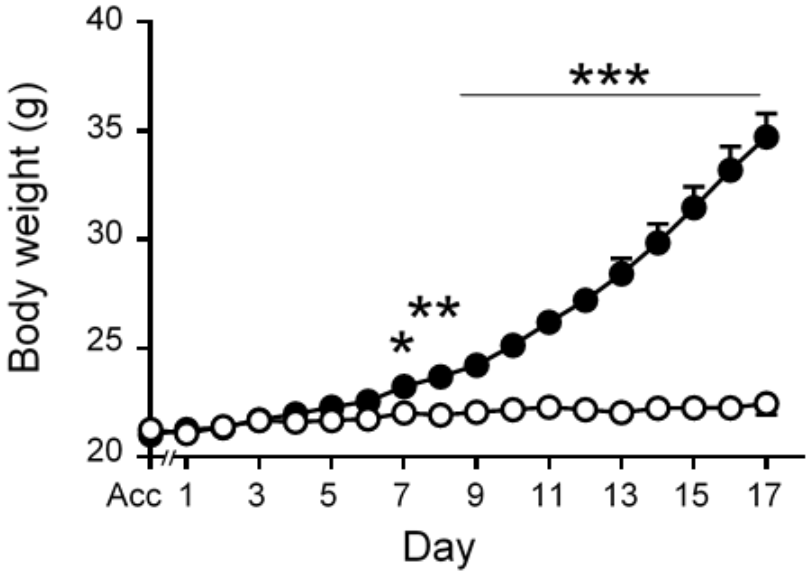


Figure 2.1. Murine body weight increased during pregnancy.

The body weights of pregnant (●, combined early-, mid- and late-pregnancy stage groups, $N \geq 11$) and non-pregnant mice (○, $N = 12$) are shown on age-matched days. Body weight shown for acclimatisation (Acc) is the average body weight in the last two days of acclimatisation. Values are mean ± SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs non-pregnant mice in specific days, one-way ANOVA.

2.7.2 Food intake and meal size were increased during pregnancy

Food intake was similar in pregnant and non-pregnant mice during the acclimatisation period. Food intake every 24-hours (**Figure 2.2 A**) increased more over time in pregnant than non-pregnant mice, increasing in pregnant, but not non-pregnant mice (**Figure 2.2**). Indeed, the 24-hour food intake was higher in pregnant mice compared to non-pregnant mice on days 8.5 - 10.5 and 14.5 - 16.5, predominantly due to an increase in light phase food intake which was higher in pregnant than non-pregnant mice on days 12.5 - 16.5. Food intake during the dark phase was not different between pregnant and non-pregnant mice. Further, food intake did not differ between litter size groups (**Supplementary Fig. 2.2**).

Meal size averaged across each 24-h period increased over time, more in pregnant than non-pregnant mice. Indeed, the average meal size (**Figure 2.2 B**) was higher in pregnant than non-pregnant mice on days 8.5 - 10.5 and 14.5 – 16.5 over 24-hours and on days 2.5 - 4.5, 10.5 – 12.5, 12.5 – 14.5 and 14.5 - 16.5 during the light phase. Meal size did not differ between litter size groups during the light phase (**Supplementary Fig. 2.2**). There was no difference in the meal size between groups during the dark phase on all days.

Pregnant mice had shorter average meal duration over a 24-hour period than non-pregnant mice which was unaffected by day (**Figure 2.2 C**). This reflected changes in dark-phase meal duration, in which pregnant mice displayed a different pattern of meal duration over time compared to non-pregnant mice. Specifically, meal duration was shorter on all days of pregnancy compared to non-pregnant mice (**Figure 2.2 C**). Conversely, meal duration during the light phase was longer in pregnant than non-pregnant mice (**Figure 2.2 C**). The total

number of meals over 24 hours was similar in pregnant and non-pregnant mice, and during both light and dark phases (**Figure 2.2 D**).

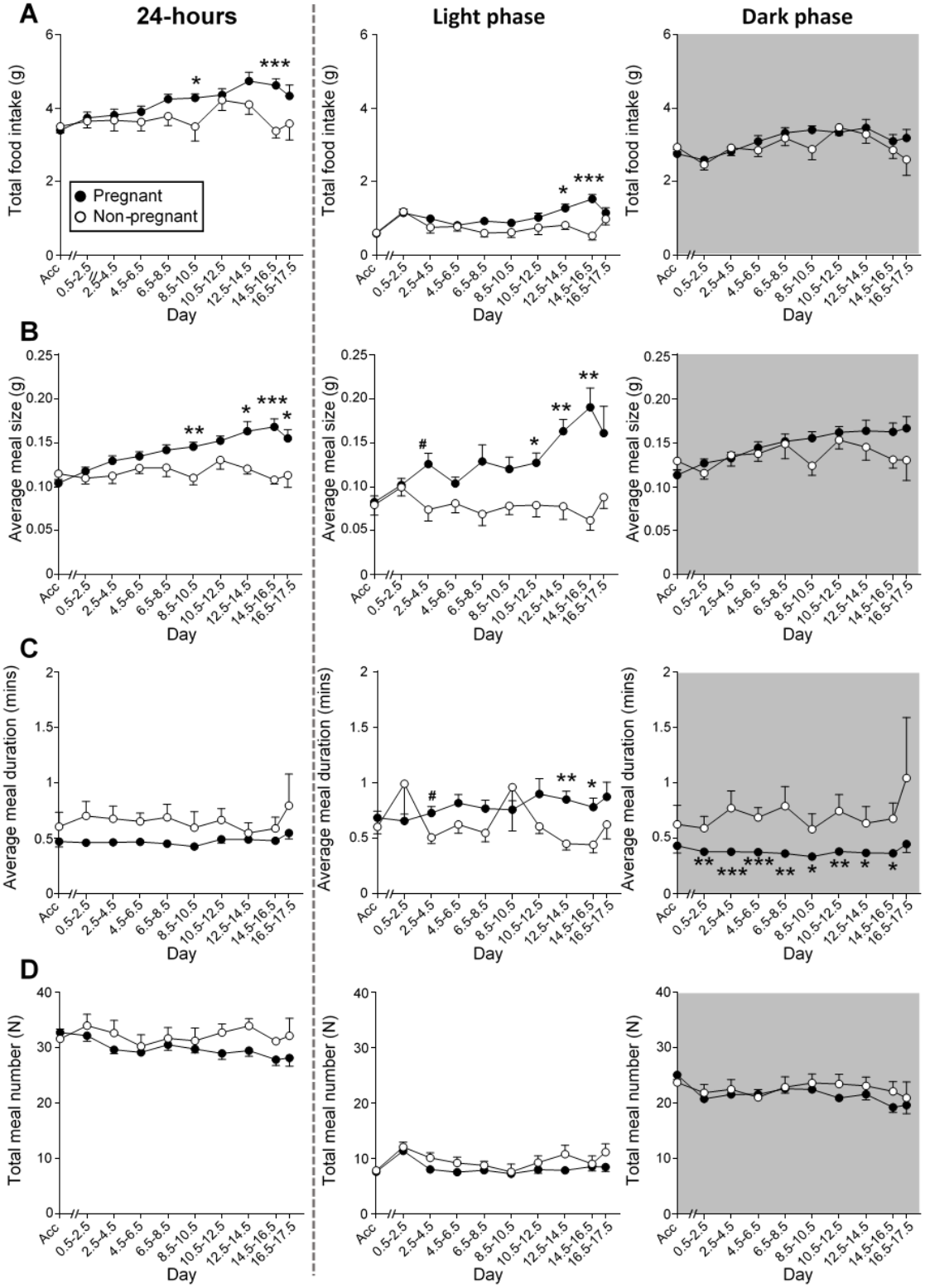


Figure 2.2. Food intake and average meal size increased in pregnant mice. Total food intake (*A*), average meal size (*B*), average meal duration (*C*) and total meal number (*D*) over 24-hours, 12-hour light phase or 12-hour dark phase (shaded graph) in pregnant mice (●, combined early-, mid- and late-pregnancy stage groups, $N \geq 11$) during pregnancy, and non-pregnant mice (○, $N = 12$) on age-matched days. All parameters shown for acclimatisation (Acc) are averaged over the last two days of acclimatisation. Each following parameter represents value averaged across two gestational or age matched days. Values represent mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs non-pregnant mice, linear mixed model and one-way ANOVA.

2.7.3. Mechanosensitivity of gastric vagal afferent tension receptors was reduced during pregnancy

The response of GVA tension receptors to circular stretch (**Figure 2.3 A, C**) increased with load and was significantly reduced during pregnancy. These responses were lower in mid- and late-pregnancy compared to non-pregnant mice. There was an inverse correlation between the response of GVA tension receptors to 5g load and the light phase meal size (**Figure 2.3 B**) The response of GVA mucosal receptors to mucosal stroking increased with load and was similar in pregnant and non-pregnant mice (**Figure 2.4 A, C**). In addition, there was no correlation between the response of GVA mucosal receptors to mucosal stroking (200 mg) and the light phase meal size (**Figure 2.4 B**).

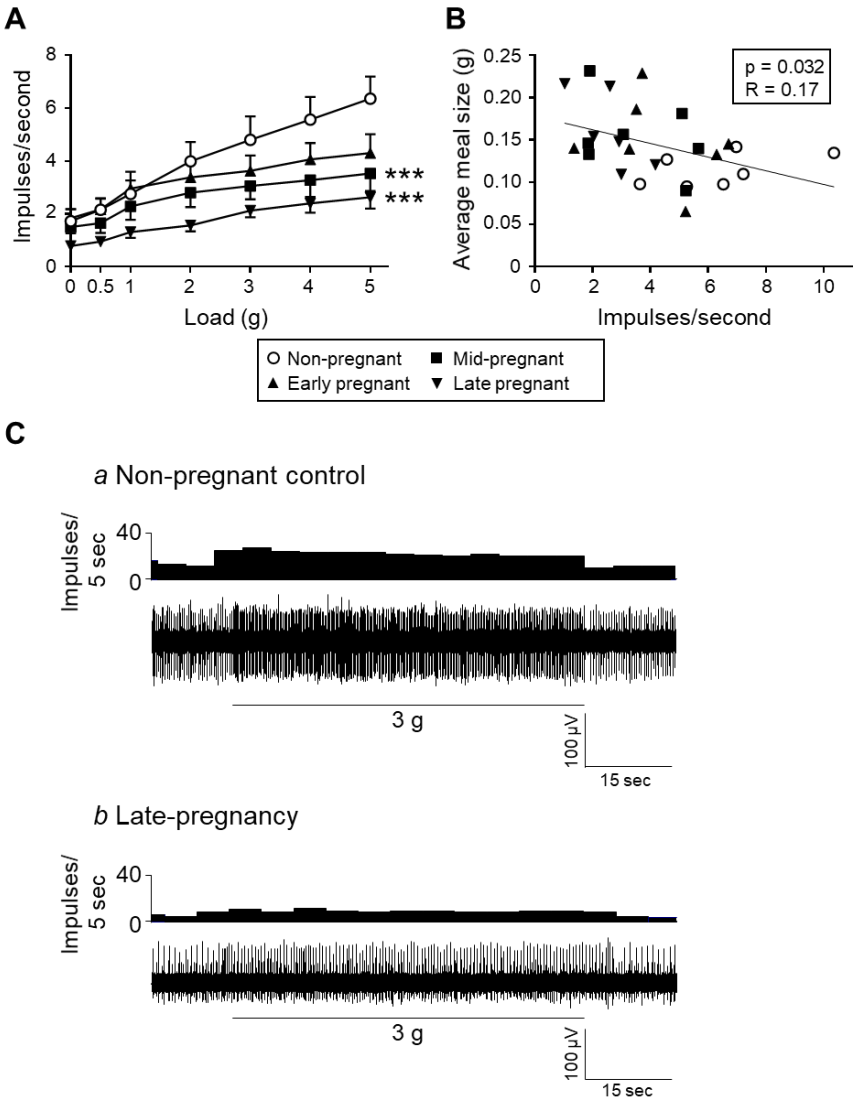


Figure 2.3. Response of gastric tension sensitive vagal afferents to distension was reduced during pregnancy.

A: The response of tension sensitive gastric vagal afferents (GVAs) to circular tension in non-pregnant mice (○), and early- (▲), mid- (■), and late-pregnant (▼) mice. Values represent mean ± SEM. *** $P < 0.001$ vs non-pregnant mice, pregnancy effect, linear mixed model. *B*: The correlation between the response of GVA tension receptors to 5 g tension and light phase average meal size. *C*: Typical recording of gastric tension sensitive vagal afferents with a circumferential stretch of 3 g in a non-pregnant mouse (*a*) and a late-pregnant mouse (*b*).

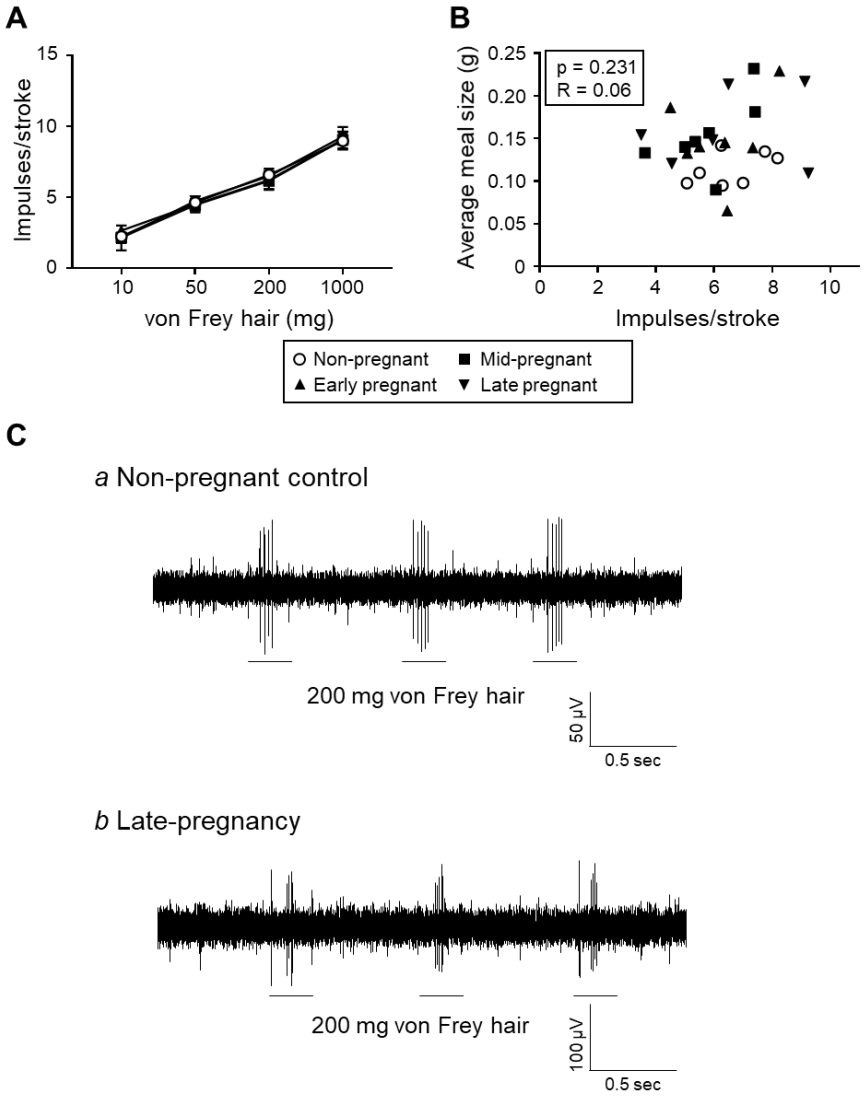


Figure 2.4. Response of gastric mucosal mechanosensitive vagal afferents to mucosal stroking was unchanged during pregnancy.

A: The response of gastric mucosal mechanosensitive vagal afferent to mucosal stroking in non-pregnant mice (○), and early- (▲), mid- (■), and late-pregnant (▼) mice. Values represent mean ± SEM. *B*: The correlation between the response of GVA mucosal receptors to stroking with a 200 mg von Frey hair and light phase average meal size. *C*: Typical recording of gastric mucosal mechanosensitive vagal afferents with a 200 mg von Frey hair in a non-pregnant mouse (a) and a late-pregnant mouse (b).

2.7.4 Mechanosensitivity of gastric vagal afferent tension receptors was increased by oestradiol and attenuated by growth hormone

The response of GVA tension receptors to circular tension (3 g) increased linearly with increasing oestradiol dose (**Figure 2.5 A**) but reduced linearly with increasing growth hormone dose (**Figure 2.5 B**). Progesterone (**Figure 2.5 C**) and prolactin (**Figure 2.5 D**) had no effect on the mechanosensitivity of GVA tension receptors.

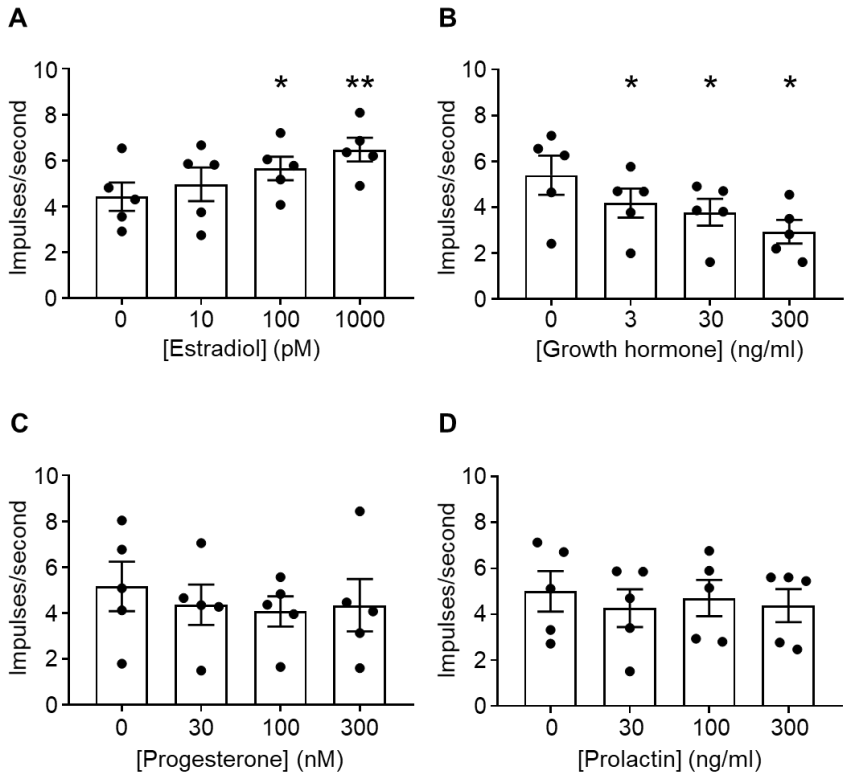


Figure 2.5. Effect of pregnancy associated hormones on the mechanosensitivity of gastric tension sensitive vagal afferents.

The effects oestradiol (*A*, *N* = 5), growth hormone (*B*, *N* = 5), progesterone (*C*, *N* = 5) and prolactin (*D*, *N* = 5) on the responses of gastric tension receptors to a circumferential stretch of 3 g. Bars represent mean ± SEM. Symbols show datum of individual animals. * $P < 0.05$, ** $P < 0.01$ vs no hormone control, general linear model and Bonferroni pairwise comparisons.

2.8. Discussion

This study has shown that the response of GVAs to distension is attenuated in the mid- and late-stages of pregnancy in mice and strongly associated with an increase in food intake, specifically meal size. Moreover, oestradiol increased, while growth hormone decreased, the response of GVAs to gastric distension, adding support that pregnancy-related hormones may drive these GVA changes.

2.8.1 Body weight, food intake and meal size increased in mice during pregnancy

Maternal body weight increases throughout pregnancy in humans and mice [6,111], facilitating a positive energy balance necessary for fetal development. This occurs through reduced energy expenditure and increased food intake [6]. In the current study, and consistent with a previous report [6], food intake increased from mid-pregnancy onwards, predominantly due to increased meal size. Following food intake, satiety signals are generated via a gut-brain axis signalling pathway. This involves the nutrient induced secretion of gastrointestinal hormones, including CCK and glucagon-like peptide-1 (GLP-1), which can act locally to activate GVA endings, which in turn signal to central satiety centers, or enter the circulation to act directly on the central regions controlling energy homeostasis [123]. Additionally, mechanical stimuli from the presence of food in the gastrointestinal tract, can activate GVAs to generate satiety signals [124]. The increased meal size in pregnant mice suggests attenuated satiety signalling along this axis; confirmed by the attenuated mechanosensitivity of GVA tension receptors in the present study, which was inversely correlated with meal size. This is likely to attenuate central satiety signalling in concert with a number of other factors, including central resistance to the satiety hormone leptin [125].

Moreover, this adaptation in satiety signalling appears to be circadian in nature as the increased food intake was observed in the light phase and not the dark phase in the current study. This is particularly relevant given that GVAs display circadian rhythmicity in response to food-related stimuli [126]. In the current study, tissue was collected between 0700 and 0800 h at a point where GVA mechanosensitivity is increasing towards a peak between 1200 and 1500 h [107]. It is possible the observed increase in food intake and meal size during the light phase in pregnancy arises due to a lower peak in GVA mechanosensitivity during this period. This would also explain why there is no effect on food intake during the dark phase nadir in GVA mechanosensitivity, when there is minimal capacity to further reduce mechanosensitivity to increase food intake. However, this is highly speculative and further investigation of circadian changes in satiety signalling pathways during pregnancy is required.

2.8.2 Responses of gastric vagal afferent tension receptors are attenuated during pregnancy

The mechanosensitivity of GVA tension receptors was reduced in mid- and late-stages of pregnancy in mice relative to non-pregnant mice. Signals generated in response to gastric distension initiate the perceptions of fullness in response to a meal and can lead to meal termination [127,128]. Accordingly, the increased food intake and meal size in pregnant mice may result from reduced GVA mechanosensitivity. Indeed, there was a significant negative correlation between the mechanosensitivity of GVA tension receptors and light phase meal size. It should be noted that the lower mechanosensitivity of GVA tension receptors coincide with the reported development of central nervous system resistance to the anorectic hormones, oxytocin [13] and leptin [129]. In contrast, the increase in

meal size was observed earlier, during early pregnancy, and possibly reflects small, cumulative changes in peripheral and central food intake pathways, during the early stages of pregnancy. This requires further investigation. Food intake remains elevated in mice during lactation [38,130], another state of high energy demand, and therefore it is possible the attenuated GVA mechanosensitivity we have reported continues postpartum, however, this requires further investigation.

2.8.3 Responses of gastric vagal afferent mucosal receptors are not changed during pregnancy

In contrast to the changes observed in GVA tension receptors, the response of GVA mucosal receptors to mechanical stimuli was not altered during pregnancy. GVA mucosal receptors are thought to be important in detecting food particle size and modulating gastric emptying [106]. The effect of pregnancy on the rate of gastric emptying is controversial, with no change in humans [48,131-133] or reduced gastric emptying reported in humans [134] and rats [135] during pregnancy. If GVA mucosal receptors have a major role in modulating the rate of gastric emptying, our findings suggest that gastric emptying may not change during pregnancy in mice. However, this requires further investigation. The mechanosensitivity of GVA mucosal receptors is known to be modulated by local or remote hormones, such as leptin [107] and apelin [109], therefore any changes in circulating hormone levels may alter the mechanosensitivity of GVA mucosal receptors. For example, circulating leptin levels increase in human [136] and murine pregnancy [40], and leptin increases the mechanosensitivity of GVA mucosal receptors in mice [137]. Therefore, interactions between GVA mucosal receptors, tissue and circulating hormones during pregnancy cannot be underestimated and require further investigation.

2.8.4 Oestradiol increases and growth hormones decreases the responses of gastric vagal afferent tension receptors

In the current study, the pregnancy-associated hormones progesterone and prolactin had no effect on the mechanosensitivity of GVA tension receptors in non-pregnant mice. Consistent with this, progesterone has been reported to not change food intake in ovariectomised rats, unless exposed at non-physiologically high concentrations [72]. However, progesterone may combine with other hormones to exert differential effects. For example, oestradiol decreases food intake in ovariectomised rats [88,138], however, this anorectic effect was attenuated in rats treated with oestradiol and progesterone compared to oestradiol alone [139]. Oestradiol levels gradually increase throughout pregnancy, and peak immediately prior to birth in mice, whereas progesterone levels initially increase more rapidly and remain elevated throughout pregnancy, falling prior to the pre-delivery peak in oestradiol levels [140]. It is therefore possible that the excitatory effects of oestradiol on GVAs may be attenuated during pregnancy due to the presence of high levels of progesterone. This is speculation, however, and future studies are required to examine the effects of these hormones in combination. In contrast to progesterone, prolactin increases food intake when administered systemically to mature female rats [117,141,142], presumably through a central mechanism of action with reports suggesting prolactin plays a role in the central leptin resistance observed during pregnancy in the rat [143-146]. However, there are suggestions that prolactin acts both peripherally and centrally in the rat [74]. For example, the prolactin receptor is highly expressed in the glandular region of the stomach and the epithelial cells of the villi in the small intestine [76]. Therefore, prolactin may act indirectly on GVAs during pregnancy through the stimulation of gut hormone release, however, this requires further investigation.

Oestradiol significantly increased the response of GVA tension receptors to gastric distension, consistent with its reported anorectic role [88]. The case for a direct effect of oestradiol on GVAs is supported by the expression of oestradiol receptors in vagal afferent cell bodies [83] and evidence that oestradiol restored loss of neuron excitability in female ovariectomised rats in a subpopulation of vagal afferents [84]. Oestradiol is produced by the placenta during pregnancy, leading to a rise in circulating levels from mid-pregnancy onwards [112], and is essential for the maintenance of pregnancy. Since oestradiol increased activity of GVA tension receptors, it is unlikely that oestradiol is driving the decrease in GVA tension receptor mechanosensitivity and the increase in food intake during pregnancy. However, as mentioned earlier, attenuation of oestradiol effects on GVAs, by other pregnancy hormones such as progesterone [139], may lead to an overall reduction in GVA mechanosensitivity. This requires further investigation.

In the current study, growth hormone attenuated the response of GVA tension receptors to circular tension. Treatment with growth hormone increases food intake in normal [117] and hypophysectomised rats [147], as well as growth hormone deficient children [148]. It is possible, considering the effects of growth hormone on GVA tension receptor mechanosensitivity, that at least part of growth hormone's effects on food intake is mediated through modulation of GVA signalling. The human placenta expresses a variant growth hormone gene, resulting in suppression of pulsatile pituitary secretion and continuously elevated placental growth hormone in maternal circulation by mid-pregnancy [93]. Basal secretion of growth hormone from the pituitary increases markedly in mice from early to mid-pregnancy, with circulating levels elevated for the remainder of pregnancy [115]. These elevated growth hormone levels during pregnancy have the potential to

attenuate mechanosensitivity of GVA tension receptors to increase food intake. However, effects of growth hormone on appetite and mechanosensitivity of GVA tension receptors require confirmation in pregnancy.

Circulating levels of all the pregnancy hormones tested are significantly elevated during pregnancy in mice [140], conceivably masking any circadian fluctuations in GVA sensitivity and subsequent food intake patterns. However, in the current study we noted an increase in food intake specifically during the light phase. As stated earlier this is possibly related to the capacity for GVA signals to be attenuated. This capacity is high during the light phase when GVA mechanosensitivity is at a peak but low during the dark phase when GVA mechanosensitivity is at nadir.

2.9. Conclusion

In conclusion, this study has established that the mechanosensitivity of GVA tension receptors to gastric distension is significantly reduced during mid- and late-pregnancy. Considering the strong negative correlation, this reduction in peripheral satiety signalling is likely to contribute to the increased food intake, particularly meal size, during pregnancy. In addition, growth hormone reduced the mechanosensitivity of GVA tension receptors and, therefore, the increased circulating levels of growth hormone during pregnancy may contribute to the reduced mechanosensitivity of GVAs during pregnancy.

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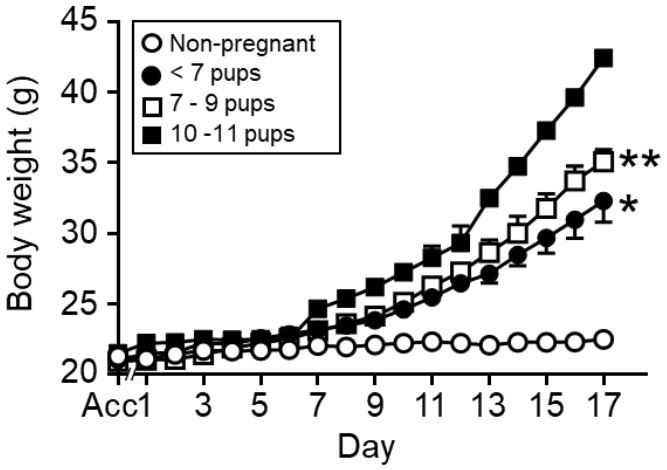
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

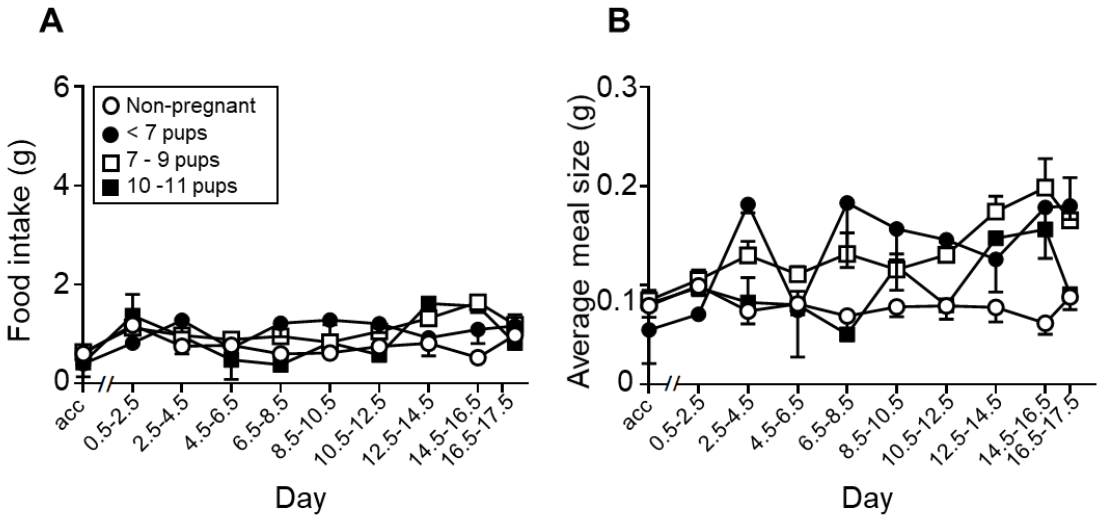
AUTHOR CONTRIBUTIONS

H.L. and G.C. wrote the manuscript. A.P., K.G. and G.C. designed and performed the pregnancy studies. H.L., S.C. and S.K. performed electrophysiology. G.C. analysed the metabolic data. K.G. and G.C. performed the statistical analysis. R.Y. and S.L. contributed to experiment design. All authors contributed to interpretation of data and editing of the manuscript.



Supplementary Fig. 2.1: Maternal body weight increased more in dams with larger litters.

Body weight of pregnant mice with small (< 7 pups, ●, *N* = 6), normal (7 - 9 pups, □, *N* = 20), or large (10 - 11 pups, ■, *N* = 5) litter sizes are shown during pregnancy, with data for non-pregnant mice (○, *N* = 12) on age-matched days. Body weight during acclimatisation (Acc) represents the average body weight in the last two days of acclimatisation. Values are mean ± SEM. * $P < 0.05$ and ** $P < 0.01$ compared to maternal body weight with a large litter size of 10 -11 pups, litter size effect, linear mixed model and Bonferroni pairwise comparisons.



Supplementary Fig. 2.2: Maternal food intake and average meal size during the light phase did not differ with litter size.

Food intake (*A*) and average meal size (*B*) during the light phase of pregnant mice with small (< 7 pups, ●, *N* = 6), normal (7 - 9 pups, □, *N* = 20), or large (10 - 11 pups, ■, *N* = 5) litter size are shown during pregnancy, with data for non-pregnant mice (○, n = 12) on age-matched days. Food intake and meal size during acclimatisation (Acc) represents the average body weight in the last two days of acclimatisation. Values are mean ± SEM.

