Gastrointestinal Appetite Hormones Change With Maternal Adaptations to Pregnancy and Lactation in the Rat

Thesis

Gastrointestinal appetite hormones change with maternal adaptations to pregnancy and lactation in the rat

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Published abstracts

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Johnson, Michelle; Saffrey, Jill and Taylor, Victoria (2014). **Sex differences in gut satiety hormones peptide-YY and glucagon-like peptide-1 in pups raised in larger lactation litter sizes.** In: *Society for Reproduction and Fertility Annual Conference 2014*, 1–2 September 2014, Edinburgh. [http://f1000.com/posters/browse/summary/1096876]

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Johnson, Michelle; Saffrey, Jill and Taylor, Victoria (2012). **Do circulating and tissue concentrations of ghrelin and peptide-YY (PYY) change during the oestrous cycle in Wistar Rats?** In: *Society for Reproduction and Fertility Annual Conference 2012*, 9-11 July 2012, Edinburgh.

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Abstract

As obesity is becoming more prevalent, there is concern for maternal health during pregnancy, parturition and beyond, alongside the impact that maternal obesity has on offspring health. Appetite regulation by gut hormones is one target for obesity 'treatment', although little is known about this fundamental system during different female reproductive stages. The main aims of this thesis were to study the orexigenic stomach-secreted hormone ghrelin and the anorexigenic colon-secreted hormones peptide-YY (PYY) and glucagon-like peptide-1 (GLP-1) in female rats during their oestrous cycle, pregnancy and lactation. Changes in gut dimensions during pregnancy and lactation were also studied. In addition, lactation litter sizes were adjusted to explore the effects of different nutritional demands on both the dams and their male and female pups.

Reduced food intake has been reported during oestrus in rats. Here, significantly reduced fasted stomach contents leading into oestrus occurred with significantly increased circulating GLP-1 during proestrus. Ghrelin-positive stomach cells were significantly higher after parturition compared with non-pregnant and pregnant dams; this change may initiate and sustain lactation-associated hyperphagia, with significantly increased plasma ghrelin evident by late lactation. Paradoxical high levels of PYY and GLP-1 were found early in lactation when food intake was high, which may be related to a significant increase in gut growth from early to late lactation. Maternal gut size was significantly increased by late lactation and lactation litter size appeared to influence these changes. Only male offspring had altered satiety

hormones, with significantly decreased descending colon PYY and GLP-1 levels when raised in large litters.

In conclusion, gut hormones and gastrointestinal modifications were altered during different reproductive states in females and in their male offspring at weaning. Further study is required to elucidate whether these changes may persist, influencing future health status, and whether they can be reliably modified for therapeutic purposes.

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%CV	coefficient of variation
°C	degrees Celsius
α-MSH	alpha-melanocyte stimulating hormone
µg	microgram
µl	microlitre
µm	micrometre
3V	third ventricle
5-HT	serotonin
ACTH	adrenocorticotropic hormone
AGA	appropriate for gestational age
AgRP	agouti-related protein
ARC	arcuate nucleus
CART	cocaine and amphetamine regulated transcript
CCK	cholecystokinin
cm	centimetre
CNS	central nervous system
d0L	day 0 of lactation (day of birth)
d5L	day 5 of lactation
d10L	day 10 of lactation
d25L	day 25 of lactation
d4P	day 4 of pregnancy
d12P	day 12 of pregnancy
d18P	day 18 of pregnancy
DPP IV	dipeptidyl peptidase IV

E_2	oestradiol
EC	enteroendocrine cell
EDTA	ethylenediaminetetraacetic acid
EMEA	European Medicines Evaluation Agency
EPI	epithelium
ERα	oestrogen receptor alpha
FSH	follicle-stimulating hormone
g	gravity (centrifugation); gram (mass)
GABA	gamma-aminobutyric acid
GABAR	gamma-aminobutyric acid receptor
GH	growth hormone
GHRH	growth hormone releasing hormone
GHSR	growth hormone secretagogue receptor
GI	gastrointestinal
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLP-2	glucagon-like peptide-2
GnRH	gonadotropin releasing hormone
GOAT	ghrelin *O*-acyl transferase
HFD	high fat diet
HRT	hormone replacement therapy
INSR	insulin receptor
IR	immunoreactive
kΩ	kilo ohm

l	litre
LC/MS-MS	liquid chromatography-mass spectrometry
LGA	large for gestational age
LH	luteinising hormone
M	molar
MBOAT	membrane-bound *O*-acyltransferase 4
MC4R	melanocortin 4 receptor
MCH	melanin-concentrating hormone
ml	millilitre
mm	millimetre
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
ng	nanogram
NPY	neuropeptide-Y
ObR	leptin receptor
OVX	ovariectomy/ovariectomised
OXM	oxyntomodulin
P_4	progesterone
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pg	picogram
PL	placental lactogen
pmol	picomole
POMC	pro-opiomelanocortin

PP	pancreatic polypeptide
PRL	prolactin
PVN	paraventricular nucleus
PYY	peptide-YY
QC	quality control
RAPID	**R**educed temperatures, **A**cidification, **P**rotease inhibition, **I**sotopic exogenous controls, and **D**ilution
RT	room temperature
RYGB	Roux-en-Y gastric bypass
SEM	standard error of the mean
SGA	small for gestational age
SIFT-MS	selected ion flow tube mass spectrometry
SPA	spontaneous physical activity
TPN	total parenteral nutrition
WAT	white adipose tissue
WWT	wet weight of tissue

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Introduction

Obesity is a leading global cause of disease and premature death and yet The World Health Organisation (WHO) describes it as preventable, given the correct interventions (WHO, 2014). Worldwide, it was estimated that 35% of adults over the age of 20 were overweight in 2008, with 11% of adults falling into the obese category (WHO, 2014). In the UK, it is estimated that by the year 2030, obesity and obesity-related disease will cost the National Health Service £2 billion a year (Ng and Wilding, 2014). Of most concern is that in excess of 40 million children worldwide, under the age of 5 years, were overweight or obese in 2012 (WHO, 2014). Maternal body mass gain is a natural consequence of pregnancy, however many women are already overweight and will further over-consume during pregnancy, which, combined with interrupted sleep and mealtime patterns, can result in additional postpartum body mass retention. The influence of pregnancy and lactation on subsequent body mass and the metabolic health of both mothers (Rooney and Schauberger, 2002) and their offspring (Heerwagen *et al.*, 2010) is now well established. To understand why body mass is gained and retained at these times and to enable the development of appropriate treatments, it is essential to determine how appetite regulation and the gastrointestinal system are altered during pregnancy, lactation and the later postpartum period. However, males have preferentially been used in many studies of appetite regulation because the reproductive cycle, pregnancy, lactation and

the later postpartum period all present complicating factors to consider experimentally (reviewed by Asarian and Geary, 2013).

The regulation of appetite is one mechanism by which whole body energy homeostasis is maintained, however the incidence of obesity is increasing in part due to the increased availability of calorie-dense, high fat foods and an increasingly sedentary lifestyle creating an 'obesogenic' environment. Appetite is regulated by both the nervous and endocrine systems and in humans, social and emotional cues can also influence appetite and food choice. Food palatability also plays a large role in when humans may choose to eat, even when not hungry, and has been referred to as 'non-homeostatic' eating (Berthoud, 2006). Short-term appetite can play a role in meal initiation and be programmed by a certain situation or time of day, whilst long-term homeostatic appetite is a result of the body sensing an energy deficit. To regulate long-term homeostatic appetite, the hypothalamus provides neural control, while the enteroendocrine cells (ECs) of the gut provide hormonal control. These are summarised below (sections 1.2, 1.3 and 1.4).

1.2. Hypothalamic regulation of appetite

The hypothalamus plays a pivotal role in the regulation of food intake, containing receptors for many of the gut-derived appetite-influencing hormones (Figure 1.1), and receives and relays information regarding nutrient availability to maintain whole body energy homeostasis. The arcuate nucleus (ARC) of the hypothalamus contains an orexigenic (appetite-stimulating) and an anorexigenic (appetite-inhibiting) subset of first order

neurons; the orexigenic neurons co-express neuropeptide-Y (NPY), agouti-related protein (AgRP) and gamma-aminobutyric acid (GABA) and the anorexigenic neurons co-express pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript peptide (CART) (reviewed by Valassi *et al.*, 2008). These neurons from the ARC have axons that extend to second order neurons of other brain regions, including the paraventricular nucleus (PVN) (Valassi *et al.*, 2008). Although not a focus of this work, proposed hypothalamic mechanisms of action for ghrelin and PYY will be summarised in section 1.6.

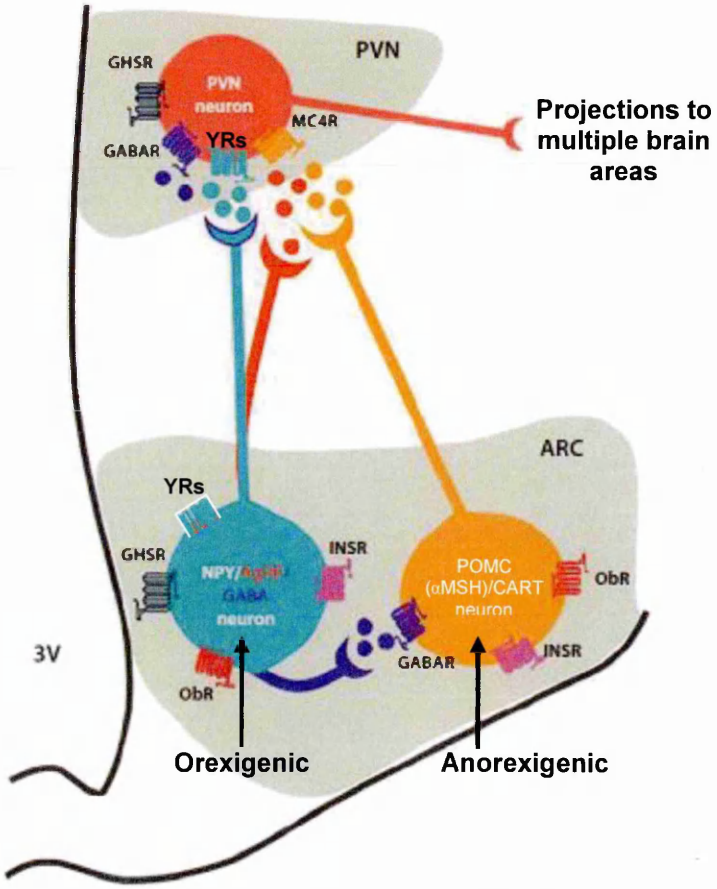

Figure 1.1. The arcuate nucleus (ARC) of the hypothalamus and appetite regulation (3V, third ventricle; PVN, paraventricular nucleus; POMC/CART, pro-opiomelanocortin (purple)/cocaine and amphetamine regulated transcript; NPY/AgRP/GABA, neuropeptide-Y (blue)/agouti-related protein (red)/gamma-aminobutyric acid (purple); α-MSH, α-melanocyte stimulating hormone; GHSR, growth hormone secretagogue receptor; INSR, insulin receptor; ObR, leptin receptor; MC4R, melanocortin 4 receptor; GABAR, gamma-aminobutyric receptor; YRs, Y receptors). (Modified from Briggs and Andrews, 2011).

1.3. The gastrointestinal tract

The mammalian gastrointestinal (GI) tract is separated into several main subdivisions, including the mouth, oesophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine, rectum and the anus, which are collectively responsible for the breakdown and absorption of food and the excretion of waste products (Figure 1.2).

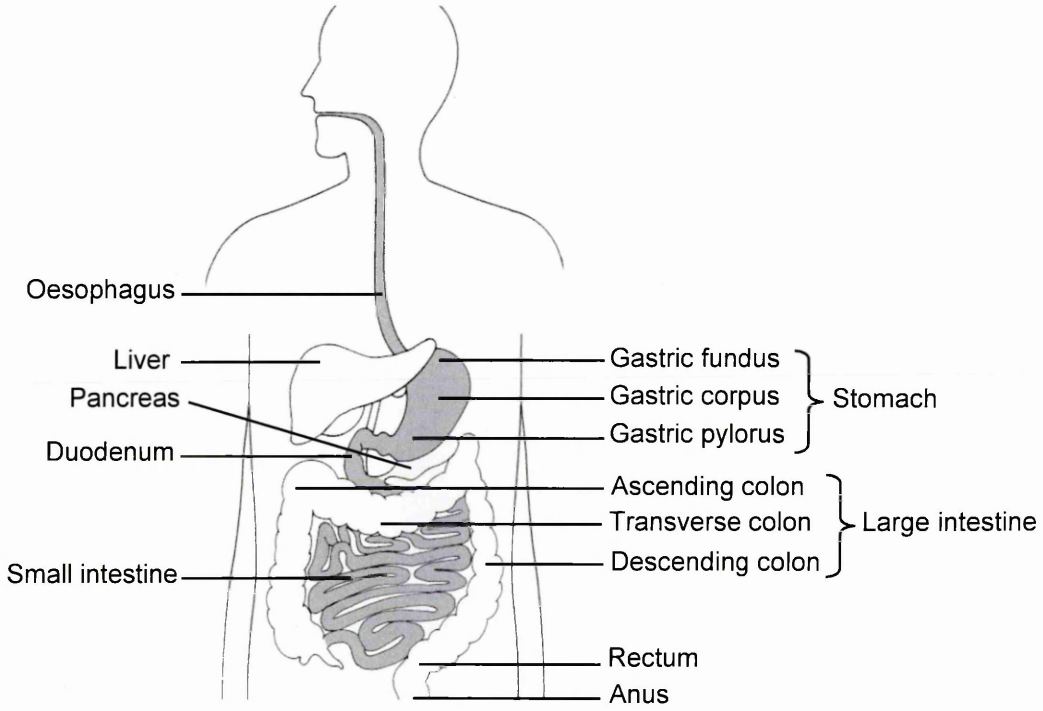

Figure 1.2. The human gastrointestinal tract
(Modified from the National Institute of Diabetes and Digestive Kidney Diseases (NIDDK) image library, NIDDK, n.d.)

In the mouth, food is broken down both physically (chewing) and enzymatically (saliva) before it is swallowed, entering the oesophagus and being delivered to the stomach. Digestion of carbohydrates, proteins and lipids begins in the mouth (Goodman, 2010). Food is further digested by the stomach mechanically mixing its contents with gastric acid, secreted by parietal cells, and a number of digestive enzymes including pepsin and lipase, secreted by gastric chief cells, that break down proteins and lipids

respectively (Goodman, 2010). Gastric emptying releases food into the small intestine, where enzymes in the brush border of gut enterocytes are involved in digestion and nutrient transportation into the bloodstream, alongside digestion by pancreatic and liver enzymes. The small intestine has a large surface area due to the presence of villi and microvilli on the epithelial mucosa and is the main gut area where water is absorbed. Any remaining water and electrolytes are absorbed in the large intestine and the gut microbiota metabolise any remaining nutrients, such as short-chain fatty acids (Goodman, 2010). Indigestible content is formed into faeces which are temporarily stored before excretion as waste.

Nutrients such as carbohydrates, proteins and lipids are absorbed by specialised gut epithelial cells, enterocytes, and pass through the portal blood and into the liver before transportation to other areas of the body (Goodman, 2010). The processes of secretion, digestion, absorption and motility depend on the co-ordinated actions of different intestinal cell types, including different types of epithelial cells and also muscle cells. This co-ordination is achieved by the intrinsic and extrinsic innervation of the gut and also by the endocrine system of the GI tract.

The gut endocrine system is of great importance due to its essential role in whole body energy homeostasis. Appetite and some gut functions, such as gastric emptying, are influenced by circulating peptide hormones that are produced and secreted by ECs located in the mucosal epithelium of the gut. These hormones act in the central nervous system (CNS), in control centres

of the brain, and peripherally in the body. Hormone signals from the gut act on neurons of the hypothalamus either by travelling across the blood-brain barrier via the circulation or via the vagus nerve by activation of gastric vagal afferents (Figure 1.3).

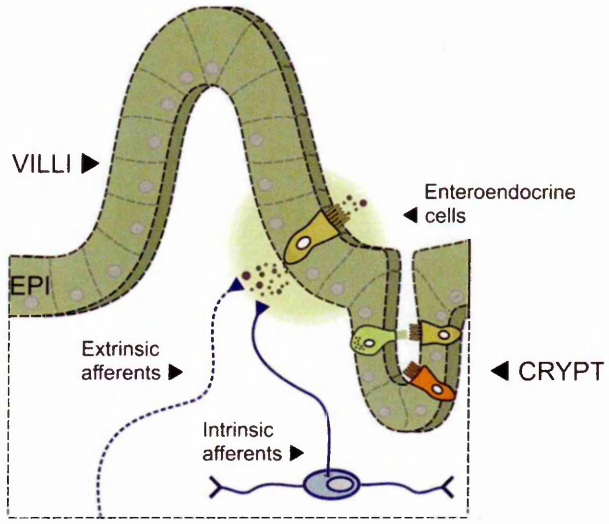

Figure 1.3. Enteroendocrine cells in gut mucosal epithelium
Cross-section of the intestinal wall, illustrating that enteroendocrine cells in the epithelium (EPI) of the gut are associated with intrinsic afferents of the enteric nervous system and extrinsic vagal afferents (Bertrand, 2009).

Figure 1.4 outlines principal areas of the human GI tract that produce and secrete key gut hormones involved in appetite regulation and their main actions, including those studied in this thesis. These GI areas (Figure 1.4) are broadly similar in rodents, although rats have a caecum, rather than an appendix, which connects the small and large intestine and is required for fermentation of cellulose. The hormones studied here are ghrelin, peptide-YY (PYY) and glucagon-like peptide-1 (GLP-1). Details of these hormones and their mechanisms of action will be described later (section 1.6).