CHAPTER 3: Effect of pregnancy on the expression of nutrient-sensors and satiety hormones in mice

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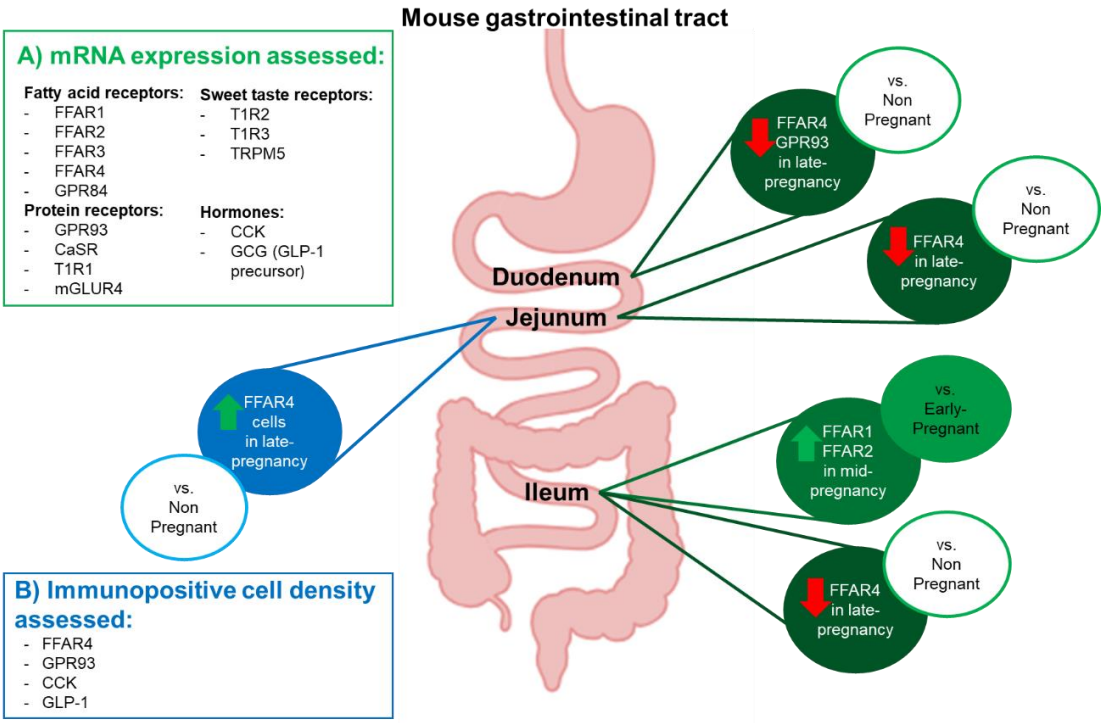
3.1. Graphical Abstract



3.2. Overview:

The pregnant mice utilised in this chapter are the same as reported in **Chapter 2**. This chapter reports intestinal nutrient-sensor and gut hormone expression during pregnancy. This work has been accepted for publication in the journal *Peptides* and has therefore been reproduced exactly as submitted with the exception of formatting.

3.3 Authorship Document

Statement of Authorship

Title of Paper	Effect of pregnancy on the expression of nutrient-sensors and satiety hormones in mice
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript DOI: 10.1016/j.peptides.2023.171114 Clarke GS, Li H, Ladyman SR, Young RL, Gatford KL, Page AJ. Effect of pregnancy on the expression of nutrient-sensors and satiety hormones in mice. Peptides. 2023; Online ahead of pre-print.

Principal Author

Name of Principal Author (Candidate)	Miss Georgia Clarke		
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Prepared figures. Edited and revised manuscript. Accepted final version of manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/08/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Hui Li		
Contribution to the Paper	Conception and design of research. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript		
Signature		Date	9/08/23

Name of Co-Author	Dr Sharon Ladyman		
Contribution to the Paper	Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	8/8/23

Name of Co-Author	A/Prof Richard Young		
Contribution to the Paper	Conception and design of research. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript		
Signature		Date	8/8/23

Name of Co-Author	A/Prof Kathryn Gatford		
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript		
Signature		Date	8/8/23

Name of Co-Author	Prof Amanda Page		
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	8/8/23

3.4. Highlights

- Adaptations in intestinal chemoreceptors and hormones during pregnancy is unknown.
- Duodenal *GPR93* and intestinal *FFAR4* expression was lower in late-pregnant mice.
- FFAR4 positive cells were greater in the jejunum of late- vs. non-pregnant mice.
- Gut hormone (CCK & GLP-1) expression and cell density was unchanged in pregnancy.

3.5. Abstract

Small intestinal satiation pathways involve nutrient-induced stimulation of chemoreceptors leading to release of satiety hormones from intestinal enteroendocrine cells (ECCs). Whether adaptations in these pathways contribute to increased maternal food intake during pregnancy is unknown. To determine the expression of intestinal nutrient-sensors and satiety hormone transcripts and proteins across pregnancy in mice. Female C57BL/6J mice (10-12 weeks old) were randomized to mating and then tissue collection at early- (6.5 d), mid- (12.5 d) or late-pregnancy (17.5 d), or to an unmated age matched control group. Relative transcript expression of intestinal fatty acid, peptide and amino acid and carbohydrate chemoreceptors, as well as gut hormones was determined across pregnancy. The density of G-protein coupled receptor 93 (GPR93), free fatty acid receptor (FFAR) 4, cholecystokinin (CCK) and glucagon-like peptide1 (GLP-1) immunopositive cells was then compared between non-pregnant and late-pregnant mice. Duodenal *GPR93* expression was lower in late pregnant than non-pregnant mice ($P < 0.05$). Ileal *FFAR1* expression was higher at mid- than at early- or late-pregnancy. Ileal *FFAR2* expression was higher at mid-pregnancy than in early pregnancy. Although *FFAR4* expression was consistently lower in late-pregnant than non-pregnant mice ($P < 0.001$), the density of FFAR4 immunopositive cells was higher in the jejunum of late-pregnant than non-pregnant mice. A subset of protein and fatty acid chemoreceptor transcripts undergo region-specific change during murine pregnancy, which could augment hormone release and contribute to increased food intake. Further investigations are needed to determine the functional relevance of these changes.

3.6. Introduction

The fetus depends on macro- and micronutrients transported from the maternal circulation and across the placenta to support development [149]. As pregnancy advances, fetal nutrient demand increases, requiring additional maternal energy intake [149]. In humans, energy intake increases by 340-540 kcal/day in the third trimester and in rodents by ~20-30% just prior to birth [150]. These increases in food intake during pregnancy are supported by changes to regulation of central (brain) pathways mediating appetite and satiety [140]. In addition, we have shown that vagal afferent responses to stretch in the stomach are attenuated during pregnancy, likely also contributing to the observed increase in meal size [42].

Like the stomach, mechanosensitive vagal afferents are also located in the duodenum [24], however, satiety pathways driven via chemosensitive receptors have also been characterized in the small intestine (SI). These chemoreceptors are present on specialized enteroendocrine cells (EECs) that face the SI and are activated by ingested nutrients [151-153]. Activation of nutrient chemoreceptors induces satiety through release of gut hormones, including glucagon-like peptide1 (GLP-1) and cholecystokinin (CCK), from EECs into the blood to act on the brain [154,155]. These hormones also act locally on vagal afferents via receptors expressed on endings adjacent to EECs in the lamina propria [24,152]. Chemoreceptors expressed by EECs include receptors for breakdown products of carbohydrates (e.g., the heterodimeric G protein-coupled sweet taste receptor T1R2-T1R3, taste-specific transient receptor potential cation channel subfamily melastatin member 5 (TRPM5)), fats (e.g., the free fatty acid receptor (FFAR) 1, 2, 3 & 4 and G-protein-coupled receptor 84 (GPR84)) and proteins (e.g., the G protein-coupled receptor 93 (GPR93), aromatic amino acid sensor (CaSR), heterodimeric G protein-coupled umami taste receptor T1R1-T1R3, and the

metabotropic glutamate receptor type 4 (mGLUR4))[151]. Chemoreceptor-expressing EECs also co-express gut hormones, for example, FFAR1 co-localizes with CCK and GLP-1 in the duodenum of mice [156] and FFAR4 co-localizes with GLP-1 in human duodenal EEC cells [157].

Knowledge of the distribution of nutrient receptors and gut hormones across species has expanded rapidly within the last decade, increasing understanding of nutrient-sensing in the context of food intake and glycaemia [153,158]. Expression of *T1R1* in tongue [54] was higher, *T1R3* in the tongue was lower [54] and *FFAR2* expression in pancreatic islet cells [53] was higher by mid-late pregnant mice compared to non-pregnant mice. However, whether intestinal nutrient-sensing changes during pregnancy is unknown. Therefore, the aim of this study was to characterise the regional expression of nutrient chemoreceptors and gut hormones in the SI across pregnancy in mice.

3.7. Methods

3.7.1. Animals and experimental design.

All animal procedures were approved by the South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee (SAM395.19) and conducted in compliance with the Australian code for the care and use of animals for scientific purposes and ARRIVE guidelines [159]. Adult female C57BL/6J mice (10–12 weeks old, 18-22 g) were obtained from SAHMRI Bioresources. Mice were housed with ad libitum access to water and food (Teklad standard diet code: 2018, Envigo, Cambridge, UK; 18.6% protein, 6.2% fat, 44.2% carbohydrate), in a 12:12 light/dark cycle (lights on at 0700 h) at 22 °C as previously described [42].

The experimental design, including mating, housing (Promethion Metabolic cages for measurement of food intake and body weight) and humane killing, has been reported previously [42]. Briefly, female mice were mated for pregnancy or unmated as non-pregnant controls. Mated mice were randomized using a simple table method following detection of a vaginal plug to early- (6.5 days, n = 10), mid- (12.5 days, n = 10) and late-pregnancy (17.5 days, n = 11) time-points. At pregnancy time-points, mice were anaesthetized by isoflurane inhalation (5% in oxygen) then humanely killed by decapitation. Humane killing of control mice was age-matched to plugging days (n = 12). Mice that were mated and plugged but did not become pregnant (n = 6) were excluded from the study. These mice were not added to the non-pregnant group, to avoid potential impacts of elevated prolactin during pseudo-pregnancy [116]. One non-pregnant mouse was excluded due to an infection at the time of tissue collection. The sample size was based off a *priori* calculation and a similar study design previously published [153]. Samples from pregnant dams had a litter size range of 4 to 11.

3.7.2. Tissue preparation.

Whole intestinal wet weight was first measured. The duodenum, jejunum and ileum were rapidly removed into cold 0.1M phosphate buffer. Intestinal segments were defined as duodenum (pylorus to the ligament of Treitz), jejunum (ligament of Treitz to the middle of SI mesenteric fan) and ileum (aboral end of the mesenteric fan to the caecum). Mucosal scrapings were collected from a ~1 cm length in the middle of each segment, frozen in liquid nitrogen and stored at -80°C for RNA analysis. Intact tissue (~1 cm) collected from the middle of each segment was immersion fixed in 4% paraformaldehyde dissolved in phosphate buffer for 2 h, including application of fixative into the lumen of the tissue,

cryoprotected in a 30% sucrose-phosphate buffer solution overnight, then embedded in optimal cutting temperature medium (Tissue-Tek, ProSciTech, QLD, Australia) and stored at -80 °C until sectioned for immunohistochemistry.

3.7.3. Quantitative RT-qPCR.

Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies, Adelaide, SA, Australia) according to manufacturer's instructions. RNA quality was assessed using the NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Adelaide, SA, Australia), estimated by the $A_{260/280}$ ratio. Qualitative real-time PCR (qRT-PCR) was conducted using TaqMan™ or SYBR® green methods depending on primer design.

For TaqMan™-based qRT-PCR, EXPRESS One-Step Superscript RT-PCR kits (Invitrogen, California, USA) and thermocycler (Applied Biosystems® 7500 Real-Time PCR System, Thermo Fisher Scientific) were used. All primers used were predesigned TaqMan™ primer assays (**Table 3.1**). β2 microglobulin (*B2M*), hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) and peptidyl-prolyl isomerase A (*PPIA*) were used as reference genes, chosen based on expression stability across the SI (0.001 duodenum/ileum, 0.002 jejunum) and during pregnancy using NormFinder software (Department of Molecular Medicine, Aarhus University Hospital, Denmark). A DNase digestion step was implemented with an ezDNase kit (Invitrogen, SA, Australia) to eliminate genomic DNA from total RNA samples.

SYBR® green qRT-PCR was performed using the QuantiTect SYBR® Green RT-PCR Kits (Qiagen, Hilden, Germany) and QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific). Predesigned SYBR® green primers (**Table 3.1**) were purchased from Qiagen, with the exception of T1R3 which was

purchased from Sigma-Aldrich. *B2M* and *HPRT* were used as reference genes. Negative controls for all genes were performed by substituting RNA template and reverse transcriptase for RNase-free water in both methods. No reverse transcriptase controls were included for *GPR84, FFAR1, FFAR2* and *PPIA* and all SYBR primers. Each assay was run in triplicate and transcript levels were calculated relative to the averaged cycle threshold (Ct) value of reference genes using the $2^{-\Delta CT}$ method [160].

Table 3.1: Primers used for qRT-PCR

GENE TARGET	PCR METHOD	NCBI ACCESSION NO.	SOURCE
		Fatty acids chemoreceptors	
FFAR1 (GPR40)	**TaqMan™**	Mm00809442_s1	Thermo Fisher Scientific
FFAR2 (GPR43)	**TaqMan™**	Mm01176527_m1	Thermo Fisher Scientific
FFAR3 (GPR41)	**SYBR® Green**	Mm_Ffar3_1_SG	Qiagen
FFAR4 (GPR120)	**TaqMan™**	Mm00725193_m1	Thermo Fisher Scientific
GPR84	**TaqMan™**	Mm00518921_m1	Thermo Fisher Scientific
		Protein and amino acid chemoreceptors	
CaSR	**TaqMan™**	Mm00443375_m1	Thermo Fisher Scientific
GPR93 (LPAR5)	**TaqMan™**	Mm01190818_m1	Thermo Fisher Scientific
mGLUR4	**TaqMan™**	Mm01306128_m1	Thermo Fisher Scientific
T1R1 (Tas1r1)	**SYBR® Green**	Mm_Tas1r1_1_SG	Qiagen
		Sweet taste chemoreceptors	
TRPM5	**SYBR® Green**	Mm_Trpm5_1_SG	Qiagen
T1R2 (Tas1r2)	**SYBR® Green**	Mm_Tas1r2_1_SG	Qiagen`
T1R3 (Tas1r3)	**SYBR® Green**	M_Tas1r3_2	Sigma-Aldrich
		Satiety hormones	
GCG	**SYBR® Green**	Mm_Gcg_1_SG	Qiagen
CCK	**TaqMan™**	Mm00446170_m1	Thermo Fisher Scientific
		Reference genes	
B2M	**TaqMan™** **SYBR® Green**	Mm00437762_m1 Mm_B2m_2_SG	Thermo Fisher Scientific Qiagen
HPRT	**TaqMan™** **SYBR® Green**	Mm01545399_m1 Mm_Hprt_1_SG	Thermo Fisher Scientific Qiagen
PPIA	**TaqMan™**	Mm02342429_g1	Thermo Fisher Scientific

3.7.4. Immunohistochemistry

Immunohistochemistry was used to compare the densities of duodenal and jejunal cells expressing differentially expressed nutrient receptors (FFAR4, GPR93) or gut hormones (CCK, GLP-1) between late-pregnant and non-pregnant mice. Experimental conditions for immunohistochemistry were adapted from previous reports [161,162]. Briefly, transverse 10 µM cross-sections of duodenum and jejunum were air dried for 1 hour and rinsed three times in phosphate buffered saline containing 0.2% Triton X-100 (PBS-TX; Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 minutes. The tissue was then blocked for 60 minutes at room temperature in 10% normal donkey serum (D9663, Sigma-Aldrich). Sections were then washed three times for 2 minutes with PBS-TX and incubated with either rabbit anti-GPR120 (FFAR4, 1:100, SAB4501490, Sigma-Aldrich), rabbit anti-LAP receptor 5 (GPR93, 1:800, ABT114, Sigma-Aldrich) or rabbit anti-human CCK (1:400, LS-C199788, LifeSpan Biosciences, Seattle, Washington, America) diluted in PBS-TX for 18 hours at 4 °C. Unbound antibody was then washed off with PBS-Tx (three times, 5 minutes) and species-specific secondary antibodies conjugated to Alexa Fluor® 488 or 568 (1:200, Invitrogen) added to the slides for 60 minutes at RT. Subsequently, cryosections were rinsed with PBS-TX (three times, 5 minutes), mounted with ProLong® Diamond Antifade reagent with DAPI (Invitrogen) and coverslipped. Detection of GLP-1 required an antigen retrieval step. Slides were rinsed three times in phosphate buffered saline (1X concentrate, pH 7.4, gibco by Life technologies) for 5 minutes, then heated to 95 °C in antigen retrieval solution for 20 minutes and transferred to room temperature antigen retrieval solution for another 20 minutes (1X concentrate, DAKO, Agilent). The sections were then rinsed and blocked as above and incubated with mouse anti-GLP-1 (1:100, NBP2-23558; Novus Biologicals). No

immunofluorescence was detected on slides where the primary antibody was omitted. Detection of antigens using these protocols was confirmed in positive control tissues of mouse pancreas (GLP-1, [163]), proximal colon (CCK, **Supplementary Figure 3.1**) and stomach (GPR93 [161], FFAR4 [164]).

3.7.5. Microscopy and cell counts

Immunofluorescence was visualised using a BX51 epifluorescence microscope (Olympus, Parkside, SA, Australia) and counts conducted as previously reported [165]. Briefly, epithelial cells positive for primary antibodies with DAPI-stained nuclei were manually counted over 9 transverse sections (non-consecutive, minimum every 10 sections cut) per region and mouse. The area of the transverse cross-section was determined using a freehand polygon tracing tool on images taken at x2 magnification with the bright field filter. The number of cells per area in each region was averaged for each mouse, for 6 mice per group and intestinal region. Images for publication were captured using an XM10 monochrome camera (Olympus) and brightness adjusted with the CellSens Dimensions Imaging Software (Olympus). GSC was blinded to groups during cell counts.

3.7.6. Statistical analysis

Statistical analyses were performed using SPSS v. 28 (IBM Cooperation, Armonk, NY). Mouse body weight, litter size and SI weight were assessed by a one-way ANOVA and Bonferroni comparison used to compare specific pregnancy stages. Effects of pregnancy stage (non-, early-, mid-, late-pregnancy), SI region (duodenum, jejunum, ileum) and interactions on nutrient receptor and hormone transcript expression were analysed using mixed models. Multiple SI regions from individual mice were treated as repeated measures. Where interactions were present, effects of pregnancy stage were assessed

separately within each region by one-way ANOVA. Bonferroni post-hoc tests were used to compare SI regions and pregnancy stages where main effects were significant. Transcript expression data was tested to ensure it met ANOVA assumptions, including equal variance and normal distribution. Detailed food intake behaviour has been reported in these mice, including light-phase meal size [42]. As SI tissue was collected at the start of the light-phase, we tested the association between light-phase meal size with changes in intestinal nutrient receptor expression during pregnancy, using a Pearsons correlation. We focussed on transcript differences noted in pregnancy for duodenal *GPR93*, intestinal (combined duodenum, jejunum and ileum as there was no SI regional effect) *FFAR4* and ileal *FFAR1* and *FFAR2*. The density of immunopositive cells in non- and late-pregnant mice in each region was compared using a one-way ANOVA. Results are expressed as mean ± standard deviation.

3.8. Results

3.8.1. Mouse phenotype and SI weight

The body weight of all mice was similar during the acclimatisation period and between early-pregnant compared to non-pregnant mice (both $P > 0.1$, **Table 3.2**). By mid-pregnancy, body weight was 1.2-fold that of early- and non-pregnant mice (both $P < 0.001$). At late-pregnancy, body weight was 1.3-fold that of mid-pregnant mice ($P < 0.001$) and 1.6-fold that of early- and non-pregnant mice (both $P < 0.001$). Litter size was similar between pregnancy stages ($P > 0.05$). SI wet weight was similar in non-pregnant and early-pregnant mice, but in mid- and late-pregnant mice was 1.1- and 1.2-fold of that in early- and non-pregnant mice respectively (all $P < 0.01$, **Table 3.2**). There was no difference in the wet weight between mid- and late-pregnant mice ($P > 0.1$).

Table 3.2: Mouse phenotype and SI weight

	Non-pregnant	Early-pregnant	Mid-pregnant	Late-pregnant	Significance
No. of mice	11	9	10	11	
Gestational age	N/A	d 6.5	d 12.5	d 17.5	
Litter size	0	9.22 ± 0.67	8.6 ± 1.17	7.36 ± 2.73	$P > 0.05$
Initial weight[1] (g)	21.1 ± 0.74	20.5 ± 0.62	20.9 ± 1.0	20.9 ± 1.22	$P > 0.1$
Final weight[2] (g)	22.1 ± 1.07[a]	22.4 ± 1.15[a]	27.6 ± 1.54[b]	34.8 ± 3.49[c]	$P < 0.001$
SI weight (g)	1.29 ± 0.15[a]	1.38 ± 0.08[a]	1.55 ± 0.08[b]	1.55 ± 0.10[b]	$P < 0.001$

[1]Initial body weight was the average of body weight measures in metabolic cages during the last 48 h of acclimatisation. [2]Final body weight was the average of body weight measures in metabolic cages during the 24 h before humane killing. Maternal body weight, litter size and small intestinal (SI) weight were analysed by one-way ANOVA, and Bonferroni comparison used to compare specific pregnancy stages. Data are presented as mean ± SD. Data that does not share a common superscript are different ([a, b, c] $P < 0.05$). d, gestational age.

3.8.2. Fatty acid chemoreceptor

Relative expression of fatty acid receptor *GPR84* and *FFAR3* differed between SI regions (each $P < 0.001$) but not between pregnancy stages (each $P > 0.1$). Expression of *GPR84* (**Figure 3.1 A**) in the jejunum was 1.4-fold and 1.5-fold of that in the duodenum ($P < 0.01$) and ileum ($P < 0.001$) respectively, and did not differ between the duodenum and ileum ($P > 0.1$). Relative *FFAR3* expression (**Figure 3.1 D**) in the ileum was 0.6-fold that of the duodenum and jejunum (each $P < 0.001$), and did not differ between duodenum and jejunum ($P > 0.1$).

Effects of pregnancy on transcript expression of *FFAR1* and *FFAR2* differed between SI regions (interaction $P < 0.05$). *FFAR1* expression (**Figure 3.1 B**) differed by pregnancy stage in the ileum ($P < 0.05$) but not duodenum or jejunum (both $P > 0.1$). In the ileum, *FFAR1* expression at mid-pregnancy was 1.8- and 1.9-fold that observed in early- and late-pregnancy (*each* $P < 0.01$) respectively. However, there was no difference in ileal *FFAR1* expression between the non-pregnant group and mice at any pregnancy stage (each $P > 0.1$). *FFAR2* expression also differed by pregnancy in the duodenum, jejunum and ileum (**Figure 3.1.C**, $P < 0.05$). Ileal expression was 1.4-fold of that in mid- compared to early-pregnancy ($P < 0.05$) but did not differ between other stages (each $P > 0.1$). Ileal *FFAR1* and *FFAR2* transcript levels were unrelated to meal size in the light phase ($P > 0.1$). There were no differences between pregnancy stages in the duodenum or jejunum (each $P > 0.1$).

Relative *FFAR4* expression (**Figure 3.1 E**) differed by pregnancy stage ($P < 0.001$) but not SI region ($P > 0.1$). Overall, *FFAR4* expression was 0.6- and 0.5-fold of that in late-pregnant mice compared to early-pregnant and non-pregnant mice respectively (both $P < 0.01$). *FFAR4* expression was also 0.7-fold of that in

mid- than early-pregnant mice ($P < 0.05$), and its expression did not differ between early- and non-pregnant mice ($P > 0.1$). This reduction in intestinal *FFAR4* transcript level was negatively correlated to meal size in the light phase ($P < 0.05$). The density of FFAR4 immunopositive cells in the jejunum was 2.2-fold of that in late-pregnant compared to non-pregnant mice (**Figure 3.1 F and 3.1 G**, $P < 0.05$). FFAR4 immunopositive cell density in the duodenum did not differ between these late- and non-pregnant mice ($P > 0.1$).

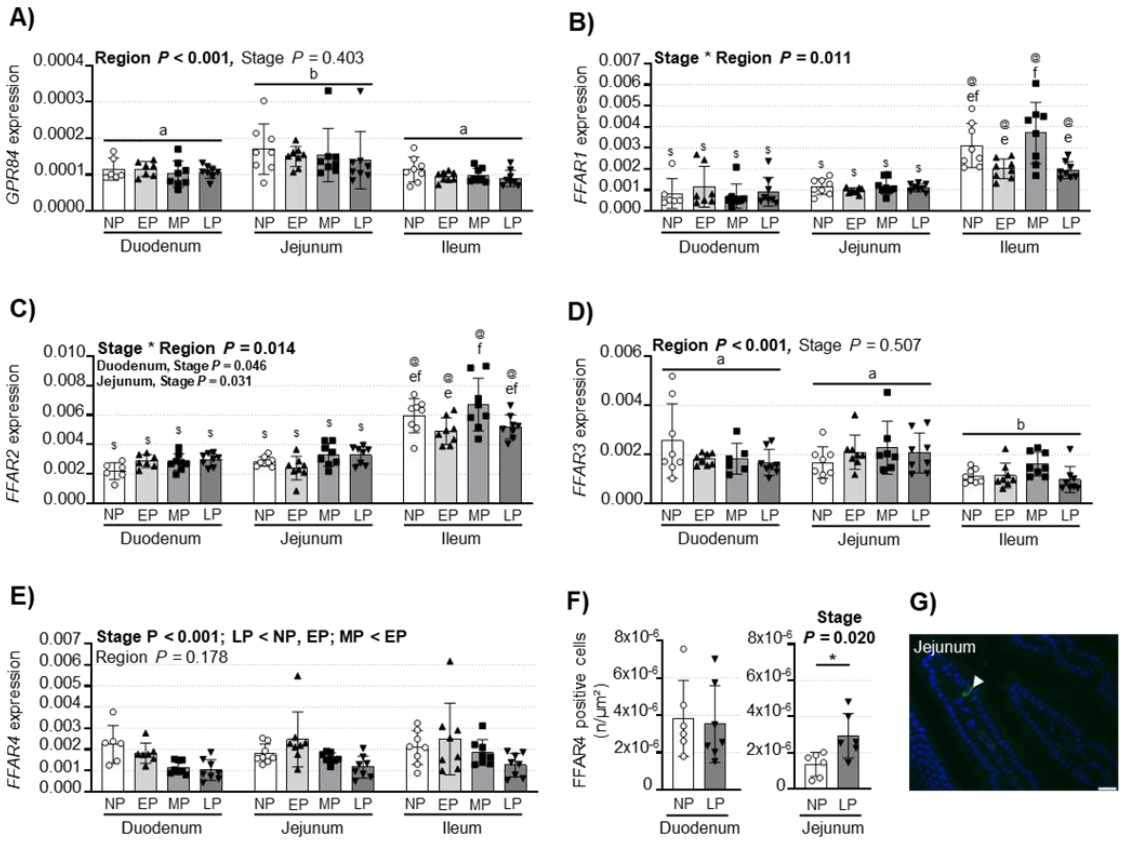


Figure 3.1. SI regional- and pregnancy-specific expression of fatty acid chemoreceptors.

Relative mRNA expression of G-protein-coupled receptor 84 (A, *GPR84*), free fatty acid receptor 1 (B, *FFAR1*), free fatty acid receptor 2 (C, *FFAR2*), free fatty acid receptor 3 (D, *FFAR3*) and free fatty acid receptor 4 (E, *FFAR4*) in early- (EP; n = 6-8, ▼), mid- (MP; n = 6-8, ■) and late-pregnant (LP; n = 6-8, ▲) mice compared to non-pregnant mice (NP; n = 6-8, ○). *GPR84*, *FFAR1*, *FFAR2* and *FFAR4* expression is relative to the average Ct of the reference genes *B2M*, *HPRT* and *PPIA*. *FFAR3* expression is relative to the average Ct of *B2M* and *HPRT*. The number of FFAR4 immunopositive cells (F) in the duodenum and jejunum was assessed in late and non-pregnant mice (n = 6-7 per region and stage). Representative immunohistochemistry images (G) are shown, with white arrows indicating positive cells for FFAR4 and the blue staining of nuclei. Scale bar = 20 µm, 40x magnification. Bars show mean ± standard deviation, symbols show data for individual mice. Different letters indicate differences between intestinal regions, different symbols indicate difference between pregnancy stages.

3.8.3. Protein chemoreceptors

The relative expression of *CaSR* (**Figure 3.2 A**) and *mGLUR4* (**Figure 3.2 B**) transcripts differed by SI region (each $P < 0.001$) with no effect of pregnancy stage (each $P > 0.05$). Duodenal *CaSR* expression was 2.2-fold and jejunal 1.9-fold that of the ileum (each $P < 0.001$), and expression did not differ between duodenum and jejunum ($P > 0.1$). Duodenal *mGLUR4* expression was 0.7-fold ($P < 0.01$) and ileal expression 0.5-fold ($P < 0.05$) that of the jejunum, and ileal expression was 0.8-fold of that in the duodenum ($P < 0.001$). Expression of *T1R1* was unaffected by SI region or pregnancy stage (**Figure 3.2 C**, each $P > 0.1$). In contrast, effects of pregnancy on expression of *GPR93* differed between SI regions (Figures 3.2 D, interaction $P < 0.01$). Relative *GPR93* expression differed by pregnancy stage in the duodenum ($P < 0.01$) but not in jejunum or ileum (each $P > 0.05$). Duodenal *GPR93* expression in late-pregnant mice was 0.6-fold that of non-pregnant mice ($P < 0.01$) and did not differ between other pregnancy stages (each $P > 0.1$). This reduction in duodenal *GPR93* transcript level was negatively correlated to meal size in the light phase ($P < 0.05$). The densities of GPR93 immunopositive cells (**Figure 3.2 E and 3.2 F**) in duodenum and jejunum were similar in late- and non-pregnant mice (each $P > 0.1$).

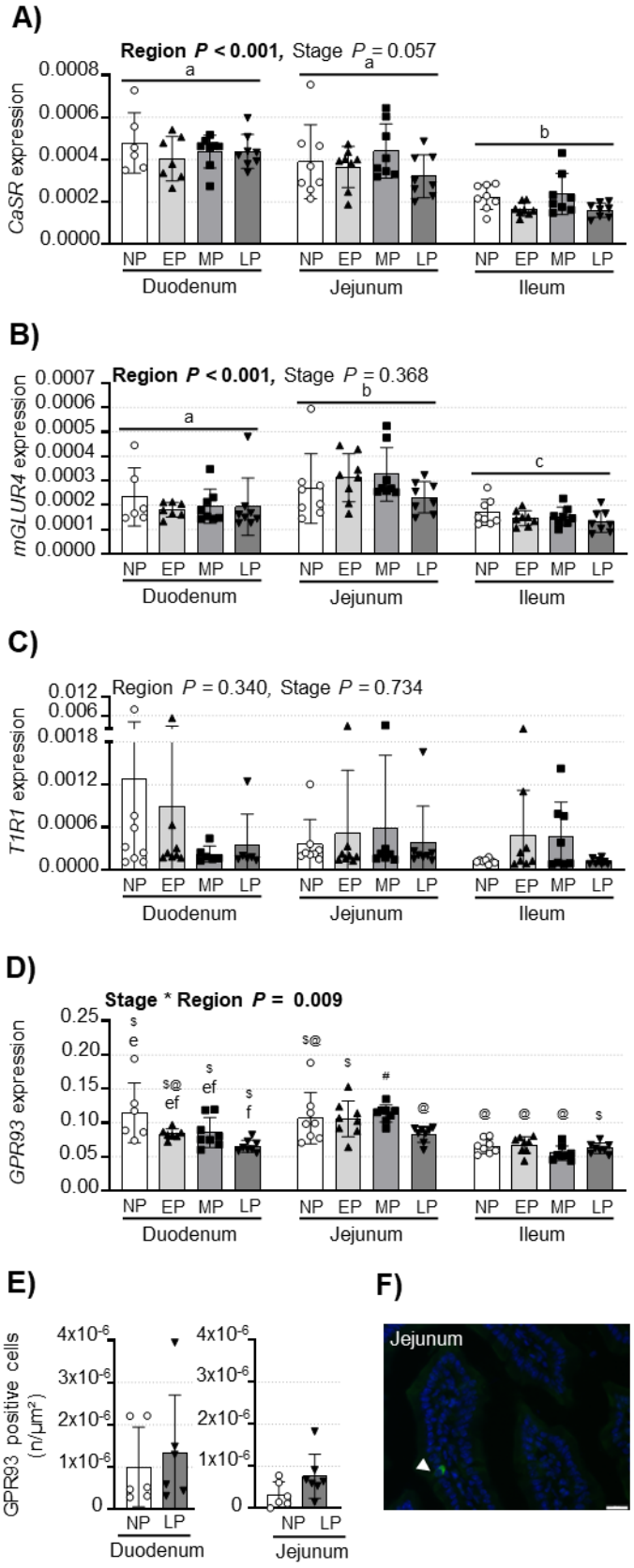

A)
Region P < 0.001, Stage P = 0.057
CaSR expression
0.0008
0.0006
0.0004
0.0002
0.0000
a
a
b
NP EP MP LP
Duodenum
Jejunum
Ileum
B)
Region P < 0.001, Stage P = 0.368
mGLUR4 expression
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
a
b
c
C)
Region P = 0.340, Stage P = 0.734
T1R1 expression
0.012
0.006
0.0018
0.0012
0.0006
0.0000
D)
Stage * Region P = 0.009
GPR93 expression
0.25
0.20
0.15
0.10
0.05
0.00
E)
GPR93 positive cells (n/µm²)
4x10-6
3x10-6
2x10-6
1x10-6
0
F)
Jejunum

Figure 3.2. SI regional- and pregnancy-specific expression of protein and amino acid chemoreceptors.