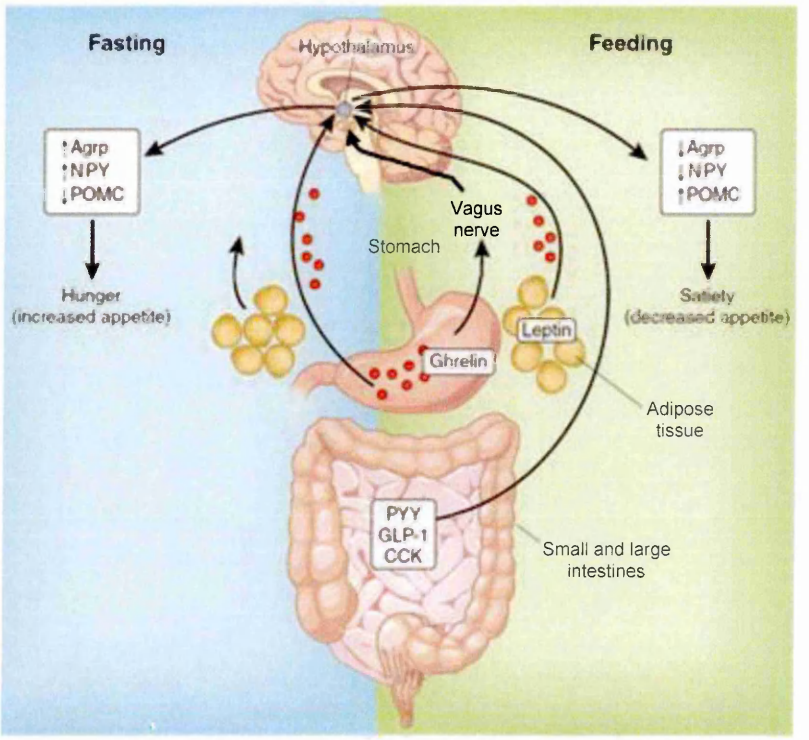

Figure 1.4. Principal sites of synthesis of the appetite-regulating peptide hormones in humans
The stomach, intestines and adipose tissue release hormones that induce hunger or satiety by interaction with the hypothalamus, via the circulation or by interaction with the vagus nerve. (PYY, peptide-YY; GLP-1, glucagon-like peptide-1; CCK, cholecystokinin; AgRP, agouti-related protein; NPY, neuropeptide-Y; POMC, pro-opiomelanocortin). (Modified from Larder and O'Rahilly, 2012).

1.4. Enteroendocrine cells of the gut

Gut ECs have been studied to understand their possible role in pathologies such as GI cancers and in obesity and eating disorders. The epithelial cells of the gut differentiate from pluripotent stem cells at the base of intestinal crypts (Gunawardene *et al.*, 2011), migrating upwards as they do so (Figure 1.5). As well as ECs, other epithelial cells of the gut, which also develop from these stem cells, include mucus-secreting goblet cells and absorptive enterocytes.

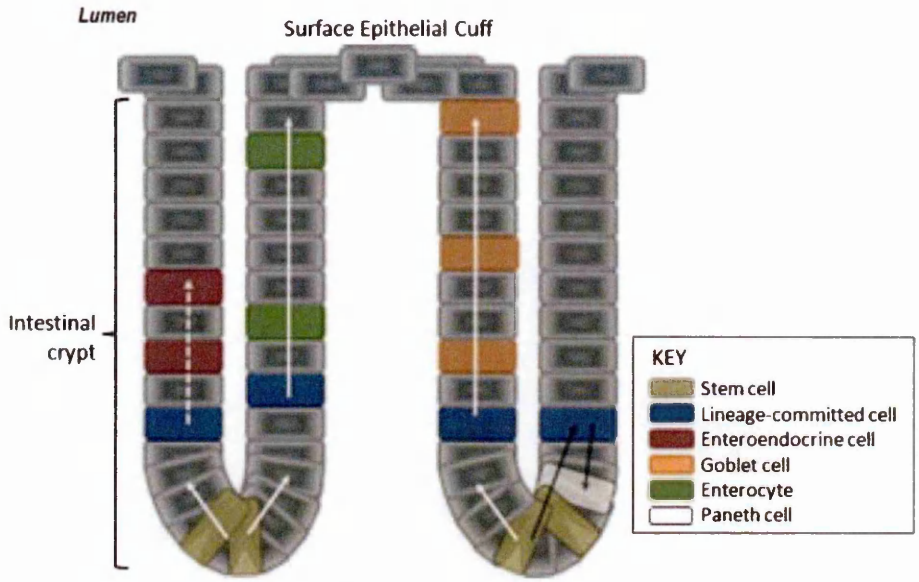

Figure 1.5. Development of endocrine cells of the mammalian gastrointestinal tract
Pluripotent stem cell daughter cells migrate upwards and differentiate into one of the four represented cellular lineages (Gunawardene *et al.*, 2011).

ECs are found throughout the entire length of the gut and can be characterised either as 'open' type cells, which have microvilli that extend into the lumen of the gut or as 'closed' type cells, which do not (Sternini *et al.*, 2008). Both of these cell types produce and secrete different products, including peptide hormones and neurotransmitters, singly or in combination and have been named as shown below (Table 1.1).

Table 1.1. Summary of some of the enteroendocrine cell types found in the gastrointestinal tract and their principal locations
(* also known as gastric inhibitory peptide). (Gunawardene *et al.*, 2011; Sternini *et al.*, 2008).

Principal Gut Locations	Cell Type	Product Secreted
Throughout the gastrointestinal tract	Enterochromaffin cells	Serotonin (5-HT)
Stomach (throughout) Stomach (pylorus) Stomach (corpus)	X/A-like cell G-cells D-cells	Ghrelin Gastrin Somatostatin
Proximal small intestine Small intestine	I-cells K-cells	Cholecystokinin (CCK) Glucose-dependent insulinotropic peptide* (GIP)
Distal large intestine	L-cells	Peptide-YY (PYY) Glucagon-like peptides (GLP) Oxyntomodulin (OXM)

1.4.1. Enteroendocrine cells during development

A number of studies have aimed to determine at what stage of development ECs appear in the GI tract, with many such studies focussing on ghrelin since its discovery as the only orexigenic gut hormone to be identified to date. Expression of ghrelin mRNA in the stomach of male and female rat pups has been detected from 1 week of age, with a gradual increase until 4 to 5 weeks of age (Sakata *et al.*, 2002). The same authors reported the presence of ghrelin-immunopositive cells in the stomach from 1 week of age, with a higher cell density in females than in males, although cell density increased with age in the males which was not the case in the females. Furthermore, ghrelin immunopositive cells showed an age-dependent localisation in the stomach, where they were predominantly located at the base of the fundic glands at 2 weeks of age and started appearing at the glandular neck by 3 weeks of age. Another study documented similar findings, with an increase in stomach tissue ghrelin concentrations from 1 to 5 weeks of age in rats (Hayashida *et al.*, 2002). They additionally reported the presence of ghrelin-immunopositive cells in the foetal stomach at days 18, 20 and 22 of gestation; these cells increased in number with gestational age. It is likely that prior to birth, ghrelin has a more important role in the rat foetal pancreas than in the stomach, due to much higher concentrations present here (Chanoine and Wong, 2004).

Colonic ECs have been reported to appear by embryonic day 14.5 in mice. All colonic ECs expressed PYY at this stage, prior to further differentiation at later stages when glucagon was found to co-express with PYY throughout

much of gestation, which the authors state is similar to the normal secretory profile of L-cells (Upchurch *et al.*, 1996). An earlier study also reported that PYY was present at an early stage, with PYY mRNA expression being evident in the foregut from embryonic day 11 (Jazin *et al.*, 1993). Together, these studies may suggest a role for PYY in the early development and growth of the gut.

The pre- and postnatal stages of development may represent critical times at which the ECs secreting appetite-regulatory hormones could be altered and could influence gut growth (Gomez *et al.*, 1995), which may change the way the gut and gut hormones respond to appetite and food intake in later life. It is also possible that changes to the gut and gut hormones may affect hypothalamic circuitry relating to appetite and body mass control, which in rodents, is not fully mature until approximately 21 days postnatally, thus the suckling period presents a critical time period during which this regulatory system could be altered. In humans, this system is less easily affected by postnatal changes, as hypothalamic maturation occurs during the third trimester of pregnancy (Prior and Armitage, 2009), however early changes in gut development still represent a time in which this system could be influenced.

1.5. Regulation of food intake and body mass

Humans and rodents are the main species utilised in the majority of studies involving appetite and food intake. Whilst humans are classed as being meal-feeders by day, rodents tend to consume multiple smaller meals, after an

initial larger meal, throughout the nocturnal period when they are active. Despite these differences, feeding regulation has been shown to be very similar between the species. One way in which the different feeding patterns has been taken into account in comparison studies is to standardise measurements by taking them at the same time of day, if multiple samples are required for a study. Due to the different secretory profiles of hormones throughout a 24 hour day, taking samples at the most relevant times for the hormone of interest is critical, as differences in their highest concentration between different days or experimental interventions may be lost if they are measured at a daily nadir, or anywhere in-between. If gut hormones are to be measured, fasting is generally used as a method to standardise measurements across days and subjects, especially in *ad libitum* fed rodents, in order to obtain a value that can be reliably compared between subjects. In rodent studies of gut appetite hormones, it is important to take samples when most physiologically relevant to them, which is during the dark phase. Taking samples from rodents during the light period arguably does not allow an accurate comparison between human and rodent studies. A degree of compromise needs to be struck between the daily profile of the peptide being measured and the possibility of standardising subjects by fasting.

Gut hormones play key roles in the regulation of feeding and body mass, although in humans, food intake is also influenced by learned behaviour and both social and emotional cues. Human studies report large variations in day-to-day food intake (Bray *et al.*, 2008), yet on average, adults will only gain around 0.5 kg in body mass each year of their lives (Bessesen, 2011),

suggesting a finely tuned regulatory system. This value, however, does not consider physiological states such as pregnancy or lactation, which temporarily alter this regulatory system (section 1.8) yet their effects may last years. Table 1.2 gives an overview of the main hormones and neurotransmitters that have been identified to be involved in appetite regulation and their expected effect on food intake in mammals. Table 1.2 also outlines the principal site of synthesis for each hormone and neurotransmitter, although many of these peptides are found in multiple locations both inside and outside the gut. For example, ghrelin and somatostatin distribution in the gut is primarily in the stomach, with decreasing amounts along the small intestine and into the large intestine. PYY and GLP-1 are located primarily in the large intestine, most concentrated in the rectum, with decreasing amounts back up through the large intestine and into the small intestine.

Alongside influencing food intake and gut function, it has been suggested that some gut hormones may also influence subconscious spontaneous physical activity (SPA), such as fidgeting. For example, Pfluger *et al.* (2011) concluded that the orexigenic hormone ghrelin further helped maintain energy homeostasis by decreasing SPA during chronic elevation of ghrelin to conserve energy. These authors have suggested that decreased SPA could be a protective mechanism to promote lipid storage in adipose tissue, thus maintaining energy stores during a period of fasting (Pfluger *et al.*, 2011).

Table 1.2. An overview of the main peripheral and central peptides and neurotransmitters involved in mammalian feeding and body mass regulation and their principal sites of synthesis
(* synthesised in many additional areas).(Volkow *et al.*, 2011, Akil *et al.*, 1984, Rodgers *et al.*, 2002, Gibson *et al.*, 2010, Wilding, 2002).

Product	Principal sites of synthesis	Effect on feeding
Peripheral		
Ghrelin	Stomach	↑
Somatostatin	Stomach	↓
Cholecystokinin (CCK)	Small intestine	↓
Peptide-YY (PYY)	Large intestine	↓
Glucagon-like peptide-1 (GLP-1)	Large intestine; pancreas	↓
Pancreatic polypeptide (PP)	Large intestine; pancreas	↓
Oxyntomodulin (OXM)	Large intestine	↓
Serotonin (5-HT)	Throughout the GI tract	↓
Insulin	Pancreas	↓
Amylin	Pancreas	↓
Leptin	Adipose tissue	↓
Adiponectin	Adipose tissue	↑
Central		
α-melanocyte stimulating hormone (α-MSH)	Hypothalamus	↓
Agouti-related protein (AgRP)	Hypothalamus	↑
Cocaine and amphetamine-regulated transcript (CART)	Hypothalamus	↓
Dopamine	Ventral tegmental area*	↑
Melanin-concentrating hormone (MCH)	Hypothalamus	↑
Neurotensin	Hypothalamus	↓
Neuropeptide Y (NPY)	Hypothalamus	↑
Noradrenaline	Central nervous system	↑
Opioids (endogenous)	Central nervous system	↑
Orexin A and B	Hypothalamus	↑
Pro-opiomelanocortin (POMC)	Hypothalamus	↓

Our current understanding of the roles of regulatory peptides in feeding and the regulation of body mass has advanced in recent years but still remains incomplete. As obesity is becoming more prevalent worldwide, these endogenous hormones are valid targets for potential obesity treatments. Due

to their complex inter-regulatory actions and the apparent redundancy of some pathways this may prove difficult to achieve, as targeting a single peptide or receptor may not have the desired effect or may even cause unwanted side-effects. One review has discussed evidence of the difficulties involved in developing a drug that has the potential to be used as an obesity treatment. PYY, GLP-1 and oxyntomodulin (OXM) have all decreased food intake and body mass when given to rodents and humans (reviewed by Chaudhri *et al.*, 2008). However, these authors point out that both PYY and GLP-1 cause nausea and vomiting at high concentrations, likely due to their effects on the inhibition of gastric emptying and like OXM, they are also rapidly broken down in the circulation by the enzyme dipeptidyl peptidase IV (DPP IV), thus requiring a DPP IV-resistant drug analogue. If a pharmacological agent was successfully manufactured to manipulate endogenous hormone actions to decrease appetite in obese individuals, it would have to maintain a physiologically acceptable hormone balance so that other body systems and pathways did not overcompensate and render any effect temporary. A good example of this problem is a study by Sainsbury *et al.* (2002) that used a knockout mouse model with a hypothalamic-specific deletion of the Y2 receptor (the receptor for NPY and PYY) and found that the expected decreases in food intake and body mass were only transient, suggesting compensatory mechanisms took place in order to maintain whole body homeostasis in the longer term (Sainsbury *et al.*, 2002).

One recent advance is the development of liraglutide, a GLP-1 receptor agonist that remains in the circulation for 11 to 13 hours (reviewed by Ng and

Wilding, 2014). Liraglutide was first approved by the European Medicines Evaluation Agency (EMEA) in 2009 for the treatment of type 2 diabetes, improving glycaemic control, but it has also been shown to cause a sustained mean body mass loss of 7.6 kg after 2 years of treatment. Due to the obvious attraction of body mass loss and increased glycaemic control, liraglutide has recently been submitted as an anti-obesity drug to both the EMEA and the US Food and Drug Administration (Ng and Wilding, 2014). However, liraglutide is similar to other GLP-1 treatments in that 14% of patients in clinical trials have reported vomiting as a side effect. An increased resting pulse rate has also been reported after 2 years of treatment with liraglutide (Ng and Wilding, 2014). More long-term studies would need to establish whether the medical benefits of obesity treatment with liraglutide outweigh these possible side effects.

The only current demonstrated long-term 'cure' for obesity is gastric bypass surgery, with Roux-en-Y bypass (RYGB) among the most well studied due its use in 70 to 75% of bariatric procedures (reviewed by Beckman *et al.*, 2010). Briefly, this procedure involves reducing stomach volume by bypassing the distal stomach and a portion of the proximal small intestine, which creates not only a restriction in food intake but also results in malabsorption of nutrients (Beckman *et al.*, 2010). Beckman *et al.* (2010) reviewed the findings of the most robust studies in this area and documented that RYGB increased circulating levels of PYY and GLP-1, with PYY levels being consistently elevated above baseline levels regardless of fed state. These authors also pointed out that most studies were in agreement that levels of both total and

acyl ghrelin (peptide forms are described in section 1.6.1.1) were decreased compared with control levels in circulation. Although gastric bypass results in long-term body mass loss that is beneficial to the health of morbidly obese individuals by improving their metabolic status, later body mass gain and health complications are not unheard of. A long-term study of obese patients that had a RYGB procedure found that body mass loss was maximal 4 years after the procedure, after which there was a tendency to regain some body mass by postoperative year 7 (Spivak *et al.*, 2012). These authors also documented that 9% of patients who underwent RYGB experienced a 'serious life-threatening complication', examples of which included intra-abdominal bleeding and bowel obstruction caused by an internal hernia. A recent review expressed concern for long-term health problems that are as yet to be fully characterised. This review proposed that not only are malabsorption of vitamin D and calcium likely to become an issue years after RYGB, despite supplementation, but that elevated PYY levels and reduced ghrelin levels may result in a decline in bone mineral density (Hage and El-Hajj Fuleihan, 2014). These authors stressed the requirement for future studies to monitor and address changes in bone mineral density in relation to gut hormones postoperatively to protect the future health of bypass patients.

1.6. Appetite hormones in the gastrointestinal tract

Whilst many peptide hormones are involved in appetite regulation (see Table 1.2), this thesis has focussed on three; the orexigenic gut peptide ghrelin, produced primarily by the stomach, and the anorexigenic gut peptides PYY and GLP-1, produced primarily throughout the large intestine. Ghrelin and

PYY can be studied together as gut peptides with some opposing actions. PYY and GLP-1 are co-secreted by L-cells in the distal gut and both are implicated in satiety. The known properties of these three peptides are described below. The anticipated benefit of studying multiple hormones together is to provide a more in-depth understanding of appetite regulation, due to the complex interaction between the gut hormones in the regulation of appetite during dynamic reproductive states.

1.6.1. Ghrelin

Ghrelin was first isolated from rat stomach as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) and was so named as 'ghre' is the root of the word 'grow', thus describing its stimulatory role in causing growth hormone (GH) release from the anterior pituitary gland (Kojima *et al.*, 1999). To date, ghrelin is the only orexigenic peptide to have been isolated that is produced outside the CNS, and its role in energy homeostasis and feeding stimulation is a major area of research. Ghrelin has also been shown to increase gastric motility and to increase body mass and adiposity (reviewed by Lim *et al.*, 2010). Ghrelin is predominantly secreted from stomach X/A-like cells and is released in response to both homeostatic (energy deficit) and non-homeostatic (no energy deficit) hunger. The secretion of ghrelin is influenced by a number of factors including stomach distension and a rise or fall in the levels of appetite-regulatory hormones such as PYY (see section 1.6.4). The GHSR has been identified in multiple locations including the pituitary and adrenal glands, hypothalamus, pancreas,

myocardium and the spleen (Lim *et al.*, 2010), although the role of ghrelin in some locations has not yet been established.