Relative mRNA expression of calcium and aromatic amino acids receptor (A, *CaSR*), glutamate receptor (B, *mGLUR4*), taste receptor type 1 (C, *T1R1*) and G-protein coupled receptor 93 (D, *GPR93*) in early- (EP; n = 6-8, ▼), mid- (MP; n = 6-8, ■) and late-pregnant (LP; n = 6-8, ▲) mice compared to non-pregnant mice (NP; n = 6-8, ○). *CaSR*, *mGLUR4* and *GPR93* expression is relative to the average Ct of the reference genes *B2M*, *HPRT* and *PPIA*. *T1R1* expression is relative to the average Ct of *B2M* and *HPRT*. The number of GPR93 immunopositive cells (E) in the duodenum and jejunum was assessed in late and non-pregnant mice (n = 6-7 per region and stage). Representative immunohistochemistry images (F) are shown, with white arrows indicating positive cells for GPR93 and the blue staining of nuclei. Scale bar = 20 µm, 40x magnification. Bars show mean ± standard deviation, symbols show data for individual mice. Different letters indicate differences between intestinal regions, different symbols indicate difference between pregnancy stages.

3.8.4. Expression of sweet tasting chemoreceptor components

Expression of *TRPM5* (**Figure 3.3 A**) and *T1R3* transcripts (**Figure 3.3 C**) differed by SI region ($P < 0.05$ and $P < 0.011$ respectively) but not by pregnancy stage (both $P > 0.1$). Ileal *TRPM5* expression was 0.8-fold that of jejunum ($P < 0.05$) with both regions similar to duodenum (each $P > 0.1$). *T1R3* expression in the jejunum and ileum was 1.2-fold ($P < 0.05$) and 1.3-fold ($P < 0.05$) respectively, of duodenal expression, and did not differ between jejunum and ileum ($P > 0.1$). Expression of *T1R2* transcripts was unaffected by SI region and pregnancy stage (**Figure 3.3.B**, each $P > 0.1$).

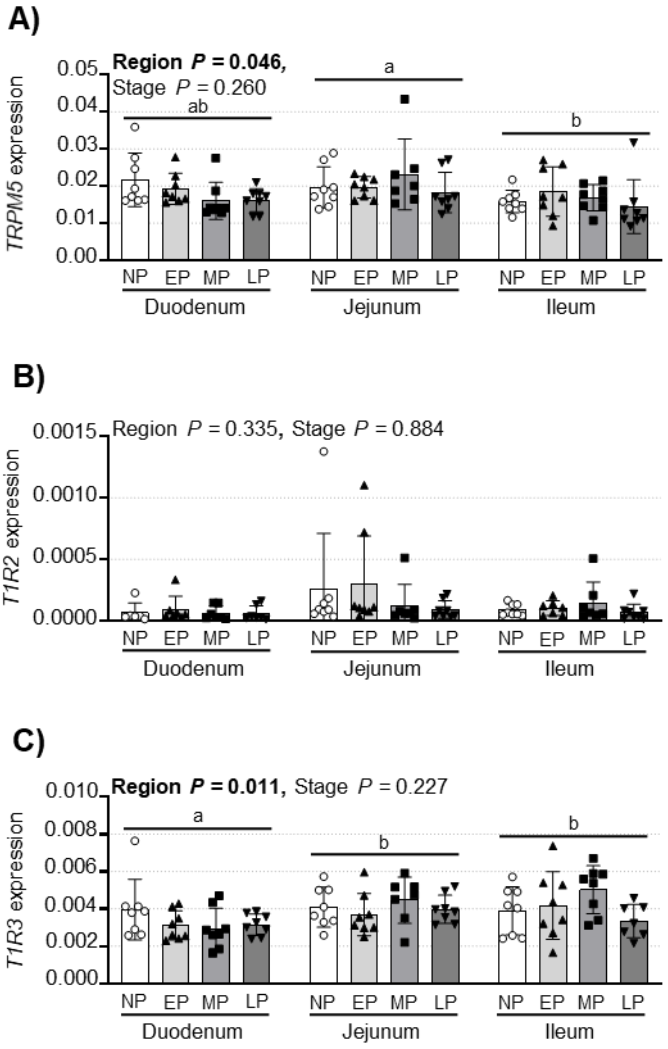


Figure 3.3. SI regional- and pregnancy-specific transcript expression of sweet taste chemoreceptors.

Relative mRNA expression of taste-specific cation channel (A, *TRPM5*), taste receptor type 2 (B, *T1R2*), umami and sweet taste receptor subunit (C, *T1R3*) to early- (EP; n = 6-8, ▼), mid- (MP; n = 6-8, ■) and late-pregnant (LP; n = 6-8, ▲) mice compared to non-pregnant mice (NP; n = 6-8, ○). Expression is relative to the average Ct of *B2M* and *HPRT*. Bars show mean ± standard deviation, symbols show data for individual mice. Different letters indicate differences between intestinal regions.

3.8.5. Expression of intestinal hormones CCK and GLP-1

Relative expression of *CCK* (**Figure 3.4 A**) and the GLP-1 precursor, glucagon (*GCG,* **Figure 3.4 B**) differed by SI region (each $P < 0.001$) but not pregnancy stage (each $P > 0.1$). Ileal *CCK* expression was 0.2-fold that of duodenum and jejunum (each $P < 0.001$), but did not differ between the duodenum and jejunum. *GCG* expression increased down the intestinal length; ileal expression was 2.7-fold that of duodenum and 1.4-fold that of jejunum (each $P < 0.001$), while jejunal *GCG* expression was 2.0-fold that of duodenum ($P < 0.001$). The densities of CCK (**Figure 3.4 C and 3.4 D**) and GLP-1 (**Figure 3.4 E and 3.4 F**) immunopositive cells in both the duodenum and jejunum were similar in late- and non-pregnant mice (each $P > 0.1$).

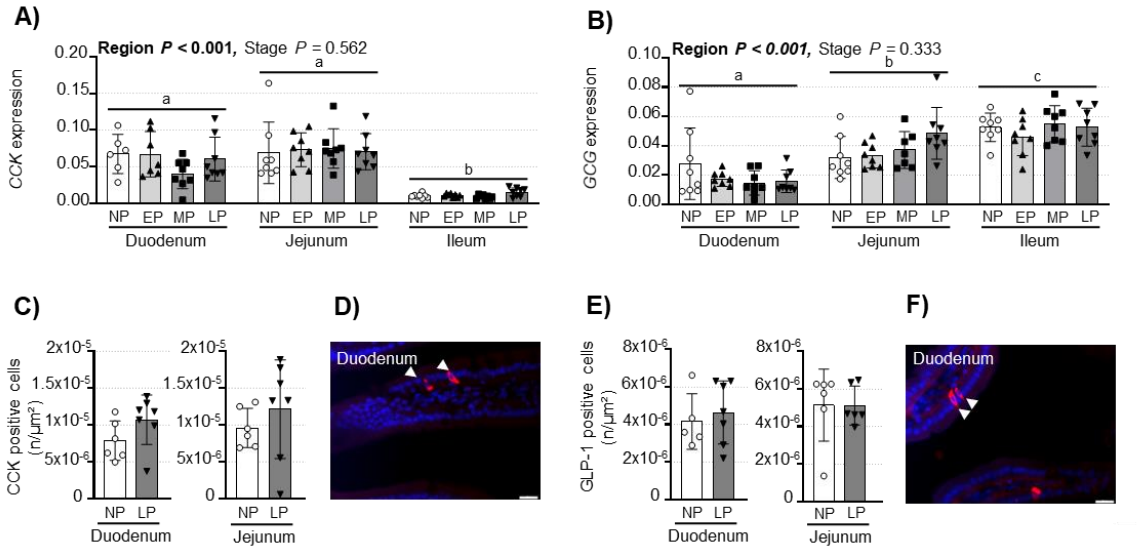


Figure 3.4. SI regional- and pregnancy-specific expression of CCK and GLP-1.

Relative mRNA expression of cholecystokinin (A, *CCK*) and Pro-glucagon (B, *GCG*) in the duodenum, jejunum and ileum in early- (EP; n = 6-8, ▼), mid- (MP; n = 6-8, ■) and late-pregnant (LP; n = 6-8, ▲) mice compared to non-pregnant mice (NP; n = 6-8, ○). *CCK* expression is relative to the average Ct of the reference genes *B2M*, *HPRT* and *PPIA*. *GCG* expression is relative to the average Ct of *B2M* and *HPRT*. The number of CCK and GLP-1 immunopositive cells (C, E) in the duodenum and jejunum was assessed in late and non-pregnant mice (n = 6-7 per region and stage). Representative immunohistochemistry images are shown, with white arrows indicating positive cells for CCK (D) and GLP-1 (F) and the blue staining of nuclei. Scale bar = 20 µm, 40x magnification. Bars show mean ± standard deviation, symbols show data for individual mice. Different letters indicate differences between intestinal regions.

3.9. Discussion

The current study has confirmed that SI weight increases during pregnancy and that there are specific adaptations in intestinal chemoreceptor expression during murine pregnancy. Intestinal *FFAR4* and duodenal *GPR93* transcripts were lower in late-pregnant mice compared to non-pregnant mice, although the densities of *FFAR4* and *GPR93* immunopositive cells density did not decrease during pregnancy. In contrast, *FFAR1* and *FFAR2* transcript expression was higher in mice at mid-pregnancy than at early-pregnancy, and not different between late-pregnant and non-pregnant mice. Both transcript expression and immunopositive cell densities of the satiety hormones CCK and GLP-1 were unchanged during pregnancy. Since *FFAR4* and *GPR93* chemoreceptors detect fatty acids and proteins, leading to secretion of satiety hormones, their reduced expression may contribute to increases in food intake during pregnancy.

The current study confirms that SI weight increases during pregnancy, as previously reported in pregnant rats [55,166]. Increases in weight could be due to an increase in intestinal length, which peaks in late-pregnant compared to non- and early-pregnant rodents [55,166]. Furthermore, it could be explained by an increase in SI mucosa weight, which increases by 30-40% in pregnant rats compared to non-pregnant rats [166], and increases in SI villi length as seen in late-pregnancy [167,168]. These structural adaptations would presumably be important in increasing surface area for nutrient absorption.

It is well established that chemoreceptors and satiety hormones are regionally expressed along the SI [153,158] and placed to trigger release of gut hormones as nutrient ligands descend the SI. The current study confirms that there is a distinct expression pattern for each chemoreceptor, with many regional expression patterns similar to previous reports, including high *GPR93* across all regions and

highest *FFAR1* in ileum [153], also reported in fed mice and in tissues collected at a similar time of day. The higher *GPR84* expression in jejunum relative to the other regions, differs from previous reports of peak expression in the ileum [153]. This may reflect differences in the exact SI location sampled, or differences in sampling time, given that some SI taste receptors, such as intestinal *T1R2* [126], display remarkable circadian rhythmicity of expression. Diet composition and feeding state of mice [169,170] could also affect expression, exemplified by gastric CaSR, which varies substantially based on diet composition [161] and region (gastric cf duodenal [161]) in mice. Lastly, these mice were fed ad libitum, and we have previously reported a significant increase in ad libitum food intake from mid-pregnancy onwards in this cohort of mice [42]. The corresponding timing of changes in transcript levels of: *FFAR1* and *2* at mid-pregnancy; *FFAR4* at mid- and late-pregnancy; and *GPR93* at late-pregnancy parallel the emergence of these food intake adaptations. Further research is now needed to determine whether gene expression changes we describe here drive or follow adaptations in food intake during pregnancy.

We report for the first time that late-pregnant mice have lower duodenal *GPR93* expression and lower *FFAR4* across all regions compared to non-pregnant control mice. However, these changes were not reflected in the density of FFAR4 and GPR93 immunopositive cells. Density of FFAR4-positive cells was in fact higher in the jejunum of late-pregnant compared to non-pregnant mice, and similar in these groups within duodenum, whilst the densities of GPR93-positive cells were similar in late- and non-pregnant mice for both regions. It is possible that the expression of these nutrient receptors and hormones are altered within individual cells, as antibodies are limited to detecting target-expressing cell pools, therefore proteomic quantitation or Western blot will be a critical next step [171].

Despite the presence of similar or increased cell densities for both sensors, we hypothesise that reduced FFAR4 and GPR93 expression, if present at the protein level, would attenuate satiety signalling in EECs to intestinal vagal afferents and increase food intake. This hypothesis is supported by the negative correlation identified between intestinal *FFAR4* and duodenal *GPR93* transcript levels and light-phase meal size, which warrants further investigation. Down-regulation of nutrient chemoreceptor expression during pregnancy was selective for *FFAR4* and *GPR93*. There was little change in expression of other nutrient chemoreceptors, other than differences in expression of ileal *FFAR1* and *FFAR2* transcripts between early- and mid-pregnancy stages (although not in comparison to non-pregnant mice). The significance of these changes across pregnancy warrant further investigation, but may reflect fatty acid demands at different pregnancy stages. For example, lipid demand and synthesis increases between weeks 10 and 30 of gestation in women, and is essential for depositing fat reserves for lactation and sustaining fetal and placental growth [172].

Unexpectedly, *GCG* transcript expression and GLP-1 positive cell density were stable throughout pregnancy in the present study. This contrasts with previous reports of higher abundance of intestinal GLP-1 positive cells in late-pregnant (d 18.5) compared to non-pregnant C57BL/6 mice [69], although the region sampled was not stated. Similarly, there is limited and inconsistent data on circulating GLP-1 concentrations during pregnancy, and it is unclear whether these relate to pregnancy stage or nutrition. For example, although total GLP-1 plasma concentrations are higher in ad libitum fed mid-pregnant (d 12) [55] and late-pregnant (d 20) rats [173] than non-pregnant controls, total GLP-1 was lower in fasted pregnant rats (all stages) compared to proestrus controls [55]. Active GLP-1 plasma concentrations in fasting women were higher in the third- than first

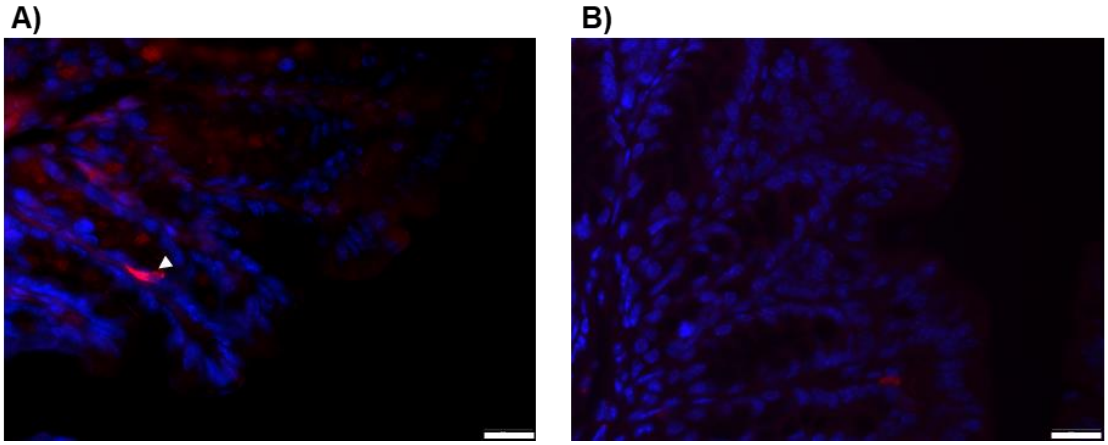
trimester, but did not differ from those of non-pregnant controls [56]. Although higher plasma GLP-1 concentrations would be expected to reduce food intake during pregnancy, it is important to acknowledge that satiating actions of active GLP-1 are likely mediated by their action at the intestine, via activation of L-cell adjacent vagal afferents. As such, circulating GLP-1 levels may be a poor surrogate for satiety changes.

This study provides the first data on *CCK* expression during pregnancy. Circulating concentrations of CCK are higher in pregnant than non-pregnant fasted dogs [174] and late-pregnant than non-pregnant women [63]. Nevertheless, both SI *CCK* expression and CCK-positive cell density were similar across all pregnancy stages in mice in the present study. Interestingly, consumption of fatty acid (as Emtobil), which activates FFAR1 and FFAR4 receptors [175], stimulated greater CCK release in late pregnant than non-pregnant women [62]. Whether fatty acid-induced CCK release also changes in mouse pregnancy, where the decreased *FFAR4* expression would be expected to down-regulate this response, and whether SI *FFAR4* expression changes in human pregnancy require further investigation. Interestingly, while elevated CCK release would be expected to induce satiety, there is evidence that CCK resistance may facilitate increased food intake in pregnancy. For example, peripherally-administered CCK did not significantly decrease food intake in mid-pregnant compared to non-pregnant rats (decrease:14% cf 47%) [39]. Multiple organs adapt to the demands of pregnancy, under the influence of rising progesterone, oestrogen, prolactin, leptin and growth hormone levels during pregnancy (previously reviewed [150]). This includes the intestine, which is a critical determinant of maternal energy and glycaemic homeostasis and expresses receptors for these pregnancy hormones (previously reviewed [150]). It is also possible that increased gut hormone release

could occur in response to an increase in intestinal length [55,166] and a larger nutrient load delivered to the intestine, regardless of chemoreceptor expression, since we know that meal size increases during pregnancy, especially within the mouse [42].

3.10. Conclusion

In summary, the SI increases in weight during pregnancy and we found distinct changes in transcript expression of specific nutrient receptors, including *FFAR1, 2, 4* and *GPR93* in murine pregnancy. Despite decreased *FFAR4* and *GPR93* expression, there was no decrease in the density of cells expressing these proteins. Fatty acids and proteins are sensed by FFAR4 and GPR93 chemoreceptors, leading to the secretion of CCK and GLP-1 and signalling satiation. Others have reported higher circulating concentrations of GLP-1 and CCK during pregnancy, but actions on SI vagal afferents have not been assessed. Future research is required to verify these transcript expression changes at a protein level and whether nutrient-evoked CCK and GLP-1 release is altered during murine pregnancy.



Supplementary Figure 3.1. Control for CCK positive cells.

Representative immunohistochemistry image of cholecystokinin (CCK) positive cell (A) and negative section (B, no antibody) in the mouse proximal colon. White arrows indicating positive cell for CCK and the blue staining of nuclei. Scale bar = 20 µm, 40x magnification.

CHAPTER 4: Pregnancy and a high-fat high-sugar diet each attenuate mechanosensitivity of murine gastric vagal afferents, with no additive effects.

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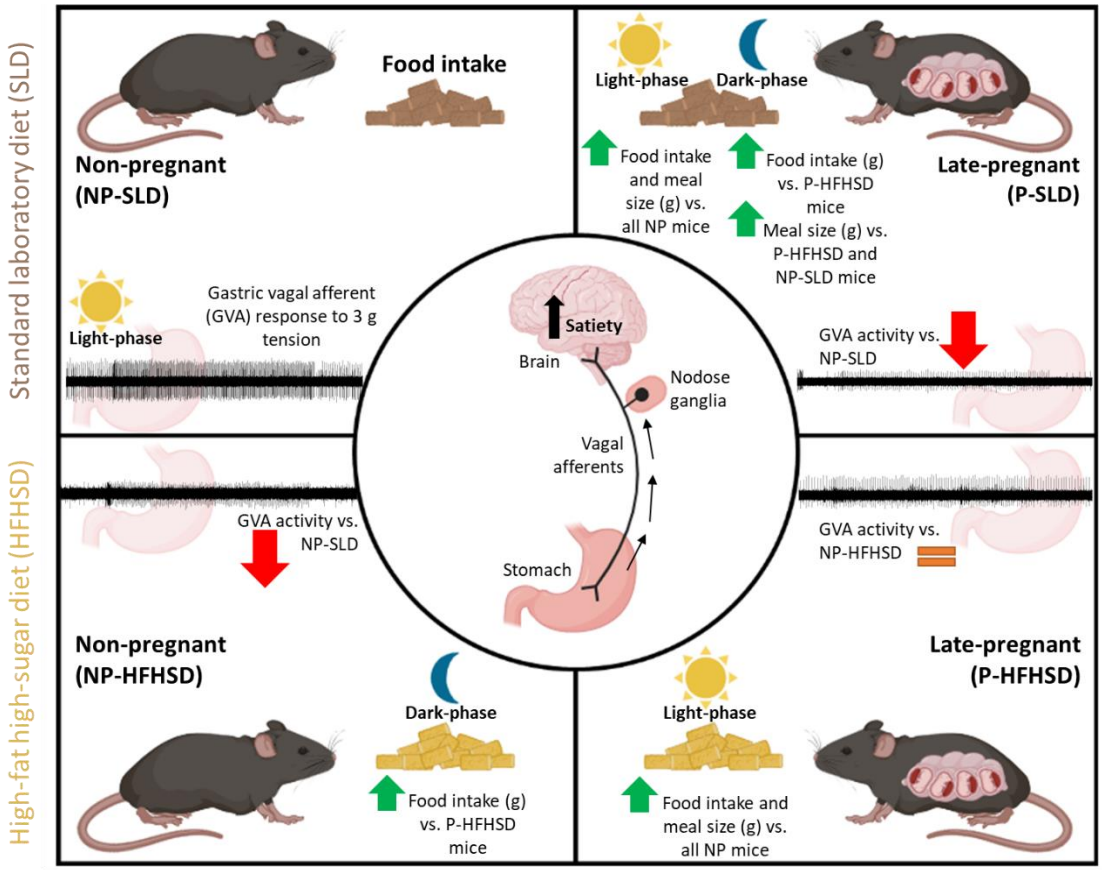
4.1. Graphical abstract



4.2. Overview

Glu Venus expressing-mice used within this study were provided under a transfer agreement with the University of Cambridge and Dr Lisa Nicholas from the Adelaide Centre for Epigenetics, University of Adelaide, Adelaide, Australia. The model of maternal obesity induced by a HFHSD was led by and characterised in this mouse genotype by Dr Nicholas. Within this current project my roles included: assisting in weekly weighing and feeding of the mice during the 12 week diet regime, assisting with management of the metabolic cages, leading the timed-mating and post-mortems, completing the majority of the gastric electrophysiology (over 60%), processing the collected tissue, analysing the metabolic data, completing the statistics, creating the figures and writing the manuscript. This paper describes the effects of pregnancy, a maternal high-fat, high-sugar diet before and during pregnancy, and their interactions, on food intake, feeding behaviours and gastric vagal afferent function. This work has been prepared in manuscript format for later submission to *Acta Physiologica*.

4.3. Authorship document

Statement of Authorship

Title of Paper	Pregnancy and a high-fat high-sugar diet each attenuate mechanosensitivity of murine gastric vagal afferents, with no additive effects.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Miss Georgia Clarke		
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Prepared figures. Wrote manuscript. Edited and revised manuscript. Accepted final version of manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	20/09/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Hui Li		
Contribution to the Paper	Conception and design of research. Performed experiments and analysed data. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	21/9/23

Name of Co-Author	Dr Lisa Marie Nicholas		
Contribution to the Paper	Conception and design of feeding regime. Bred Glu-Venus mouse line in Australia. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	20/9/23

Name of Co-Author	Dr Sharon Ladyman
Contribution to the Paper	Edited and revised manuscript. Accepted final version of manuscript.
Signature	Date: 21/9/23

Name of Co-Author	A/Prof Kathryn Gatford
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript
Signature	Date: 20/9/23

Name of Co-Author	Prof Amanda Page
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript.
Signature	Date: 20/9/23

4.4. Abstract

Gastric vagal afferents (GVA) sense food-related mechanical stimuli and signal to the central nervous system to initiate meal termination. Pregnancy and diet-induced obesity are independently associated with dampened GVA mechanosensitivity and increased food intake. Whether a high-fat, high-sugar diet (HFHSD) diet impacts pregnancy-related adaptations in GVA signalling is unknown and was investigated in this study. Three-week-old female Glu Venus-expressing mice, on a C57BL/6 background, were fed a standard laboratory diet (SLD) or HFHSD for 12 weeks, then half of each group were mated to generate late pregnant (d17.5; P-SLD *N*=12, P-HFHSD *N*=14) or non-pregnant (NP-SLD *N*=12, NP-HFHSD *N*= 16) groups. Body weight and food intake were monitored in Promethion metabolic cages from before mating until d 17.5 of pregnancy or equivalent ages in non-pregnant mice, prior to tissue collection at 0700 h for *in vitro* single fibre GVA recording. Pregnant mice gained more weight than non-pregnant mice but weight gain was unaffected by diet. By mid-pregnancy, food intake (in kJ and g) was higher in pregnant than non-pregnant mice during the light-phase (each $p < 0.001$) due to larger meals (kJ and g, each $p < 0.001$), irrespective of diet. The effect of diet and pregnancy on GVA function was selective to tension-sensitive afferents. Although both pregnancy and HFHSD-feeding reduced GVA mechanosensitivity to stretch (each $p < 0.01$), pregnancy did not further down-regulate GVA stretch responses within HFHSD-mice ($p = 0.652$). Larger light-phase meals in pregnant compared to non-pregnant HFHSD-mice may therefore reflect down-regulation of other satiety pathways.

4.5. Highlights

- In SLD-fed mice gastric tension-sensitive vagal afferent mechanosensitivity was attenuated in pregnant compared to non-pregnant mice, which is concurrent with increases in total food intake and meal size.
- In non-pregnant mice, tension-sensitive gastric vagal afferent mechanosensitivity was selectively attenuated in HFHSD- compared to SLD-mice. Despite this, HFHSD-mice ate less food and smaller meals compared to the SLD-fed mice, suggesting other satiety mechanisms are limiting food intake.
- Despite higher food intake, there was no further reduction in mechanosensitivity in pregnant HFHSD-mice compared to non-pregnant HFHSD-mice and further studies are required to increase understanding of food intake regulation across pregnancy.

4.6. Introduction

Food intake is highly regulated and remains relatively stable during steady-state conditions [176], whilst highly plastic regulatory mechanisms allow food intake to adapt rapidly to changing metabolic demands. The gastrointestinal tract (GIT) plays a key role in the regulation of food intake by sensing food intake and signalling via vagal afferents to the central nervous system to modulate satiety [176]. Gastrointestinal vagal afferents are sensory fibres located primarily in the stomach and intestinal wall that detect the arrival, volume and chemical composition of a meal [103]. In the stomach, gastric vagal afferents (GVAs) primarily respond to mechanical stimuli. Mechanosensitive tension-sensitive GVAs respond to distension following meal intake and are thought to act centrally to induce satiation [103] and also as feedback signals to regulate gut function such as gastric accommodation and motility [177]. The other subtype of mechanosensitive GVA are mucosal afferents, which respond to mucosal stroking and are thought to detect particle density and regulate gastric emptying [103]. GVAs are highly plastic, responding to circadian cues and nutritional status [107]. For example, tension-sensitive GVA responses to stretch are attenuated after fasting [22], which is consistent with the increase in the size of the first meal after a fast [22,178]. Furthermore, GVA signalling adapts to allow changes in food intake in response to long-term changes in energy demand, such as pregnancy, where increased maternal energy intake is required to support maternal adaptations and increased metabolic rate, fetal and placental growth and to prepare for future lactation [150]. To meet these demands, daily food intake increases by around 200-300 calories in the third trimester in pregnant women and by around 25% from mid-pregnancy onwards in mice [150]. In parallel with increasing food intake, GVA responses to distension are attenuated from mid-pregnancy onwards, allowing greater food

intake before induction of satiety signals, compared to non-pregnant mice [42]. Indeed, the same pregnant mice display altered eating behaviours, with the consumption of larger meals over a longer meal duration during the light-phase [42]. Interestingly, tension-induced signalling by murine gastric [22] and jejunal vagal afferents [179] are also attenuated in high-fat diet (HFD)-induced obesity, with reduced signalling likely to promote increased food intake. Similar to pregnancy, HFD-fed mice also exhibit altered feeding patterns, with higher energy consumption and meal number during the light-phase compared to those fed standard chow, suggesting their inability to sense satiation signals [47]. In developed countries, increasing rates of obesity are occurring in the context of diets that are high in both fat and sugar. For example, in countries including the United States, Australia, New Zealand and parts of Europe, 70% of calories arise from animal foods, oils, fat and sweeteners [180]. The impacts of a high-fat, high-sugar diet (HFHSD) on GVA responses to mechanical food-related stimuli have not been reported to date.

The combination of obesity and pregnancy is increasingly common. Almost 50% of women in developed countries are overweight or obese prior to pregnancy [181,182]. Obesity during pregnancy increases the risks of short-term complications and predisposes offspring to metabolic diseases in later life [183]. In addition, more than 50% of women gain excessive weight during pregnancy [184], which is itself associated with increased risks of complications including gestational diabetes, the need for caesarean-section delivery and infant macrosomia [185]. Excessive weight gain during pregnancy may in part reflect impacts of obesity on appetite-regulatory pathways. In both lean and overweight/obese women, pregnancy is associated with altered main meal patterns, with increasing meal frequency and snack-dominant meal patterns as gestation progresses [44]. Greater weight gain in

obese than lean pregnant women may be attributed to diet composition, as they consume a diet higher in processed foods and confectionary snacks [186,187]. Furthermore, the increase in energy intake during pregnancy is higher in Western diet-fed than standard chow-fed mice [188,189]. Given that obese women are entering pregnancy at an increased risk of pregnancy complications, strategies to reduce energy intake or enable adherence to nutritional guidelines are clinically important. The mechanisms permitting the overconsumption of food during HFHSD feeding in pregnancy are unknown, including possible changes in GVA signalling. We investigated this question using a mouse model of HFHSD feeding.

4.7. Materials and methods

4.7.1 Ethics

All studies were approved by the animal ethics committee (SAM-21-048) of the South Australian Health and Medical Research Institute (SAHMRI) and carried out in accordance with the Australian code for the care and use of animals for scientific purposes, 8th edition 2013 and adhere to the Arrive 2.0 guidelines [159].