An early study of ghrelin levels in relation to appetite demonstrated that ghrelin levels were highest in humans (1 male and 9 females) 1 to 2 hours prior to meal onset and rapidly decreased to their lowest levels approximately 1 hour after meal onset (Cummings *et al.*, 2001). Further studies have also explored the role of ghrelin in other areas (Table 1.3) including reproduction, which will be discussed in detail later (section 1.7.2).

Table 1.3. Some of the additional effects of ghrelin in mammals
(Modified from Kojima and Kangawa, 2005).

Target	Effect of ghrelin
Gastric acid	↑
Overall gastric motility	↑
Gut mucosa turnover	↑
Growth hormone (GH)	↑
Adrenocorticotropic hormone (ACTH)	↑
Cortisol	↑
Prolactin	↑
Insulin	↑/↓
Blood glucose	↑
Cardiac output	↑
Blood pressure	↓

1.6.1.1. The ghrelin peptide forms and ghrelin O-acyl transferase (GOAT)

Encoded by the ghrelin gene, which in humans is on chromosome 3p25-26, the 117-amino acid preproghrelin (reviewed by Kojima and Kangawa, 2005) is cleaved to produce the 28-amino acid peptide ghrelin (Kojima *et al.*, 1999).

Ghrelin is present in two main forms, acyl ghrelin, known as 'physiologically active' ghrelin and desacyl ghrelin, which is known as the 'inactive form'. Total ghrelin is a combination of the acyl and desacyl forms of the ghrelin peptide. Both acyl and desacyl ghrelin forms are present in gut tissue and in the circulation of rodents and humans (Hosoda *et al.*, 2000). There is also a third ghrelin fragment cleaved from the C-terminal end known as obestatin (Kobelt *et al.*, 2008), which has been suggested to act as an antagonist to the orexigenic properties of ghrelin, although its role remains controversial. A number of other minor fragments are derived from the ghrelin peptide, although again, their roles are not well characterised (Kojima and Kangawa, 2005).

Acyl ghrelin is the endogenous ligand for the GHSR, thus stimulating GH secretion (Kojima *et al.*, 1999). Briefly, high levels of ghrelin can stimulate hypothalamic release of growth hormone releasing hormone (GHRH) which in turn releases GH from the pituitary; GH production can be inhibited by high levels of somatostatin (reviewed by Zizzari *et al.*, 2011). Total ghrelin has been found to induce GH secretion both directly, by stimulating the release of GHRH (Wren *et al.*, 2002) and indirectly, by inhibition of hypothalamic somatostatin release (Tolle *et al.*, 2001). Tolle *et al.* (2001) additionally tested desacyl ghrelin in their experiments and found no effect on GH levels, suggesting that acylation by ghrelin *O*-acyl transferase (GOAT) was necessary for their reported effects. GOAT modifies the ghrelin peptide at its third serine residue by the addition of a fatty acid to its hydroxyl group (Figure 1.6), so that it can bind to the GHSR (Kojima and Kangawa, 2005). GOAT,

also known as MBOAT4, belongs to the membrane-bound *O*-acyltransferase (MBOAT) super-family of enzymes and was first identified in the mouse genome (Yang *et al.*, 2008). The fatty acid transferred to ghrelin by GOAT is usually octanoic acid, however one study demonstrated the ability of GOAT to additionally utilise decanoic and tetradecanoic acids in their cell culture system (Gutierrez *et al.*, 2008).

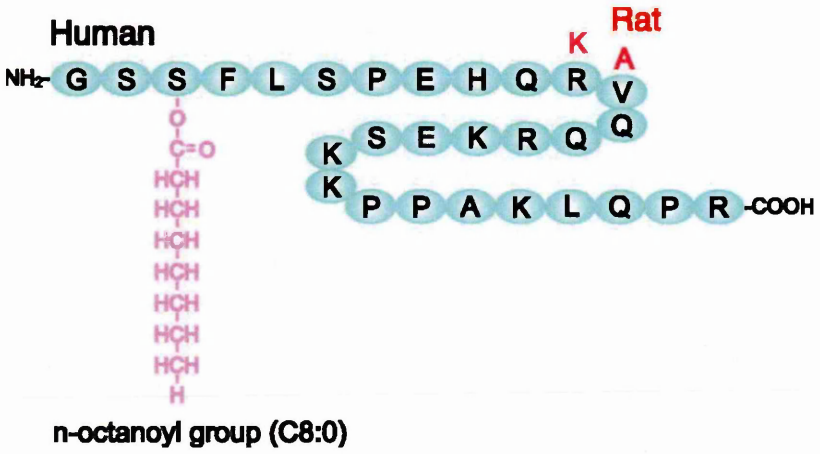

Figure 1.6. Ghrelin is modified at serine 3 by addition of n-octanoic acid
This image shows the difference between human and rat ghrelin (Kojima and Kangawa, 2005).

One study in GOAT knockout mice reported no change in food intake or body or fat mass (Zhao *et al.*, 2010). However after severe calorie restriction in that study, the GOAT knockout mice were moribund after 1 week and the authors suggest that acyl ghrelin may play a vital role in maintaining glucose levels at a viable level via GH secretion, thus preventing death from hypoglycaemia (Zhao *et al.*, 2010). This once again stimulated interest in exploring additional roles of ghrelin, after earlier conflicting observations from other knockout models. For example, ghrelin knockout mice have been reported to have the same food intake, body mass, blood chemistry and organ masses and pathology as wild-type littermates and responded in a

similar way to a 24 hour fasting period (Sun *et al.*, 2003), with another study reporting that ghrelin knockouts additionally did not have an altered gastric emptying rate (De Smet *et al.*, 2006). Additional work by Sun *et al.* using a GHSR knockout model reported similar findings in that there was no difference in either food intake or body composition of these mice and that serum ghrelin levels increased after fasting, although an injection of ghrelin failed to increase food intake in the knockouts as it did in the wild-type mice (Sun *et al.*, 2004). These authors suggested that their findings indicated alternative pathways through which growth and body composition can be mediated in the absence of GHSR. Alternative pathways adopted during development to compensate for the lack of the only peripherally derived orexigenic hormone seem plausible as a safeguard to the system, which, if such pathways exist, demonstrates the essential requirement of this part of the appetite regulatory system.

1.6.1.2. Actions of ghrelin in the hypothalamus

The exact mechanisms by which ghrelin acts within the hypothalamus are not completely established, but Figure 1.7 portrays a currently accepted likely model. In the regulation of feeding, ghrelin stimulates the orexigenic NPY/AgRP/GABA neurons in the ARC of the hypothalamus, stimulating NPY/AgRP/GABA peptide release from their nerve terminals in the PVN (reviewed by Briggs and Andrews, 2011). GABA release from the same ARC neurons inhibits the POMC/CART production of the anorectic peptide α-MSH and therefore decreases the anorexigenic tone within the hypothalamus

(Briggs and Andrews, 2011). It is the combined actions of a stimulation of NPY/AgRP/GABA and an inhibition of POMC/CART that increases appetite.

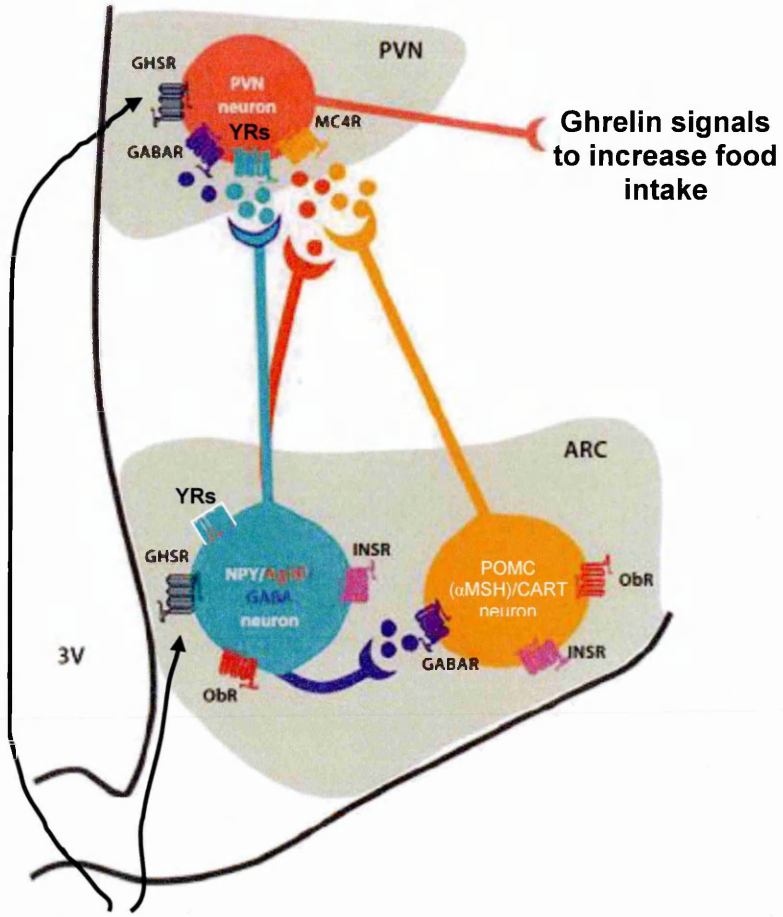

Figure 1.7. Simplified model for ghrelin action in the hypothalamus, resulting in an increase in appetite
(3V, third ventricle; ARC, arcuate nucleus; PVN, paraventricular nucleus; POMC/CART, pro-opiomelanocortin (purple)/cocaine and amphetamine regulated transcript; NPY/AgRP/GABA, neuropeptide-Y (blue)/agouti-related protein (red)/gamma-aminobutyric acid (purple); α-MSH, α-melanocyte stimulating hormone; GHSR, growth hormone secretagogue receptor; INSR, insulin receptor; ObR, leptin receptor; MC4R, melanocortin 4 receptor; GABAR, gamma-aminobutyric receptor; YRs, Y receptors). (Modified from Briggs and Andrews, 2011).

1.6.2. Peptide-YY (PYY)

PYY is an anorexigenic gut peptide secreted postprandially as a satiety hormone and is released in a biphasic manner, with levels increasing before L-cells are in physical contact with nutrients in the gut lumen and a larger sustained peak when nutrients reach the distal small intestine (reviewed by

De Silva and Bloom, 2012). PYY plasma levels increase in proportion to meal size from approximately 15 minutes after meal initiation, with levels remaining increased for up to 5 hours compared to baseline levels after a large evening meal in humans (Adrian *et al.*, 1985). Studies using PYY knockout mice have since confirmed PYY's role as a satiety regulating peptide, with knockouts being both hyperphagic and obese, a phenotype which was reversed with intraperitoneal PYY injections (Batterham *et al.*, 2006). PYY has also been demonstrated to have an inhibitory effect on gut motility, delaying gastric emptying, inhibiting gastric acid secretion and mediating an 'ileal-brake mechanism', which occurs due to the presence of fat in the lumen of the ileum and inhibits motility in the upper GI tract (reviewed by Ueno *et al.*, 2008). See Table 1.4 for additional examples of the physiological actions of PYY.

Table 1.4. Some of the additional effects of PYY in mammals
(Modified from Ballantyne, 2006).

Target	Effect of PYY
Gastric acid	↓
Intestinal secretions	↓
Overall gastric motility	↓
Insulin	↓
Blood pressure	↑
Vasoconstriction	↑
Glomerular filtration rate	↓
Postprandial natriuresis	↑

In addition to its established role in satiety signalling, reduced levels of PYY have been suggested to facilitate pubertal maturation in humans, with PYY levels being at their lowest levels in both sexes at the middle stages of puberty when GH levels are maximal (Lloyd *et al.*, 2010).

1.6.2.1. The PYY peptide

The 36-amino acid PYY peptide was first isolated from the mucosa of porcine small intestine, and was so named due to the tyrosine (Y) residues at its C and N terminals (Tatemoto and Mutt, 1980). PYY is secreted from L-cells located predominantly in the colon and rectum and is a member of the pancreatic polypeptide family, which also includes the peptides NPY and pancreatic polypeptide (PP) (reviewed by Batterham and Bloom, 2003). PYY_{1-36} is the full length peptide, which is cleaved by dipeptidyl peptidase IV (DPP IV) in the circulation to form PYY_{3-36}, which is known as the 'active' form (Batterham and Bloom, 2003); both forms are found in gut tissue (Eberlein *et al.*, 1989) and in the circulation (Grandt *et al.*, 1994). PYY_{1-36} acts as an agonist for all five mammalian Y receptors discovered to date with different affinities, while PYY_{3-36} preferentially binds to Y2 and has also been shown to bind Y5, although to a lesser extent (reviewed by Blomqvist and Herzog, 1997). A study using a Y2 receptor knockout mouse model demonstrated the importance of this receptor, with an intraperitoneal injection of PYY_{3-36} having no effect on inhibiting food intake, as it did dose-dependently in wild-type mice (Batterham *et al.*, 2002).

Some studies have reported that when delivered directly to the brain, PYY can have an orexigenic effect (Hagan and Moss, 1995, Morley *et al.*, 1985). One study reported that intracerebroventricular infusion of PYY increased food and water intake by significantly more than the potent orexigenic hormone NPY, with which PYY shares hypothalamic receptors (Hagan and Moss, 1995). A review article documenting the differences between PYY_{1-36}

and PYY_{3-36} suggested that PYY_{1-36} may act through the Y1 receptor to elicit such an effect, which is not seen with peripheral infusion due to PYY_{1-36} breakdown into PYY_{3-36} in the circulation (Ballantyne, 2006). It remains unclear whether PYY may have additional roles in supporting appetite stimulation and whether the above studies are representative of what may occur with endogenous PYY_{1-36} in the peripheral circulation.

1.6.2.2. Actions of PYY in the hypothalamus

PYY is released postprandially from the GI tract and activates Y receptors on the NPY/AgRP/GABA neurons in the ARC of the hypothalamus. Our knowledge is far from complete on the exact mechanisms by which PYY reduces appetite and the following is provided as an overview (Figure 1.8).

PYY_{3-36} induces satiety by binding to hypothalamic Y2 receptors on NPY neurons within the ARC, initiating a number of processes. Firstly, PYY_{3-36} bound to Y2 receptors blocks the binding of its orexigenic family member NPY, which binds to the same receptor (Blomqvist and Herzog, 1997). Secondly, it has been reported that PYY_{3-36} reduces GABA release from the orexigenic NPY/AgRP/GABA neurons in the ARC, which was thought to disinhibit the anorexigenic POMC/CART neurons, a hypothesis that was supported by a non-significant increase in POMC mRNA after ARC injection of PYY_{3-36} (Batterham *et al.*, 2002). An increase in POMC activity, in turn, increased the levels of α-MSH, further increasing the anorexigenic tone within the ARC (Batterham *et al.*, 2002).

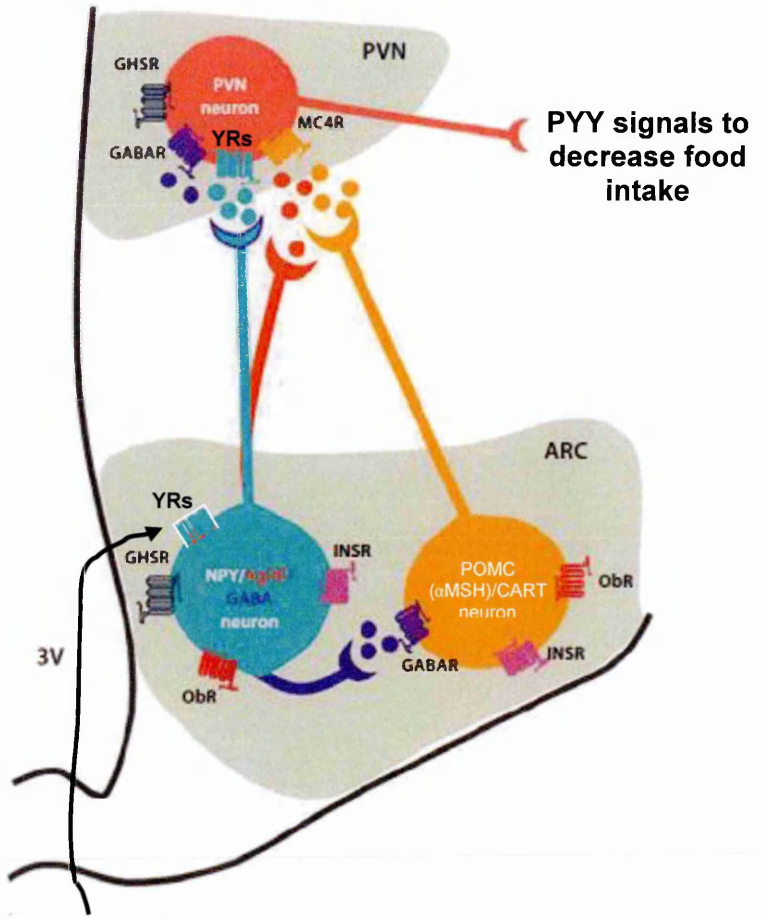

Figure 1.8. Simplified model for PYY action in the hypothalamus, resulting in a reduction of appetite
(3V, third ventricle; ARC, arcuate nucleus; PVN, paraventricular nucleus; POMC/CART, pro-opiomelanocortin (purple)/cocaine and amphetamine regulated transcript; NPY/AgRP/GABA, neuropeptide-Y (blue)/agouti-related protein (red)/gamma-aminobutyric acid (purple); α-MSH, α-melanocyte stimulating hormone; GHSR, growth hormone secretagogue receptor; INSR, insulin receptor; ObR, leptin receptor; MC4R, melanocortin 4 receptor; GABAR, gamma-aminobutyric receptor; YRs, Y receptors). (Modified from Briggs and Andrews, 2011).

1.6.3. Glucagon-like peptide-1 (GLP-1)

GLP-1 is an incretin hormone involved in glycaemic control (Edwards *et al.*, 1999) with an important role in the enhancement of pancreatic β-cell insulin secretion in response to an increase in circulating glucose (Schmidt *et al.*, 1985). In the gut, the vast majority of L-cells co-secrete GLP-1 and PYY, with one recent study reporting no detectable human L-cells in culture positive for

only one of these peptides (Habib *et al.*, 2013). Like PYY, GLP-1 is also released postprandially in a biphasic manner as a satiety signal in proportion to meal size (De Silva and Bloom, 2012). It is well established that intravenous infusion of GLP-1_{7-36} reduces energy intake in humans in a dose-dependent manner, likely partly due to a reduction in gastric emptying rate (Delgado-Aros *et al.*, 2002, Verdich *et al.*, 2001). In response to a meal, plasma GLP-1_{7-36} levels are elevated 15 minutes from the start of food consumption and GLP-1_{7-36} infusion does not affect food palatability, indicating that GLP-1_{7-36} causes a true reduction in appetite (Flint *et al.*, 1998) rather than causing food aversion. Additional actions of GLP-1 are listed below (Table 1.5). Some studies have implicated a role for GLP-1 in cardiovascular health, with protective effects and increased myocardial performance after myocardial infarction in both rodent and human studies (reviewed by Holst, 2007).