4.7.2. Animals and experimental design

Glu Venus-expressing mice [190], maintained on a C57BL/6 background, were obtained under a material transfer agreement from Cambridge Enterprise Limited, United Kingdom and bred at the SAHMRI bioresources facility. Glu Venus mice express fluorescent green intestinal L cells [190], which will be utilised in characterising changes in intestinal L cell populations during pregnancy. Mice were housed at 22°C, in a 12:12 light/dark cycle with lights on at 0700 h. Female mice (3 - 4 weeks old, 9 – 20 g) were randomised to be fed a standard laboratory diet (SLD, *n* = 24: Teklad standard diet: 13 kJ/g, digestible energy from protein

24%, fat 18% and carbohydrates 58%, CAT #: 2018, Envigo, Cambridgeshire, United Kingdom,) or a HFHSD (*n* = 30: Specialty Feeds: 23 kJ/g, digestible energy from protein 17.6%, fat 58.4% (derived from soya bean and coconut oil) and carbohydrates 24% (sucrose 175 g/kg), CAT #: SF21-003, Glen Forrest, Western Australia, Australia) for 12 weeks (diet phase). Mice were housed in groups of 2-5 litter mates and weighed weekly during the feeding phase of the study, from weaning until 14-15 weeks of age. Mice were then single housed in metabolic cages for a 7 d acclimatisation period (Promethion Sable System, Las Vegas, USA). Following acclimatisation, female mice were pair-housed at 1700 h with a male mouse in a home cage for mating, remaining on their diets during the mating period. Female mice were checked daily at 0700 h and pregnancy was confirmed by the presence of a vaginal plug (assigned as d 0.5 of pregnancy). Plugged females were then returned to individual metabolic cages until the late-pregnancy end point at d 17.5 (SLD *N* = 12*,* HFHSD *N* = 14). Control (non-pregnant) female mice (SLD *N* = 12, HFHSD *N* = 16) were pair-housed with another female in a normal home cage and returned to metabolic cages on age-matched days. Mice were kept on respective diets during the 17.5 d period. On d 17.5, mice were anaesthetised between 0700 and 0730 h by isoflurane inhalation (5% in oxygen) and humanely culled by decapitation prior to tissue collection for electrophysiology experiments described below. Maternal gonadal and perirenal fat pads and individual fetuses were dissected and weighed.

Mice that were mated, with vaginal plugs present, but did not become pregnant (*N* = 11) were excluded from the study and not included in the final mating or pregnancy numbers. These mice were not added to the non-pregnant group, to avoid potential impacts of elevated prolactin during pseudo-pregnancy [116]. One non-pregnant mouse was excluded from the study due to over-barbering and

another was unexpectedly found dead in the cage. The planned sample size of *N* = 12 was based on variation in GVA function in previous studies within our laboratory [22,42].

4.7.3. Metabolic monitoring

Metabolic cages were used to continuously measure body weight and record real-time feeding events, including total food intake, average meal size and duration and total meal number, and analysed as previously described [42]. Briefly, metabolic data was transformed using the Promethion data software package ExpeData version 1.9.14 (Promethion Sable System, Las Vegas, USA) using analytical macro 6. Data from each day of the study was divided into 12 h time periods corresponding to the light- and dark-phases. Body weight is presented for each study day (averaged across 24 h) and food intake parameters (food and energy intake, meal size in energy and grams, meal duration, meal number) are presented as averages across two gestational days or age matched days. All mice were included in the analysis, but data points were excluded if they did not include a full 12 h worth of data for each photoperiod, e.g. due to cage changes. Data was therefore averaged across two gestational days (starting with the first two days from mating) to account for excluded time points. These two gestational day time points also align with key developmental stages of blastocyst formation and placental development [191].

4.7.4. *In vitro* mouse gastric vagal afferent electrophysiology

The electrophysiological methods used to record mouse GVA activity have been described in detail previously [103,120]. Briefly, the thorax was opened to remove the stomach and oesophagus and the vagal nerves were separated from the oesophagus. The stomach was opened with the vagal nerves attached and

placed mucosal side up in an organ bath filled with a modified Krebs solution, including nifedipine (1 µM) to prevent smooth muscle contraction. The vagal nerves were placed into another chamber filled with liquid paraffin. The nerves were teased apart into small bundles and placed onto a platinum recording electrode for single fibre recording. Nerve impulses were amplified (DAM50, World Precision Instruments, Sarasota, FL, USA), filtered (Band-pass filter 932, CWE, Ardmore, PA, USA), and recorded.

GVA mechanosensitivity was identified by locating receptive fields on the stomach, where tension-sensitive GVAs respond to mucosal stroking and tension stimuli, whilst mucosal GVAs respond to mucosal stroking only [103]. To record the responses of tension-sensitive afferents to stretch, a threaded hook was attached adjacent to the receptive field and to a cantilever system. Tension stimuli were created by placing weights (0.5 - 5 g) on the cantilever system for 1 minute. To record the responses of mucosal afferents, the receptive field was stroked with calibrated von Frey hairs (10 - 1000 mg). Up to five individual tension-sensitive or mucosal afferents were recorded per mouse. Action potentials of single units were analysed using Spike 2 software (Cambridge Electronic Design, UK). When recordings were obtained from more than one GVA subtype in an individual mouse, data was averaged to create one data point per GVA subtype per mouse.

4.7.5. Statistical Analysis

All data are presented as mean ± SD with *N* = number of animals. Statistical analyses were conducted using SPSS v. 28 (IBM Corporation, Armonk, NY). Effects of diet on body weight and weight gain (weeks 1-12) were analysed by one-way ANOVA. Body weight during acclimatisation (week 13) and during the 17-d period from mating (and the equivalent period in non-pregnant age-matched

controls) was analysed by linear mixed model to assess the effect of pregnancy (pregnant vs non-pregnant) and diet (SLD vs HFHSD) with day as a repeated factor. Where a pregnancy*diet*day interaction was significant, mixed repeated models were used to assess effects of diet and day separately in non-pregnant and pregnant groups, and effects of pregnancy and day separately in SLD and HFHSD mice. Where diet*day and/or pregnancy*day interactions were significant, two-way ANOVAs were used to assess effects of pregnancy and diet separately for each day of the study. Effects of pregnancy and diet on body weight gain (acclimatisation – d 17), and fat pad weights (gonadal and perirenal) were analysed by two-way ANOVA. Where diet*pregnancy interactions were significant, effects of pregnancy within each diet group, and effects of diet within non-pregnant and pregnant groups were separately analysed by one-way ANOVA. Effects of diet on litter size and average pup weight was analysed by one-way ANOVA.

Full day, light- and dark-phase food intake parameters were analysed using linear mixed models to assess the effect of pregnancy (pregnant vs non-pregnant) and diet (SLD vs HFHSD) with day as a repeated factor, as described above for body weight analyses. Because the diets differed in composition, we analysed food intake and meal size in terms of both weight and energy content. We also separately analysed effects of pregnancy and diet on average food intake and meal size over the final two study days (closest to the time of GVA assessment), using a two-way ANOVA. Full day, light- and dark-phase food intake parameters were included and where a diet*pregnancy interaction was significant, we then analysed effects of pregnancy within each diet group and effects of diet within each pregnancy group using one-way ANOVA.

The mechanosensitivity of gastric tension-sensitive and mucosal afferents were analysed using a linear mixed model to assess the effect of pregnancy (pregnant vs non-pregnant) and diet (SLD vs HFHSD), and with load (circular tension (grams) or von Frey hair (milligrams), respectively) as a repeated factor. Where a pregnancy*diet*load interaction was significant, we used mixed repeated models to assess effects of diet and load separately within each pregnancy group, and to assess effects of pregnancy and load separately within each diet group. Where diet*load and/or pregnancy*load interactions were significant, we ran a two-way ANOVA for each level of load, to assess the effects of pregnancy and diet. Where a diet*pregnancy interaction was significant for a given load, we used one-way ANOVA to assess effects of pregnancy within each diet group and of diet within each pregnancy group. To determine if there was a correlation between GVA sensitivity and meal size, the responses to GVA tension (5 g) and mucosal (200 mg) afferents, recorded during the light-phase, were plotted against meal size during the light-phase and a Pearson correlation performed.

4.8. Results

4.8.1 Phenotype

The body weight of mice was not different between SLD and HFHSD groups prior to starting the diet (**Table 4.1**). After 12 weeks on the diet, body weights of HFHSD- and SLD-mice were similar, however, HFHSD-mice gained more weight than SLD-mice (**Table 4.1**). From acclimatisation onwards, the effects of pregnancy and diet on body weight differed with day of study, including within diets and pregnancy groups (3-way and 2-way interactions, each $p < 0.01$, **Figure 4.1 A**). HFHSD-mice were heavier than SLD-mice from acclimatisation (week 13) until the end of the study, except on day 15 (all $p < 0.05$, **Figure 4.2 A**). Pregnant

mice were heavier than non-pregnant mice from d 9 onwards (**Figure 4.1 A**). Effects of diet on total weight gain differed between pregnant and non-pregnant mice (diet*pregnancy interaction, p = 0.027, **Figure 4.1 B)**. Not surprisingly, pregnant mice gained more weight than non-pregnant mice within both diet groups (**Figure 4.1 B**). Diet did not affect weight gain within pregnant mice, whilst NP-HFHSD mice gained more weight than NP-SLD mice (p = 0.016, **Figure 4.1 B**).

At the end of the study, gonadal fat pad was heavier in HFHSD- than SLD-mice independent of pregnancy (**Table 4.1**). The effect of pregnancy on perirenal fat mass differed between diets (diet*pregnancy interaction, p = 0.025, **Table 4.1**). The perirenal fat pad was heavier in NP-HFHSD than NP-SLD mice but was not affected by diet in pregnant mice. Perirenal fat pad weight was also higher in P-SLD compared to NP-SLD mice, but not different between pregnant and non-pregnant mice fed HFHSD (**Table 4.1)**. Maternal diet had no effect on litter size and average pup weights (**Table 4.1**).

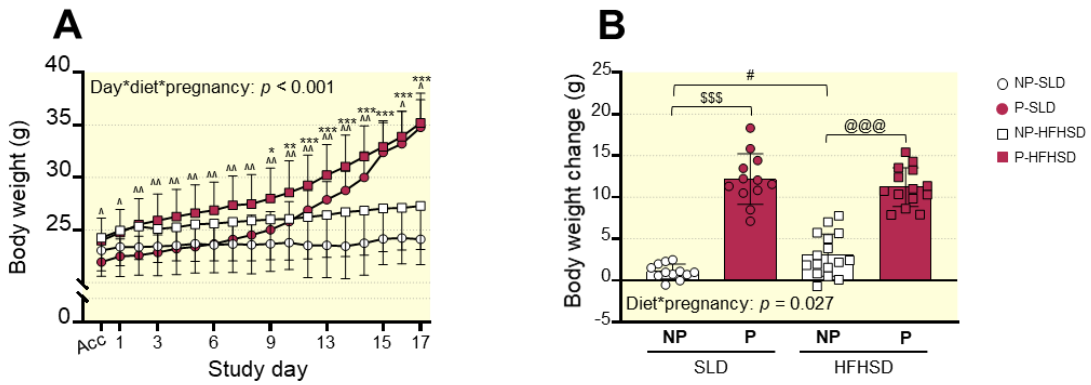

Figure 4.1 Impact of diet and pregnancy on body weight.

Part (A): Daily body weight from acclimatisation to d 17.5 of non-pregnant (NP) and pregnant (P) mice fed a standard laboratory diet (SLD; non-pregnant, NP, ○: *N* = 12; pregnant; P, ●: *N* = 12) or high-fat high-sugar diet (HFHSD; NP, □: *N* = 16; P, ■: *N* = 14) for 12 weeks prior and during the study period. Data are presented as mean ± SD and were analysed using a linear mixed model to assess the effect of pregnancy (pregnant vs non-pregnant) and diet (SLD vs HFHSD) with day as a repeated factor. Where a pregnancy*diet*day interaction was significant, mixed repeated models were used to assess effects of diet and day separately in non-pregnant and pregnant groups, and effects of pregnancy and day separately within SLD and HFHSD mice. Where diet*day and/or pregnancy*day interactions were significant, a two-way ANOVA was run to assess effects of pregnancy and diet separately for each day of the study. Diet effect represented by ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ and pregnancy effect by, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Part (B): Change in body weight of P- and NP-SLD mice and P- and NP-HFHSD mice from acclimatisation to d 17.5 of study. Bars and whiskers show mean ± SD, with data from each mouse indicated by symbols. Data were analysed using a two-way ANOVA. Where diet*pregnancy interactions were significant, one-way ANOVA were used to assess effects of pregnancy within each diet group, and the effects of diet within non-pregnant and pregnant groups. NP-SLD vs P-SLD, $^{\$\$\$}$ $p < 0.001$; NP-HFHSD vs P-HFHSD, $^{@@@}$ $p < 0.001$; NP-SLD vs NP-HFHSD, $^{\#}$ $p < 0.05$.

Table 4.1. Mouse phenotype.

	Treatment groups				P (ANOVA)		
Diet phase	**SLD (*N* = 24)**		**HFHSD (*N*=30)**		**Diet**		
Body weight at week 0 (g)	15.4 ± 2.1		14.8 ± 2.1		NS		
Body weight at week 12 (g)	22.5 ± 2.1		23.9 ± 2.8		NS		
Weight gain, in diet phase (g)	7.1 ± 1.9		9.1 ± 2.4		0.002		
End of study	**NP-SLD (*N* = 12)**	**P-SLD (*N* = 12)**	**NP-HFHSD (*N* = 16)**	**P-HFHSD (*N* = 14)**	**Diet**	**Pregnancy**	**Diet x Pregnancy**
Final weight (g)	24.1 ± 2.4	34.8 ± 2.6	27.3 ± 4.1	35.2 ± 2.8	0.046	<0.001	NS
Gonadal fat mass (g)	0.35 ± 0.22	0.34 ± 0.08	0.65 ± 0.48	0.48 ± 0.26	0.014	NS	NS
Perirenal fat mass (g)	0.11 ± 0.09	0.26 ± 0.13$^{\$\$}$	0.29 ± 0.25$^{\#}$	0.21 ± 0.11	NS	NS	0.025
Litter size (*N*)	N/A	7.3 ± 2.3	N/A	6.6 ± 2.2	NS	-	-
Average pup weight (g)	N/A	0.87 ± 0.12	N/A	0.86 ± 0.15	NS	-	-

Mice were fed either a standard laboratory diet (SLD) or high-fat high-sugar diet (HFHSD) for 12 weeks from weaning, then randomised to mating (pregnant group, P) or to be unmated controls (non-pregnant group, NP), and remained on their diets for a further 17 days from mating or age-matched days. Data are mean ± SD. N/A, not applicable; NS, not significant. Effects of diet on body weights at week 0 and 12 and weight gain were analysed using a one-way ANOVA. Effects of pregnancy and diet on body weight at d 17 and fat pad weights (gonadal and perirenal) were analysed using a two-way ANOVA. Where diet*pregnancy interactions were significant, one-way ANOVA were used to assess effects of pregnancy within each diet group, and the effects of diet within non-pregnant and pregnant groups. Effects of diet on litter size and average pup weight was analysed by one-way ANOVA. NP-SLD vs P-SLD, $^{\$\$}$ $p < 0.01$; NP-SLD vs NP-HFHSD, $^{\#}$ $p < 0.05$.

4.8.2 Impacts of diet and pregnancy on food intake behaviours

4.8.2.1. Energy intake

Within the entire 24 h period, effects of diet on energy intake (kJ) differed with both day (day*diet interaction: $p = 0.038$) and pregnancy (pregnancy*diet interaction: $p = 0.017$), and we therefore analysed effects of diet and pregnancy for each 2-day period throughout the study (**Figure 4.2 Ai**). HFHSD-mice consumed more energy than SLD-mice between d 0.5-8.5 and d 12.5-14.5 (all $p < 0.05$, **Figure 4.2 Ai**), but there was no effect of pregnancy (all $p > 0.05$). On d 8.5-10.5 and d 12.5-14.5, effects of diet depended on pregnancy status (pregnancy*diet interactions: each $p < 0.05$). On both days, NP-HFHSD mice consumed more food than NP-SLD-mice (both $p < 0.01$, **Figure 4.2 Ai**). Pregnancy did not affect food intake on d 8.5-10.5 and d 12.5-14.5 within each diet group and diet did not affect food intake within pregnant mice (all $p > 0.05$).

During the light-phase, effects of diet on energy intake (kJ) differed with both day (day*diet interaction: $p = 0.035$) and pregnancy (pregnancy*diet interaction: $p < 0.001$), and we therefore analysed effects of diet and pregnancy for each 2-day period throughout the study (**Figure 4.2 Aii**). Energy intake was greater in SLD- than HFHSD-mice during acclimatisation only ($p < 0.05$, **Figure 4.2 Aii)**. Irrespective of diet, light-phase energy intake was greater in pregnant than non-pregnant mice, throughout d 8.5-12.5 and 14.5-17.5 (all $p < 0.05$, **Figure 4.2 Aii)**.

During the dark-phase, effects of pregnancy and diet on energy intake differed between days (day*pregnancy*diet interaction: $p = 0.043$, **Figure 4.2 Aiii**), and we therefore analysed effects of diet and pregnancy for each 2-day period throughout the study. From acclimatisation until d 8.5, energy intake was greater in the HFHSD- than SLD-mice (all $p < 0.05$), and did not differ between pregnant

and non-pregnant mice (**Figure 4.2 Aiii**). Neither diet nor pregnancy status affected energy intakes on d 10.5-12.5 or 16.5-17.5 (**Figure 4.2 Aiii**). Effects of pregnancy on dark-phase energy intakes differed between diets on d 8.5-10.5, 12.5-14.5 and 14.5-16.5 (pregnancy*diet interaction: each $p < 0.05$, **Figure 4.2 Aiii**). On d 8.5-10.5 and 12.5-14.5, energy intakes were greater in non-pregnant than pregnant mice within HFHSD-mice (all $p < 0.05$) and not affected by pregnancy status within SLD-mice (**Figure 4.2 Aiii**). Conversely, on d 16.5-17.5, energy intakes were greater in pregnant than non-pregnant mice within SLD-mice ($p < 0.05$) and not affected by pregnancy status within HFHSD-mice (**Figure 4.2 Aiii**). On d 8.5-10.5, 12.5-14.5 and 14.5-16.5, energy intakes were greater in HFHSD-fed than SLD-fed within non-pregnant mice (all $p < 0.05$) and not affected by diet within pregnant mice (**Figure 4.2 Aiii**).

4.8.2.2. Food intake

Within the entire 24 h period, effects of diet on food intake (g) differed with both day (day*diet interaction: $p = 0.003$) and pregnancy (pregnancy*diet interaction: $p = 0.003$), and effects of pregnancy also changed across the study (day*pregnancy interaction: $p = 0.044$). We therefore analysed effects of diet and pregnancy for each 2-day period throughout the study (**Figure 4.2 Bi**). Food intake was higher in SLD- than HFHSD-mice during acclimatisation, from d 2.5 until d 6.5 and again at d 10.5-12.5 and 16.5-17.5 (all $p < 0.05$, **Figure 4.2 Bi**). Food intake from acclimatisation until d 6.5 and at d 10.5-12.5 and 16.5-17.5 did not differ between non-pregnant and pregnant groups (**Figure 4.2 Bi**). Effects of diet on food intake between d 6.5-10.5 and d 12.5-16.5 differed between pregnant and non-pregnant mice (pregnancy*diet interactions: all $p < 0.05$). On d 6.5-8.5 and 8.5-10.5, food intake was not altered by pregnancy status within either diet group, or by diet within non-pregnant mice (**Figure 4.2 Bi**). Within pregnant mice,

food intake during this period was higher in those fed SLD than HFHSD (both $p < 0.001$, **Figure 4.2 Bi**). On d 12.5-14.5, food intake was not altered by pregnancy status within either diet group, and was greater in SLD than HFHSD groups within both non-pregnant and pregnant groups (all $p < 0.05$, **Figure 4.2 Bi**). On d 14.5-16.5, pregnant mice ate more than non-pregnant mice within the SLD groups ($p < 0.001$), but not within HFHSD groups (**Figure 4.2 Bi**). Food intake on d 14.5-16.5 remained higher in SLD than HFHSD groups within both non-pregnant ($p = 0.015$) and pregnant groups ($p < 0.001$, **Figure 4.2 Bi**).

During the light-phase, effects of pregnancy and diet on food intake varied with study day (day*pregnancy interaction: $p < 0.001$; day*diet interaction: $p = 0.043$, **Figure 4.2 Bii**). Within each study day, SLD-mice ate more than HFHSD-mice (all $p < 0.05$, **Figure 4.2 Bii**). From d 8.5 onwards, food intake was higher in pregnant than non-pregnant mice (all $p < 0.05$, **Figure 4.2 Bii**).

During the dark-phase, effects of pregnancy on food intake differed between diets (pregnancy*diet interaction: $p = 0.009$) and did not differ between study days (**Figure 4.2 Biii**). Food intake was higher in SLD- than HFHSD-mice within pregnant mice ($p < 0.001$), but did not differ between diet groups within non-pregnant mice (**Figure 4.2 Biii**). Food intake was higher in non-pregnant than pregnant mice within the HFHSD groups ($p < 0.001$,) but conversely was higher in pregnant than non-pregnant mice with SLD groups ($p = 0.016$, **Figure 4.2 Biii**).

4.8.2.3 Energy per meal

Across the 24 h period, the amount of energy consumed per meal was higher in HFHSD- than SLD-mice ($p = 0.015$), was unaffected by pregnancy and did not change across study days (**Figure 4.2 Ci**). During the light-phase, effects of pregnancy and diet on energy intake per meal differed between days

(day*pregnancy and day*diet interactions: each $p < 0.01$, **Figure 4.2 Cii**), and we therefore analysed effects of diet and pregnancy for each 2-day period throughout the study. HFHSD-mice consumed more energy per light-phase meal than SLD-mice on d 0.5-2.5, but this pattern reversed with time, such that SLD-mice consumed more energy per meal than HFHSD-mice on d 8.5-10.5 and 14.5-16.5 (all $p < 0.05$, **Figure 4.2 Cii**). Pregnant mice consumed more energy per light-phase meal than non-pregnant mice on most days from d 2.5 onwards (all $p < 0.05$, **Figure 4.2 Cii**).

During the dark-phase, effects of pregnancy and diet on energy intake per meal differed between days (day*pregnancy*diet interaction: $p = 0.023$, **Figure 4.2 Ciii**). Early in the study, during acclimatisation, on d 0.5-2.5 and 6.5-8.5, energy intake per dark-phase meal was higher in HFHSD-mice than SLD-mice (each $p < 0.05$, **Figure 4.2 Ciii**). Pregnancy did not affect energy intake per dark-phase meal independent of diet at any day, but had diet-dependent effects on d 14.5-16.5 (pregnancy*diet interaction: $p = 0.049$, **Figure 4.2 Ciii**). On d 14.5-16.5, energy intakes per dark-phase meal were higher in pregnant than non-pregnant mice within SLD-mice ($p = 0.004$), but not HFHSD-mice, and did not differ between diet groups within either pregnant or non-pregnant groups (**Figure 4.2 Ciii**).

4.8.2.4. Meal size

Across 24 h, the effect of diet on meal size (in g) differed with pregnancy and day (day*pregnancy*diet interaction: $p = 0.007$, **Figure 4.2 Di**), and we therefore analysed effects of diet and pregnancy at each day of the study. Meal size was higher in the SLD than HFHSD group during acclimatisation and on d 0.5-2.5, 2.5-4.5, 4.5-6.5, 8.5-10.5, 10.5-12.5 and 16.5-17.5 (all $p < 0.05$, **Figure 4.2 Di**).

On study days 6.5-8.5, 12.5-14.5 and 14.5-16.5, effects of diet on meal size differed with pregnancy status (pregnancy*diet interactions: all $p < 0.05$, **Figure 4.2 Di**). On d 6.5-8.5 and 12.5-14.5, SLD-mice consumed larger meals than HFHSD-mice within pregnant groups (both $p < 0.001$), but meal size was similar between diet groups within non-pregnant mice, and similar between non-pregnant and pregnant groups within each diet group (**Figure 4.2 Di**). On d 14.5-16.5, SLD-mice consumed larger meals than HFHSD-mice within pregnant and non-pregnant groups (both $p < 0.01$). Meal size on d 14.5-16.5 was greater in pregnant than non-pregnant mice within SLD groups ($p <0.001$), but did not differ with pregnancy within HFHSD-mice (**Figure 4.2 Di**).

During the light-phase, effects of diet on meal size (g) differed with both day (day*diet interaction: $p < 0.001$) and pregnancy (pregnancy*diet interaction: $p = 0.013$), and effects of pregnancy also changed across the study (day*pregnancy interaction: $p < 0.001$). We therefore analysed effects of diet and pregnancy for each 2-day period throughout the study (**Figure 4.2 Dii**). SLD-mice ate larger light-phase meals than HFHSD-mice on most study days (acclimatisation, d 0.5-2.5, 4.5-6.5, 6.5-8.5, 8.5-10.5, 10.5-12.5, 12.5-14.5 and 16.5-17.5, diet effects: each $p < 0.05$, **Figure 4.2 Dii**). Pregnant mice consumed larger light-phase meals than non-pregnant mice from d 4.5 until d 10.5 and again on d 16.5-17.5 (pregnancy effects, each $p < 0.05$, **Figure 4.2 Dii**). On d 2.5-4.5 and 14.5-16.5 effects of pregnancy on meal size differed between diet groups (pregnancy*diet interactions: both $p < 0.05$, **Figure 4.2 Dii**). On d 2.5-4.5, pregnant mice ate larger meals than non-pregnant mice in SLD-mice ($p = 0.014$), but not in HFHSD-mice (**Figure 4.2 Dii**). In addition, on d 2.5-4.5, SLD-mice ate larger meals than HFHSD-mice in non-pregnant groups ($p =0.023$), but not within pregnant groups (**Figure 4.2 Dii**). On d 14.5-16.5, pregnant mice ate larger meals than non-

pregnant mice in both SLD ($p < 0.001$) and HFHSD groups ($p = 0.013$). In addition, on d 14.5-16.5, SLD-mice ate larger meals than HFHSD-mice within both non-pregnant ($p = 0.001$) and pregnant groups ($p < 0.001$, **Figure 4.2 Dii**).

During the dark-phase, the effect of diet on meal size (in g) differed with pregnancy and day (day*pregnancy*diet interaction: $p = 0.007$, **Figure 4.2 Diii**), and we therefore analysed effects of diet and pregnancy at each day of the study. For all study days except d 14.5-16.5, SLD-mice consumed larger dark-phase meals than HFHSD-mice (all $p < 0.05$), and meal size did not differ between pregnant and non-pregnant mice (**Figure 4.2 Diii**). Effects of pregnancy on dark-phase meal size differed between diet groups on d 14.5-16.5 (pregnancy*diet interaction: $p = 0.010$). On d 14.5-16.5, pregnant mice ate larger dark-phase meals than non-pregnant mice in SLD groups ($p = 0.004$), but not in those fed HFHSD, while SLD-mice ate larger meals than HFHSD-mice within both non-pregnant ($p = 0.028$) and pregnant groups ($p < 0.001$, **Figure 4.2 Diii**).

4.8.2.5. Meal duration

Across 24 h, meal duration was shorter in pregnant than non-pregnant mice ($p = 0.005$), and in HFHSD- than SLD-mice ($p < 0.001$), and decreased across time ($p < 0.001$), with no interactions between factors (**Figure 4.2 Ei**). During the light-phase, meal duration was similarly shorter in in HFHSD- than SLD-mice ($p < 0.001$), but did not differ between pregnant and non-pregnant mice or study day (**Figure 4.2 Eii**). During the dark-phase, meal duration did not change with study day, and effects of diet differed between pregnant and non-pregnant groups (diet*pregnancy: $p = 0.017$, **Figure 4.2 Eiii**). Within non-pregnant and within pregnant groups, dark-phase meal duration was shorter in in HFHSD- than SLD-mice (both $p < 0.001$, **Figure 4.2 Eiii**). Within SLD- ($p < 0.001$) and HFHSD ($p =$

0.004) groups, dark-phase meal duration was shorter in pregnant than non-pregnant mice.

4.8.2.6. Meal number

Across 24 h, the number of meals eaten changed between days (p = 0.034), being greater on d 0.5-2.5 compared to acclimatisation for all mice (p = 0.016) and not different between other days (**Figure 4.2 Fi**). During the light-phase, effects of diet differed between study days (diet*day interaction: p = 0.024, **Figure 4.2 Fii**). During acclimatisation, mice fed SLD ate more light-phase meals than mice fed HFHSD (p = 0.007) with no differences between mice subsequently mated and non-mated (**Figure 4.2 Fii**). On d 14.5-16.5, effects of diet differed between pregnant and non-pregnant mice (diet*pregnancy interaction: p = 0.012). HFHSD-mice ate more light-phase meals than SLD-mice within pregnant (p = 0.044) but not non-pregnant groups. Pregnant mice ate more light-phase meals than non-pregnant mice on d 14.5-16.5 within the HFHSD groups (p = 0.006), but not in those fed a SLD. Light-phase meal number was unaffected by diet or pregnancy on other study days (**Figure 4.2 Fii**).

During the dark-phase, effects of pregnancy and diet on dark-phase meal number differed between days (day*pregnancy*diet interaction: p = 0.028, **Figure 4.2 Fiii**), and we therefore analysed effects of diet and pregnancy at each day of the study. No main effects of pregnancy were observed for any period of the study. On d 2.5-6.5, 12.5-14.5 and 16.5-17.5 the HFHSD group consumed more dark-phase meals than the SLD group (each $p < 0.05$, **Figure 4.2 Fiii**). On d 0.5-2.5 and 8.5-10.5, effects of pregnancy differed between diets (pregnancy*diet interaction: each $p < 0.05$). On d 0.5-2.5, dark-phase meal number did not differ between pregnant and non-pregnant mice within SLD groups, but was greater in

NP-HFHSD than P-HFHSD mice (p = 0.001, **Figure 4.2 Fiii**). Within non-pregnant mice, those fed HFHSD ate more meals during the dark-phase (p < 0.001), but meal number did not differ between diet groups within pregnant mice (**Figure 4.2 Fiii**). On d 8.5-10.5, dark-phase meal number did not differ between non-pregnant or pregnant groups within mice fed either SLD or HFHSD. Within non-pregnant mice, mice fed HFHSD ate more meals during the dark-phase than mice fed SLD (p = 0.013), but meal number did not differ between diet groups within pregnant mice (**Figure 4.2 Fiii**).

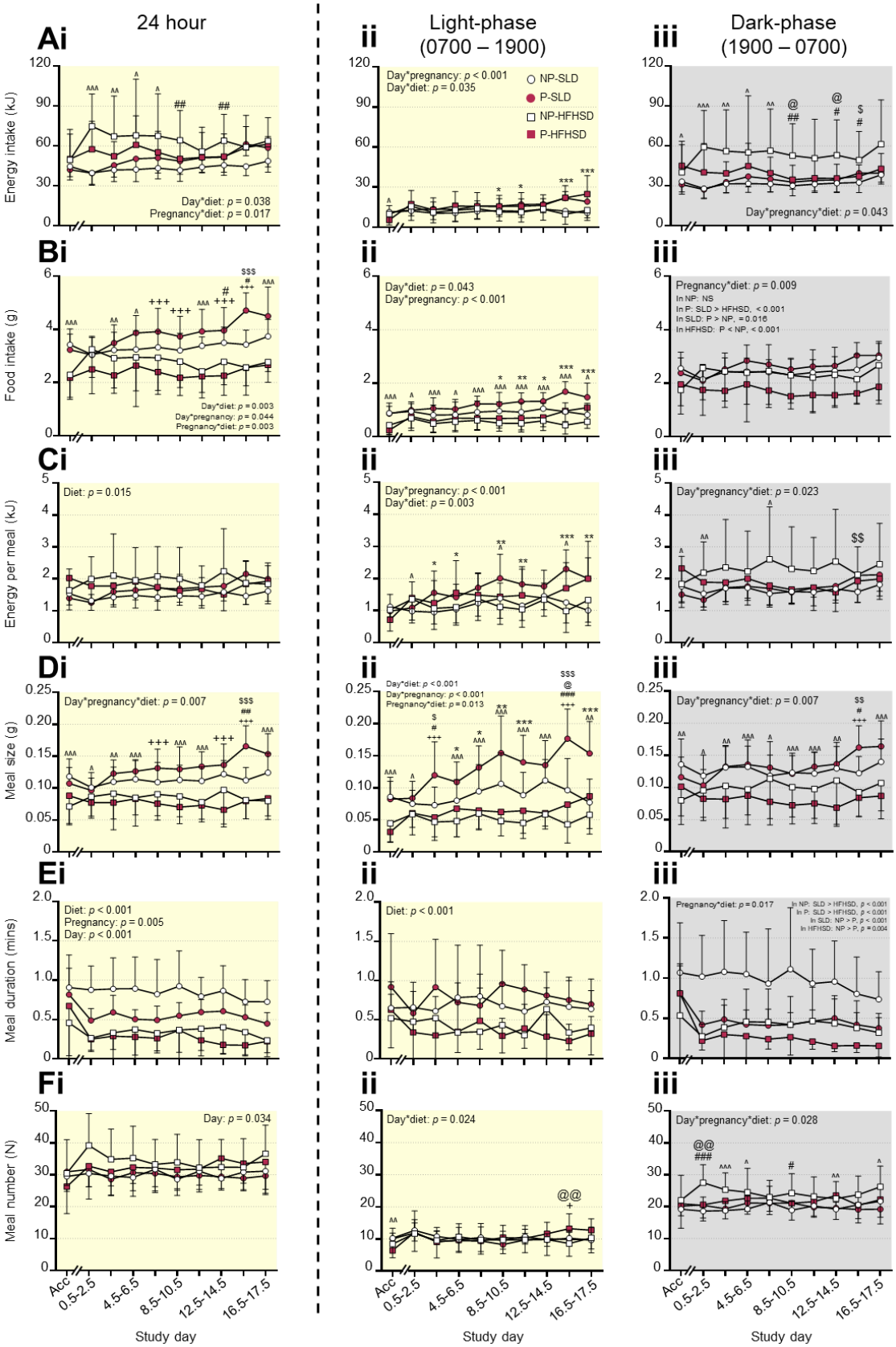
24 hour
Light-phase (0700 – 1900)
Dark-phase (1900 – 0700)
Ai
ii
iii
Energy intake (kJ)
Day*diet: p = 0.038
Pregnancy*diet: p = 0.017
Day*pregnancy: p < 0.001
Day*diet: p = 0.035
NP-SLD
P-SLD
NP-HFHSD
P-HFHSD
Day*pregnancy*diet: p = 0.043
Bi
Food intake (g)
Day*diet: p = 0.003
Day*pregnancy: p = 0.044
Pregnancy*diet: p = 0.003
Day*diet: p = 0.043
Day*pregnancy: p < 0.001
Pregnancy*diet: p = 0.009
In NP: NS
In P: SLD > HFHSD, < 0.001
In SLD: P > NP, = 0.016
In HFHSD: P < NP, < 0.001
Ci
Energy per meal (kJ)
Diet: p = 0.015
Day*pregnancy: p < 0.001
Day*diet: p = 0.003
Day*pregnancy*diet: p = 0.023
Di
Meal size (g)
Day*pregnancy*diet: p = 0.007
Day*diet: p < 0.001
Day*pregnancy: p < 0.001
Pregnancy*diet: p = 0.013
Day*pregnancy*diet: p = 0.007
Ei
Meal duration (mins)
Diet: p < 0.001
Pregnancy: p = 0.005
Day: p < 0.001
Diet: p < 0.001
Pregnancy*diet: p = 0.017
Fi
Meal number (N)
Day: p = 0.034
Day*diet: p = 0.024
Day*pregnancy*diet: p = 0.028
Acc
0.5-2.5
4.5-6.5
8.5-10.5
12.5-14.5
16.5-17.5
Study day

Figure 4.2. Impacts of diet and pregnancy on food intake behaviours.

Food intake behaviours of pregnant (P) and non-pregnant (NP) mice exposed to a standard laboratory diet (SLD, non-pregnant, NP, ○: $N \leq 12$; pregnant; P, ●: $N \leq 12$) and high-fat high-sugar diet (HFHSD, NP, □: $N \leq 16$; P, ■: $N \leq 14$). Total food intake in energy content (kJ, Ai, ii and iii) and grams (Bi, ii and iii), meal size in energy content (kJ, Ci, ii, and iii) and grams (g, Di, ii and iii), meal number (Ei, ii and iii) and meal duration (Fi, ii and iii) across 24 h (i), light-phase (ii) and dark-phase (iii, shaded). Data are presented as mean ± SD, and were analysed using linear mixed models to assess the effect of pregnancy (pregnant vs non-pregnant) and diet (SLD vs HFHSD) with day as a repeated factor. Where a pregnancy*diet*day interaction was significant, mixed repeated models were run separately within non-pregnant and pregnant mice to assess the effects of diet and day, and separately within the SLD and HFHSD mice to assess the effects of pregnancy and day. Where diet*day and/or pregnancy*day interactions were significant, data for each day was analysed separately using a two-way ANOVA to assess effects of pregnancy and diet. Diet effect, ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$. Pregnancy effect, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Within days, where a diet*pregnancy interaction was significant, one-way ANOVAs were used to assess effects of diet within each pregnancy group, and also effects of pregnancy within each diet group. NP-SLD vs P-SLD-, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$; NP-HFHSD vs P-HFHSD, $^{@}$ $p < 0.05$, $^{@@@}$ $p < 0.001$; NP-SLD vs NP-HFHSD, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$; P-SLD vs P-HFHSD, $^{+++}$ $p < 0.001$.

4.8.3. Food intake and meal size at the end of the study

In addition to investigating how feeding behaviours changed across the study, we analysed daily food intake **(Figures 4.3 Ai, ii and iii)** and average meal size in grams **(Figures 4.3 Bi, ii and iii)** during the final two study days separately, as GVA function is expected to correlate with current feeding behaviours and satiation. Across 24 h, the effects of diet on food intake and meal size differed between pregnancy groups (pregnancy*diet interactions, $p = 0.007$ and $p = 0.003$ respectively, **Figure 4.3 Ai and Bi**). We therefore separately analysed effects of diet within each pregnancy group and effects of pregnancy within each diet group for each outcome. Food intake was higher in SLD- than HFHSD-mice within pregnant *(*$p < 0.001$) but not non-pregnant groups (**Figure 4.3 Ai**). Food intake was also higher in pregnant than non-pregnant mice within SLD ($p = 0.003$) but not HFHSD groups (**Figure 4.3 Ai**). Meal size was larger in SLD- than HFHSD-mice within both pregnant *(*$p < 0.001$) and non-pregnant groups ($p = 0.029$, **Figure 4.3 Bi**). Meal size was also higher in pregnant than non-pregnant mice within SLD- ($p = 0.002$) but not HFHSD groups (**Figure 4.3 Bi**).

During the light-phase, food intake was higher in SLD-than HFHSD-mice (diet: $p < 0.001$) and in pregnant than non-pregnant mice (pregnancy: $p < 0.001$), with no interactions (**Figure 4.3 Aii**). Similarly, light-phase meal size was larger in SLD-than HFHSD-mice (diet: $p < 0.001$) and in pregnant than non-pregnant mice (pregnancy: $p < 0.001$), with no interactions (**Figure 4.3 Bii**).

During the dark-phase, the effects of diet on food intake differed with pregnancy status (pregnancy*diet interaction: $p = 0.007$, **Figure 4.3 Aiii**). Dark-phase food intake was higher in SLD-than HFHSD-mice within pregnant *(*$p < 0.001$) but not non-pregnant groups (**Figure 4.3 Aiii**). Dark-phase food intake was higher in non-

pregnant than pregnant mice within HFHSD (p = 0.022) but not SLD groups (**Figure 4.3 Aiii**). During the dark-phase, the effects of diet on meal size also differed with pregnancy status (pregnancy*diet interaction: p = 0.005, **Figure 4.3 Biii**). Dark-phase meal size was higher in SLD- than HFHSD-mice within pregnant (p < 0.001) but not non-pregnant groups (**Figure 4.3 Biii**). Dark-phase food intake was higher in pregnant than non-pregnant mice within SLD-mice (p = 0.016) but did not differ between pregnant and non-pregnant HFHSD groups (**Figure 4.3 Biii**).

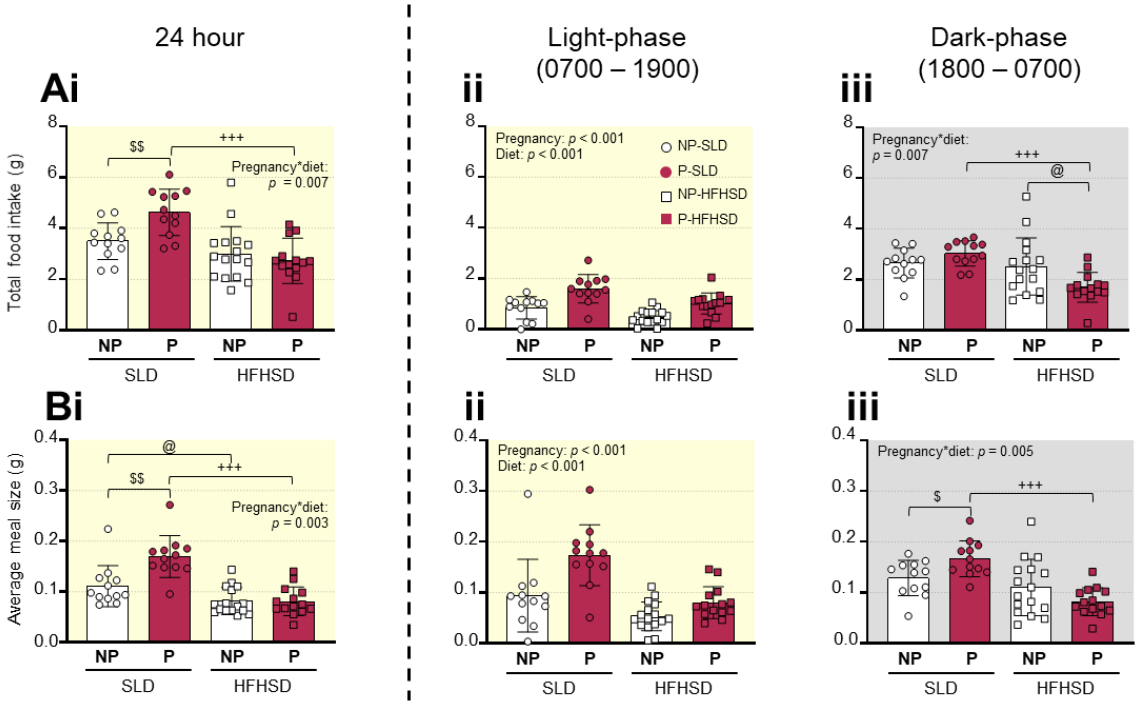

Figure 4.3. Impact of diet and pregnancy on total food intake and average meal size during the last two days of study.

Total food (g, Ai, ii and iii) and average meal size (g, Bi, ii and iii) of pregnant (P) and non-pregnant (NP) mice exposed to a standard laboratory diet (SLD, non-pregnant, NP, ○: $N \leq 12$; pregnant; P, ●: $N \leq 12$) and high-fat high-sugar diet (HFHSD, NP, □: $N \leq 16$; P, ■: $N \leq 14$). Data are presented separately for the entire 24 h, light-phase and dark-phase (shaded). Bars and whiskers show mean ± SD, with data from each mouse indicated by symbols. Data were analysed using a two-way ANOVA. Where diet*pregnancy interactions were significant, one-way ANOVA were used to assess effects of pregnancy within each diet group, and the effects of diet within non-pregnant and pregnant groups. NP-SLD vs P-SLD, $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$; NP-HFHSD vs P-HFHSD, $^{@}$ $p < 0.05$; P-SLD vs P-HFHSD, $^{+++}$ $p < 0.001$.

4.8.4. Impacts of diet and pregnancy on the mechanosensitivity of gastric vagal afferents and correlation with meal size.

The response of mucosal afferents to stroking increased as the von Frey hair weight increased ($p < 0.001$), but was unaffected by pregnancy or diet (both $p > 0.1$, **Figure 4.4 A)**. Responses of GVA mucosal afferents to stroking (200 mg) were not correlated with light-phase meal size within either diet group (**Figure 4.4 C**).

Effects of pregnancy and diet on tension-sensitive GVA mechanosensitivity differed with load (load*pregnancy*diet interaction: $p = 0.003$, **Figure 4.4 B**). At loads of 0 to 2 g, tension-sensitive GVA responses were unaffected by diet or pregnancy. At greater loads, effects of diet on tension-sensitive GVA responses differed between pregnancy groups (pregnancy*diet interactions: each $p < 0.05$, **Figure 4.4 B**). Within non-pregnant groups, tension-sensitive GVA responses to loads of 3 g ($p = 0.039$), 4 g ($p = 0.009$) and 5 g tension ($p = 0.026$) were greater in SLD- than HFHSD-mice. In contrast, tension-sensitive GVA responses did not differ between diet groups within pregnant mice, **Figure 4.4 B**). Within SLD-mice, tension-sensitive GVA responses were greater in non-pregnant than pregnant mice at loads of 3 g ($p = 0.048$), 4 g ($p = 0.030$) and 5 g ($p = 0.022$). In contrast, within HFHSD-mice, tension-sensitive GVA responses did not differ between non-pregnant and pregnant mice (**Figure 4.4 B**). Responses of tension-sensitive GVA to load (5 g) were not correlated with light-phase meal size within either diet group (**Figure 4.4 D**). Representative recordings of tension-sensitive GVA responses in each group at 3 g load are shown in **Figure 4.4 E-H**.

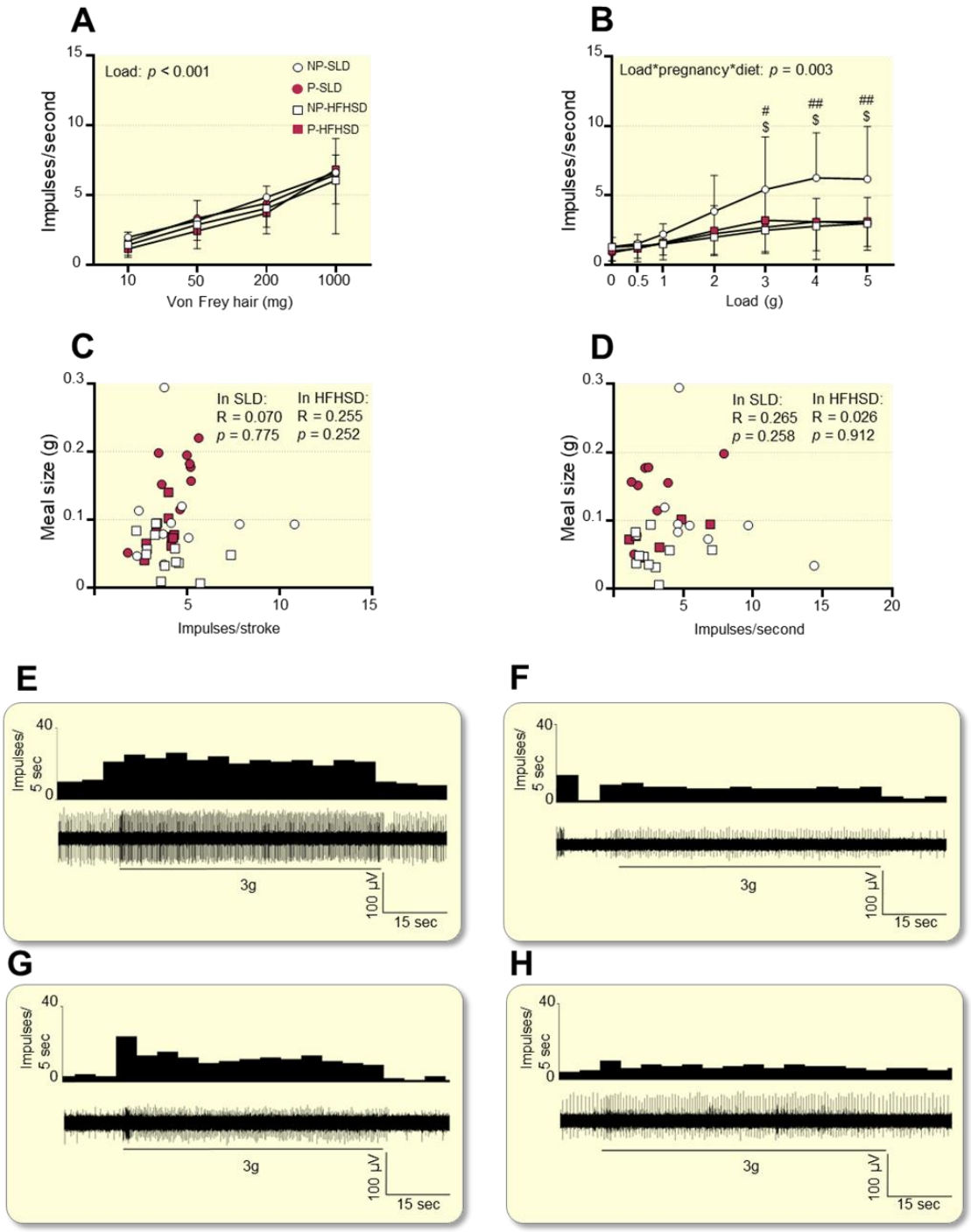
A
Load: p < 0.001
NP-SLD
P-SLD
NP-HFHSD
P-HFHSD
Impulses/second
Von Frey hair (mg)
B
Load*pregnancy*diet: p = 0.003
Impulses/second
Load (g)
C
In SLD: R = 0.070 p = 0.775
In HFHSD: R = 0.255 p = 0.252
Meal size (g)
Impulses/stroke
D
In SLD: R = 0.265 p = 0.258
In HFHSD: R = 0.026 p = 0.912
Meal size (g)
Impulses/second
E
F
G
H
Impulses/ 5 sec
3g
100 µV
15 sec

Figure 4.4. Impact of diet and pregnancy on gastric vagal afferent mechanosensitivity and correlation to meal size.

The response of mucosal gastric vagal afferents (GVAs) to mucosal stroking (10 mg – 1000mg, A) and tension-sensitive GVAs to circular tension (0 g – 5 g, B) in pregnant (P) and non-pregnant (NP) mice fed standard laboratory diet (SLD, non-pregnant, NP, ○: N = 9; pregnant; P, ●: N = 11) or high-fat high-sugar diet (HFHSD, NP, □: N = 10; P, ■: N = 10). Data are presented as mean ± SD and were analysed using a linear mixed model to assess the effect of pregnancy (pregnant vs non-pregnant) and diet (SLD vs HFHSD) with load (circular tension (grams) or von Frey hair (milligrams), respectively) as a repeated factor. Where a pregnancy*diet*load interaction was significant, mixed repeated models were used to assess effects of diet and day separately in non-pregnant and pregnant groups, and effects of pregnancy and day separately within SLD and HFHSD mice. Where diet*load and/or pregnancy*load interactions were significant, a two-way ANOVA was run to assess effects of pregnancy and diet separately for each level of load. Where a diet*pregnancy interaction was significant for a given load, we ran a one-way ANOVA to assess effects of pregnancy within each diet group and of diet within each pregnancy group. NP-SLD vs P-SLD, $^{\$}$ $p < 0.05$; NP-SLD vs NP-HFHSD, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$.

The correlation between the response of (C) mucosal afferents to mucosal stroking with a 200 mg von Frey hair and (D) tension-sensitive GVA to 5 g tension and light-phase meal size. Data were analysed using a Pearson's correlation. Symbols indicate outcomes for individual animals.

Typical response of a tension-sensitive GVA to 3 g load in NP-SLD (C), P-SLD (D), NP-HFHSD (E) and P-HFHSD (F) mice.

4.9. Discussion

The current study investigated the effects of a HFHSD and pregnancy on food intake and GVA signalling. The HFHSD-mice consume more energy across a 24 h period than the SLD-mice up until d 8.5, due to greater dark-phase energy intake. Daily food intake in g was higher in P-SLD than P-HFHSD mice from d 6.5-16.5, predominantly due to greater food intake during the dark-phase. From mid-pregnancy onwards, pregnant mice consumed more food (in g and kJ) than non-pregnant mice during the light-phase due to greater meal size, independent of diet. We also demonstrated that the response of GVAs to stretch was attenuated by pregnancy within mice fed a SLD, and by HFHSD-feeding within non-pregnant mice, but that there was no further reduction in GVA mechanosensitivity during pregnancy within mice fed a HFHSD. A previous report demonstrated dampened GVA responses to stretch and increase in meal size in the setting of lean pregnancy [42], which is consistent with GVA findings and greater meal size in the last two days of the current study in pregnant compared to non-pregnant SLD-mice. Since tension-sensitive GVA mechanosensitivity is reduced by HFHSD-feeding in non-pregnant mice, which would be expected to permit larger meals, the smaller meal sizes of all HFHSD relative to all SLD mice likely involves other satiety mechanisms.

Greater body weight and food intake during pregnancy occurred concurrent with down-regulation of tension-sensitive GVAs in SLD-fed mice.

Maternal body weight increases during pregnancy in humans and rodents [150], and consistent with this, pregnant mice gained more weight than non-pregnant mice in both diet groups. Within the SLD group, mice ate more food (in g) later in pregnancy (d 14.5-16.5) than non-pregnant mice, predominantly due to an increase in light-phase meal size. This is consistent with our previous findings in SLD-mice [42] and occurred without adaptions in meal number [6,42]. Pregnant SLD mice also had a shorter meal duration than NP-SLD mice across the dark-phase compared to non-pregnant SLD mice, as previously reported [42]. On the final two days of study (d 16.5 – 17.5) total food intake and meal size (in g) were greater in pregnant compared to NP-SLD mice. These increases in food intake and meal size are consistent with down-regulation of satiety signals during pregnancy, including the attenuated mechanosensitivity of tension-sensitive GVAs in P-SLD mice in our previous [42] and current study. However, unlike our previous study [42], there was no association between meal size and GVA responses to stretch in the present cohort. This is likely due to a smaller sample size, although the increased meal size during pregnancy may reflect down-regulation of multiple satiety pathways contributing to meal size, including intestinal satiety signals [39,176,192] and central food intake regulatory pathways (e.g. leptin resistance, as reviewed [150]). Dampened tension-sensitive GVA mechanosensitivity during pregnancy is likely driven by changes in hormone levels [150]. For example, growth hormone (GH) increases from early- to mid-pregnancy and then remains elevated during late-pregnancy in mice [115], and *ex vivo* administration of GH decreased the response of murine tension-sensitive GVAs to stretch [42]. Whether GH receptor is

expressed on GVAs is unknown, and the potential role of GH as a mechanism underlying down-regulation of tension-sensitive GVA responses during pregnancy requires further investigation.

Weight gain and energy intake were increased by feeding a high fat, high sugar diet in non-pregnant mice, concurrent with down-regulation of tension-sensitive GVAs.

In the current study, HFHSD-mice gained more weight than SLD-mice during the 12 weeks from weaning, but at slower rates than in previous studies [193] and without reaching heavier weights 12 weeks after diet commencement, although they were heavier than SLD-fed mice throughout most of the final 17 d (pregnancy phase) of the study. This lower weight gain may reflect genotype differences between studies, although Glu Venus mice in the present study were maintained on a C57BL/6 background, an established strain for diet-induced obesity [194]. Others have reported that HFHS diets similar to those used in the current study induce weight gain in rats [195] and mice [193], and that increased weight can be detected from as early as four weeks of feeding in female C57BL/6J mice [193]. Differences in the timing and extent of weight gain between studies could also reflect the age at which the mice started the diet, which was introduced soon after weaning at 3-4 weeks old in the current study compared to 7 weeks old in the previous study in mice [193].

In the current study, 24 h energy intake was ~50% higher in the NP-HFHSD than NP-SLD mice on selected days, predominantly due to an increase in total energy intake and meal number during the dark phase. Consequently, diurnal rhythmicity in feeding patterns were preserved in both diet groups, with HFHSD- and SLD-mice consuming ~75% and ~70% of their diet respectively during the dark phase.

This contrasts with effects of HFD-induced obesity, which dampened diurnal rhythms in food intake in mice, increasing food intake during the light-phase such that these mice only consumed ~60% of their food during the dark phase, while controls ate ~80% of their diet during the dark phase [47,196]. This might reflect different satiating responses to HFD (animal lard based) compared to HFHSD (plant fat based) composition or the differing sugar content, however, it still remains unclear whether fats or carbohydrates are more satiating [197]. Furthermore, the combination of a diet both high in fat and sugar alters appetite, through altering brain function (previously reviewed [198]), however, it is yet to be determined whether these macronutrients have a synergistic effect on satiety responses *in vivo*.

In the current study, a chronic HFHSD also dampened tension-sensitive GVA responses to stretch in non-pregnant mice. This is consistent with the reduced sensitivity of GVAs in female [22] and intestinal [179] VAs in male mice fed a HFD (60% of energy from fat fed as lard). Despite the dampened tension-sensitive GVA signalling in HFHSD-mice in the present study, they ate less food and smaller meals (in g) during the light-phase in the 2 days prior to the electrophysiology experiments, compared to the SLD-mice. Interestingly, effects of diet on food intake were independent of pregnancy status, with lower food intake in HFHSD- than SLD-mice despite down-regulation of GVA responses, suggesting that other satiety mechanisms are limiting overall food intake. For example, there are high levels of fat in the HFHSD and fat is a strong satiety mediator, with signals arising peripherally in the small intestine [40]. Fat-induced release of gut satiety hormones, such as cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1), can then either enter the blood stream to bind to CCK and GLP1 receptors directly in the brain and/or act locally by binding to these

receptors on VA's to reduce food intake [150,175]. In HFD-mice, responses of jejunal VAs to CCK are dampened compared to responses in SLD-mice [179] but this may not be the case with the HFHSD-mice.

Weight gain during pregnancy was unaffected by diet and in HFHSD-mice there was no further down-regulation of GVA responses during pregnancy.

Within the current study, maternal weight gain, litter size, average pup weight and perirenal fat pad weight of pregnant mice were unaffected by diet, while gonadal fat mass was heavier in HFHSD- than SLD-mice regardless of pregnancy status. This contrasts finding from Park *et al*, where HFHSD-fed mice were heavier than SLD-fed mice at d 7, 14 and 20 of pregnancy, with greater fat mass and lower lean mass as measured by EchoMRI in late-pregnancy (d 16) [193]. Greater weight gain reported by Park *et al* [193] could reflect average litter size/weight or more acute effects of a HFHSD on food intake, since female mice were on the diet for 6 weeks before mating compared to 12 weeks in the current study.

There were no differences in 24 h energy intake between P-HFHSD and NP-HFHSD mice across the 17.5 d of pregnancy in the current study. In addition, there was no further reduction in tension-sensitive GVA mechanosensitivity during pregnancy in the HFHSD-mice. This is consistent with no effect of pregnancy on meal size during the light-phase in the HFHSD groups, 2 days prior to the electrophysiology recordings. However, food intake during the dark-phase in the final 2 study days was significantly reduced in P-HFHSD compared to NP-HFHSD mice, which may reflect other pregnancy-related adaptations. GVAs exhibit circadian rhythmicity, with the mechanosensitivity of tension-sensitive GVAs exhibiting the greatest responsiveness during the light-phase, aligning with

lower energy demands and reduced food intake [107]. These rhythms are lost in HFD-induced obese mice, due to attenuated mechanosensitivity of tension-sensitive GVAs during the light-phase compared to SLD controls [47]. Recently, we have shown an increase in light-phase food intake during pregnancy, predominantly due to an increase in food intake bouts occurring late in the light-phase (Zeitgeber time 8-12) [199]. Since tissue was collected early in the light-phase for GVA recordings in the current study, it is possible that adaptations in P-HFHSD mice were not captured and therefore future research should characterise daily variation in GVA sensitivity in response to a HFHSD and pregnancy. Lastly, the stage of pregnancy at which pregnancy-adaptations in GVA mechanosensitivity are oollost is unknown. We therefore suggest that future research should measure GVA sensitivity across early-, mid- and late-pregnancy in HFHSD-mice, similar to our prior study using SLD-mice [42].

Responses of mucosal gastric vagal afferents are not changed during pregnancy or by diet.

The response of mucosal afferents to mucosal stroking was unchanged during pregnancy or by a HFHSD, consistent with our prior findings in P-SLD compared to NP-SLD mice [42] and the similar mucosal GVA responses in non-pregnant female HFD and SLD mice [22]. Mucosal afferents are located within the gastric mucosa and are thought to modulate gastric emptying through discrimination of particle size [103]. Reported changes in rates of gastric emptying during pregnancy are inconsistent. Studies have reported no change in humans [48,131-133] or slower gastric emptying in both humans [134] and rats [135]. Similar controversies exist in relation to effects of obesity on gastric emptying, with no difference in gastric emptying rates between obese and lean individuals [200,201], and increased [202] or decreased [110] gastric emptying in obese compared to lean mice. Even less is

understood about the dual effects of obesity and pregnancy; in one study, gastric emptying of water was similar in obese compared to lean late-pregnant women [203]. Overall, if mucosal afferents do play a major role in gastric emptying, it appears that pregnancy and obesity may not alter this response, however, this requires further investigation.

4.10. Conclusion

In conclusion, a HFHSD reduces tension-sensitive GVA responses to stretch in non-pregnant mice, similar to observations in HFD-induced obesity. However, meal size (g) is reduced rather than increased in HFHSD-fed mice, suggesting other satiety mechanisms are contributing to food intake behaviour. This study also confirmed previous findings that mechanosensitivity of tension-sensitive GVAs is selectively attenuated during a lean murine pregnancy, with concurrent increases in total food intake and meal size [42]. Although there is a reduction in the mechanosensitivity of tension-sensitive GVAs in HFHSD-fed compared to SLD-fed non-pregnant mice, there was no further reduction in mechanosensitivity of tension-sensitive GVAs in P-HFHSD compared to NP-HFHSD mice. Further studies are required to increase understanding of food intake

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTION:

L.M.N. established and provided access to the experimental model of HFHSD-feeding during pregnancy in the Glu Venus mice. All authors contributed to experimental design. G.C. and H.L. conducted electrophysiology. G.C. analysed data and wrote the manuscript. All authors contributed to interpretation of data and editing of the manuscript.

CHAPTER 5: Circadian patterns of behaviour change during pregnancy in mice

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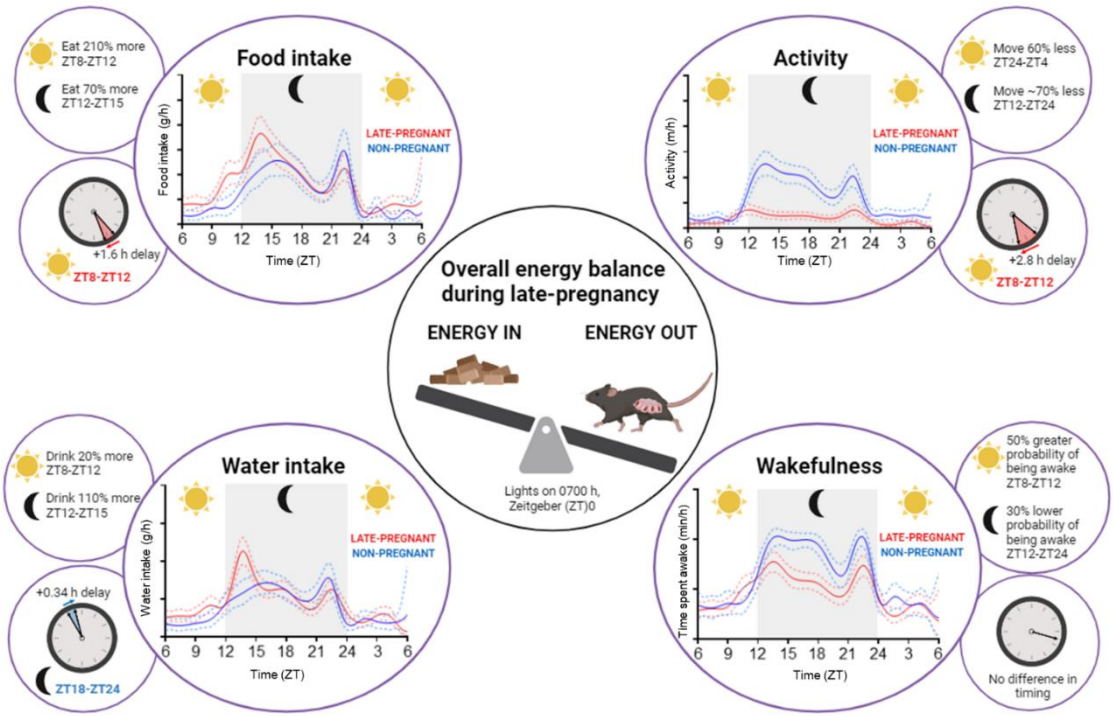
5.1. Graphical abstract



5.2. Overview

The pregnant mice utilised in this chapter are the same as in **Chapters 2 and 3**. This chapter reports circadian patterns of food and water intake, activity and wakefulness across each week of pregnancy. **Chapter 2** reports on daily food intake during pregnancy, where the data is separated into 24 h, light- and dark-phase consumption across each 2-day block days. **Chapter 5** differs from this analysis approach, as it focusses on the timing of food intake and the amount of food consumed each hour, analysed across each study week in pregnant and non-pregnant mice. The work in this chapter aimed to determine whether the timing of the peak behavioural events are altered during pregnancy. I contributed to the extraction of the raw data, which was then modelled by statistician Andrew Vincent to describe behavioral rhythms and timing. I wrote the manuscript, including preparation of figures and tables.

This work below has been submitted to the *Journal of Physiology*, and has therefore been reproduced in the thesis as submitted, except for formatting.