Table 1.5. Some of the additional effects of GLP-1 in mammals
(Modified from Ng and Wilding, 2014).

Target	Effect of GLP-1
Gastric acid	↓
Intestinal secretions	↓
Overall gastric motility	↓
Insulin	↑
Blood glucose	↓
Blood pressure	↑
Heart rate	↑
Vasodilatation	↑
Postprandial natriuresis	↑

1.6.3.1. The GLP-1 peptide

GLP-1 is derived from the 160-amino acid proglucagon in the gut alongside GLP-2, glicentin and OXM (reviewed by Holst, 2007). In the pancreas, proglucagon is cleaved into glucagon, amongst other fragments (Holst, 2007). In gut tissue, GLP-1 is found predominantly in the intact and 'biologically active' form ($GLP\text{-}1_{7\text{-}36}$) (Hansen *et al.*, 1999). In the circulation, $GLP\text{-}1_{7\text{-}36}$ has a very short half-life of approximately 2 minutes due to rapid degradation by DPP IV, resulting predominantly in the 'biologically inactive' $GLP\text{-}1_{9\text{-}36}$ form (Kieffer *et al.*, 1995, Mentlein *et al.*, 1993). It is estimated that only approximately 10 to 15% of intact $GLP\text{-}1_{7\text{-}36}$ secreted by the gut reaches the systemic circulation after degradation in both the gut and liver (Holst and Deacon, 2005). This is partly due to the presence of DPP IV in the brush border and capillary endothelium of the gut (Hansen *et al.*, 1999).

GLP-1 acts through the GLP-1 receptor (GLP-1R), which has been found in multiple locations including the heart, pancreatic islets, stomach parietal cells and in the hypothalamus (Holst, 2007, Schmidtler *et al.*, 1994, Shughrue *et al.*, 1996). $Exendin_{9\text{-}39}$ is a well defined specific antagonist for GLP-1R and studies utilising these properties have confirmed GLP-1's role as an anorexigenic gut hormone. The Bloom group have demonstrated a significant increase in both food intake and body mass after daily intracerebroventricular injection of $exendin_{9\text{-}39}$ in schedule-fed male rats, whereas rats injected with $GLP\text{-}1_{7\text{-}36}$ had significantly reduced food intake and body mass compared to saline-treated controls (Meeran *et al.*, 1999). GLP-1R knockout mice have not been shown to have altered appetite or body mass, however they do not

show inhibition of food intake after intracerebroventricular injection of GLP-1, which the wild-type mice did (Scrocchi *et al.*, 1996). It remains to be established whether GLP-1 could be acting through another receptor and/or pathway that is yet to be identified or whether GLP-1's anorexigenic effects, in terms of the whole appetite regulatory system, are dispensable.

1.6.3.2. Proposed actions of GLP-1 in the regulation of food intake

In contrast to ghrelin and PYY, the central mechanisms by which GLP-1 exerts its inhibitory effects on appetite remain largely uncharacterised. GLP-1 receptors have been located in multiple regions of the rat hypothalamus (Shughrue *et al.*, 1996) but it seems to be unlikely that intact $GLP\text{-}1_{7\text{-}36}$ secreted by the gut reaches these receptors due to its rapid degradation by DPP IV (section 1.6.3.1). Further work needs to be done to fully elucidate the mechanisms by which GLP-1 induces satiety. A better understanding of GLP-1 action may be gained from studying its effects alongside other gut hormones, for example PYY, with which it is co-secreted from gut L-cells.

1.6.4. Interactions between ghrelin, peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) in appetite regulation

The possible interactions between orexigenic and anorexigenic hormones in the regulation of appetite have been studied far less than the peptides have been individually. Intravenous co-infusion of ghrelin with either $PYY_{3\text{-}36}$ or with GLP-1 in male rats has demonstrated ghrelin's ability to increase both food intake and the rate of gastric emptying compared to rats that received $PYY_{3\text{-}36}$ or GLP-1 only, suggesting that ghrelin can attenuate the effects of

PYY and GLP-1 (Chelikani *et al.*, 2006). Another study in male rats reported that GLP-1_{7-36} significantly attenuated the increased food intake caused by ghrelin, upon intracerebroventricular injection of these peptides (Schusdziarra *et al.*, 2008). In support of the inhibitory role of GLP-1 on ghrelin levels was the finding that GLP-1_{7-36} significantly reduced the secretion of pre-stimulated ghrelin in isolated male rat stomach (Lippl *et al.*, 2004). One study looked at the combined effects of PYY_{3-36} and GLP-1_{7-36} in both rodents and humans. In male lean, obese (*ob/ob*) and diabetic (*db/db*) mice and in male rats, intraperitoneal co-injection of PYY and GLP-1 significantly enhanced feeding inhibition compared to saline control or separate injection of the peptides, although the effects were not as long lasting in the *ob/ob* and *db/db* mice (Neary *et al.*, 2005). These results were replicated in the human part of the study, with 4 men and 6 women studied. Co-infusion of PYY and GLP-1 reduced energy intake at a buffet meal by 27%, compared to 15% with PYY only infusion and no effect was seen with GLP-1 only infusion (Neary *et al.*, 2005). The findings of that study suggest that co-secreted PYY and GLP-1 may act together to reduce food intake to a homeostatically appropriate level.

Infusion of GLP-1_{7-36} significantly reduced postprandial plasma total ghrelin levels in human males 150 to 360 minutes after a meal compared to when participants had received the saline control infusion (Hagemann *et al.*, 2007). These authors also measured plasma insulin, with which ghrelin had an inverse relationship (Hagemann *et al.*, 2007). They suggested that ghrelin

levels may have been altered by GLP-1 indirectly, with GLP-1 affecting insulin levels, which in turn may have affected ghrelin levels.

Ghrelin is known to increase gastric acid secretion in rats in a dose-dependent manner (Masuda *et al.*, 2000); it is likely that ghrelin also regulates gastric acid secretion in humans. One study has shown that intravenous infusion of PYY or GLP-1_{7-36} reduces gastric acid secretion significantly, by 68% and 67% respectively in men and women (Wettergren *et al.*, 1994), which suggests that ghrelin, PYY and GLP-1 may all interact to regulate gastric acid secretion.

In human males, intravenous infusion of GLP-1 was not found to reduce plasma total ghrelin levels compared to controls, however after 120 minutes of GLP-1 infusion, plasma total PYY levels were significantly decreased. Furthermore, the increase in plasma PYY caused by CCK infusion was attenuated by CCK-GLP-1 co-infusion (Brennan *et al.*, 2007). It seems unlikely that GLP-1 would have an inhibitory effect on PYY due to their similar postprandial release and effects. One explanation for the finding of Brennan *et al.* (2007) could be that GLP-1 infusion inhibited endogenous and exogenous CCK, thus attenuating the PYY increase caused by CCK. This explanation could be supported by the fact that GLP-1 infusion alone only caused a significant reduction in plasma PYY 120 minutes into the infusion.

In conclusion, evidence exists for the direct and indirect interactions of ghrelin, PYY and GLP-1 in the regulation of food intake, however more work

is needed to clarify the precise mechanisms by which this occurs. Moreover, more studies need to include females to further elucidate appetite regulation by gut hormones when changes in food intake are known to occur during the reproductive cycle, pregnancy and lactation. Many developed treatments to control appetite and reduce food intake and body mass would be aimed at and used by females of reproductive age, with potential unintended effects on fertility and offspring development, yet only males are used in the majority of such studies. Specific examples of studies that have established differences in appetite hormones during the oestrous cycle (Chapter 3), pregnancy (Chapter 4) and lactation (Chapter 5) will be described in the introductions to the relevant chapters.

1.7. The female rodent reproductive cycle

The rodent reproductive cycle, the oestrous cycle, comprises the stages of proestrus, oestrus, metestrus and diestrus and is typically a 4 day cycle (Figure 1.9), although an extra day spent in diestrus is common (Adler and Feder, 1981). Rodents are nocturnally active mammals that eat the majority of their food during the dark phase and Figure 1.9 illustrates the relationship of the light/dark cycle to the oestrous cycle. An undisturbed lighting schedule is vital to preserve a normal cycling pattern in rodents, so it is important to minimise any light that may enter the room during the active (dark) period if attempting to monitor the cycle or to successfully mate the rodents (Goldman *et al.*, 2007).

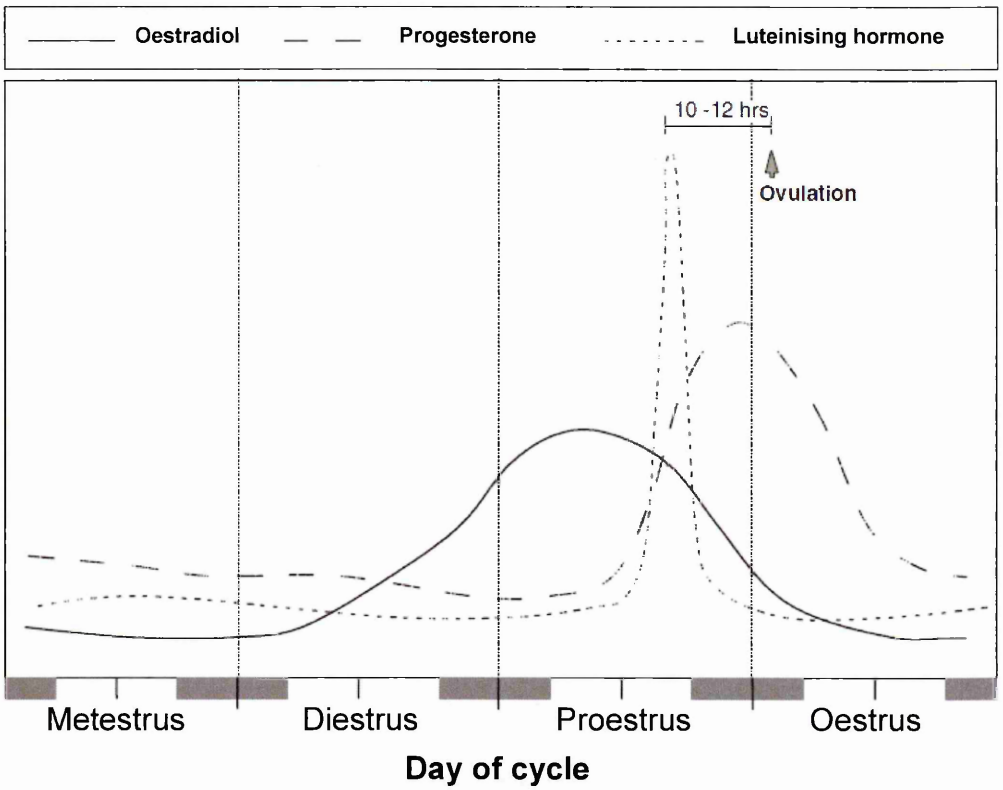

Figure 1.9. Representation of a standard 4 day rodent oestrous cycle
Demonstrating the hormonal events that occur during the light and dark phases (white and shaded blocks along the X axis) leading up to ovulation at oestrus. (Modified from Goldman *et al.*, 2007).

Leading up to ovulation, metestrus and diestrus are the luteal phase of the cycle governed by luteinising hormone (LH) which, alongside oestrogens, stimulates ovulation and prepares the uterus for implantation, with follicle-stimulating hormone (FSH) acting on the ovaries to secrete oestrogens. During diestrus, ovarian secretion of oestradiol increases and then peaks during proestrus, which stimulates the hypothalamic release of gonadotropin releasing hormone (GnRH). In turn, GnRH stimulates the pituitary gland to secrete LH and bring about ovulation, together with an increase in circulating progesterone 10 to 12 hours later. The stage of oestrus can vary in length from 12 to 20 hours, depending on whether mating occurs. Ovulation will typically occur just after the onset of the dark period (Becker *et al.*, 2005). Dams can commonly spend a second day in the stage of diestrus, where this

stage is prolonged for approximately 30 hours before ovulation begins, which extends the cycle to 5 days in duration (Adler and Feder, 1981).

The most accurate method by which cycle stage can be determined is to quantify plasma or serum hormones (Becker *et al.*, 2005), however many researchers make use of less invasive, costly and time consuming methods. Vaginal cytology (methodology described in section 2.2) is the gold standard for staging the oestrous cycle, although there are other indicators of cycle stage that can be used to augment this technique, such as the oestrus dance. Despite its name, behavioural oestrus typically begins during cytologically identified proestrus, between 6 and 12 hours before ovulation (Adler and Feder, 1981). Lordosis behaviour (ear-wiggling, hopping, darting, freezing) can even be assessed without a male rat being present, by stimulation of the flanks of the rat (Adler and Feder, 1981). A further, but terminal, method of proestrus confirmation (Adler and Feder, 1981) is the analysis of uterine wet mass, which is heaviest during proestrus between 12 and 16 hours prior to ovulation.

1.7.1. Oestrogens and appetite

In addition to known effects on appetite, it is well established that sex steroid hormones, particularly oestrogens, play a role in sustaining GH secretion (reviewed by Ho *et al.*, 1996). This is partly what fuelled the interest in investigating appetite hormones, such as ghrelin, in relation to ovarian hormones. Research linking food intake and reproductive cycle stage has been published on rodents, pigs, goats, sheep, primates and humans

(reviewed by Dye and Blundell, 1997). A link has now been established between oestrogens and food intake, and many species including rats, mice and humans have reduced food intake leading up to ovulation due to the latent effects of an earlier ovarian peak in oestradiol secretion (reviewed by Asarian and Geary, 2006). Putting this into context, in rodents, food intake is reduced during oestrus due to high levels of oestradiol at proestrus. One study has additionally established that rats show increased locomotor activity during oestrus, but that this did not contribute to the decrease in food intake in that study (Eckel *et al.*, 2000). These authors suggested that there are distinct independent mechanisms by which food intake is reduced at oestrus.

1.7.2. Ghrelin and the reproductive cycle

Many studies (reviewed by Butera, 2010) have examined the effects of oestradiol on ghrelin's orexigenic properties by artificial manipulation of these hormones, with a main focus on the effects on food intake. One of the earlier studies in this area reported that ghrelin cell density in the stomach and ghrelin levels in plasma rapidly increased soon after ovariectomy (OVX) in rats, and that these effects were reversed by oestradiol treatment (Matsubara *et al.*, 2004). This finding suggests that the well established body mass gain after OVX/menopause may be due to decreased restraint of ghrelin by a lack of oestrogen, resulting in increased hunger signalling. The study by Matsubara *et al.* (2004) also established that oestrogen receptor-α (ERα) is present in the nuclei of ghrelin-immunoreactive ECs, which they suggested was evidence for a direct relationship between oestrogen and ghrelin in the stomach. These authors also suggest that the existence of ERα in the

stomach could further implicate differential ghrelin expression and release by the stomach in relation to the cyclical changes in oestrogen. One other key study in this area is from the Geary group, which established that intraperitoneal injection of ghrelin significantly increased appetite in males and OVX females, but had no significant effect in normally cycling (combined stages) females, and that changes in appetite were attenuated by oestrogen treatment, suggesting attenuation by oestrogen levels (Clegg *et al.*, 2007). When cycle stage was taken into account, there was a significant increase in food intake caused by ghrelin only during metestrus and diestrus, when endogenous oestrogen levels are lowest (Clegg *et al.*, 2007).

There are fewer human studies that have measured ghrelin levels throughout the course of a menstrual cycle available for comparison. One group has reported that there was no difference in serum total ghrelin concentrations between the early follicular (approximately proestrus in rats), late follicular (approximately oestrus) and mid-luteal (approximately early diestrus) phases of the menstrual cycle in regularly cycling women and that injection of total ghrelin did not alter serum concentrations of oestradiol (Messini *et al.*, 2009). Observation across a whole menstrual cycle in one study reported no significant difference in either acyl ghrelin, desacyl ghrelin or the ratio between the two forms through the cycle (Dafopoulos *et al.*, 2009). A later study by this group found that plasma acyl ghrelin was not changed by acute treatment of oestradiol in normally cycling women in the follicular phase (cycle days 3 to 9), post-menopausal women or women following OVX (Dafopoulos *et al.*, 2010). However, peri-menopausal women have been

found to have higher serum acyl ghrelin concentrations than in pre- and post-menopausal women (Sowers *et al.*, 2008). Moreover, continued combined hormone replacement therapy (HRT; oestrogen plus progesterone) reduced circulating total ghrelin compared to women who only briefly underwent HRT (Soni *et al.*, 2011). These latter two studies do suggest a link between ghrelin levels and the hormones involved in the menstrual cycle, but due to conflicting evidence from human studies as described above, more in-depth study would be required to further our understanding of ghrelin's possible role in the reproductive cycle in women.

1.7.3. PYY and the reproductive cycle

Until now, there has been no study to investigate the potential changes in endogenous PYY levels during a natural and complete reproductive cycle, however one study has investigated the effect of PYY_{3-36} treatment on food intake in OVX non-human primates treated with reproductive hormones. This study reported that intracerebroventricular injection of PYY_{3-36} significantly reduced food intake in weeks 1 and 2 of oestradiol only treatment, but only reduced food intake in week 1, not week 2, of oestradiol plus progesterone treatment (Papadimitriou *et al.*, 2007). Indeed, a study in OVX rats implicated a role for the Y2 receptor in reproduction, with a 3 day treatment of oestradiol increasing Y2 binding, and oestradiol plus progesterone treatment on day 4 reducing Y2 binding in some hypothalamic regions (Parker *et al.*, 1996). Taken together, these two studies provide evidence that high levels of oestradiol could support a PYY-induced reduction in appetite.

1.7.4. GLP-1 and the reproductive cycle

Limited work has looked at the possible relationships between GLP-1 levels and the reproductive cycle. One study (Brennan *et al.*, 2009) has compared the follicular and luteal phases of the menstrual cycle of women and reported that fasted baseline levels of GLP-1 were not significantly different but that during the follicular phase, both gastric emptying rate and energy intake at a buffet meal were significantly reduced. However, when the participants of that study were given a glucose load, GLP-1 levels were significantly reduced during the follicular phase when compared to the luteal phase, which may suggest a difference in GLP-1 regulation between the two phases of the cycle.

Despite limited available data, it seems entirely plausible that ovarian hormones affect appetite by interaction with gut appetite hormones. Accordingly, females and information about their reproductive cycle stage should also be used in studies exploring the hormonal regulation of appetite in order for results to be representative of the population. Further work needs to establish the effects of the reproductive cycle on levels of ghrelin, PYY and GLP-1 in order to elucidate the potential role of these gut hormones in appetite modulation during these events.

1.8. Pregnancy and lactation

Pregnancy and lactation are times of maternal adaptation in order to support foetal and postnatal development. It is well known that hyperphagia occurs during these periods in order to meet the additional energy demands required

by the growing offspring, but females can often gain surplus body mass due to over-consumption (reviewed by Ladyman *et al.*, 2010). Obesity can lead to the development of hypertension and insulin resistance, which can both also occur during pregnancy, meaning that a pre-existing condition can be compounded and cause multiple maternal health complications. A study which highlights the multiple complications that can occur due to maternal obesity through a high fat diet (HFD) in pregnant non-human primates reported a stillbirth rate of 35% compared to 6% in those fed a control diet (Frias *et al.*, 2011). This study found a reduction in uterine and placental volume blood flow, with increased placental infarction sites, which are all likely to increase the risk of stillbirth. Furthermore, the females that developed insulin resistance during pregnancy were found to be more at risk of complications in their study.

1.8.1. Maternal diet: effects on offspring health

Many studies have explored the role of maternal diet on offspring metabolic programming and adult health, with focus often on either dietary deficit or excess. The Barker hypothesis, which is also referred to as the 'thrifty phenotype' hypothesis, outlines how maternal under-nutrition during pregnancy can lead to poor foetal and childhood growth, with a predisposition to the development of metabolic disorders in adult life (reviewed by Hales and Barker, 2001). It is well established that offspring of under-nourished mothers have impaired insulin secretion, which is suggested to occur because the offspring are developmentally and/or epigenetically programmed to a postnatal environment similar to that *in utero* (i.e. nutritional deficit).

Exposure to a more than adequate diet in adulthood can then increase the incidence of type 2 diabetes and obesity in these offspring (Hales and Barker, 2001) as nutrients are 'unexpectedly' available in excess. So far, less work has been done to investigate epigenetic modifications to offspring when mothers are over-nourished during pregnancy. It is thought that an increase in circulating glucose and lipid in overweight and obese mothers can lead to foetal epigenetic modifications to genes involved in lipid metabolism, and many others, increasing the risk of insulin resistance and obesity in childhood and later life (reviewed by Heerwagen *et al.*, 2010). Below in this brief review, focus will be placed on some of the effects of a maternal HFD during pregnancy and lactation, or offspring over-nutrition, on offspring health due to its relevance to the growing incidence of obesity.

An observation that hypertension is more likely to occur in offspring of HFD fed mothers has led to examination of the effects on vascular tissue. Impaired femoral artery endothelium-dependent relaxation and abnormal fatty acid composition of the aorta has been described in adult (160 days old) female offspring of rat dams that were fed a HFD during pregnancy and the lactation period, which the authors suggest could increase the risk for the development of hypertension and atherosclerosis (Ghosh *et al.*, 2001). Another study of adult (180 days old) male and female offspring of rat dams fed a HFD found that feeding the offspring a HFD after weaning prevented the changes they reported in vascular endothelial function in the offspring fed a control diet from weaning (Khan *et al.*, 2004). These authors suggested that their findings could demonstrate foetal adaptations to a maternal HFD,

with preparation for receiving the same nutrition during postnatal life, similar to the 'thrifty phenotype' hypothesis as described previously. However, hypertension was still evident in the female offspring, suggesting only a partial adaptation to the foetal environment (Khan *et al.*, 2004).

A maternal HFD has also been found to affect the liver of offspring. One study in mice reported that from one week after weaning onwards, male offspring of dams fed a HFD throughout pregnancy and lactation had significantly increased levels of liver triglycerides and increased hepatic liver droplet size (Ashino *et al.*, 2012). Examination of the same male offspring at 28 and 82 days of age found that they had significantly increased fat mass compared to the control offspring, despite no difference in food intake between the groups (Ashino *et al.*, 2012). A study in rats examined the generational effects of a maternal HFD on male offspring, with grandparent dams fed either a control or a HFD throughout pregnancy and lactation and offspring parents fed a control diet throughout. Male offspring exposed to a HFD through their grandmothers were found to have larger hepatic lipid droplets, combined with a reduction in their liver expression of genes encoding antioxidant defence proteins and an increased level of several cellular senescence markers in the liver when compared to the controls (Zhang *et al.*, 2011). These two studies demonstrate that a HFD can affect hepatic health in male offspring in both mice and rats, which could lead to increased incidence of non-alcoholic fatty liver disease in adulthood.

It has also been found that a low level of maternal ghrelin concentration in mice appeared to have an adverse effect on the uterine programming of their female offspring and caused subnormal fertility rates through reduced implantation, despite normal ovarian function and embryo production (Martin *et al.*, 2011). These authors suggested that their findings could have serious implications for the fertility of women born to obese mothers who would have had blunted ghrelin levels due to their obesity.

Using a model of pup over-nutrition by decreasing litter size in rodents, without changing maternal diet, studies have determined that offspring body mass and adiposity are increased in comparison to control/standard litter sizes (Balonan and Sheng, 2000, Faust *et al.*, 1980, Fiorotto *et al.*, 1991, Plagemann *et al.*, 1999, Plagemann *et al.*, 1992, Schmidt *et al.*, 2001, Widdowson and McCance, 1963). These studies will be described in more detail in the introduction to Chapter 7.

The findings of the above studies, and many others not cited here, clearly demonstrate that diet-induced obesity during pregnancy and lactation increases the likelihood of a number of clinical complications during these times for both the mothers and their offspring. There is not space to fully describe the mechanisms of foetal programming here, but evidence is growing that epigenetic changes are important. Such changes are beyond the scope of this thesis and are not discussed further here (see reviews by Hales and Barker, 2001, Heerwagen *et al.*, 2010). Possible effects of

maternal diet on the EC populations and regulation of appetite by gut hormones has not yet been investigated.

1.8.2. Maternal health in the later postpartum period

A long-term study of women, with an average follow-up period of 8.5 years, reported that the mean gain in body mass 6 months postpartum was 1.7 kg and increased to 6.3 kg at the long-term follow-up appointment (Rooney and Schauberger, 2002). These authors also described that women who were able to return to their pre-pregnancy body mass by 6 months postpartum were lighter than women who had retained their 'pregnancy weight' in the long term. Furthermore, it was reported that breastfeeding provided no short-term body mass reduction, but at the long-term follow-up appointment, women who breastfed for a minimum period of 3 months gained significantly less body mass than women who breastfed for a shorter length of time (Rooney and Schauberger, 2002). An earlier study found that Caucasian women who gained below the recommended body mass during pregnancy retained as much of their pregnancy body mass as those who gained the recommended amount, however this was race-dependant (Keppel and Taffel, 1993). These findings demonstrate the possibility that some part of the maternal adaptation to facilitate pregnancy and lactation may result in long-term maternal changes, but information to explain this phenomenon is limited. More work needs to be done to establish whether physical maternal adaptations to pregnancy and lactation, such as increased gut size (Burdett and Reek, 1979, Campbell and Fell, 1964, Crean and Rumsey, 1971, Cripps and Williams, 1975, Datta *et al.*, 1995, Taylor *et al.*, 2009) persist

postpartum. It would also be of interest to determine whether these maternal changes could potentially contribute to retention of increased body mass after the lactation period has ended. Studies on maternal gut adaptations to pregnancy and lactation in rats will be described in Chapters 4, 5 and 6.

1.8.3. Hormonal control of appetite in pregnancy and lactation

Hyperphagia during pregnancy and lactation in humans and other mammals occurs to support these metabolically demanding states (reviewed by Ladyman *et al.*, 2010) and the role of leptin in supporting hyperphagia has been well established.

Leptin is an anorexigenic hormone released by adipose tissue; circulating leptin concentrations are proportionate to the total adipose tissue mass (reviewed by Friedman, 2011, Ladyman *et al.*, 2010). Leptin levels rise throughout pregnancy as the female accumulates adipose reserves, as they would in an obese state. However, adipose deposition is beneficial during pregnancy to build lipid reserves which are then utilised during lactation. Developing a resistance to leptin supports hyperphagia and allows further adipose accumulation. Leptin resistance has been widely documented as an adaptation to support pregnancy (reviewed by Friedman, 2011, Ladyman *et al.*, 2010) and is established to begin during mid-gestation. Leptin resistance during pregnancy has been shown to occur by multiple mechanisms in the pregnant rat. Of primary importance to leptin resistance is the failure of the

NPY/AgRP/GABA and POMC/CART neurons in the ARC to respond in the usual way to increases in leptin levels (reviewed by Ladyman *et al.*, 2010).

A study using the leptin-deficient *ob/ob* mouse model treated pregnant mice with leptin until withdrawal of treatment at various stages of gestation and found no difference in the outcome of the pregnancy (in terms of litter size and pup mass and appearance), concluding that the main role of leptin resistance in pregnancy is to increase maternal food intake (Mounzih *et al.*, 1998). Intracerebroventricular administration of leptin had been shown to decrease both food intake and body mass in nulliparous rats, but had no effect in rats at days 13 and 18 of pregnancy (Trujillo *et al.*, 2011), demonstrating a central leptin resistance at those stages of pregnancy. That study also documented that leptin resistance in day 13 and 18 pregnant rats resulted from a reduction in the levels of leptin crossing the blood-brain barrier combined with alterations to the normal hypothalamic cascade mechanisms (Trujillo *et al.*, 2011), a finding consistent with others (Ladyman *et al.*, 2009).

A reduction in leptin transport across the blood-brain barrier in late pregnancy has been described in rats, caused by high prolactin levels (ready to stimulate lactation) (Trujillo *et al.*, 2011). That study also reported that hypothalamic mRNA for NPY and AgRP increased in relation to the development of pregnancy-associated hyperleptinaemia and POMC mRNA decreased. However, Ladyman *et al.* (2009) found no difference in NPY,

AgRP or POMC mRNA in response to hyperleptinaemia during pregnancy in the same breed of rat.

High leptin levels at time of parturition (Figure 1.10) are also suggested to increase maternal investment in the pregnancy by reduced infanticide in Siberian hamsters (French *et al.*, 2009). These authors suggest that increased leptin acts to signal to the dams that there are adequate energy reserves to invest in feeding their offspring.

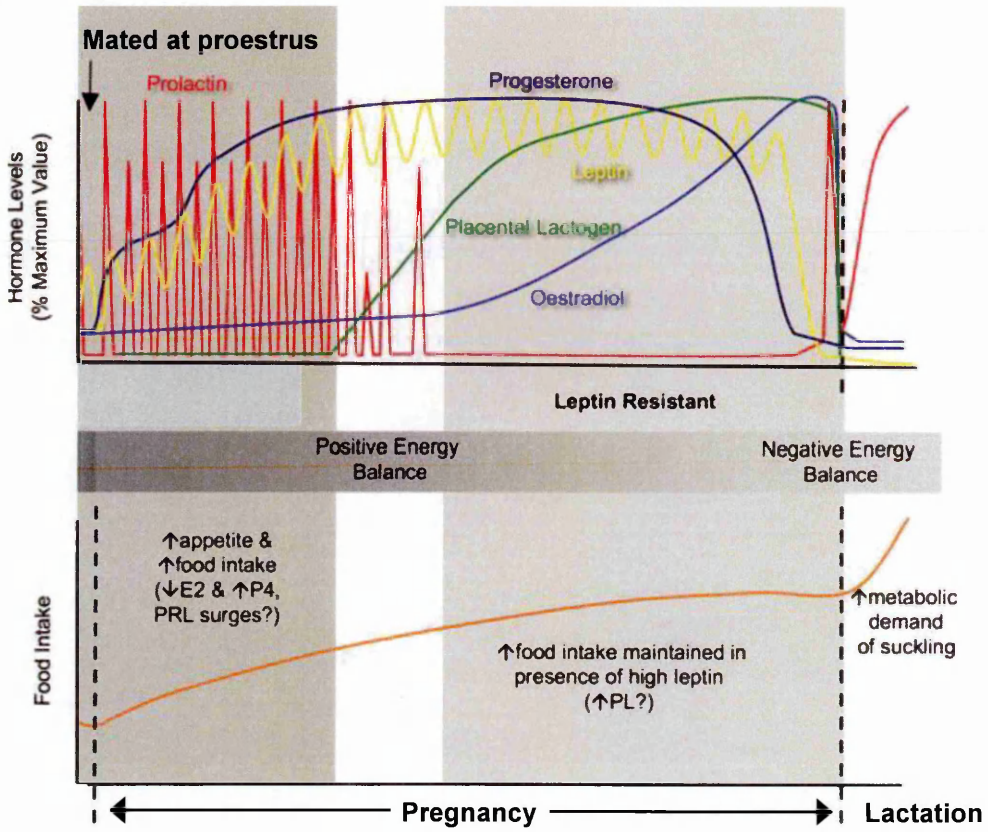

Figure 1.10. A schematic depicting the hormones that support pregnancy alongside energy balance and food intake in rodents
(E_2, oestradiol; P_4, progesterone; PRL, prolactin; PL, placental lactogen). (Modified from Augustine *et al.*, 2008).

The hyperphagia of lactation is likely to be facilitated by a rapid reduction in leptin levels (Figure 1.10). One study has demonstrated the ability of central

and peripheral leptin administration to reduce appetite and body mass in lactating rats from day 8 postpartum (earliest point studied), showing that there is no lactation-associated leptin resistance as is present during pregnancy (Woodside *et al.*, 2000). As leptin concentration is proportional to adiposity, mobilisation of adipose reserves during lactation is the likely cause of a reduction in leptin levels, and rats prevented from lactating had significantly higher plasma leptin and adiposity compared to control lactating dams (Woodside *et al.*, 2000).

Leptin has also been studied with other appetite-regulatory hormones. In one study, leptin injection decreased plasma total ghrelin levels in male mice (Ueno *et al.*, 2004) and another study reported that leptin-induced satiety in male rats was reversed by injection of total ghrelin (Shintani *et al.*, 2001). These studies suggest another way in which leptin may be regulated during pregnancy and lactation in rodents. Some work has been done examining the gut hormones ghrelin, PYY and GLP-1 in relation to appetite modulation during pregnancy and lactation, but evidence for their involvement is still limited. Below is a summary of the main findings of others, with a more detailed review given in the introductions to Chapters 4 (pregnancy) and 5 (lactation).

1.8.3.1. Ghrelin in pregnancy and lactation

The orexigenic properties of ghrelin have led to investigation of its possible role in the hyperphagia of pregnancy and lactation, although many findings have been contradictory. Two studies that have measured total ghrelin levels

in the stomach tissue of pregnant rats reported no significant differences between the stages of pregnancy studied (Shibata *et al.*, 2004, Taylor *et al.*, 2009). Both of these studies also quantified plasma total ghrelin levels, however one study found a significant difference between days 10 and 15 of pregnancy (Shibata *et al.*, 2004) and the other found no difference between these days and additional pregnancy time-points (Taylor *et al.*, 2009). What these two studies do have in common is that they demonstrate that total ghrelin levels remained unsuppressed during pregnancy, demonstrating a dissociated relationship between ghrelin and leptin during a rat pregnancy. Taylor *et al.* (2009) went on to explore maternal total ghrelin levels at various lactation time-points and reported that both ghrelin and leptin levels appeared to normalise from the onset of weaning (Taylor *et al.*, 2009). Neither the study by Taylor *et al.* nor Shibata *et al.* found a significant difference in total ghrelin levels at the lactation time-points studied.

Human studies are also often contradictory and difficult to compare due to different stages of pregnancy being studied. One study has reported no change to levels of fasted plasma acyl ghrelin in early pregnancy (Moshtaghi-Kashanian *et al.*, 2011) and another documented a significant reduction in both fasted plasma total and acyl ghrelin during later pregnancy (Tham *et al.*, 2009). A further study compared women at each trimester of their pregnancy and reported no significant differences in acyl ghrelin levels (Valsamakis *et al.*, 2010). In lactating women, one study found that acyl ghrelin levels were highest at the start of lactation, with a gradual drop in the levels as lactation progressed (Ilcol and Hizli, 2007).

Another difficulty in the comparison of studies measuring ghrelin levels is the difference between which form of ghrelin is quantified, whether it is the total form or the acyl or desacyl portion. A study in rats found a non-significant trend for acyl ghrelin to decrease with pregnancy, however, there was a significant increase in desacyl ghrelin by day 19 of pregnancy (Nakahara *et al.*, 2006), demonstrating that although total ghrelin concentrations may not be different in some studies, it is possible that the ratio of acyl and desacyl ghrelin may have been altered by the pregnant or lactating state.

1.8.3.2. PYY in pregnancy and lactation

Information regarding possible roles of PYY in the appetite changes occurring in pregnancy and lactation is still very limited. In rats, there is agreement that circulating levels of PYY increase throughout pregnancy (Taylor *et al.*, 2009, Tovar *et al.*, 2004) with a PYY peak reported at day 5 of lactation (Suzuki *et al.*, 2014, Taylor *et al.*, 2009). Why PYY levels increase during pregnancy and into early lactation remains to be established, as hyperphagia is evident from mid-pregnancy onwards in rats. As an endogenous satiety factor, it seems reasonable to anticipate that overall PYY levels would decrease in order to facilitate hyperphagia. Furthermore, high PYY levels have been reported in gut tissue (Taylor *et al.*, 2009) and in the hypothalamus (Suzuki *et al.*, 2014) at day 5 of lactation, suggesting a systemic increase in PYY during this time of negative energy balance. It is possible that there could be a level of maternal resistance to PYY during pregnancy, as previously described for leptin (section 1.8.3), or increased PYY could be due to the reported physical increase in the size of the gut

(Taylor *et al.*, 2009) but this remains to be established. Human studies have not reported a difference in circulating PYY levels during pregnancy (Larson-Meyer *et al.*, 2010) and lactation (Valsamakis *et al.*, 2010). What is apparent from these observations is the need for further study of PYY during pregnancy and lactation in order to elucidate its roles and establish what changes may be taking place at these times.