5.3. Authorship Document

Statement of Authorship

Title of Paper	Pregnancy induces altered circadian patterns of behaviour in mice.
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Publication to be submitted to: The journal of physiology, circadian timing of behaviour and biology (special call).

Principal Author

Name of Principal Author (Candidate)	Miss Georgia Clarke		
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Prepared figures. Edited and revised manuscript. Accepted final version of manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/8/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Andrew Vincent		
Contribution to the Paper	Analysed data. Wrote statistical methods. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	22/8/23

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Contribution to the Paper	Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	22/8/23

Name of Co-Author	A/Prof Kathryn Gatford		
Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript		
Signature		Date	22/8/23

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Contribution to the Paper	Conception and design of research, performed experiments. Interpreted results of experiments. Edited and revised manuscript. Accepted final version of manuscript.		
Signature		Date	22/8/23

5.4. Highlights

- Circadian rhythms synchronise daily behaviours including eating, drinking and sleep, but how these change in pregnancy is unclear.
- Food intake increased, with delays in peaks of food intake behaviour late in the light phase from the second week of pregnancy, compared to non-pregnant group. Activity decreased by ~70% in pregnant group, particularly in the dark (active) phase, with delays in peaks of wakefulness evident from the first week of pregnancy onwards.
- These behavioural changes contribute to positive energy balance during pregnancy.
- Delays in circadian behaviours during mouse pregnancy were time-period and pregnancy stage specific, implying different regulatory mechanisms.

5.5. Abstract

Food intake and activity are altered in pregnancy to meet the increased energy demands of fetal and placental growth, and to deposit energy reserves for lactation. Compared to non-pregnant females, pregnant mice consume more food, eating larger meals during the light-phase, and reduce physical activity. How pregnancy changes circadian timing of behaviour was less clear. We therefore randomised female C57BL/6J mice to mating for study until early- (N = 10), mid- (N = 10) or late-pregnancy (N = 11), or as age-matched, non-pregnant controls (N = 12). Mice were housed individually in Promethion cages under a 12 h light: 12 h dark cycle (lights on 0700 h, Zeitgeber (ZT) 0) for behavioural analysis. Food intake between ZT10 and ZT11 was greater in pregnant than non-pregnant mice in weeks 2 and 3. In mice that exhibited a peak in the last 4 h of the light-phase (ZT8-ZT12), peaks were delayed by 1.6 h in pregnant compared to non-pregnant group. Food intake just after dark-phase onset (ZT13-ZT14), was greater in pregnant than non-pregnant group during week 3. Water intake patterns corresponded to food intake. From week 1 onward, the pregnant group moved less during the dark-phase, with decreased probability of being awake, compared to non-pregnant group. Peaks in activity and wakefulness were also delayed during pregnancy. In conclusion, increased food intake during pregnancy reflects increased amplitude of eating behaviour, without longer duration. Decreases in activity also contribute to positive energy balance in pregnancy, with delays to all measured behaviours evident from mid-pregnancy onwards.

5.6. Introduction

Pregnancy demands an increased energy supply to support fetal and placental development, deposit energy reserves for lactation and support maternal physiological adaptations [150]. Dietary intake increases by around 10% in the third trimester in women and by ~20-30% in late pregnancy in mice and rats (reviewed in [150]). Physical activity tends to be lower in the third trimester of human pregnancy [5] and is dramatically reduced across the entire duration of pregnancy in mice [6], also increasing the availability of energy. An important component of energy balance regulation is its strong circadian rhythmicity, enabling the coordination of behaviours including food intake, energy expenditure and sleep.

The circadian system is comprised of a series of circadian clocks that exist as a hierarchy. The superchiasmatic nucleus of the anterior hypothalamus is entrained by light and acts as the “master” clock to entrain other tissues, whilst beneath this, peripheral tissues including the gastrointestinal tract contain clock mechanisms to maintain local rhythmicity [126]. These molecular clocks generate circadian rhythms, enabling the daily repeating or synchronisation of events in response to the light: dark cycle and to feeding activity during the “active” period [126]. These events ultimately regulate physiological functions, optimising energy homeostasis relative to the current environmental demand. Circadian rhythms are important during pregnancy with disruption of these rhythms increasing the risk of miscarriage, preterm birth and intrauterine growth restriction in women [204], as well as reducing implantation rates and impairing placental development and fetal growth in rodents [205]. Disruption of circadian rhythms during pregnancy also adversely impacts progeny health, with impaired neurobehavioral and cognitive outcomes as well as poorer metabolic health reported in animal models of maternal circadian disruption [206].

Although the importance of circadian rhythms for pregnancy is appreciated, how pregnancy itself affects circadian rhythms of behaviour and physiological functions is unclear, due to limited and contradictory evidence. For example, Martin-Fairey *et al.* [207] reported up to a 4 h advancement in the onset of running wheel activity between gestational day 3 and d 10 in pregnant compared to non-pregnant mice, while Yaw *et al.* [208] reported a delayed onset of running wheel activity between d 8 and d 13. In both studies, the timing of running wheel activity returned to pre-pregnancy patterns by late pregnancy [207,208]. Consistent with their results in mice, Martin-Fairey *et al.* [207] reported advanced sleep onset by 24 minutes in the first and 18 minutes in the second trimester of human pregnancy. Altered circadian regulation is also evident in the observed changes in feeding behaviours during pregnancy. We have reported that mid- and late-pregnant mice eat more than non-pregnant mice during the light-phase, in association with increased meal size [42]. In rats, food and water intake during the dark-phase is greater in pregnant than non-pregnant females [209]. During rat pregnancy, maximum food intake occurs within a shorter feeding window (between 11 and 15 hours after lights on, or Zeitgeber (ZT) 11 – 15 cf. ZT 11 – 19) and follows a bimodal rather than unimodal pattern [209]. However, circadian patterns of sleep/wake and water intake behaviours have not been reported in mice. Furthermore, it is unknown whether the circadian window of feeding is altered during pregnancy in mice. To address some of these gaps in knowledge, we assessed the circadian rhythm of food and water intake, activity and wakefulness across healthy pregnancy compared to non-pregnant control mice.

5.7. Methods

5.7.1. Ethical approval

All experimental procedures were approved by the South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee (SAM395.19) and were conducted in compliance with the Australian code for the care and use of animals for scientific purposes, 8th edition 2013. We also confirm that we understand the Journals of Physiology's ethical principles and we comply with the checklists given to authors [159,210].

5.7.2. Animals and experimental design

Housing, nutrition and mating of mice has been described previously [42]. Briefly, adult female C57BL/6 mice (10-12 weeks, 18-22 g) were exposed to a 12 h light/dark cycle (lights on 0700 h; ZT 0) and fed a standard chow diet *ad libitum*. All mice were single-housed in metabolic cages (Promethion Sable System; Las Vegas, USA) and acclimatised for 7 days. Following this period, mice were randomised using a simple table method to be either mated with a stud male to generate pregnancies (N=31) or unmated for study as age-matched, non-pregnant controls (N=12). Mice with vaginal plugs indicative of mating and non-pregnant mice were then placed back into metabolic cages and data was collected until mice were terminated at various time points for use in a previously published study of gastric vagal afferent function [42]. Pregnant mice were anaesthetised by isoflurane inhalation (at 5% in oxygen) before humane killing via decapitation at early-pregnancy (6.5 days after mating, N=10), mid-pregnancy (12.5 days after mating, N=10) or late pregnancy (17.5 days after mating, N=11). Pregnant mice were randomised using a block method, avoiding weekends and with no more than two mice killed each day, to permit electrophysiological studies

previously reported [42]. Non-pregnant mice were killed on age-matched days (N= 4 early, N=2 mid, N=6 late) and randomised using the same process. The mice were monitored daily and displayed a behavioural phenotype consistent with reports in other healthy pregnancy studies. This includes a significant increase in maternal body weight by day 7, increases in food intake during mid-pregnancy, primarily due to meal size and duration rather than meal number [6,42], and a dramatic reduction in physical activity after mating [6]. Fetal number was counted in all pregnancies at termination to ensure fetal number was within the expected range. For analysis, behavioural data was utilised for all available non-pregnant or pregnant mice at each day after the start of the study.

Mice that were mated and showed vaginal plugs but did not become pregnant (N=6) were excluded from the study and not included in the final group numbers. These mice were not added to the non-pregnant group, to avoid potential impacts of elevated prolactin during pseudo-pregnancy [116]. One non-pregnant mouse was excluded due to an infection at the time of tissue collection and was not included in the final non-pregnant group number. The sample size was calculated based on variation in gastric vagal afferent function, which was the primary outcome in the previous study where we collected the detailed data on behaviours of these mice [42].

5.6.3. Metabolic monitoring and data preparation

The following outcomes were recorded by the metabolic cage system: food intake (reduction in hopper weight, intake < 0.002 g excluded), water consumption (reduction in hopper weight), activity (sum of all distances and including fine movement such as grooming and scratching) and sleep (defined as stillness lasting ≥ 40 second, converted to wakefulness and modelled as % of time spent

sleeping). Metabolic data was recorded continuously at 1-second intervals throughout the study. Raw data was collected by Sablescreens (Promethion Sable System; Las Vegas, USA), extracted using ExpeData V. 1.9314 (Promethion Sable System; Las Vegas, USA) and Macro Interpreter V. 2.44 (Promethion Sable System; Las Vegas, USA). Food and water intake and activity data was analysed using the Universal Macro Collection 10.1.1.2 (macro 13) and sleep data was analysed using OneClickMacro 5 minute intervals V2.50.4.4 (macro 13). These macros provided food and water intake, activity and wake data for each individual mouse averaged across 5 minute blocks. Data was then averaged within each mouse for each hour of study before circadian analysis. Data for each outcome was analysed for each week of the study, corresponding to thirds of mouse pregnancy in the pregnant group: week 1: from day 0.5 to day 6.5; week 2 from day 6.5 to day 12.5; and week 3 from day 12.5 to day 17.5 of the study. There were 12, 8 and 6 non-pregnant and 31, 21 and 11 pregnant mice with data for weeks 1, 2 and 3 respectively. All data points were utilised for each animal.

5.6.4. Statistical methods

Raw data for food and water intake, activity and time spent awake is presented in mean ± SD for each mouse, averaged across each week of study within non-pregnant and pregnant groups. Due to data distributions we modelled the fraction of time awake/asleep (range 0-1) with a beta distribution. For the other three outcomes (range ≥ 0) analyses on the original scale produced residual distributions that were clearly not normally distributed, a square root transformation was used to resolve this problem. Square-rooted outcomes were back transformed as the square of the square root mean plus the square root

variance. To capture the rapid changes in mean levels over the time period, natural splines were employed with 13 knots evenly spaced from ZT 8.5 to ZT 4.5.

A two-stage approach was used to determine effects of pregnancy within each week of the study. To compare differences in peak time and location between treatment groups (pregnancy vs non-pregnancy) requires a complex model that allows rapid variation in behaviour and peak location. The traditional two-way ANOVA (time x group) analysis does not allow for assessment of the timing of peak behaviour. Therefore, we explored models of behaviour change that allowed activity to vary continuously over time using splines and aimed to capture the variation in behaviour observed in the raw data. This model is a not mechanistic model, but rather an exploratory model and allowed us to identify differences in behaviour between groups and over time (days-since-pregnant (DSP)). Initially we explored three way interaction models of time (spline hr) x group x linear (DSP). However it became apparent that non-linear modelling for the third component (DSP) would be required. The three-way interaction: spline (time, hr) x group x spline (DSP) had too many parameters, and simpler models using polynomials for the third term resulted in poor estimation at the extremes (day 0 and day 17). Hence, we present the simpler model with days-since-pregnancy discretised into three categories. These time points represent stages of developmental progression including implantation of the blastocyst and placental development such that, day 5-8: blastocyst implantation site grows, day 10-11: definite placenta structure present and day 15-17: placenta at maximum size [191]. From the acclimatisation period was modelled to generate within-individual estimates (on the logit scale for wake/sleep model and square-root scale for activity, food and water intake models) for each mouse. These models

included the natural splines (13 knots) with interaction with pregnancy group, a random intercept per mouse, and were estimated using the R package *glmmTMB* [211].

Secondly, similar multi-level models were applied in order to compare pregnant and non-pregnant mice. These models included the same natural splines (13 knots) with a three-way interaction with pregnancy status and study week as fixed effects and nested random intercepts per week within mouse. Individual variation in behaviour was corrected for by inclusion of the within-individual acclimatisation period estimates of each behaviour across time. Bayesian STAN [212] model code was generated using package *brms* [213] and estimated using the package *rstan* [214]. The non-informative priors recommended by *brms* were employed. Each model was estimated using three chains with 10k iterations, half used for burn-in and thinned by a quarter. We note that the differences in the timing of peak activity could have been analysed using bootstrapped multilevel mixed effects models, however model convergence is often a problem for inference via likelihood, hence our choice to use the Bayesian Monte Carlo methodology.

Visual inspection of the raw and modelled data identified five time periods where differing local behaviour occurred: Period I: ZT8 to ZT12 (late light-phase), II: ZT12 to ZT15 (early dark-phase), III: ZT15 to ZT18 (mid dark-phase), IV: ZT18 to ZT24 (late dark-phase) and V: ZT24 to ZT4 (early light-phase). For each behaviour outcome within each time period we assessed whether a peak in behaviour occurred (local maximum), and peak time and amplitude were extracted for detected peaks. Mean and 2.5 and 97.5 quantiles across the Bayesian model iterations are reported for point and interval estimates for each group (non-pregnant and pregnant) and by week of the study. Peak time and

location were considered statistically significant if the credible interval did not cross over 0.

5.7. Results

5.7.1. Mouse phenotype

As previously reported, there was no initial difference in the starting body weight of mice subsequently allocated to pregnant and non-pregnant groups [42]. From day 7.5 of the study (all $P < 0.05$), pregnant mice were heavier than non-pregnant mice [42]. All mice had between 7 and 11 fetuses, which was within our expectation of what would be seen in a healthy pregnancy, with the exception of one mouse having 4 fetuses. Fetal number was similar (P = 0.085) between mice culled at early- (9.22 ± 0.67 pups), mid- (8.60 ± 1.17 pups) and late-pregnancy (7.36 ± 2.73 pups) [192].

5.7.2. Effects of pregnancy on food intake

Modelled food intake patterns corresponded to raw data in each week of the study (**Figures 5.1 A-5.1 C**). Modelled food intake was similar in non-pregnant and pregnant mice at all time points in the first week of the study (**Figure 5.1 D**). In the second week of study, modelled food intake was greater in pregnant than non-pregnant mice only between ZT10 and ZT11 (**Figure 5.1 E**). In the final week, modelled food intake was greater in pregnant than non-pregnant mice between ZT10 and ZT11 and also between ZT13 and ZT14 (**Figure 5.1 F**).

Week 1: Within the first time period of interest (ZT8-ZT12), 57% of non-pregnant and 33% of the pregnant group (model iterations) exhibited a peak in food intake (**Table 5.1**). The timing and amplitude of peaks in food intake, for those animals that exhibited a peak, was similar in the non-pregnant and pregnant group (**Table 5.1**). In the second time period of interest (ZT12-ZT15), 65% of non-pregnant and

99% of the pregnant group exhibited a peak (**Table 5.1**). Within this time period, food intake was 1.3-fold higher in pregnant than the non-pregnant group, but peak timing was similar between groups. In later time periods (ZT15-ZT18, ZT18-ZT24 and ZT24-ZT4), 89%, 99% and 99% of the non-pregnant group and 25%, 100% and 99% of the pregnant group respectively, exhibited peaks, and peak timing and amplitude did not differ between non-pregnant and pregnant groups (**Table 5.1**).

Week 2: Within the first time period of interest (ZT8-ZT12), 85% of non-pregnant and 36% of the pregnant group exhibited peaks in food intake (**Table 5.1**). Food intake in the pregnant group was 2.0-fold greater and the timing was 1.62 h later in pregnant than the non-pregnant group. In the second time period of interest (ZT12-ZT15), 72% of non-pregnant and 100% of the pregnant group exhibited peaks, and again food intake was 1.4-fold greater in pregnant than the non-pregnant group, although peak timing was similar between groups (**Table 5.1**). In the third, fourth and fifth time periods of interest (ZT15-ZT18, ZT18-ZT24 and ZT24-ZT4), 74%, 99% and 100% of the non-pregnant group, and 35%, 100% and 92% of the pregnant group, respectively, exhibited peaks and there was no difference in peak timing or amplitude between groups (**Table 5.1**).

Week 3: Within the first time period of interest (ZT8-ZT12), 66% of non-pregnant and 57% of the pregnant group exhibited a peak in food intake (**Table 5.1**). Within the mice that exhibited this peak, food intake was 3.10-fold greater and the timing was 1.60 h later in pregnant than non-pregnant mice. In the second time period of interest (ZT12-ZT15), 48% of non-pregnant and 99% of the pregnant group exhibited peaks (**Table 5.1**). Food intake at this peak was 1.7-fold greater during pregnancy, whilst peak timing was similar in the pregnant and non-pregnant groups. In the third, fourth and fifth time periods of interest (ZT15-ZT18, ZT18-

ZT24 and ZT24-ZT4), 80%, 100% and 95% of non-pregnant group, and 5%, 100% and 79% of the pregnant group, respectively, exhibited peaks (**Table 5.1**). For each time period, within the mice with peaks, there were no differences between the pregnant and non-pregnant groups in the timing or amplitude of these peaks (**Table 5.1**).

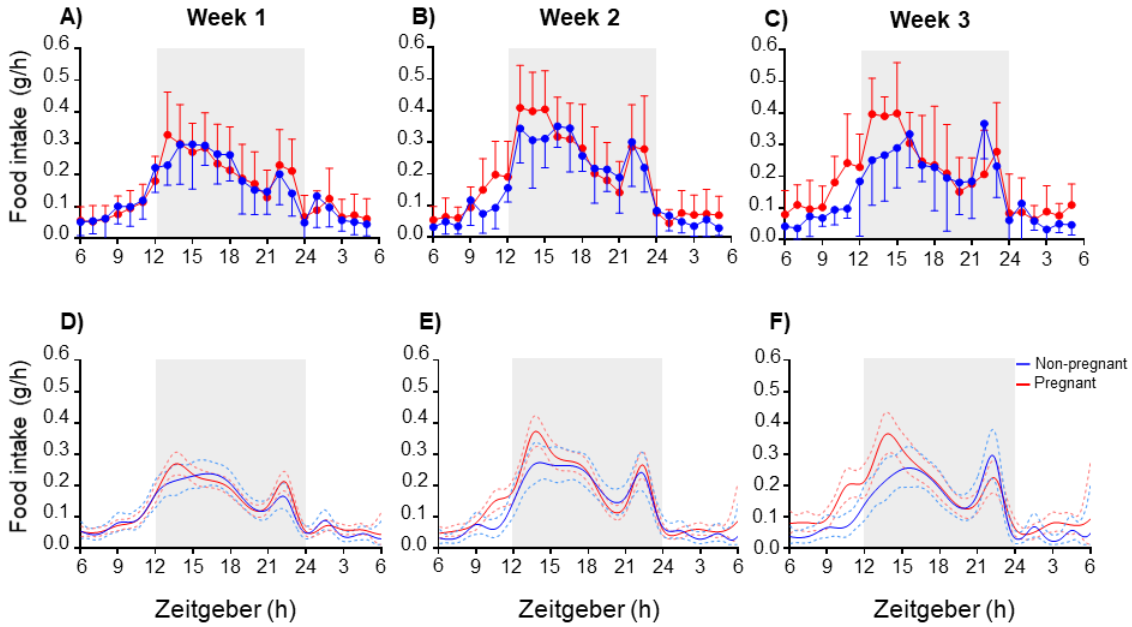


Figure 5.1. Food intake pattern of non-pregnant and pregnant mice for each week of study.

Food consumption (A-C) is the mean ± SD of raw data for each mouse, averaged across each week of study within non-pregnant (blue line, N ≤ 12) and pregnant (red line) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (A, day 0.5-6.5, N = 31), week 2 (B, day 6.5-12.5, N = 21) and week 3 (C, day 12.5-17.5, N = 11). The fitted model of food consumption (D-F) across each week of study indicates fitted means (solid lines) and 95% credible intervals (dashed lines) for all non-pregnant (blue) and pregnant (red) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (D, day 0.5-6.5), week 2 (E, day 6.5-12.5) and week 3 (F, day 12.5-17.5).

Table 5.1: **The effect of pregnancy on timing and amplitudes of peaks in food intake.**

Time periods of interest	Study Week	Percent of iterations per group with detected peak (%)		Peak characteristics for mice with detected peak					
				Peak Time (ZT) [95% CrI]			**Peak Amplitude (g/ h) [95% CrI]**		
		Non-pregnant	**Pregnant**	**Non-pregnant**	**Pregnant**	**Difference**	**Non-pregnant**	**Pregnant**	**Difference**
I (ZT8-ZT12)	1	57.1	33.1	9.22 [8.79, 9.81]	9.18 [8.52, 9.58]	-0.05 [-0.90, 0.58]	0.083 [0.057, 0.115]	0.074 [0.058, 0.092]	-0.009 [-0.044, 0.023]
	2	85.5	36.0	9.12 [8.58, 9.87]	10.74 [10.47, 11.05]	**1.62 [0.70, 2.22]**	0.077 [0.046, 0.115]	0.156 [0.126, 0.189]	**0.079 [0.030, 0.124]**
	3	66.1	57.2	9.20 [8.30, 10.73]	10.79 [10.52, 11.16]	**1.60 [0.07, 2.47]**	0.067 [0.036, 0.106]	0.205 [0.159, 0.256]	**0.138 [0.077, 0.197]**
II (ZT12-ZT15)	1	65.1	99.1	13.31 [12.27, 14.64]	13.74 [13.41, 14.29]	0.43` [-0.94, 1.62]	0.212 [0.169, 0.260]	0.270 [0.234, 0.307]	**0.058 [0.000, 0.114]**
	2	71.9	100	13.89 [12.74, 14.79]	13.83 [13.65, 14.11]	-0.06 [-0.97, 1.11]	0.273 [0.212, 0.337]	0.373 [0.325, 0.0423]	**0.101 [0.018, 0.179]**
	3	48.1	99.3	13.60 [12.23, 14.94]	13.93 [13.59, 14.67]	0.32 [-1.13, 1.88]	0.214 [0.152, 0.283]	0.365 [0.305, 0.431]	**0.152 [0.058, 0.244]**
III (ZT15-ZT18)	1	89.0	25.4	16.38 [15.21, 17.48]	16.88 [15.32, 17.44]	0.50 [-1.16, 1.87]	0.238 [0.201, 0.278]	0.213 [0.190, 0.236]	-0.026 [-0.071, 0.017]
	2	73.7	35.3	16.64 [15.15, 17.69]	16.98 [16.46, 17.40]	0.34 [-0.90, 1.91]	0.263 [0.218, 0.0311]	0.274 [0.244, 0.306]	0.011 [-0.047, 0.067]
	3	80.6	5.3	15.99 [15.10, 17.41]	16.80 [15.04, 17.58]	0.81 [-1.22, 2.19]	0.254 [0.196, 0.318]	0.244 [0.205, 0.284]	-0.010 [-0.087, 0.060]
IV (ZT18-ZT24)	1	99.3	100	22.12 [21.76, 22.32]	22.24 [22.14, 22.33]	0.11 [-0.11, 0.48]	0.167 [0.127, 0.208]	0.213 [0.183, 0.245]	0.047 [-0.003, 0.096]
	2	99.9	100	22.23 [21.95, 22.41]	22.35 [22.26, 22.42]	0.12 [-0.08, 0.40]	0.242 [0.184, 0.306]	0.266 [0.255, 0.311]	0.024 [-0.053, 0.098]
	3	100	100	22.16 [21.95, 22.60]	22.27 [22.05, 22.42]	0.10 [-0.15, 0.35]	0.297 [0.223, 0.379]	0.225 [0.177, 0.282]	-0.071 [-0.167, 0.023]
V (ZT24-ZT4)	1	99.7	99.4	1.53 [1.31, 1.79]	1.90 [1.53, 2.87]	0.37 [-0.12, 1.37]	0.089 [0.060, 0.123]	0.073 [0.058, 0.090]	-0.016 [-0.052, 0.017]
	2	69.9	92.4	1.56 [1.03, 3.65]	2.59 [1.60, 3.73]	1.02 [-1.08, 2.36]	0.055 [0.031, 0.086]	0.060 [0.043, 0.080]	0.005 [-0.030, 0.037]
	3	99.5	79.0	1.55 [1.33, 1.88]	2.96 [1.56, 3.91]	1.41 [-0.02, 2.38]	0.069 [0.036, 0.112]	0.081 [0.052, 0.116]	0.012 [-0.039, 0.060]

CrI, 95% credible interval; ZT, Zeitgeber; Study week = week 1 (days 0.5-6.5, N = 31), week 2 (days 6.5-12.5, N = 21), week 3 (days 12.5-17.5, N = 11). Non-pregnant mice, N ≤ 12. Intervals of group differences that exclude 0 are highlighted with bold text. Positive values present delayed timing or increased amplitude. Negative values present advanced timing or decreased amplitude.

5.7.3. Effects of pregnancy on water intake

Modelled water intake patterns corresponded to raw data in each week of the study (Figures **5.2 A-5.2 C**). Modelled water intake was similar in non-pregnant and pregnant mice at all time points in the first and second weeks of the study (**Figure 4.2 D, E).** In the final week, modelled water intake was greater in pregnant than non-pregnant mice between ZT13 and ZT14 (**Figure 5.2 F**).

Week 1: Within the first, second, fourth and fifth time periods of interest (ZT8-ZT12, ZT12-ZT15, ZT18-ZT24 and ZT24-ZT4) 64%, 57%, 100% and 100% of the non-pregnant group and 26%, 99%, 100% and 100% of pregnant group respectively, exhibited peaks in water intake (**Table 5.2**). Within mice with a peak in these time periods there were no differences in the timing or amplitude of water intake peaks between the pregnant and non-pregnant group (**Table 5.2**). In the third time period of interest (ZT15-ZT18), 98% of non-pregnant and 92% of the pregnant group exhibited a peak in water intake, with similar timing in both groups and with a 0.2-fold lower peak amplitude in pregnant than the non-pregnant group (**Table 5.2**).

Week 2: Within the first time period of interest (ZT8-ZT12), 72% of non-pregnant and 60% of the pregnant group exhibited peaks in water intake (**Table 5.2**). Within mice exhibiting a peak, the peak occurred 1.36 h later in pregnant than non-pregnant group, with similar amplitude in both groups (**Table 5.2**). In the third time period of interest (ZT15-ZT18), 98% of non-pregnant and 47% of the pregnant group exhibited a peak, with a 0.2-fold lower peak amplitude in pregnant than non-pregnant group, and no difference in peak timing (**Table 5.2**). In the second, fourth and fifth time periods of interest (ZT12-ZT15, ZT18-ZT24 and ZT24-ZT4), 69%, 100% and 97% of non-pregnant group and 99%, 100%, 84% of pregnant

group respectively, exhibited peaks, with no differences in peak amplitude or timing between the pregnant and non-pregnant groups for these peaks (**Table 5.2**).

Week 3: Within the first and second time period of interest (ZT8-ZT12 and ZT12-ZT15), 39% and 43% of non-pregnant and 86% and 100% of pregnant group exhibited a peak in water intake (**Table 5.2**). Within mice exhibiting these peaks, the amplitude of the water intake peak was 2.3-fold and 2.1-fold greater, respectively, in pregnant than the non-pregnant group, and peak timing was similar between groups (**Table 5.2**). In the third and fifth time periods of interest (ZT15-ZT18 and ZT24-ZT4), 91% and 97% of the non-pregnant group and 73% and 85% of the pregnant group respectively, exhibited peaks (**Table 5.2**), and peak timing and amplitude were similar in pregnant and non-pregnant groups. In the fourth time period of interest (ZT18-ZT24), all non-pregnant and pregnant groups exhibited a peak (**Table 5.2**). This peak in water intake occurred 0.34 h later in pregnant than the non-pregnant group, and peak amplitude was similar between groups.

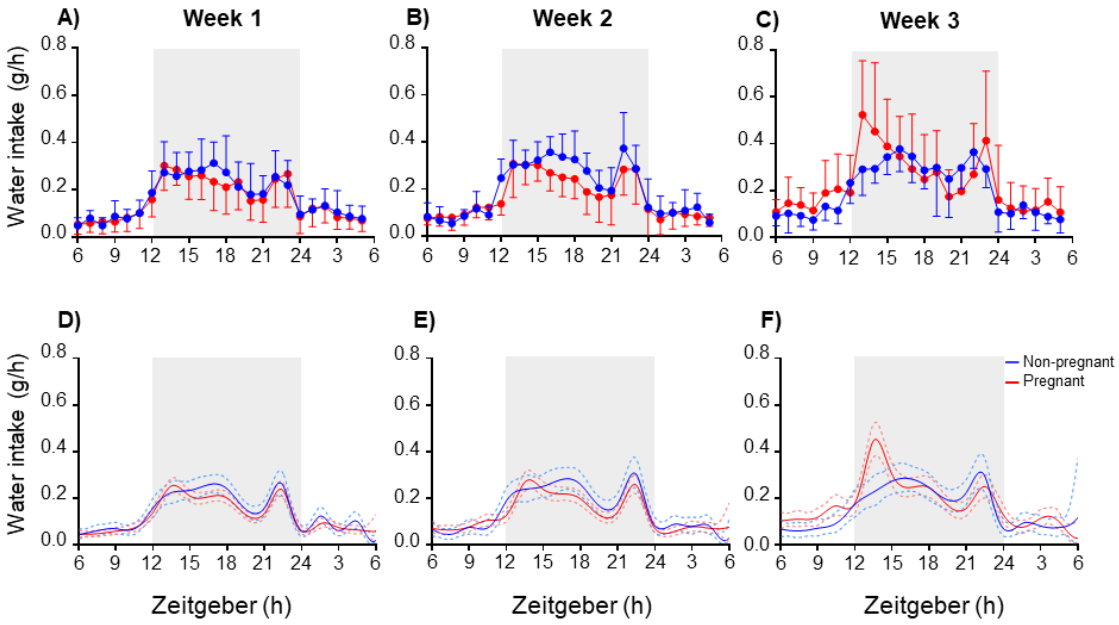


Figure 5.2. Water intake pattern of non-pregnant and pregnant mice across each week of study.

Water consumption (A-C) is the mean ± SD of raw data for each mouse, averaged across each week of study within non-pregnant (blue line, N ≤ 12) and pregnant (red line) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (A, day 0.5-6.5, N = 31), week 2 (B, day 6.5-12.5, N =21) and week 3 (C, day 12.5-17.5, N = 11). The fitted model of water consumption (D-F) across each week of study indicates fitted means (solid lines) and 95% credible intervals (dashed lines) for all non-pregnant (blue) and pregnant (red) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (D, day 0.5-6.5), week 2 (E, day 6.5-12.5) and week 3 (F, day 12.5-17.5).