1.8.3.3. GLP-1 in pregnancy and lactation

Few studies have examined the levels of GLP-1 during pregnancy and lactation. The majority of studies involving GLP-1 are in relation to its role as an incretin. A level of insulin resistance is known to develop during pregnancy in both rats and women (Leturque *et al.*, 1984, Vrachnis *et al.*, 2012), which is likely to affect GLP-1 levels. Future studies will need to address how the pregnant and lactating state may alter GLP-1 levels and how this may link with appetite regulation during these times.

1.9. Concluding statement

It is clear that there are complex changes occurring in appetite-regulating hormones throughout the reproductive cycle, pregnancy and lactation. The studies described above, and others, have provided answers to some questions, but more extensive characterisation of the changes that occur is still required. Further understanding of the underlying hormonal control regulating appetite at these times may lead to a greater comprehension of the underlying physiology of pregnancy and lactation, which may in turn

uncover possible medical treatments to prevent some of the aforementioned complications associated with maternal over-eating in both mothers and their offspring.

1.10. Aims

The main aim of the work in this thesis was to start to address the sex bias that is evident in the existing literature on gut appetite hormones by exploring the roles that ghrelin, PYY and GLP-1 may have in appetite changes during the rodent oestrous cycle (Chapter 3), pregnancy (Chapter 4) and lactation (Chapter 5). The lactation period was further investigated by manipulation of postpartum litter size and its effects on both dams (Chapter 6) and their male and female offspring (Chapter 7). Specific aims included:

- To investigate and compare concentration changes of the gut appetite hormones ghrelin, PYY and GLP-1 in both fed and fasted plasma and (fasted) gut tissue concentrations during the reproductive cycle, pregnancy and lactation.

- To further elucidate reported changes in maternal gut size during pregnancy and lactation, with inclusion of lactation litter size manipulation to ascertain whether increased gut size may be related to maternal demand.

- To determine whether relationships exist between any changes in plasma or gut hormone concentrations and changes in gut size.

- To explore whether male and female pups from different lactation litter sizes have different levels of ghrelin, PYY and GLP-1 in gut tissues and/or the peripheral circulation.

CHAPTER 2: METHODS

2.1. Animals

This work was licensed under the Home Office Animals (Scientific Procedures) Act 1986 and had approval from The Open University Ethics Committee. Female Wistar rats were housed in groups of 4 and maintained under a 12 hour reverse light cycle (lights off between 11.00 and 23.00) in the Biomedical Research Unit (The Open University) with free access to standard diet and water. All procedures were carried out during the dark phase.

Female nulliparous Wistar rats (Harlan, UK) used in the work described in Chapter 3 (n=43) were housed in standard cages and given time to adjust to the reverse light cycle prior to the study. Rats were regularly handled prior to all studies to reduce stress during regular weighing, oestrous monitoring and when briefly separating dams from their newborn litters. All cages of reproductively cycling females were kept in the same Scantainer along with 1 cage of male rats to keep females cycling normally.

2.1.1. Breeding

Rats used in the work described in research Chapters 4, 5, 6 and 7 were bred in-house from an additional group of animals (2 to 3 females per cage, n=14; n=3 males) purchased from Harlan (UK). To obtain initial litters from the colony, females were introduced to a male (1 to 2 females per male) in

his home cage for a period of 5 days and litters (un-manipulated) were kept to maturity for future pregnancy and lactation time points. Original breeding females had 1 to 2 litters each before dissection.

2.1.2. Study design

Nulliparous females were culled at each stage of the oestrous cycle (Chapter 3) to determine changes to gut appetite hormones during the oestrous cycle. The oestrus dance (section 2.2.1) was used for accurately time mating females for the pregnancy time points of days 4, 12 and 18 of pregnancy (Chapter 4), and for some lactation time points when appropriate females presented with the dance. Dams for the lactation time points of days 0, 5, 10 and 25 of lactation (Chapter 5) had litters standardised to 8 ± 1 pups each by day 2 postpartum and pups remained with dams throughout the study. Additional groups of dams had their postpartum litter size manipulated to either 4 or 12 pups and were dissected at day 25 of lactation to investigate the effects of lactation litter size on both the dams (Chapter 6) and their male and female offspring (Chapter 7). Figure 2.1 illustrates where the pregnancy and lactation time points studied fall within the context of a typical 21 day rat pregnancy and 25 day lactation period. Sample groups were optimised based on the findings of Taylor *et al.* (2009).

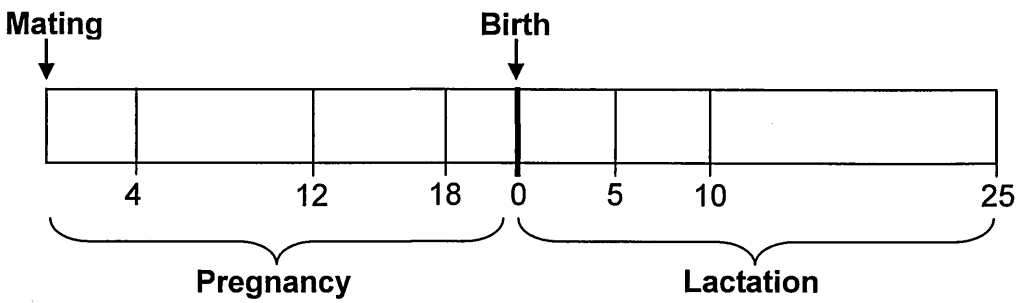

Figure 2.1. Timing of sample groups during a standard rat pregnancy and lactation period after mating at proestrus

2.2. Oestrous monitoring

The gold standard of cycle stage determination in rodents is vaginal cytology, with preference towards vaginal lavage rather than a cotton swab due to collection of an increased number of cells with undisturbed morphology (Becker *et al.*, 2005). Daily oestrous monitoring was undertaken at 24-hourly intervals to observe all cycle stages (Chapter 3).

As guided by the literature (Becker *et al.*, 2005, Goldman *et al.*, 2007, Marcondes *et al.*, 2002), proestrus (Figure 2.2 A) was identified by the presence of a large number of spherical nucleated cells, which resulted in a very cloudy cell suspension. Leucocytes were often present but in very small numbers, with cornified (keratinised, non-nucleated) cells being rare. A predominance of cornified cells confirmed oestrus (Figure 2.2 B), with a number of these cells becoming spindle-shaped in appearance. Metestrus (Figure 2.2 C) was characterised by an abundance of leucocytes with a small number of cornified, particularly spindle-shaped, cells present. During diestrus (Figure 2.2 D), few cells of any cell type were present, which was characteristic of the stage in itself. A diestrus lavage would typically contain a lot of mucus, sticking leucocytes together in lines.

Many study animals were found to be in transition stages of the cycle, so in cases where no clear stage existed, the ratio of nucleated to cornified to leucocytic cells (Ramos *et al.*, 2001), alongside the observed cycle pattern of the rat, was used to stage the cycle.

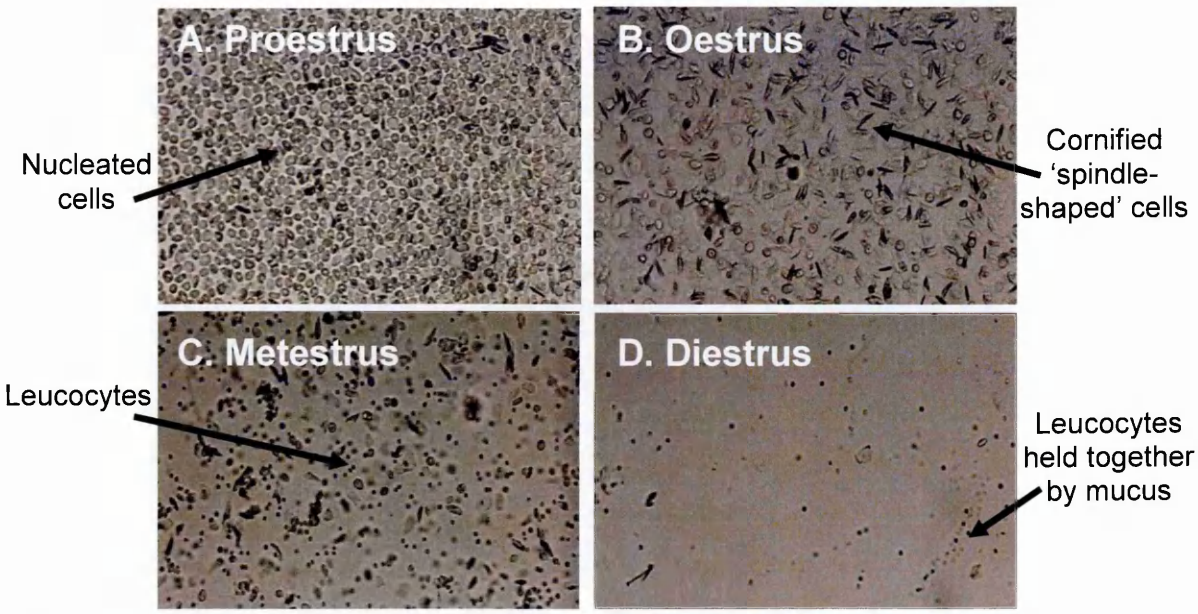

Figure 2.2. Representative images for each stage of the rat oestrous cycle (x100 magnification).

In addition to cytology, a number of groups have demonstrated that the electrical resistance of the inner lining of the rat vagina can be used to help identify oestrus, with a significantly increased electrical resistance at this stage (Ramos *et al.*, 2001, Singletary *et al.*, 2005). As described by Ramos *et al.* (2001), a standard electrical meter with resistance measurement capability was adapted to accompany vaginal lavage as a method of cycle stage determination. Prior to vaginal lavage, the male terminal of the meter was inserted into the vagina until the reading was stable and was repeated 3 to 5 times to record an average resistance reading. The meter was set to 2000 kΩ to obtain the reading, as Ramos *et al.* (2001) demonstrated successful readings within this range.

2.2.1. The oestrus dance

Whilst handling the animals prior to oestrous monitoring, a distinct behaviour was observed, which was identified to be an oestrus dance (Figure 2.3). When in proestrus (confirmed by vaginal lavage), stimulation of the back of the rat near the hind legs would result in presentation of lordosis with ear wiggling, hopping, darting and freezing, without a male rat being present or even visible, as described by Adler and Feder (1981). The majority of females would present an obvious oestrus dance, so it was used as a further confirmation of proestrus. Alongside this, animals were observed to have a general agitated demeanour and would be a lot more physically active during proestrus. For the breeding colony of Wistars used in this study, the females would begin showing this sexual behaviour shortly after lights out.

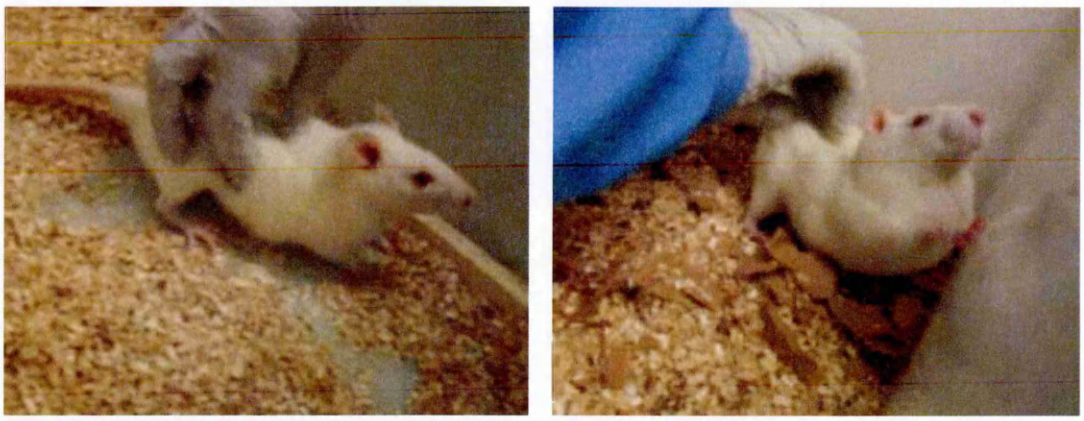

Figure 2.3. The oestrus dance occurred during proestrus

2.3. Sample collection

2.3.1. Protocol for animal fasting

All adult rats used in this study were fasted for 4 to 5 hours prior to culling, as a way to standardise gut hormone measurements. Rodents consume most of their food, approximately 85%, during the hours of darkness (Asarian and

Geary, 2002, Eckel *et al.*, 2000, Murakami *et al.*, 2002). Therefore to study appetite hormones during the most physiologically relevant dark phase, these animals were placed on a reverse lighting schedule (lights off at 11.00) and it was possible to remove the diet prior to the beginning of dark period at approximately 08.00. It was planned so that removing the diet before lights-off would provide a longer period of fasting than simply the 4 to 5 hours between removal of diet and culling. All but 1 of the nulliparous animals lost body mass (3.5 ± 0.22%, n=43; Chapter 3) during the fasting period, which supported this possibility. Figure 2.4 shows the protocol for fasting.

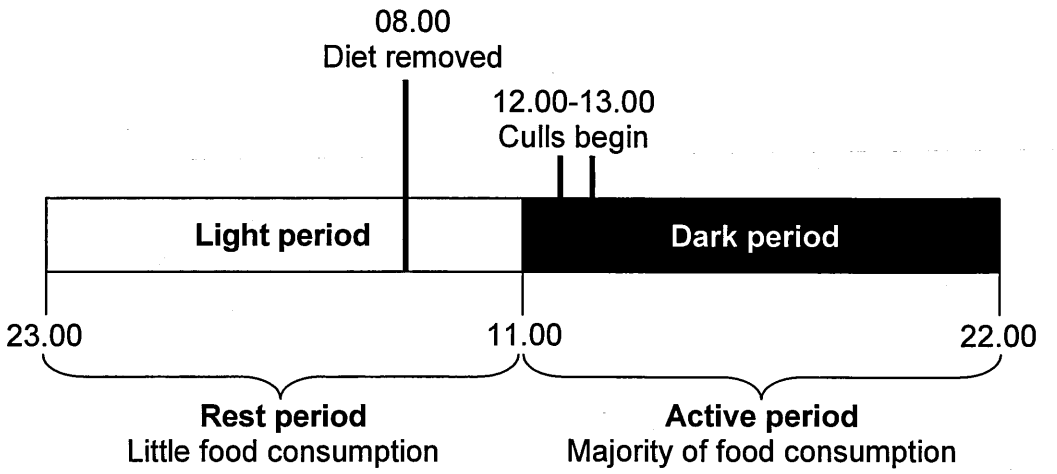

Figure 2.4. Protocol for fasting of study animals on a reverse lighting schedule

Other studies quantifying levels of ghrelin have fasted animals for a much longer time period than in the current study, due to the finding that ghrelin is increased in the fasted state. It is common for studies using both rats and mice to fast the animals for 24 hours (Lim *et al.*, 2009, Stengel *et al.*, 2009, Sun *et al.*, 2007, Toshinai *et al.*, 2001, Tschop *et al.*, 2000), with some studies extending the fasting time to 48 hours (Toshinai *et al.*, 2001, Tschop *et al.*, 2000, Zhao *et al.*, 2008), or even to 72 hours (Tovar *et al.*, 2004). Studies in humans often use an overnight fast prior to serum or plasma

hormone measurement (Sowers *et al.*, 2008, Tham *et al.*, 2009, Valsamakis *et al.*, 2010).

In this study, it was important that all of the animals were all fasted for an equal time period for comparison purposes, but at the same time it was important to minimise fasting length due to the use of pregnant dams. In the very early stages of pregnancy, such as the day 4 time point, the stress caused by an extended period of fasting may be sufficient for foetal resorption due to maternal stress.

2.3.2. Matched fed and fasted blood sample collection

Matched fed and fasted plasma samples were obtained from each dam to allow a fed and fasted comparison of ghrelin, PYY and GLP-1 concentrations in each individual animal. Fed blood samples were obtained from each rat by a small incision with a scalpel blade to one tail vein at the tail tip. Rats used for the work described in Chapter 3 had a fed blood sample taken from 13.00, after completion of oestrous monitoring. Rats were introduced into a tube-type restrainer (Vet-Tech Solutions Ltd) and topical cryo-analgesia (Cryogesic, Ethyl Chloride BP fine spray, Acorus Therapeutics Ltd) was applied to the tail tip. Dams used for work described in all other results chapters were anaesthetised using isofluorane (IsoFlo, Abbott) in an anaesthetic chamber until no pedal reflex was present. Rats were then transferred to a mask and eyes were lubricated (Lubrithal, VetXX Ltd) to prevent them from drying out. A small volume of blood was then collected through a minor incision to the tail as outline above. For all study animals, a

small amount of finger pressure was applied to the tail to accelerate this procedure and reduce time spent either in the restraining tube or under anaesthetic. When blood started to clot, or enough blood was collected, tails were gently cleaned using water and wounds coated with a spray-on dressing (OpSite, Smith & Nephew Medical Ltd). After the procedure, rats were placed into a clean cage, with free access to food and water.

Fed blood samples were all immediately acidified 1:10 in buffer (0.1 M ammonium acetate, 0.5 M NaCl, pH 3.6) as recommended (Stengel *et al.*, 2009) for optimal peptide preservation and recovery (see section 2.3.2.1). 1 ml of the fasted trunk blood (see below) collected from each animal was also immediately acidified 1:10 in buffer, with the remainder left un-acidified. Animals were left overnight after the tail bleed and food was removed from the cage at 08.00 the following day, prior to culling from 12.00. Rats were culled by anaesthetised decapitation, upon which a fasted blood sample was obtained from trunk blood. All blood was collected into 10 ml EDTA coated tubes (TekLab) with additional protease inhibitor (aprotinin; Trasylol, Bayer) and blood volume was recorded.