Table 5.2: **The effect of pregnancy on timing and amplitudes of peaks in water intake.**

Time periods of interest	Study Week	Percent of iterations per group with detected peak (%)		Peak characteristics for mice with detected peak					
				Peak Time (ZT) [95% CrI]			**Peak Amplitude (g/ h)[95% CrI]**		
		Non-pregnant	**Pregnant**	**Non-pregnant**	**Pregnant**	**Difference**	**Non-pregnant**	**Pregnant**	**Difference**
I (ZT8-ZT12)	1	64.4	26.4	8.77 [8.07, 9.29]	9.08 [8.15, 9.55]	0.30 [-0.53, 1.15]	0.071 [0.048, 0.097]	0.060 [0.046, 0.076]	-0.011 [-0.039, 0.016]
	2	72.6	60.5	9.06 [8.62, 9.49]	10.42 [9.27, 10.89]	**1.36 [0.15, 2.04]**	0.076 [0.047, 0.011]	0.106 [0.081, 0.132]	0.030 [-0.014, 0.071]
	3	39.2	86.5	9.24 [8.22, 11.05]	10.40 [9.31. 10.80]	1.16 [-0.68, 2.30]	0.074 [0.042, 0.011]	0.167 [0.125, 0.215]	**0.094 [0.039, 0.150]**
II (ZT12- ZT15)	1	57.9	99.9	13.62 [12.56, 14.40]	13.71 [13.55, 13.91]	0.09 [-0.71, 1.16]	0.027 [0.018, 0.028]	0.256 [0.224, 0.289]	0.029 [-0.029, 0.085]
	2	69.2	99.9	13.19 [12.34, 14.21]	13.90 [13.71, 14.22]	0.71 [-0.37, 1.61]	0.236 [0.186, 0.291]	0.280 [0.241, 0.320]	0.043 [-0.022, 0.107]
	3	43.3	100	13.27 [12.14, 14.79]	13.71 [13.60, 13.83]	0.44 [-1.07, 1.58]	0.220 [0.164, 0.284]	0.454 [0.383, 0.526]	**0.234 [0.140, 0.331]**
III (ZT15-ZT18)	1	98.4	92.4	16.90 [15.82, 17.56]	17.23 [16.61, 17.60]	0.32 [-0.59, 1.51]	0.262 [0.226, 0.301]	0.213 [0.190, 0.236]]	**-0.049 [-0.095, -0.006]**
	2	98.2	47	16.86 [15.67, 17.61]	17.06 [16.45, 17.52]	0.20 [-0.82, 1.48]	0.284 [0.239, 0.333]	0.218 [0.191, 0.246]	**-0.067 [-0.124, -0.012]**
	3	91.7	73.2	16.28 [15.20, 17.61]	17.53 [17.01, 17.89]	1.25 [-0.18, 2.39]	0.286 [0.232, 0.345]	0.254 [0.214, 0.297]	-0.032 [-0.106, 0.039]
IV (ZT18-ZT24)	1	100	100	22.24 [22.13. 22.33]	22.31 [22.24, 22.36]	0.06 [-0.05, 0.19]	0.267 [0.217, 0.320]	0.240 [0.210, 0.273]	-0.028 [-0.088, 0.032]
	2	100	100	22.31 [22.17, 22.42]	22.35 [22.27, 22.42]	0.04 [-0.10, 0.20]	0.309 [0.246, 0.377]	0.260 [0.222, 0.300]	-0.049 [-0.126, 0.024]
	3	100	100	22.14 [21.85, 22.31]	22.48 [22.31, 22.74]	**0.34 [0.09, 0.72]**	0.313 [0.242, 0.390]	0.251 [0.202, 0.303]	-0.062 [-0.155, 0.029]
V (ZT24-ZT4)	1	100	100	1.58 [1.44, 1.76]	1.90 [1.61, 2.48]	0.32 [-0.02, 0.92]	0.124 [0.091, 0.162]	0.095 [0.077, 0.114]	-0.029 [-0.070, 0.010]
	2	96.9	84.2	2.24 [1.36, 3.94]	2.99 [1.67, 3.91]	0.74 [-1.47, 2.26]	0.086 [0.058, 0.119]	0.079 [0.058, 0.102]	-0.007 [-0.046, 0.031]
	3	97.8	85.2	1.08 [1.46, 3.42]	1.42 [1.66, 3.96]	1.34 [-0.46, 2.31]	0.097 [0.063, 0.137]	0.122 [0.090, 0.159]	0.026 [-0.025, 0.075]

CrI, 95% credible interval; ZT, Zeitgeber; Study week = week 1 (days 0.5-6.5, N = 31), week 2 (days 6.5-12.5, N = 21), week 3 (days 12.5-17.5, N = 11). Non-pregnant mice, N ≤ 12. Intervals of group differences that exclude 0 are highlighted with bold text. Positive values present delayed timing or increased amplitude. Negative values present advanced timing or decreased amplitude.

5.7.4. Effects of pregnancy on activity

Modelled total activity patterns corresponded to raw data in each week of the study (Figures **5.3 A-5.3 C**). For all weeks of the study, modelled total activity was similar in non-pregnant and pregnant mice throughout the light period (ZT0-ZT12), and activity throughout the majority of the dark period (ZT13-ZT23) was lower in pregnant compared to non-pregnant mice (**Figure 5.3 D-5.3 F**).

Week 1: Within all time periods of interest (ZT8-ZT12, ZT12-ZT15, ZT15-ZT18, ZT18-ZT24 and ZT24-ZT4), more than 95% of the non-pregnant group and more than 88% of the pregnant group exhibited a peak in activity (**Table 5.3**). Within mice that exhibited a peak in activity, the peak amplitude was lower for all time periods (0.4-, 0.4-, 0.7-, 0.5- and 0.4-fold respectively) in pregnant than the non-pregnant group (**Table 5.3**). In the fourth time period of interest (ZT18-ZT24) the timing of the peak occurred 0.20 h later in the pregnant than non-pregnant group, but peaks occurred at similar times for all other periods of interest (**Table 5.3**).

Week 2: Within the first and fifth time period of interest (ZT8-ZT12 and ZT24-ZT4), 76% and 65% of the non-pregnant group and 33% and 83% of the pregnant group respectively exhibited peaks in activity, and detected peaks were of similar timing and amplitudes in both groups (**Table 5.3**). In the second, third and fourth time periods of interest (ZT12-ZT15, ZT15-ZT18 and ZT18-ZT24), 97%, 99% and 100% of the non-pregnant group and 100%, 54% and 100% of the pregnant group respectively, exhibited activity peaks (**Table 5.3**). The amplitude of peak activity was lower (0.5-, 0.7- and 0.6-fold respectively) in the pregnant than non-pregnant group for all these time periods during the dark period (**Table 5.3**). The activity peak in time period four (ZT18-ZT24) occurred 0.13 h later in the pregnant than non-pregnant group, whilst the timing of activity peaks between ZT12-ZT15

and ZT15-ZT18 were similar in the pregnant and non-pregnant groups (**Table 5.3**).

Week 3: Within the first time period of interest (ZT8-ZT12), 87% of non-pregnant and 41% of the pregnant group exhibited a peak in activity (**Table 5.3**). Within mice that exhibited a peak between ZT8 and ZT12, peak amplitude was similar but occurred 2.78 h later in the pregnant compared to non-pregnant group (**Table 5.3**). In the second, third, fourth and fifth time periods of interest (ZT12-ZT15, ZT15-ZT18, ZT18-ZT24 and ZT24-ZT4), 96%, 47%, 100% and 88% of the non-pregnant group and 62%, 77%, 100% and 39% of the pregnant group respectively, exhibited activity peaks (**Table 5.3**). Across these time periods, the peak amplitude was lower (0.7-, 0.8, 0.6- and 0.6-fold respectively) in pregnant than the non-pregnant group and the timing of peaks was similar between groups (**Table 5.3**).

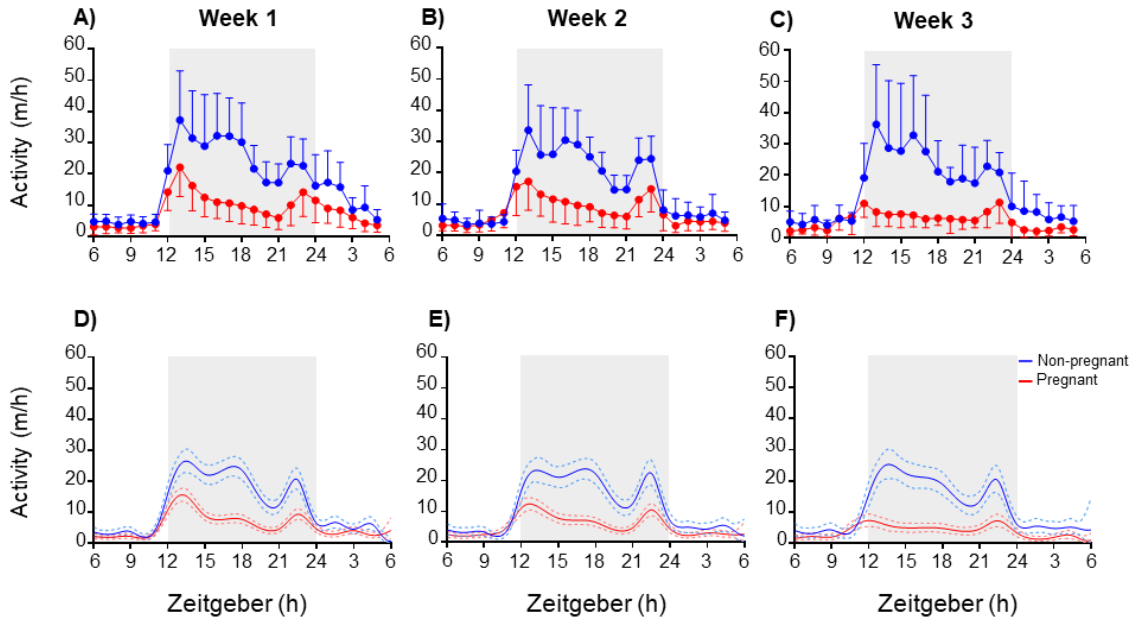


Figure 5.3. Activity pattern of non-pregnant and pregnant mice across each week of study.

Activity (A-C) is the mean ± SD of raw data for each mouse, averaged across each week of study within non-pregnant (blue line, N ≤ 12) and pregnant (red line) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (A, day 0.5-6.5, N = 31), week 2 (B, day 6.5-12.5, N = 21) and week 3 (C, day 12.5-17.5, N = 11). The fitted model of activity (D-F) across each week of study indicates fitted means (solid lines) and 95% credible intervals (dashed lines) for all non-pregnant (blue) and pregnant (red) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (D, day 0.5-6.5), week 2 (E, day 6.5-12.5) and week 3 (F, day 12.5-17.5).

Table 5.3: **The effect of pregnancy on timing and amplitudes of peaks in activity.**

Time periods of interest	Study Week	Percent of iterations per group with detected peak (%)		Peak characteristics for mice with detected peak					
				Peak Time (ZT) [95% CrI]			**Peak Amplitude (m/ h) [95% CrI]**		
		Non-pregnant	**Pregnant**	**Non-pregnant**	**Pregnant**	**Difference**	**Non-pregnant**	**Pregnant**	**Difference**
I (ZT8- ZT12)	1	95.1	88.6	8.67 [8.17, 8.91]	8.63 [8.11, 8.93]	-0.04 [-0.60, 0.53]	3.96 [2.70, 5.73]	2.33 [1.61, 3.16]	**-1.64 [-3.26, -0.12]**
	2	76.5	33.5	8.60 [8.09, 8.93]	9.03 [8.42, 9.35]	0.43 [-0.27, 1.02]	3.59 [2.23, 5.21]	2.51 [1.64, 3.52]	-1.08 [-2.90, 0.59]
	3	87.4	41.1	8.81 [823, 9.15]	11.60 [9.35, 11.98]	**2.78 [0.92, 3.58]**	4.35 [2.56, 6.50]	7.02 [5.25, 9.02]	2.67 [-0.14, 5.30]
II (ZT12-ZT15)	1	99.7	100	13.48 [13.12, 13.75]	13.17 [12.94, 1.34]	-0.31 [-0.67, 0.09]	26.5 [22.8, 30.4]	15.6 [13.6, 17.8]	**-10.9 [-15.3, -6.66]**
	2	97.2	100	13.23 [12.69, 13.77]	12.71 [12.38, 13.14]	-0.52 [-1.18 0.17]	23.3 [19.6, 27.4]	12.4 [10.5, 14.5]	**-10.9 [-15.4, -6.72]**
	3	96.1	62.3	13.64 [12.94, 13.49]	12.44 [12.01, 13.56]	-1.20 [-2.13, 0.22]	25.3 [20.7, 30.2]	7.12 [5.31, 9.10	**-18.2 [-23.5, -13.1]**
III (ZT15- ZT18)	1	99.5	91.2	17.28 [16.69, 17.61]	17.42 [16.99, 17.70]	0.13 [-0.40, 0.80]	24.8 [21.7, 28.0]	8.03 [6.66, 9.51]	**-16.8 [-20.2, -13.4]**
	2	99.2	54.3	17.09 [16.24, 17.57]	17.07 [16.46, 17.54]	-0.02 [-0.84, 0.98]	23.8 [20.5, 27.2]	7.14 [5.76, 8.55]	**-16.7 [-20.4, -13.1]**
	3	47.4	77.7	16.83 [15.18, 17.57]	16.92 [15.18, 17.92]	0.09 [-2.06, 2.23]	21.0 [17.6, 24.4]	5.0 [3.74, 6.43]	**-16.0 [-19.8, -12.3]**
IV (ZT18- ZT24)	1	100	100	22.33 [22.24, 22.41]	22.53 [22.44, 22.63]	**0.20 [0.09, 0.33]**	20.7 [17.5, 24.3]	9.4 [7.77, 11.2]	**-11.3 [-15.4, -7.61]**
	2	100	100	22.37 [22.28, 22.46]	22.50 [22.42, 22.60]	**0.13 [0.01, 0.26]**	22.6 [18.6, 26.8]	10.5 [8.57, 12.5]	**-12.1 [-16.8, -7.60]**
	3	100	100	22.27 [22.11, 22.39]	22.44 [22.21, 22.71]	0.17 [-0.09, 0.48]	20.5 [16.4, 25.0]	7.24 [5.35, 9.33]	**-13.3 [-19.7, -12.3]**
V (ZT24- ZT4)	1	97.2	99.8	1.56 [1.27, 3.52]	2.92 [2.50, 3.30]	1.35 [-0.49, 1.86]	67.4 [49.4, 87.8]	41.3 [30.6, 53.3]	**-26.1 [-49.0, -4.0]**
	2	65.8	83.2	2.03 [1.19, 3.94]	3.20 [1.93, 3.93]	1.17 [-0.87, 2.44]	46.0 [31.1, 63.5]	30.6 [20.8, 42.0]	-15.5 [-35.8, 3.82]
	3	88.5	39.7	2.01 [1.28, 3.82]	3.53 [1.47, 3.97]	1.52 [-0.46, 2.51]	54.0 [35.2, 74.9]	23.8 [14.5, 35.4]	**-30.2 [-53.4, -8.10]**

CrI, 95% credible interval; ZT, Zeitgeber; Study week = week 1 (days 0.5-6.5, N = 31), week 2 (days 6.5-12.5, N = 21), week 3 (days 12.5-17.5, N = 11). Non-pregnant mice, N ≤ 12. Intervals of group differences that exclude 0 are highlighted with bold text. Positive values present delayed timing or increased amplitude. Negative values present advanced timing or decreased amplitude.

5.7.5. Effects of pregnancy on wakefulness

Modelled wakefulness probabilities corresponded to raw data for time spent awake in each week of the study (**Figures 5.4 A-5.4 C**). Modelled probabilities of being awake were similar in pregnant and non-pregnant mice throughout the light period and early and late in the dark periods (ZT22.5- ZT13.5) across all weeks of the study (**Figure 5.4 D-5.4 F**). The probability of being awake between ZT13.5 and ZT22.5 was lower in pregnant than non-pregnant mice in the first and second week of study (**Figure 5.4 D and 5.4 E**). In the final study week, the probability of being awake was lower in pregnant than non-pregnant mice between ZT13.5-ZT20 and ZT21.5-ZT22.5 (**Figure 5.4 F**).

Week 1: Within the first and fifth time periods of interest (ZT8-ZT12 and ZT24-ZT4), 68% and 98% of the non-pregnant group and 64% and 98% of the pregnant group respectively, exhibited peaks in wakefulness which did not differ in timing or amplitude (**Table 5.4**). In the second, third and fourth time periods of interest (ZT12-ZT15, ZT15-ZT18 and ZT18-ZT24) more than 99% of the non-pregnant group and more than 95% of the pregnant group exhibited peaks in wakefulness (**Table 5.4**). The probability of being awake was lower (0.1-, 0.4- and 0.2-fold respectively) in pregnant than the non-pregnant group for all of these time periods during the dark period (**Table 5.4**). The timing of peaks in wakefulness between ZT12 and ZT15 and between ZT15 and ZT18 did not differ between groups, whilst the peak in wakefulness in the second half of the dark period (ZT18-ZT24) was 0.11 h later in the pregnant than non-pregnant group (**Table 5.4**).

Week 2: During the light phase, (first time period of interest: ZT8-ZT12 and fifth time period of interest: ZT24-ZT4), 74% and 72% of the non-pregnant group and 25% and 82% of the pregnant group respectively, exhibited peaks in

wakefulness, and these were similar in timing and amplitude between groups (**Table 5.4**). During the dark phase (second time period of interest: ZT12-ZT15, third time period of interest: ZT15-ZT18 and fourth time period of interest: ZT18-ZT24) more than 96% of the non-pregnant group and 100%, 86% and 100% of the pregnant group respectively, exhibited wakefulness peaks (**Table 5.4**). The probability of being awake was lower (0.2-, 0.4- and 0.2-fold respectively) in the pregnant than non-pregnant group across these time periods (**Table 5.4**). The timing of peaks in wakefulness for the second and third time periods of interest did not differ between groups, whilst the peak in wakefulness in the second half of the dark period (ZT18-ZT24) was 0.11 h later in the pregnant than non-pregnant group (**Table 5.4**).

Week 3: Within the first time period of interest (ZT8-ZT12), 54% of the non-pregnant group and 20% of the pregnant group exhibited a peak in wakefulness (**Table 5.4**). Within mice that exhibited a peak in the probability of wakefulness, the amplitude of wakefulness was 1.5-fold greater in the pregnant than non-pregnant group, but the peaks occurred at similar times (**Table 5.4**). Throughout the dark phase, in the second, third and fourth time periods of interest (ZT12-ZT15, ZT15-ZT18 and ZT18-ZT24) 87%, 73% and 100% of the non-pregnant group and 99%, 46% and 100% of the pregnant group respectively, exhibited wakefulness peaks (**Table 5.4**). Within mice that exhibited a peak in behaviour, the probability of being awake was lower (0.2-, 0.4- and 0.3-fold respectively) in the pregnant than non-pregnant group for each of these time periods, but the timing of the peaks did not differ between groups (**Table 5.4**). Within the fifth time period of interest (ZT24-ZT4) 97% of non-pregnant and 59% of the pregnant group exhibited a peak in wakefulness and there was no difference in timing or amplitude of peaks within mice that exhibited this peak (**Table 5.4**).

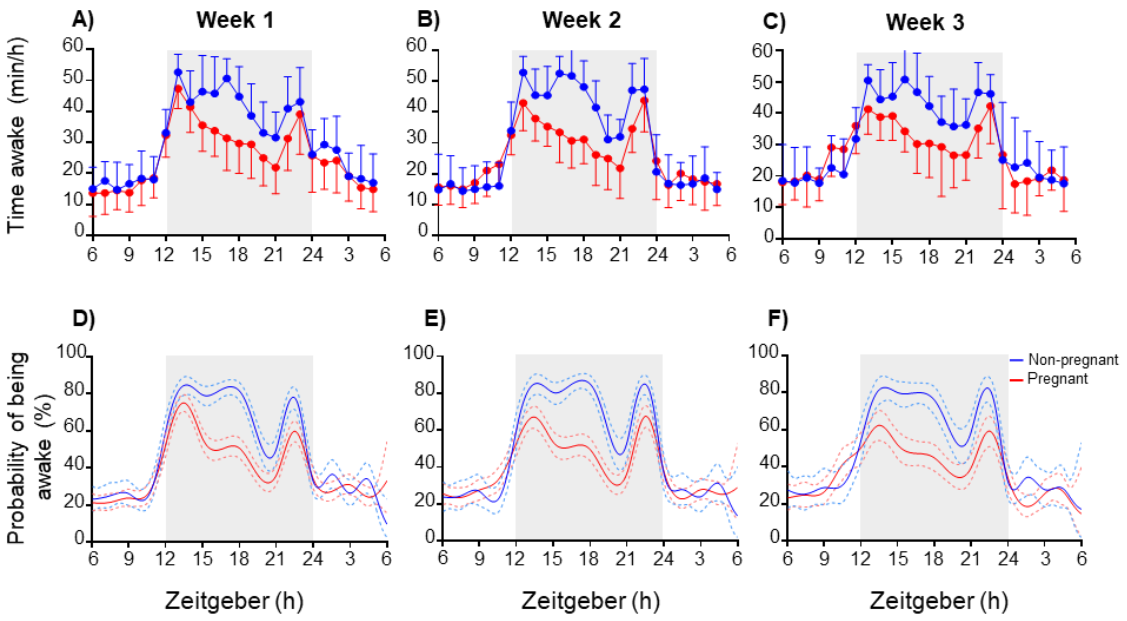


Figure 5.4. Sleep/wake behaviour of non-pregnant and pregnant mice across each week of study.

Time spent awake (A-C) is the mean ± SD of raw data for each mouse, averaged across each week of study within non-pregnant (blue line, N ≤ 12) and pregnant (red line) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (A, day 0.5-6.5, N = 31), week 2 (B, day 6.5-12.5, N = 21) and week 3 (C, day 12.5-17.5, N = 11). The fitted model of sleep probability (D-F) across each week of study indicates fitted means (solid lines) and 95% credible intervals (dashed lines) for all non-pregnant (blue) and pregnant (red) groups. Light phase (ZT6-ZT12 and ZT24-ZT6, no shading) and dark-phase (ZT12-ZT24, shaded) are shown for week 1 (D, day 0.5-6.5), week 2 (E, day 6.5-12.5) and week 3 (F, day 12.5-17.5).

Table 5.4: **The effect of pregnancy on timing and amplitudes of peaks in probability of being awake.**

Time periods of interest	Study Week	Percent of iterations per group with detected peak (%)		Peak characteristics for mice with detected peak					
				Peak Time (ZT) [95% CrI]			**Peak Amplitude (%) [95% CrI]**		
				Non-pregnant	**Pregnant**	**Difference**	**Non-pregnant**	**Pregnant**	**Difference**
I (ZT8-ZT12)	1	68.8	64.0	8.67 [8.08, 9.10]	8.88 [8.10, 9.32]	0.21 [-0.64, 0.96]	26.8 [20.6, 33.4]	23.7 [19.3, 28.5]	-3.1 [11.1, 4.8]
	2	74.9	25.6	8.70 [8.10, 9.10]	9.13 [8.24, 9.53]	0.41 [-0.51, 1.13]	27.6 [20.0, 35.9]	28.0 [22.5, 33.8]	0.39 [-9.8, 8.9]
	3	54.4	20.4	9.05 [8.16, 10.62]	10.77 [8.0, 11.27]	1.71 [-0.22, 2.77]	28.8 [19.7, 39.1]	43.0 [35.1, 51.4]	**14.2 [1.2, 27.0]**
II (ZT12-ZT15)	1	99.1	100	13.71 [13.49, 14.02]	13.47 [13.35, 13.56]	-0.24 [-0.56, 0.00]	84.7 [79.5, 89.2]	75.0 [70.5, 79.5]	**-9.67 [-16.4, -2.9]**
	2	96.8	100	13.70 [13.42, 14.07]	13.41 [13.10, 13.59]	-0.29 [-0.74, 0.06]	85.4 [79.5, 90.5]	67.2 [61.1, 72.9]	**-18.2 [-26.6, -9.9]**
	3	87.1	99.9	13.97 [13.62, 14.63]	13.51 [12.86, 13.86]	-0.46 [-1.28, 0.09]	82.7 [75.6, 88.8]	62.3 [54.2, 70.3]	**-20.4 [-30.6, -9.5]**
III (ZT15-ZT18)	1	99.8	95.9	17.26 [14.69, 17.57]	17.46 [17.06, 17.71]	0.21 [-0.31, 0.85]	83.8 [79.4, 87.6]	51.9 [46.5, 57.2]	**-31.9 [-38.5, -25.1]**
	2	99.9	86.2	17.33 [16.80, 17.63]	17.23 [16.71, 17.59]	-0.09 [-0.73, 0.56]	87.0 [82.7, 90.7]	52.1 [46.4, 57.9]	**-35.0 [-42.0, -27.7]**
	3	73.9	46.7	16.87 [15.32, 17.58]	17.35 [16.67, 17.83]	0.47 [-0.54, 2.08]	79.9 [74.1, 85.2]	46.6 [39.9, 53.2]	**-33.3 [-41.9, -24.3]**
IV (ZT18-ZT24)	1	100	100	22.38 [22.31, 22.45]	22.50 [22.42, 22.57]	**0.11 [0.02, 0.22]**	78.1 [71.8, 83.7]	59.8 [54.1, 65.3]	**-18.4 [-26.6, -10.0]**
	2	100	100	22.43 [22.35, 22.50]	22.54 [22.46, 22.62]	**0.11 [0.01, 0.22]**	85.1 [79.2, 90.0]	67.6 [61.6, 73.4]	**-17.5 [-25.4, -9.2]**
	3	100	100	22.33 [22.22, 22.42]	22.49 [22.35, 22.68]	0.16 [-0.01, 0.37]	82.4 [74.8, 88.7]	59.2 [50.8, 67.2]	**-23.2 [-34.0, -12.1]**
V (ZT24-ZT4)	1	98.9	98.9	1.59 [1.35, 1.99]	2.72 [1.66, 3.32]	1.13 [-0.06, 1.82]	36.5 [29.2, 44.3]	31.1 [25.9, 36.5]	-5.44 [-15.2, 3.91]
	2	72.1	82.4	1.86 [1.20, 3.92]	3.17 [2.50, 3.92]	1.30 [-0.80, 2.45]	27.0 [19.7, 35.1]	28.1, 33.8]	1.02 [-9.0, 10.6]
	3	97.3	59.1	1.88 [1.36, 3.76]	3.51 [2.93, 3.97]	1.64 [-0.33, 2.46]	34.0 [22.0, 44.1]	27.9 [21.6, 34.8]	-6.1 [-17.8, 5.74]

CrI, 95% credible interval; ZT, Zeitgeber; Study week = week 1 (days 0.5-6.5, N = 31), week 2 (days 6.5-12.5, N = 21), week 3 (days 12.5-17.5, N = 11). Non-pregnant mice, N ≤ 12. Intervals of group differences that exclude 0 are highlighted with bold text. Positive values present delayed timing or increased amplitude. Negative values present advanced timing or decreased amplitude.

5.8. Discussion

We identified that mice exhibit diurnal patterns in food and water intake, activity and wakefulness behaviours, regardless of pregnancy status. In this study, the circadian pattern of behaviour during pregnancy, relative to non-pregnant mice, was characterised by: 1) a reduction in physical activity and decreased in time spent awake during the dark-phase, beginning in the first week of pregnancy; 2) an increase in food intake at the end of the light-phase during the second and third weeks of pregnancy; and 3) an increase in food and water intake at the start of the dark-phase during the final week of pregnancy. It is also apparent that pregnancy delays circadian rhythms of behaviours evidenced by later peaks in behaviours within the groups that displayed peaks in time periods of interest, particularly at the end of the dark-phase (activity and wakefulness) and end of light-phase (food intake). Thus, both the timing and total amounts of each behaviour are altered during mouse pregnancy.

5.8.1. Changes in food and water intake behaviour during pregnancy

Rodents used in biomedical research are nocturnal and consume around 65 – 80% of their daily food [196,215] and 78 – 90 % of their daily water in the active dark-phase [215,216]. In the present study, we replicate with our data that food and water intake followed strong circadian patterns aligning with the sleep/wake cycle. Furthermore, our data, together with others, shows conservation of nocturnally-dominated patterns of food intake during pregnancy in rodents. We also observed that the timing of water intake in the pregnant and non-pregnant female group was closely linked to peaks in eating. This is also consistent with reports that in male rats 70% of water intake is associated with food intake [215] and, more specifically, that 57% of total water consumption occurs within 20 minutes of

eating [216]. Although the nocturnal bias in food intake was preserved, the timing of food intake changed during pregnancy. We have reported previously that the increased food intake during mouse pregnancy [6,42,209] reflects increased food intake during the light-phase, due to larger meal size, rather than substantial increases in dark-phase food intake [42]. In the present study, we identified that this increase in light-phase food intake during pregnancy was due to pregnant mice consuming a greater amount of food shortly before the dark-phase. Interestingly, in groups that exhibited a peak in the last four hours of the light phase (ZT8-ZT12), this peak was delayed compared to the peak observed in the non-pregnant group from the second week of pregnancy, the first evidence for delayed feeding behaviours during pregnancy. Furthermore, a similar delay in water intake behaviours was also observed during the second week of pregnancy. Although the timing of food and water intake peaks early in the dark period (second time period of interest: ZT12-ZT15), at times when food intake is greater in male non-pregnant mice [196], was unaltered by pregnancy, the timing of maximal food intake differed. Our observation that more of the pregnant group exhibited a peak in food and water earlier in the dark-phase, at the second time period of interest (ZT12-ZT15), while the non-pregnant group consume food between the second and third time periods of interest (ZT12-ZT15 and ZT15-ZT18), is consistent with changes in the timing of maximal food intake in rat pregnancy [209]. Maximum food intake occurs within a shorter feeding window in pregnant than non-pregnant rats (ZT11-ZT15 cf. ZT11-ZT19), where food intake events occur within two peaks during pregnancy rather than a single peak in non-pregnant rats [209]. The mechanisms underlying changes in food and water intake during pregnancy are not clear, although the delayed onset in weeks 2 and 3 after mating suggest rising concentrations of pregnancy hormones may be responsible

[150]. Although plasma oestrogen increases during mid-pregnancy and remains elevated during late-pregnancy in mice, we do not consider this hormone a likely candidate mechanism for advancement of food intake behaviour during mouse pregnancy, since oestradiol does not alter the phase timing of food intake in female rats [217]. Maternal circulating growth hormone (GH) concentrations also increase by mid-pregnancy and remains elevated in the pregnant mouse [115]. Although the impact of GH on circadian patterns of food intake has not been directly assessed, administration of GH-releasing factor directly into the brain stimulates food intake during the inactive but not the active phase in male rats and hamsters [218,219]. We therefore hypothesise that elevated maternal GH during mouse pregnancy may underlie increased light-phase food intake. However, this remains to be determined.

5.8.2. Changes in physical activity and sleep patterns in pregnant mice

From early-pregnancy, we observed that mice slept significantly more during the dark-phase, replacing the time spent active around the cage. This pattern of rapid reduction in movement around the metabolic cages during pregnancy is consistent with the rapid reduction in voluntary running wheel activity and increased sleep beginning at the start of pregnancy reported in the same strain of mice by Ladyman *et al.* [6]. The timing of this rapid decrease in activity and increase in sleep even before implantation at ~4 days after mating [220], implies that the drivers for reduced activity are maternal in origin and do not originate from the fetus or placenta. It has been hypothesised that reduction in activity in early-pregnancy is driven by prolactin [6,221], since prolactin is one of the first maternal hormones to increase following mating [116]. Although in other studies maternal activity did not decrease until mid-pregnancy in mice [207,208], this may

reflect different methodologies including activity analysis (Yaw *et al.,* Ladyman *et al.* and current study: 5.0 minute bins, Martin-Fairey *et al.*: 6 minute bins) and cage systems (Yaw *et al.*: clocklab, Martin-Fairey *et al.*: circadian cabinets, Ladyman *et al.* and current study: Promethion cages). Reduced activity late in mouse pregnancy likely reflects the impact of both hormonal changes and body weight, since maternal body weight increases rapidly in the second half of pregnancy. At the end of the present study, pregnant mice at d 17.5 were 55% heavier than their age-matched non-pregnant controls [42].

In contrast to the consistent reports of decreased activity during mice pregnancy, the reported changes in timing of activity are inconsistent. In the present study, we observed an almost 3 h delay in the activity peak late in the light period in the last week of pregnancy. The activity and wakefulness peak that occurred late in the dark period was also delayed in weeks 1 and 2 of pregnancy, although to a lesser extent (11-20 minutes) and did not persist in late pregnancy. This is the first detailed report of circadian patterns of activity and time spent awake during mouse pregnancy. Activity onset in running wheels occurs early in the dark period in male and non-pregnant female mice reviewed in [222], and is used as a single daily measure of activity timing. Data on the timing of running wheel activity in pregnancy is inconsistent. Yaw *et al.* [208] reported delayed running wheel onset in mid-pregnant (d 8 - d 13) compared to non-, early- and late-pregnant mice. Conversely, Martin-Fairey *et al.* [207] reported earlier (up to 4 h) running wheel activity between d 3 and d 10, relative to non-pregnant controls, whilst in later pregnancy the timing of activity returned to that of non-pregnant mice. Effects of pregnancy on different types of behaviour may differ with experimental factors, for example, the measure of activity. Since total cage movement reflects spontaneous activity, whereas mice find running wheel exercise rewarding [223],

changes in running wheel activity may reflect altered reward motivation. Furthermore, increased body size in late pregnancy might restrict the ability of mice to access the wheel in late pregnancy and confound measures of activity using this approach.