2.3.2.1. The RAPID methodology for optimal peptide recovery

The RAPID (Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls, and Dilution; Figure 2.5) processing for blood samples is recommended by Stengel *et al.* (2009) as a method by which endocrine peptide recovery can be improved by reducing the peptide breakdown that occurs during standard blood processing. Stengel *et al.*

(2009) reported that the acyl to total ghrelin ratio was 1:19 using standard processing, but when using RAPID, they reported a 1:5 ratio, which they state demonstrated a more accurate ghrelin measurement as a result of RAPID processing. That study also measured PYY_{1-36} and PYY_{3-36}, reporting that almost 40% more of both peptide forms were recovered using the RAPID methodology compared to standard processing. For these reasons, it was decided to use the RAPID methodology to improve peptide yield and allow analysis of different peptide forms in plasma for the studies in this thesis. It was intended from the study onset to quantify the acyl portion of ghrelin alongside the total form by LC/MS-MS but although the samples exist, it was not possible to complete this aspect of the study.

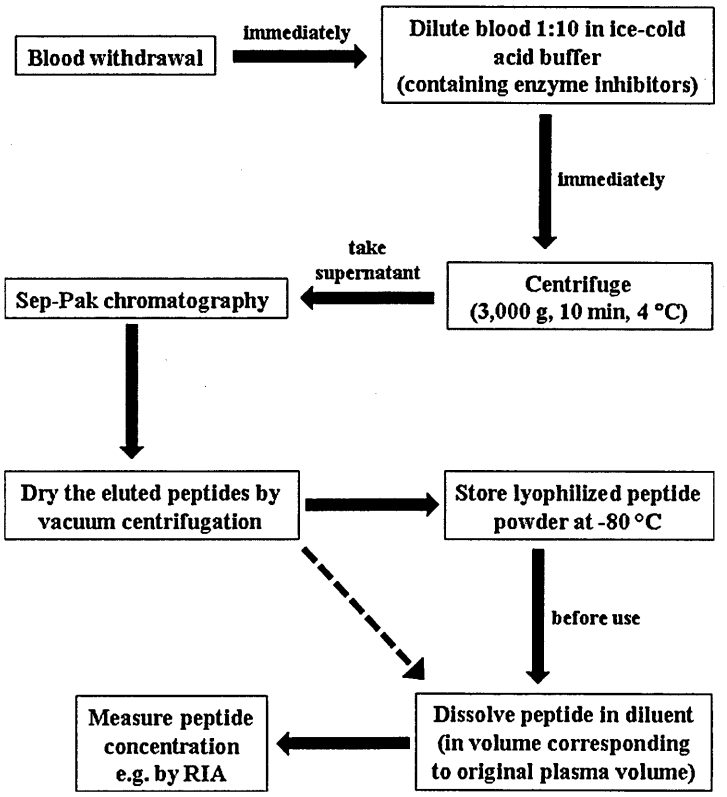

Figure 2.5 RAPID methodology flow diagram
(Modified from Stengel *et al.*, 2009).

2.3.3. Centrifugation of blood samples

All blood samples were kept on ice and then centrifuged immediately after dissection had been completed, using a bench-top refrigerated centrifuge (Heraeus Labofuge 400R) at 3000 g for 10 minutes at 4 °C. Plasma was aliquotted into Eppendorf tubes using sterile 3 ml plastic pipettes and stored at -80 °C. A vacuum centrifuge (Genevac SF50 or Concentrator Plus) was used overnight to dry down a small proportion of all fasted acidified plasma samples and the total volume of fed acidified plasma from animals in Chapters 4, 5 and 6 immediately after aliquotting; cycling females from Chapter 3 did not have fed acidified plasma dried down. All dried plasma powder was stored at -20 °C until assayed.

2.3.4. Gastrointestinal tissue sample collection

Samples of stomach and descending colon were taken from animals used in the work described in Chapter 3, and samples of stomach, ascending colon and descending colon were taken from animals used in all other chapters (Table 2.1). For pups (Chapter 7), whose colon tissue was smaller, measurements were amended accordingly, with care taken for consistency between litters.

Table 2.1. Dissection protocol for gut sample collection

Gut area	Protocol for sample collection
Stomach	Locate and dissect out
Ascending colon	Dissect out 5 cm of colon tissue immediately below caecum
Descending colon	Dissect out 5 cm of colon tissue 2 cm above the rectum

2.4. Physical measurements

Live body mass (in g) of all animals in the colony was recorded a minimum of twice per month prior to their use in the study. Additional body mass recordings were taken from animals being studied, with measurements always taken before the tail bleed and again before dissection. Regular measurement of body mass was also used as a method to improve the animal/handler relationship prior to any experimental procedure beginning. Body length was measured after culling, measuring from between the front paws to the anus. In dams used for the pregnancy and lactation time points, rectal temperature was recorded using a digital thermometer whilst animals were anaesthetised prior to the tail bleed.

2.4.1. Measurement of tissues and gut contents

Multiple measurements were taken during dissection of all adult females to obtain information about body composition and how this changed between sample groups. Table 2.2 outlines the measurements taken during dissections, however in Chapter 3, the small intestine was not fully emptied before weighing and so no comparisons were possible between small intestine tissue mass of proestrus controls and subsequent pregnant and lactating sample groups. Dams used for the day 25 of lactation time point also did not have fully emptied small intestine tissue (Chapters 5 and 6). Small and large intestines were free-floated in phosphate-buffered saline (PBS; pH 7.4), with care taken not to stretch the tissue, in order to obtain more accurate length measurements. Due to the use of the intestinal tissue for multiple methods of analysis (peptide extraction; immunostaining) and

time limitations, it was not possible to use nicardipine hydrochloride to maximally relax smooth muscle for complete standardisation of length measurements. Measurements of gut circumference, however, were standardised by measurement after 20 minute incubation in PBS containing the calcium channel blocker nicardipine hydrochloride (10^{-6} M; Sigma). Gut tubes were then cut open and measured using graph paper, with the aid of a light microscope.

Table 2.2. Protocol for measurements made of the gastrointestinal tract and other organs in each adult female

Tissue	Protocol
Stomach	Full stomach weighed, opened along greater curvature, emptied and re-weighed
Small intestine	Length recorded Full small intestine weighed, emptied and re-weighed
Duodenum	~1 cm of mid duodenum maximally relaxed in nicardipine for circumference measurement
Caecum	Length recorded Full caecum weighed, emptied and re-weighed
Large intestine	Length recorded Full large intestine weighed, emptied and re-weighed
Ascending colon	(Proximal 5 cm of large intestine) ~1 cm of proximal tissue for circumference measurement
Descending colon	(Distal 5 cm of large intestine) ~1 cm of proximal tissue for circumference measurement
Uterus	Full uterus weighed, emptied if fluid-filled, and re-weighed
Abdominal cavity adipose tissue	All white adipose tissue in the abdominal cavity removed and weighed

For pups (Chapter 7) only stomach tissue was examined for size changes due to time restrictions, with colon only being collected for peptide level analysis (see above).

In dams used for pregnancy and lactation studies, stomach and caecum contents were collected into Universal tubes at the appropriate stages of the dissection protocol and frozen (-20 °C) until use. Measurement of pH was made for each thawed sample using a digital pH meter (HI 5424, Hanna Instruments). Caecum contents were analysed using selected ion flow tube mass spectrometry (SIFT-MS; see appendix 1) from one group of pregnant and one group of lactating dams, as guided by significant pH differences. The caecum contents of two proestrus controls and the day 12 of pregnancy and day 10 of lactation groups were separated into portions of roughly equal mass (~1 g) for SIFT-MS analysis. Samples were placed into a sealed bag, which was filled with zero air before incubation at 37 °C for 1 hour in order to optimise the release of the volatile compounds from the samples for the analysis. Sample bags were then loaded onto the SIFT and each sample was analysed using 3 different precursor ions: H_3O^+, NO^+ and O_2^+. All compounds were measured in parts per billion.

2.5. Radioimmunoassays

Radioimmunoassay kits were purchased for the total peptide forms of ghrelin, PYY and GLP-1 (Millipore, UK). The total forms of each peptide were chosen for analysis to allow quantification regardless of any peptide degradation that may have either occurred during sample processing or by natural causes

(see section 1.6). Biologically active GLP-1 (GLP-1_{7-36}), for example, is rapidly truncated at the N terminal into the biologically inactive form (GLP-1_{9-36}) by dipeptidyl peptidase IV (DPP IV) (Kieffer *et al.*, 1995, Mentlein *et al.*, 1993). Thus, measuring only the biologically active forms of GLP-1 may not be a good representation of the true peptide concentration from the gut L-cells due to rapid degradation even before reaching the peripheral circulation.

2.5.1. Samples

Half of each rat stomach and a small section of mid-ascending colon (Chapter 4 onwards) and mid-descending colon from both adults and pups were collected into cryovials and immediately frozen on dry ice and stored at (-80 °C) after dissection to quantify tissue peptide content. All samples were later thawed and diluted to 1 ml per 100 g of tissue in 0.5 M glacial acetic acid and were then placed (in cryovials) in a boiling 100 °C water bath for 20 minutes. After cool enough to touch, the liquid portion of the samples was aliquotted into Eppendorf tubes using sterile 3 ml pipettes and stored at -20 °C until assayed. When tissue samples had to be diluted prior to their addition to a radioimmunoassay, they were diluted using distilled water.

As all fed plasma collected was acidified, this was analysed for each peptide. Fasted acidified plasma was used in ghrelin assays as recommended (see section 2.3.2.1) to stabilise the peptide form and for PYY and GLP-1 assays, neat un-acidified fasted plasma was used because concentrations of both of these peptides were expected to be lowest in the fasted state.

2.5.2. Assay procedure

All radioimmunoassays were performed according to the manufacturer's protocols provided and were used to analyse both plasma and tissue extracts, as outlined above. Briefly, the assay was set up using the recommended volumes of standards and quality controls and samples were added to the tubes at optimised volumes (see below). Counts from the standards provided the standard curve for the assay and the quality controls were a known low and a high concentration of the peptide, provided by the manufacturer, to check assay performance. All tubes (excluding total count and non-specific binding tubes) were incubated (4°C) overnight with the antibody for the appropriate peptide followed by a further overnight incubation (4°C) with the radiolabelled (^{125}I) tracer. The secondary antibody contained polyethylene glycol to allow separation of bound and unbound antibody prior to counting bound radioactivity using a gamma counter. This method of radioimmunoassay is competition based, so the number of binding sites available to the radiolabelled tracer is limited by the number of antibody binding sites occupied by the standard/sample after the first incubation. Thus, the lower the radioactive count obtained at the end of the assay, the higher in concentration the standard or sample, which is then quantified using the standard curve generated from the percentage of total binding calculated for each standard.

Some sample dilutions and appropriate calculation modifications were made so that samples had good separation on the standard curve. Sample dilutions were optimised in pilot assays, resulting in dilution factors as described below

for each peptide. For all calculation modifications, a calculation for the change in sample volume was made first, followed by a separate calculation for any dilution made to that sample prior to its addition to the assay tubes.

2.5.3. Total ghrelin radioimmunoassay

Samples of fed and fasted acidified plasma and stomach tissue extract were analysed for total ghrelin concentration. The ghrelin radioimmunoassay kits had a lower and upper detection limit of 93 pg/ml and 6000 pg/ml respectively in a sample size of 100 µl. During preliminary testing, ghrelin was found to be extremely concentrated in all sample types, so they were all diluted 10 times by the addition of 10 µl of sample to each assay tube, rather than 100 µl as suggested in the protocol. One ghrelin radioimmunoassay kit was further tested and replicate tubes were spiked separately with a known concentration (1 µg/ml) of an acyl and a desacyl ghrelin standard to confirm the assay was properly recovering both forms of the peptide, using low sample volumes (5 µl). Results from the ghrelin spiking confirmed that the assay was equally efficient at recognising both acyl and desacyl ghrelin and recovering the expected amounts.

No difference was found in the concentration of ghrelin between the fed and fasted plasma samples in Chapter 3, so fed plasma was not analysed for ghrelin concentration from Chapter 4 onwards, due to cost. Dams from Chapter 6 who were mothers of the pups used in Chapter 7 did have fed plasma ghrelin quantified in order to match a sample between dam and pup, as pups were assumed to be fed due to keeping them with the dams during

the fasting period. However, due to different sample preparation at this stage of the study (fed plasma samples were dried down after Chapter 3) no direct comparison could be made between matched fed and fasted plasma ghrelin concentrations in Chapter 6. Adding to the 10 times dilution described above, stomach tissue extract was additionally diluted 1:250 for animals in Chapter 4 and 7, and 1:400 for animals in Chapters 3, 5 and 6 prior to its addition to the assay.

The intra-assay and inter-assay coefficients of variation (%CV; Table 2.3) were calculated separately for each assay batch, with a batch change between analysis of the oestrous cycle samples and the remaining samples. Intra-assay variation was calculated from total binding reference (B_0) tubes at the beginning and end of assays. The inter-assay %CV was calculated from the quality control (QC) used in each assay that was closest in concentration to the samples analysed. A %CV of ≤15% was considered acceptable and any samples outside this were reanalysed. The kit protocol stated that the assays do not have any cross-reactivity with other related hormones.

Table 2.3. Ghrelin radioimmunoassay intra- and inter-assay coefficients of variation (n=number of assays)

	Intra-assay coefficient (%)	**Inter-assay coefficient (%)**
Oestrous cycle (batch 1)	10.7 (n=3)	4.7 (n=3)
Pregnant/lactating/pups (batch 2)	13.6 (n=4)	12.4 (n=4)

2.5.4. Total peptide-YY (PYY) radioimmunoassay

Fasted non-acidified plasma and fed acidified plasma were analysed using the PYY radioimmunoassay kits, alongside ascending and descending colon extracts. The manufacturer's stated sensitivity of the PYY kits was 15.6 pg/ml with a 100 µl sample volume (78.1 pg/ml with a 20 µl sample volume). Fasted non-acidified plasma samples were added to the assay as suggested in the kit protocol. Fed (acidified) plasma samples from animals used in Chapter 3 were added to kits at 150 µl, rather than 20 µl, because they were more dilute from the acidification process, creating a concentration factor of 0.67. Pup plasma (Chapter 7) was treated in a similar way and added to kits at 180 µl volume (x 0.56 concentration). Animals used in Chapters 4, 5 and 6 had fed acidified plasma samples that were dried using vacuum centrifugation and so each sample was reconstituted to its original equivalent plasma volume (minus the buffer volume) with distilled water. After this, 70 µl (Chapters 5 and 6) or 90 µl (Chapter 4) of the reconstituted fed plasma was added to each assay, resulting in a concentration factor of either 1.43 or 1.11 respectively. Sample dilutions for ascending colon (Chapter 4 onwards) and descending colon (all animals) are outlined below (Table 2.4). The kit protocol for the PYY radioimmunoassays stated that there was no detectable cross-reactivity with other related peptides, including GLP-1. The intra-assay and inter-assay %CVs for PYY radioimmunoassays are shown in Table 2.5 and were calculated per kit batch.

Table 2.4. Optimal dilutions made for PYY quantification
Dilutions were optimised for each sample group and are displayed by chapter number. Ascending colon tissue was not taken from study animals in Chapter 3.

	Ascending colon extract	Descending colon extract
Chapter 3	No data	1:20
Chapters 4, 5 and 6	1:80	1:80
Chapter 7	1:60	1:60

Table 2.5. PYY radioimmunoassay intra- and inter-assay coefficients of variation
(n=number of assays)

	Intra-assay coefficient (%)	Inter-assay coefficient (%)
Oestrous cycle (batch 1)	6.0 (n=1)	2.9 (n=3)
Pregnant/lactating/pups (batch 2)	8.1 (n=7)	13.4 (n=7)

2.5.5. Total glucagon-like peptide-1 (GLP-1) radioimmunoassay

Fasted non-acidified plasma samples were extracted and added to each kit as outlined in the assay protocol. Stated detection limits for the GLP-1 radioimmunoassays were 3-333 pM with a 300 µl extracted sample size. Double the volume of fed acidified plasma underwent the kit extraction protocol in order to double the concentration and bring these samples onto the standard curve. Briefly, for extraction, 95% ethyl alcohol was added to each sample which was then incubated on ice for 30 minutes and centrifuged at 10,000 rpm for 10 minutes in a microfuge. The supernatants were decanted and dried down using a vacuum centrifuge (Genevac SF50 or Concentrator Plus) before reconstitution in sample hydrating solution (provided in the kit). Colon tissue concentrations of GLP-1 were sufficiently

concentrated and did not require extraction (Table 2.6). In order to convert concentrations from pmol to pg/ml, all concentrations were multiplied by 3.297, as 1 pmol/l of GLP-1 is equivalent to 3.297 pg/ml. The intra-assay and inter-assay %CVs for GLP-1 radioimmunoassays are shown in Table 2.7 and were calculated per kit batch. The manufacturer stated that there was a <0.01% detection of GLP-2 and 0.2% detection of glucagon in cross-reactivity tests.

Table 2.6. Optimal dilutions made for GLP-1 quantification
Dilutions were optimised for each sample group and are displayed by chapter number. Ascending colon was not taken from study animals in Chapter 3.

	Ascending colon extract	Descending colon extract
Chapter 3	No data	1:30
Chapters 4, 5 and 6	1:80	1:70
Chapter 7	1:80	1:50

Table 2.7. GLP-1 radioimmunoassay intra- and inter-assay coefficients of variation
(n=number of assays)

	Intra-assay coefficient (%)	Inter-assay coefficient (%)
Oestrous cycle (batch 1)	4.3 (n=3)	6.3 (n=3)
Pregnant/lactating/pups (batch 2)	7.2 (n=6)	10.6 (n=3)

2.6. Immunofluorescence

2.6.1. Tissue preparation

After dissection, half of each rat stomach was pinned flat (pinned around the tissue edges), mucosa side down in a glass petri dish containing Sylgard.

Stomach and approximately 2 cm of proximal ascending colon (Chapter 4 onwards) and distal descending colon were fixed intact overnight in 4% paraformaldehyde. After 3 x 10 minute PBS washes, tissue was transferred into 30% sucrose, 0.1% sodium azide solution in PBS and maintained at 4 °C until blocking.