The mechanisms underlying the delay in activity and wakefulness during pregnancy are unknown, although there is evidence for impacts of both progesterone and oestrogen. Progesterone levels increase from d 4 in the mouse and remain relatively high for the duration of pregnancy [224]. In cycling female rats, progesterone implants delayed the onset of running wheel activity at the start of the dark phase by 22 minutes compared to cholesterol-implanted controls [225]. Albers *et al.* [225] hypothesised that progesterone antagonises oestrogen, since increases in oestrogen in female rats during pro-oestrus occurred concurrent with advanced activity onset and increased running wheel activity [225]. Similarly, oestrogen implants in hamsters advanced the timing of activity onset and consolidated activity bouts to earlier in the active phase [226]. It is therefore likely that activity and sleep are altered by a combination of rising progesterone and prolactin in early-pregnancy, and progesterone antagonises oestrogen in later pregnancy to delay activity onset, but these hypotheses are yet to be tested.

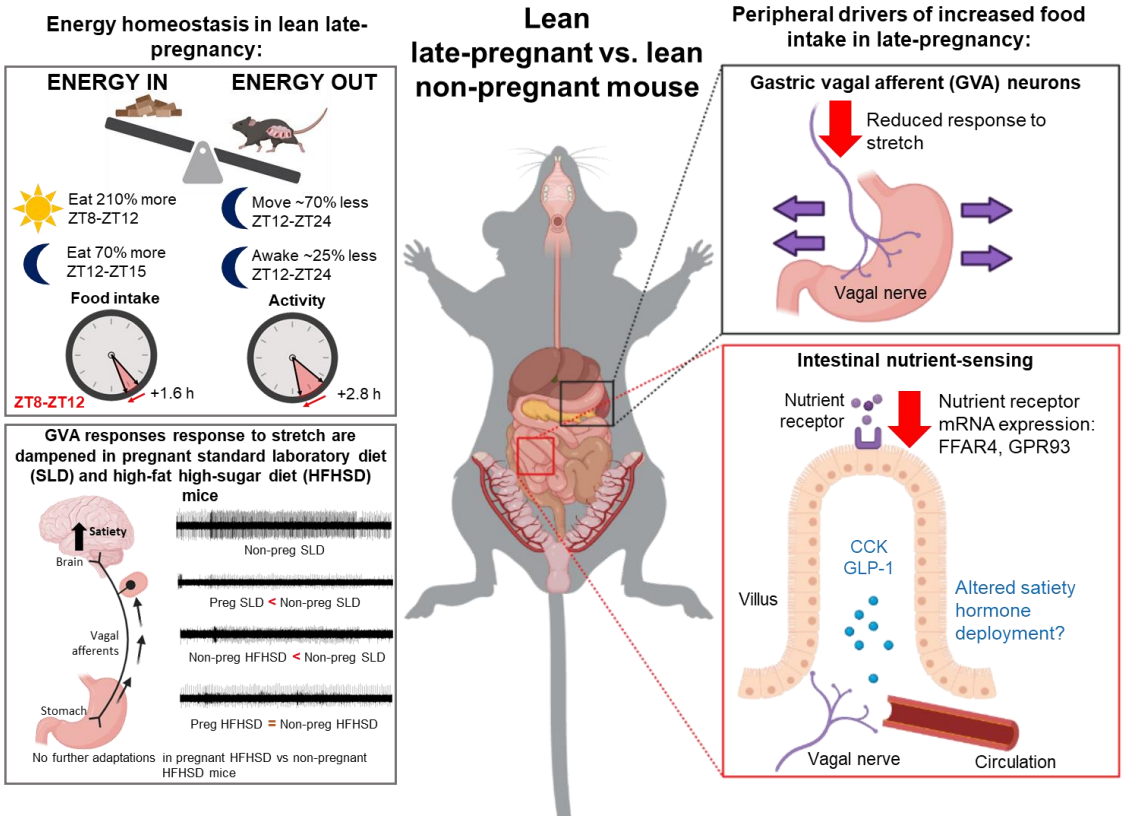
5.9. Conclusion

This study confirms and extends on previous observations that normal circadian rhythms of behaviour are altered during pregnancy and the differences in timing and amplitude of each behaviour likely reflects the role of different pregnancy hormones. Increased food intake at the start of the light-phase and end of the dark-phase during pregnancy reflects increased amplitude of eating behaviour, without longer duration. Marked decreases in activity and probability of being awake also contribute to positive energy balance in pregnancy, with delays to all

measured behaviours evident from mid-pregnancy onwards. Further research is required to determine whether pregnancy complications observed, for example in maternal obesity, result from disruption in these adaptations in circadian behaviour.

CHAPTER 6: General conclusions

6.1. Graphical abstract



6.2. General discussion

6.2.1 Introduction

Pregnancy is a time of numerous maternal physiological adaptations to support adequate fetal and placental growth, development of fat reserves for lactation and to support increased maternal metabolism [150]. One important adaptation to meet the increased nutritional demand during pregnancy is through increased food intake [150]. Satiation is a normal physiological response which leads to termination of meal intake [176] and, therefore, the increase in food intake during pregnancy could be due to down regulation of satiety signalling. Prior studies have characterised downregulation of central satiety pathways including the development of leptin resistance [150] but how peripheral satiety mechanisms change had not been investigated. The GIT receives ingested food and is important in relaying satiation signals to the brain [176]. This thesis aimed to characterise changes in gastric and intestinal satiety mechanisms during a lean and HFHSD-fed pregnancy. Furthermore, to determine the effects of pregnancy on circadian rhythms of food and water intake, sleep and activity behaviour. A summary of the main thesis findings is reported visually in section 6.1.

6.2.2. Food intake behaviour during pregnancy

It is well established that food intake increases during pregnancy in animals. A prior study in mice [6], supported by data in **Chapters 2 and 4**, reported that food intake increased during mid-pregnancy due to an increase in meal size not meal number. Furthermore, in pregnant rats, there was a small increase in meal duration with no change in meal frequency compared to pre-mating values [43]. **Chapters 2 and 4** extended this research and demonstrated that pregnant mice have a shorter meal duration without a change in meal size compared to non-

pregnant mice in the dark-phase, suggesting they are consuming food at a faster rate. Differences between rats and mice could reflect the different methodologies/cage systems (Strubbe *et al.*: meal patterns registered by movements of a bar situated in front of a food hopper [43], Ladyman *et al.* and current study: automated interaction with food hopper in Promethion cages [6,42]).

Chapter 4 also details how a HFHSD alters pregnancy adaptations compared to SLD-fed lean mice. Firstly, there were no differences in 24 h energy intake between pregnant and non-pregnant HFHSD-mice (**Chapter 4**). The timing of food intake did differ, however, with pregnant HFHSD-mice consuming less food (in g) than non-pregnant HFHSD-mice in the dark phase across the whole study. To offset lower food intake during the dark-period, pregnant mice were eating more than non-pregnant mice during the light-phase by mid-pregnancy, which is an adaptation preserved in both SLD- and HFHSD-mice. Furthermore, there was no difference in energy intake between pregnant HFHSD- and SLD-mice. This conflicts with prior studies in pregnant rats (34.42% lard) [227] and mice (20% lard) [188] and 41% fat (milk and corn oil) [228]), where 24 h energy intake was greater in pregnant HFHSD- compared to SLD-mice. Differences between studies likely reflects the fat source of the diet (e.g. plant in the current study vs animal based in previous reports). Lastly, an interesting finding from **Chapter 4** was that HFHSD-mice had a shorter meal duration than SLD-mice in both the light- and dark-phase. This could be explained by earlier meal termination due to lipid-induced satiety or the malleability/softness of the HFHSD diet making it easier to remove from the hopper than standard chow.

6.2.3. Food intake regulation

6.2.3.1. Adaptations in GVA signals in response to a HFHSD and pregnancy

The GIT is densely innervated by sensory vagal nerves that innervate the mucosa and muscle layer [24]. Gastric satiety signals arise from the activation of tension-sensitive GVAs in response to mechanical distension of the stomach wall as food fills the stomach [24]. The gastric electrophysiology study presented in **Chapter 2** revealed that the response of tension-sensitive GVAs to stretch was attenuated in mid- and late-pregnant compared to non-pregnant mice within the light-phase [42]. Furthermore, these adaptations were specific to tension-sensitive GVAs as we saw no change in the response of gastric mucosal afferents to mucosal stroking [42]. We know that meal size and frequency is related to tension-sensitive GVA mechanosensitivity, and consistent with the expected effects of reduced GVA mechanosensitivity, meal size [6,42] and duration increased during pregnancy in the mouse [42]. Dampened GVA signalling could be one mechanism enabling pregnant mice to eat more food for a longer duration before meal termination. Whilst mechanisms driving adaptations in GVA signalling in pregnant SLD-mice are yet to be determined, **Chapter 2** suggests a potential role for GH, based on increasing GH plasma concentrations during pregnancy [115] together with our finding that GH attenuated tension-sensitive GVA responses to stretch in non-pregnant mice [42]. In addition to their role in sensing the quantity of food in the stomach, VA also innervate the SI, with a high VA density in the duodenum [24]. Future research is required to determine whether duodenal VAs adapt in a similar way as GVAs during pregnancy.

Chapter 4 extends on data from **Chapter 2** and Kentish *et al* [22], by investigating whether a HFHSD impacts adaptations in GVA function during pregnancy. Firstly,

our study verified that tension-sensitive GVAs are dampened in pregnant SLD-mice [42] and are dampened in non-pregnant mice in response to a HFHSD, similar to GVA down-regulation in non-pregnant mice fed a HFD [22]. For the first time, we found that there were no further adaptions in GVA signalling in pregnant mice fed a HFHSD. This was reflected by the lack of major differences in light-phase meal patterns in the pregnant versus non-pregnant HFHSD group on the final 2 study days and could be because either pregnancy or HFHSD maximally suppresses tension-sensitive GVA responses, with no further adaptation possible. Despite their similar GVA responses, food intake during the dark-phase on the final 2 days of study was significantly lower in the pregnant compared to the non-pregnant HFHSD-mice, which may reflect the contribution of other pregnancy factors. Furthermore, since GVA recordings were from mice taken early in the light-phase and the greatest increases in light-phase feeding in pregnant SLD-mice were late in the light-phase, it is possible that differences in GVA sensitivity might be more evident at the later light-phase time points e.g. ZT8-ZT12. Future research is required to characterise the daily variation in GVA mechanosensitivity during pregnancy and in response to a HFHSD.

Lastly, there was no effect of diet on GVA sensitivity to tension in the pregnant groups, which is reflected by the lack of difference in meal size during the light-phase on the final two days of study. This could suggest that a western diet may not impair pregnancy adaptation of GVA satiety but future research is required.

6.2.3.2. Adaptations in intestinal enteroendocrine responses during pregnancy in SLD-mice

The intestinal epithelium contains specialised nutrient-sensing EECs which are important in satiety signalling [153]. These cells make direct contact with the luminal

content and at the molecular level, breakdown products of proteins, carbohydrates and fatty acids can bind to specific nutrient chemoreceptors, triggering the release of satiety hormones including GLP-1 and CCK [153]. In **Chapter 3**, the expression profile of these chemoreceptors and gut hormones was similar in the non-pregnant mice as previously reported in female mice, while a similar expression distribution profile was also observed in the human intestinal mucosa [153]. Although duodenal *GPR93* and intestinal *FFAR4* expression were lower in late-pregnant than non-pregnant mice, this was not reflected at the protein level in terms of the density of GPR93 and FFAR4 immunolabelled cells which was similar or increased, respectively, in pregnant compared to non-pregnant mice. It is possible the abundance of GPR93 and FFAR4 protein within each cell is lower in comparison to non-pregnant mice, however, this remains to be determined. We also observed subtle increases in ileal *FFAR1* and *FFAR2* expression in mid- compared to early-pregnant mice. Although it is unknown why the expression of these genes is upregulated at mid-pregnancy, it could reflect specific fatty acid demands rather than a role in food intake regulation. For example, fatty acids are essential for sustaining fetal and placental growth and depositing fat reserves for future lactation. This is supported by an increase in lipid demand and synthesis increases between weeks 10 and 30 of gestation in women [172]. Furthermore, there is an increase in respiratory quotient measurements (index for carbohydrate and lipid utilisation for energy) at second and third trimester compared to first-trimester and non-pregnant women (reviewed by Melzer *et al*. [229]), indicating lipid sparing and carbohydrate utilisation for energy. Since binding of fatty acids to these receptors leads to increased GLP-1 secretion [230], it could be that intestinal signals (e.g., GLP-1) to the adipose tissue increase adipocyte formation and inhibit apoptosis during pregnancy. It has

previously been shown *in vitro* that GLP-1 treatment of 3T3-L1 cells (preadipocyte cell line) increased adipocyte differentiation compared to vehicle controls, whilst a twice daily intraperitoneal injection of liraglutide, a GLP-1 agonist, increased *in vivo* adipogenesis compared to PBS controls [231]. Furthermore, **Chapter 3** adds more complexity to the current limited knowledge regarding gut hormones, since *GCG* and *CCK* transcript expression and GLP-1 and CCK positive cell density were stable throughout pregnancy. Plasma CCK and GLP-1 plasma concentrations are mostly increased during pregnancy in the rat, dog and human [55,56,63,173,174]. If these plasma concentrations are conserved in mice, it appears that expression is not necessarily linked to hormone secretion during pregnancy. A disconnect between gut hormone expression and secretion has been shown in a different context by Liddle *et al.*, where the neuropeptide, bombesin, stimulated CCK release without altering mRNA expression within male rat intestinal tissue [232]. An increase in nutrient-evoked GLP-1 and CCK in pregnancy seems counterintuitive to increases in food intake [42]. We hypothesise that this may reflect resistance to nutrient-evoked hormones, as shown by the failure of CCK administration to significantly suppress food intake in rats at mid-pregnancy (~14% decrease), although CCK reduced food intake by ~47% in non-pregnant rats [39]. Whether similar resistance to GLP-1 occurs during pregnancy is unknown. The satiating actions of CCK and GLP-1 are mediated through CCKA and GLP1R receptors [152], which are expressed peripherally on VAs and in the central nervous system [154,155]. In the SI, chemosensitive VAs are activated by gut hormones, and it is possible that their response to chemical stimulation are attenuated during pregnancy, similar to the reduced stretch response in GVAs in mid- and late-pregnant mice compared to non-pregnant controls (**Chapter 2**) [42]. Furthermore, pregnancy could be a state of central resistance to CCK and GLP-1. In mid-

pregnant rats (d 14) the increase in c-Fos expression in the NTS was significantly lower than non-pregnant rats following the administration of CCK (via an intraperitoneal injection) and could explain the lack of c-Fos activation within the paraventricular nucleus and supraoptic nucleus (nuclei involved in inducing satiety) as compared to non-pregnant rats [39]. Future studies are needed to investigate the expression of CCKA and GLP-1R receptors in other central nuclei associated with food intake regulation including the arcuate nucleus, paraventricular nucleus, lateral hypothalamic area and dorsomedial nucleus [15].

6.2.4. Adaptations in behavioural patterns during pregnancy

It is widely accepted that pregnancy demands a positive energy balance, which is achieved through physiological adaptations, as reported above, and through behavioural adaptations. During pregnancy in mice, behavioural changes that lead to a positive energy balance include the consumption of more food during the inactive phase (**Chapter 2 and 4**) in combination with substantial decreases in activity and time spent awake during their active phase (**Chapter 5**), consistent with a report from Ladyman *et al.* [6]. A positive energy balance is also conserved in pregnant women where food intake is increased by around 10% [31] and activity reduced or remains unchanged [5] by the third trimester. Recent research has indicated the importance of circadian timing of food intake, activity and sleep as determinants of energy balance [233]. Prior research in human and rodent pregnancy has focussed on adaptations in individual circadian behaviours such as activity [207,208], water [209] and food intake [42]. **Chapter 5** builds on this knowledge and integrates concurrently-recorded changes in behaviour, including food and water intake, time spent awake and activity. From this study, we identified that early-pregnant mice not only decrease activity and wakefulness during the dark-

phase but there is also a modest delay in the final dark-phase peak in activity and wakefulness, by 11-20 minutes, compared to the non-pregnant group, although this only lasts until week 2 of pregnancy. Furthermore, we identified that the increase in light-phase food intake during pregnancy was due to greater food intake late in the light-phase (**Chapter 2**). Interestingly for groups that displayed a peak at the end of the light-phase (first time period of interest, ZT8-ZT12), the food intake event was also delayed from week 2 onwards in the pregnant compared to the non-pregnant group. This delay was also conserved during the third week of pregnancy for peak activity within the same time period of interest (first: ZT8-ZT12). Previous studies have focussed on the onset of running wheel activity, which was later in mid-pregnant (d 8 – d 13) compared to non-, early- and late-pregnant mice [208] in one study, but conversely advanced (up to 4 h) between d 3 and d 10 and normalised in later pregnancy, relative to non-pregnant controls in a separate study [207]. A strength of **Chapter 5** was that the measure of activity timing was not confounded by either the reward aspect of running wheels or by confounding accessibility problems in late pregnant mice due to increasing abdominal size. These behavioural changes and altered timing likely result from increasing plasma sex hormone levels occurring during different stages of pregnancy [150]. For example, the reduction in activity during early-pregnancy in mice could result from the rapid rise in circulating prolactin concentrations within the first few days after mating [6]. Furthermore, the timing of food intake changes during the second week coincides with the rise in GH secretion during that time [115], therefore elevated maternal GH may underlie increased light-phase food intake. The delayed activity later in pregnancy could result from progesterone and prolactin, since administration of these hormones delays activity onset during the oestrous phase in non-pregnant rats [225]. However, this is speculative and further

research is required to confirm these hormonal mechanisms underlying changes in circadian rhythms of behaviours during mouse pregnancy.

6.3. Strengths and limitations

A primary strength of the studies in **Chapters 2, 4 and 5** is the use of the Promethion metabolic cages. Firstly, these cages measure changes in food intake every second and include fine precision scales with a 3 mg resolution. These cages therefore eliminate sources of error arising from manual measurements (e.g. inclusion of any faeces and urine in the hoppers) or a video camera for meal patterns (e.g. behavioural misclassification) [234]. These cages can also transform data in various formats including data presentation every 5 minutes to every 12 h to enable analyses of circadian and photoperiod-specific behaviours, as utilised in the studies presented in this thesis. The studies presented in **Chapters 2 and 5** are the first to present a detailed analysis of feeding behaviour across 24 h, light- and dark-phase in either SLD- or HFHSD-fed pregnant mice. The Promethion cage system also measures various behavioural parameters including but not limited to detailed water intake patterns, activity and sleep, as utilised in **Chapter 5**. There are however some limitations to this equipment. Firstly, the cage algorithm defines sleep as being still for 40 second rather than using more accurate measures such as electromyography [235]. Furthermore, particularly for the standard diet, mice gnaw on food pellets until the remaining pellet falls through the food hopper; a decrease in weight that is too large to be included as a meal. We eliminated these errors by using the largest SLD chow pellets and removing any small, partially-eaten pellets every 4 days. Furthermore, the malleability of the HFHSD makes it easier for crumbs or chunks of food to be taken at a single time. We minimised this potential source of errors by allowing the HFHSD to come to room temperature before moulding this diet

into the base of the food hopper, when replacing food during the study. A final limitation of the Promethion system is that the cage system needs to be shut down every 4 days for data collection, therefore creating blocks of time with incomplete data.

Another strength of the studies reported in this thesis was using a HFHSD regime rather than a standard HFD. Although the mice in **Chapter 4** were not obese, the HFHSD is more similar in composition than HFD to diets reported in developed countries, which are rich in animal foods, oils, fat and sweeteners [180]. Furthermore, overweight and obese pregnant women report consuming a diet high in processed foods and confectionary snacks [186,187], likely contributing to additional weight gain, since women who report a lower BMI during the first trimester consumed a diet rich in wholefoods such as fruit, vegetables, low-fat milk and white meat [236]. Furthermore, the model in **Chapter 4** was a chronic 12 week feeding regime rather than a brief feeding period in the week before mating as used in a similar study [193]. The longer feeding duration is more likely to represent women entering pregnancy on a chronic Westernised high-fat high-sugar diet than studies with shorter durations of diet exposures [193,237].

Lastly, all studies use mice to investigate changes during pregnancy, therefore care needs to be taken when translating the data to women. There are several strengths to using mice to investigate adaptations of satiety mechanisms during pregnancy. For example, as reported in **Chapter 2**, pregnant mice eat more during pregnancy, which is similar to humans [150]. The expression pattern of SI chemoreceptors (**Chapter 3**) are also well conserved between mice and humans [153,238], thereby making the mouse a good comparative model for intestinal physiology. Limitations of mice include the inability to consider effects of psychosocioeconomic factors on outcomes. For example in women, education

level, social norms and changes in hedonic control of food intake could alter eating patterns including restricting certain foods or succumbing to cravings [239]. Mice are restricted to the diet provided, whereas women can change their diet daily or between trimesters of pregnancy. Furthermore, in relation to **Chapter 5**, changes in circadian behaviour in women such as sleep disturbances are thought to be related to an enlarged abdomen and bladder, lower back pain, hunger/thirst and fetal movement [240], and whether mice experience the same symptoms is unknown. It is also important to consider pregnancy differences including the total mass of the progeny relative to maternal size and therefore energy demand associated with progeny growth. A greater increase in maternal energy demand in mice is exemplified through relative weight gain, such that late-pregnant mice gain around 50% of their pre-pregnancy body weight in comparison to women who gain around 10-15% of their pre-pregnancy weight [150].

6.4. Future directions

The results from this thesis put us in a good position to further investigate the mechanisms behind adaptations in GI satiety signalling and their functional relevance to food intake. Changes in intestinal expression of chemoreceptors reported in **Chapter 3** were evaluated by qRT-PCR and immunofluorescence. Whilst immunofluorescence can quantify the immunopositive cell pool, it is possible that the expression of these nutrient receptors and hormones are altered within individual cells. Proteomic quantitation or Western blot will be a critical next step to confirm whether the increase, decrease and unchanged mRNA expression in nutrient receptors and hormones translate to changes in protein expression [171]. Furthermore, it could also be hypothesised that despite unchanged density of positive cells for the chemoreceptors there could be an increase in their co-localisation with CCK and GLP-1 during pregnancy, with

consequent increases in nutrient-evoked hormone release. Assessing co-localisation remains a challenge however, as available antibodies for G-protein receptors are often not effective in labelling [241], or they are raised in the same species as those for hormone antigens, and hence do not allow dual labelling of the receptors and hormones. This limitation could be addressed by utilising cell-specific transgenic mouse lines such as Glu-Venus expressing mice where the co-localisation of nutrient receptors with the Venus fluorescence green L cells could be made [190].

It would also be beneficial to investigate the effect of specific nutrients on gut hormone release. From prior studies it is known that activation of FFAR1, 2, 4 and GPR93 stimulate GLP-1 and CCK secretion. For example, the FFAR4 ligand, linoleic acid, triggers GLP-1 secretion from the human L-cell line (H716 cells), an effect blocked by the FFAR4 antagonist, Xanthene 39 [242]. Accordingly, I attempted to develop an *ex vivo* biorelease protocol to allow testing of effects of nutrients on gut hormone release. Unfortunately, when intestinal segments of ~ 1 cm in length from fed non-pregnant mice, humanely killed at the start of the light-phase, were incubated in 20% intralipid or vehicle, there was no detectable nutrient-induced increase in CCK secretion. Similarly, denatonium benzoate, a potent stimulator of GLP1 secretion [243] failed to stimulate *ex vivo* GLP-1 release from intestinal segments, leading us to discard this methodology. It may be possible to assess nutrient-evoked gut hormone release using a different *ex vivo* protocol based on static culture or using isolated SI regions mounted in an Ussing chamber.

Further, elevated plasma concentrations of the satiety hormones CCK and GLP-1 [55,56,63,173,174] during pregnancy is inconsistent with the observed increase in food intake in these and our studies. Small intestinal VA are chemosensitive and it is

possible that the responses of SI VAs to chemical stimulation are also attenuated during pregnancy, similar to GVAs. Activation of neurones in the NTS (measured via c-Fos expression), where GI VAs terminate, was lower in mid-pregnant than non-pregnant rats following CCK administration (intraperitoneal) [39] raising the possibility that the response of intestinal VAs are attenuated during pregnancy. Future research should assess whether pregnancy changes the responses of duodenal and jejunal VAs to CCK and GLP-1 or whether CCKA and GLP1R receptors expressed on VAs are altered. Lastly, resistance to satiety hormones may also occur at the central level. Therefore, it would be important to determine effects of pregnancy on transport of these hormones into the brain, the expression of these receptors within hypothalamic nuclei and the response of first order and downstream neurons to administration of CCK and GLP-1 directly into the cerebral ventricles. Despite SI VAs being chemosensitive, a subset also respond to mechanical distension [24]. Therefore, it would be important to characterise whether the mechanosensitivity of tension-sensitive duodenal and jejunal VAs are also dampened from mid-pregnancy onwards and if they would contribute to increased food intake.

Like most physiological processes, GVA signals exhibit circadian rhythmicity with decreased sensitivity during the dark-phase when energy demand and food intake are highest in mice [107]. Furthermore, these rhythms are lost in HFD-induced obese mice, where the tension-sensitivity is dampened at time points ZT6 and ZT9 compared to SLD controls [47]. Since GVA recordings were from mice taken early in the light-phase (ZT0-ZT1**, Chapter 2 and 4**), and increases in light-phase feeding occurred at ZT10-ZT11 and dark–phase feeding increased at ZT13-ZT14 in pregnant SLD-mice (**Chapter 5**), future research should

characterise daily variation in GVA sensitivity in response to a HFHSD and pregnancy.

Overall it is critical we gain a better understanding of food intake regulation for weight management, since around 50% of women are overweight or obese prior to pregnancy [181,182] and more than 50% of women experience excessive weight gain during pregnancy [184]. Exploring the mechanisms regulating GI satiety signalling, including adaptations in SI satiety signalling (same methods as **Chapter 3**) and including the future experiments described above, could similarly be conducted in mice fed a chronic western diet.

Finally, progeny exposed to excess energy before birth or are from pregnancies complicated by diabetes are at increased risk for adverse fetal programming and transgenerational obesity and cardiovascular disease [244]. Experiments in rodents (reviewed by McMillen *et al*. [245]) have shown that glucose, insulin and leptin from the maternal circulation influences the development of progeny appetite regulatory centres therefore programming long-term food intake. For example, increased food intake in offspring was observed in a mouse model of gestational diabetes [246] and from HFD-fed pregnant animals (review by Chaves *et al.* [247]). By understanding mechanisms behind programmed hyperphagia we may be able to manipulate diet or find targeted therapies to lower food intake and reduce the risk of adulthood consequences and transmission of obesity to future generations.

6.5. Conclusion

In summary, this thesis highlights the extensive adaptations in GIT-mediated satiety signalling and food intake behaviours during pregnancy. We know that food intake involves a complex network of integrated signals and based on this, adaptations in GVA, nutrient chemoreceptors and hormone expression and behaviour can guide more targeted studies focussed on understanding exactly how food intake is regulated during pregnancy. Overall, the GIT provides a good therapeutic target to optimise pregnancy and fetal health. Manipulating components of these GI pathways through maternal dietary interventions, developing site-specific gastrointestinal drugs or altering the timing of behaviour may improve nutrient supply/delivery during pregnancy. In women, avoiding excessive gestational weight gain could involve reducing the caloric density of their diet, as it may induce similar satiety responses compared to lean pregnant women, or to use dietary strategies such as time-restricted feeding.

CHAPTER 7: References

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CHAPTER 8: Appendices

Appendix 1: Maternal adaptations to food intake across pregnancy: Central and peripheral mechanisms.

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REVIEW

Obesity Biology and Integrated Physiology

Maternal adaptations to food intake across pregnancy: Central and peripheral mechanisms

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Abstract

A sufficient and balanced maternal diet is critical to meet the nutritional demands of the developing fetus and to facilitate deposition of fat reserves for lactation. Multiple adaptations occur to meet these energy requirements, including reductions in energy expenditure and increases in maternal food intake. The central nervous system plays a vital role in the regulation of food intake and energy homeostasis and responds to multiple metabolic and nutrient cues, including those arising from the gastrointestinal tract. This review describes the nutrient requirements of pregnancy and the impact of over- and undernutrition on the risk of pregnancy complications and adult disease in progeny. The central and peripheral regulation of food intake is then discussed, with particular emphasis on the adaptations that occur during pregnancy and the mechanisms that drive these changes, including the possible role of the pregnancy-associated hormones progesterone, estrogen, prolactin, and growth hormone. We identify the need for deeper mechanistic understanding of maternal adaptations, in particular, changes in gut–brain axis satiety signaling. Improved understanding of food intake regulation during pregnancy will provide a basis to inform strategies that prevent maternal under- or overnutrition, improve fetal health, and reduce the long-term health and economic burden for mothers and offspring.

INTRODUCTION

Effective regulation of food intake and energy expenditure is essential for the optimal functioning of an organism and is achieved through complex integration of appetite control regions of the central nervous system (CNS) with peripheral metabolic and nutrient cues. In very basic terms, peripheral feedback to the CNS is composed of two components: post-ingestive signals arising from the gastrointestinal (GI) tract, which contribute to meal termination, and long-term adiposity signals that signal the level of body energy storage (1).

Although energy homeostasis is tightly controlled, the underlying processes show a high level of plasticity in response to environmental

Appendix 2: Pregnancy-related plasticity of gastric vagal afferent signals in mice.

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RESEARCH ARTICLE

Neurogastroenterology and Motility

Pregnancy-related plasticity of gastric vagal afferent signals in mice

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Abstract

Gastric vagal afferents (GVAs) sense food-related mechanical stimuli and signal to the central nervous system, to integrate control of meal termination. Pregnancy is characterized by increased maternal food intake, which is essential for normal fetal growth and to maximize progeny survival and health. However, it is unknown whether GVA function is altered during pregnancy to promote food intake. This study aimed to determine the mechanosensitivity of GVAs and food intake during early, mid-, and late stages of pregnancy in mice. Pregnant mice consumed more food compared with nonpregnant mice, notably in the light phase during mid- and late pregnancy. The increased food intake was predominantly due to light-phase increases in meal size across all stages of pregnancy. The sensitivity of GVA tension receptors to gastric distension was significantly attenuated in mid- and late pregnancy, whereas the sensitivity of GVA mucosal receptors to mucosal stroking was unchanged during pregnancy. To determine whether pregnancy-associated hormonal changes drive these adaptations, the effects of estradiol, progesterone, prolactin, and growth hormone on GVA tension receptor mechanosensitivity were determined in nonpregnant female mice. The sensitivity of GVA tension receptors to gastric distension was augmented by estradiol, attenuated by growth hormone, and unaffected by progesterone or prolactin. Together, the data indicate that the sensitivity of GVA tension receptors to tension is reduced during pregnancy, which may attenuate the perception of gastric fullness and explain increased food intake. Further, these adaptations may be driven by increases in maternal circulating growth hormone levels during pregnancy.

NEW & NOTEWORTHY This study provides first evidence that gastric vagal afferent signaling is attenuated during pregnancy and inversely associated with meal size. Growth hormone attenuated mechanosensitivity of gastric vagal afferents, adding support that increases in maternal growth hormone may mediate adaptations in gastric vagal afferent signaling during pregnancy. These findings have important implications for the peripheral control of food intake during pregnancy.

food intake; gastric vagal afferents; growth hormone; pregnancy; satiety signals

INTRODUCTION

Gastric vagal afferents (GVAs) sense food-related mechanical stimuli and transfer this information to the brain to modulate food intake and gastric function (1). There are two functional classes of mechanosensitive GVAs, tension and mucosal receptors (2, 3). Tension receptors, with afferent endings distributed in the muscular layers, sense the level of gastric distension and transfer signals to the brain to generate feelings of satiety and fullness (4). Mucosal receptors, with afferent endings distributed in the gastric mucosal layers, are activated by movement of food particles over the receptive field in the stomach. Although there is no direct evidence, mucosal receptors are thought to be involved in the regulation of gastric motor function delaying gastric emptying (5). This mechanism may allow large food particles to be sufficiently digested before transit to the small intestine. The subsequent accumulation of undigested food in the stomach could activate GVA tension receptors and, therefore, activation of mucosal receptors may indirectly inhibit food intake. Together, evidence indicates that both types of GVAs play distinct but complementary roles in the generation of satiety signals and control of food intake.

GVAs are highly plastic and can adapt to physiological changes to assist with the maintenance of energy homeostasis. For example, the sensitivity of GVAs to gastric distension is modulated by nutritional status in mice, with reduced mechanosensitivity observed after short-term food restriction

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