2.6.2. Tissue blocking and cryosectioning

Tubes of colon were pinned to cork disks (Figure 2.6 A) and coated (inside and outside) in OCT-embedding medium (Fisher). Colon samples were then frozen in isopentane cooled in liquid nitrogen, with care taken so that tissue did not come into direct contact with the isopentane. The body of each stomach was cut out (Figure 2.6 B and C) and this portion was placed into a plastic rectangular mould containing OCT, before freezing in isopentane cooled in liquid nitrogen. Sections of colon were made cross-sectionally. Frozen stomach tissue blocks were transversely sectioned on a cryostat (Leica CM1900), starting from the straight edge of the tissue adjacent to the oesophageal opening. All sections were cut at a thickness of 10 µm and collected onto electrostatically charged slides (SuperFrost Plus, Fisher). If not for immediate use, slides were stored at -80 °C until staining.

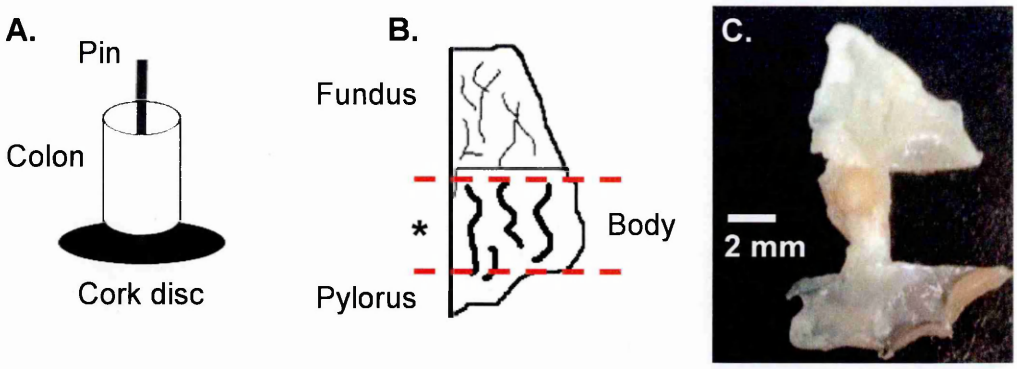

Figure 2.6. Fixation protocol for colon and stomach tissue
Tubes of colon tissue **(A)** were pinned to cork for cryoblocking and **(B)** the body of each stomach was removed **(C)** and blocked for cryosectioning, with notation (*) of tissue orientation made on each plastic mould.

2.6.3. Immunofluorescence protocol

All slides were allowed to adjust to room temperature (RT) before application of normal serum (10%) for 90 minutes to block background fluorescence. Serum was washed from slides with 3 x 10 minutes PBS before primary antibody incubation overnight (18 to 24 hours) at RT. Slides were thoroughly washed (3 x 10 minutes PBS) and the secondary antibody was applied for 120 minutes (RT), followed by a further 3 x 10 min PBS wash. When a fluorescently labelled secondary antibody was used, slides were then mounted with Citifluor (Agar Scientific) and coverslipped. When a biotinylated secondary had been applied, sections were incubated with streptavidin fluorescein for 120 minutes (RT) before a final 3 x 10 min PBS wash and mounting with Citifluor. Negative controls were performed for each test, which included slides incubated with antibody-dilution solution only, primary antibody only, secondary antibody only and, when appropriate, streptavidin fluorescein only.

2.6.4. Optimisation of antibody dilutions

All primary antibodies were optimised for each tissue type prior to EC quantification. Table 2.8 details both optimal antibody dilutions and the combinations of primary and secondary antibodies used for all immunofluorescence experiments.

Table 2.8. Optimised concentrations of each primary and secondary antibody
Table shows pairings used between primary and secondary antibodies.

Primary antibody	Dilution	Secondary antibody	Dilution
Goat anti-ghrelin *Santa Cruz Biotechnology*	1:800	Biotinylated horse anti-goat IgG *Vector*	6 ug/ml
		Streptavidin fluorescein *Vector*	4 ug/ml
Rabbit anti-PYY *Abcam*	1:600	Goat anti-rabbit IgG Alexa Fluor 488 *Invitrogen*	1:200
Rabbit anti-GLP-1 *Phoenix Pharmaceuticals*	1:600	Goat anti-rabbit IgG Alexa Fluor 488 *Invitrogen*	1:200

2.6.5. Groups selected for immunofluorescence

To identify whether the immunoreactive (IR) cell densities of ghrelin (stomach), PYY (colon) and GLP-1 (colon) changed between pregnancy and lactation in comparison with proestrus controls, animals from one group of pregnant dams and one group of lactating dams were selected. Groups were selected to have one sample group that had a high tissue peptide concentration and one sample group that had a low tissue peptide concentration. Tissue peptide concentrations were determined by

radioimmunoassays. For stomach ghrelin cell quantification, day 12 pregnant (low stomach ghrelin concentration) and dams on the day of birth (high ghrelin stomach concentration) were chosen as samples with a low and a high stomach tissue ghrelin concentration.

Experiments to determine PYY and GLP-1 immunopositive cells in ascending and descending colon tissue were performed using day 12 pregnant dams (low tissue peptide concentrations) and day 5 lactating dams (high tissue peptide concentrations). Immunostaining for PYY and GLP-1 in ascending and descending colon tissue did not yield quantifiable results, because staining for both peptides was inconsistent. The way in which the tissue was treated was consistent and the tissue had typical morphology. It is possible that due to the standardised method of sample collection, whereby the same section of gut was taken for staining from each dam, the most informative regions of gut for this method of analysis may have been missed. Cell density may vary along the length of the gut tissue, with gut growth also likely to affect cell density in different gut regions. Due to gut growth, it is likely that the above protocol for gut dissection would need to be standardised per rat, rather than the use of a standard protocol for all rats. Future work to determine the most suitable gut regions for staining of PYY and GLP-1 would have to explore using the full length of the colon to characterise the best areas for immunostaining.

2.6.6. Quantification of immunofluorescence

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

Automated cell counting was trialled for this study using the ImageJ tool that counts between set intensities. This was found not to be suitable due to large differences in background intensity caused by mucosal folding, which was prevalent in both stomach and colon sections. Thus, criteria had to be set to determine positive cells for manual counting.

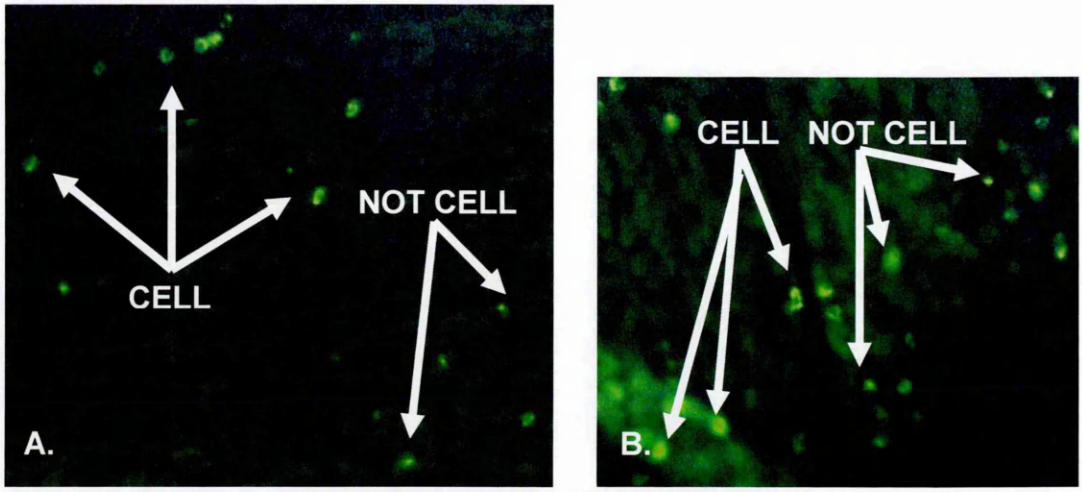

Figure 2.7. Ghrelin immunoreactive cells in rat stomach tissue
Cells counted could either be **(A)** clear or **(B)** obscured as long as cell appearance was consistent (x100 magnification).

Ghrelin cells in stomach tissue (Figure 2.7 A) were counted as positive when they were of consistent size, had clear edges and visible nuclei. In sections of tissue where the mucosa had folded over and obscured positive cells (Figure 2.7 B), nuclei were not always visible and so cells were counted when cell edges were still clearly defined and the cells were of a size consistent with IR cells in non-folded regions of the same section.

In colon tissue (Figure 2.8), PYY and GLP-1 immunopositive cells had a characteristic 'tail' and all cells with this morphology and a visible nucleus were counted. However, it was not possible to conduct this work for this thesis for reasons stated above (section 2.6.5).

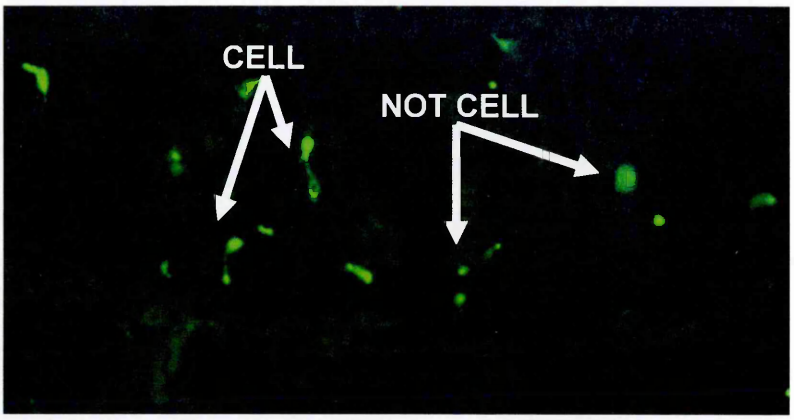

Figure 2.8. A representative image of L-cell staining for PYY/GLP-1 in descending colon
(x100 magnification).

For measurement of mucosal area, serial images were also taken of each section using a x4 objective lens in order to measure mucosal area more easily. ImageJ was additionally utilised for tissue section measurements; the freehand drawing tool was calibrated to the scale bar on images and used to draw around the mucosal epithelium in each image, so that cells could be expressed per mucosal area (cells per mm^2). The maximum mucosal and muscle (all muscle layers) thickness in each image for each tissue section, as judged by eye, was measured using ImageJ to obtain a mean maximal mucosal and muscle thickness (in mm) for each section. Thickness was measured in this way because gut mucosa is quite difficult to section uniformly, due to the natural folds of the tissue. By obtaining the mean maximal mucosal and muscle thicknesses, there is a lesser likelihood of measurements being influenced by amount of gut contents present at dissection or by changes in tissue orientation when sections were cut. In order to partially control for this, it would have been desirable to use nicardipine (see section 2.4.1) to maximally relax the tissue and standardise measurements.

2.7. Statistical analysis

All means in the results chapters are expressed ± standard error of the mean (SEM). Unless otherwise specified, data sets were analysed by one-way ANOVA with a Tukey post-hoc test and $P<0.05$ was considered significant. On figures, post-hoc significance is denoted as stars where *** is $P<0.001$, ** is $P<0.01$ and * is $P<0.05$. Within columns on tables, a>b is $P<0.001$, c>d is $P<0.01$ and e>f is $P<0.05$.

Non-homogeneous data sets underwent natural log transformation to normalise distribution and allow parametric analysis. When log transformation did not normalise the data, results were analysed by the non-parametric Kruskal-Wallis test with follow-up pairwise comparisons (Mann-Whitney), with Bonferroni correction. Matched fed and fasted concentrations of peptides were analysed using a paired samples t-test and SIFT-MS analysis of caecum contents was analysed using an independent samples t-test.

CHAPTER 3: ANALYSIS OF CHANGES IN GUT APPETITE HORMONES DURING THE OESTROUS CYCLE

3.1. Introduction

There is a well documented link between the hormones of the reproductive cycle and cyclic changes to appetite (reviewed by Asarian and Geary, 2006, Butera, 2010, Eckel, 2011). Food intake has been associated with levels of oestradiol in the circulation and rats reportedly consume less during oestrus, following the earlier peak in oestradiol secretion by the ovaries during proestrus (Asarian and Geary, 2006). In one study, a significant reduction in food intake at oestrus was found to be accompanied by a reduction in water consumption and was due to a decrease in intake, but not a decrease in meal frequency in *ad libitum* fed rats (Eckel *et al.*, 2000). Eckel *et al.* (2000) also reported a significant decrease in body mass in *ad libitum* fed rats during oestrus. Wade (1975) hypothesised that oestradiol transiently alters the homeostatic set point to which body mass is regulated and causes an indirect reduction in body mass by causing hypophagia. This hypothesis was tested by injecting ovariectomised (OVX) rats with oestradiol, which reduced food intake and subsequently body mass, but only for the first 3 weeks of treatment. Thereafter, food intake recovered to control levels and body mass stabilised to approximately 12% below that of the non-treated OVX control rats. On withdrawal of oestradiol treatment, both food intake and body mass increased above control levels (Wade, 1975). One additional study in OVX

female rats further demonstrated that cyclic administration of 'near physiological' oestradiol levels allowed maintenance of feeding patterns and body mass, both of which were lost in non-treated OVX control rats (Asarian and Geary, 2002).

Despite the described observations of cyclic changes to food intake and body mass during the reproductive cycle, the possible roles of appetite-regulating gut hormones have been little explored. Cholecystokinin (CCK) has been the most widely investigated appetite hormone in relation to the oestrous cycle. A review by Asarian and Geary (2006) summarises that during oestrus, oestradiol increases the anorexigenic actions, although not concentrations of CCK, thus reducing food intake, and that ERα is required for this effect (Geary, 2001). Ghrelin concentrations during the oestrous cycle have also been investigated and a relationship between ghrelin and oestrogen is established, but the mechanisms of action are not well explored.

3.1.1. Effects of ghrelin during the oestrous cycle

Many studies (reviewed by Butera, 2010) have examined the effects of oestradiol on ghrelin's orexigenic properties by artificial manipulation of these hormones, with a main focus on the effects on food intake. One study that provides the most comprehensive picture of the inhibitory effect of oestradiol on ghrelin in rats by Clegg *et al.* (2007) demonstrated that infusion of ghrelin increased food intake in males and in OVX females, but not in either oestrogen-treated males or intact females, suggesting that oestrogen somehow attenuates ghrelin's ability to stimulate appetite. In OVX females

treated with cyclic oestradiol to mimic the oestrous cycle, ghrelin infusion increased food intake on the equivalent days of metestrus and diestrus (low oestrogen levels), but had no effect during proestrus and oestrus (high oestrogen levels), which was also replicated in normally cycling rats (Clegg *et al.*, 2007). Butera's review (2010) of the work in their lab also concluded that injection of ghrelin during diestrus, but not proestrus, increased food intake and that this occurred due to increased meal frequency but not individual meal size. These findings suggest that during times of high oestradiol (proestrus and oestrus), endogenous ghrelin levels may be reduced, or ghrelin's actions could be attenuated by high oestradiol levels. The finding of increased meal frequency but not meal size during diestrus (Butera, 2010) is contrary to what Eckel *et al.* (2000) reported, described previously, where there is a decrease in meal size but not frequency at oestrus. Taken together, these studies may suggest that independent mechanisms are responsible for the increase in food intake at diestrus and the decrease in food intake at oestrus.

As well as assessing the influence of oestradiol levels on the effects of ghrelin on food intake, one study has shown that an injection of ghrelin significantly decreased serum oestradiol at each stage of the cycle (Fang *et al.*, 2012), suggesting that the relationship between oestradiol and ghrelin may be reciprocal.

The relationship between oestradiol and ghrelin expression in the rat stomach has been explored by a number of groups. One of the first studies

reported that ghrelin cell density in the stomach tissue of young rats increased significantly just 3 days post-OVX in 4 week old females, and 5 days post-OVX in 9 week old females, with a significant sustained increase in plasma ghrelin immediately after OVX (Matsubara *et al.*, 2004). From 7 days post-OVX in that study, ghrelin cell density and plasma ghrelin started to reduce in both groups, but it remains to be established whether this reduction would continue with time and reach intact control levels. Matsubara *et al.* (2004) also reported that the OVX-induced ghrelin increase in plasma and in both stomach ghrelin cell density and mRNA were reversed by treatment of oestradiol, although it remains to be seen whether these effects are reproducible in fully mature adult female rats. Another study using female rat isolated stomach cells demonstrated that oestrogen treatment upregulated stomach ghrelin mRNA expression in a dose-dependent manner (Sakata *et al.*, 2006). Using both male and female rats, these authors further demonstrated that ghrelin and oestrogen cells are located in close proximity in the stomach by analysis of cells immunopositive for acyl ghrelin and aromatase (Sakata *et al.*, 2006). Together, these findings suggest that oestrogen from the stomach could potentially have the ability to upregulate stomach ghrelin expression *in vivo*, but without knowing the effect of oestrogen treatment on stomach cells from male rats, this may only be the case in females. In male rats, stomach ghrelin mRNA is reported to be 2.5 fold higher in fasted (48 hours) male rats compared with those in the fed state, but that portal-vein oestradiol was not changed with fasting (Zhao *et al.*, 2008). This finding suggests that oestradiol does not contribute to gastric

ghrelin mRNA expression in fasted male rats; this study did not include females.

Although the studies described here have established that there are relationships between oestradiol, the reproductive cycle and ghrelin, no studies have yet quantified ghrelin concentration in both fed and fasted plasma and in gut tissue from rats allowed to cycle normally. In addition, there is very little research looking into the potential changes of PYY and GLP-1 concentrations during a naturally occurring reproductive cycle, despite the relationships between ghrelin, PYY and GLP-1 in appetite regulation (see section 1.6.4). One may expect that during oestrus, when food intake is lowest, PYY and GLP-1 may contribute to the anorexigenic tone, whilst ghrelin levels remain attenuated due to the peak of oestradiol during proestrus.

3.2. Objectives

The objectives for this part of the study were:

- to investigate if there are changes in the concentrations of the appetite hormones ghrelin, PYY and GLP-1 during the rodent oestrous cycle by quantification of both fed and fasted plasma and (fasted) gut tissue hormone concentrations at each stage of the oestrous cycle;
- to establish whether concentrations of these appetite hormones change in accordance with the appetite changes reported by others.

This work was also carried out as a baseline for further experimental work described in Chapters 4, 5 and 6. To investigate whether circulating and/or gut concentrations of ghrelin, PYY and GLP-1 change during pregnancy and lactation, it was necessary to explore their interactions in nulliparous females and the role they have in the regulation of appetite during the reproductive cycle. It was also necessary to establish the baseline body and gut measurements in these reproductively cycling animals in order to determine how the gut architecture might change to accommodate increased food intake during pregnancy and lactation.

3.3. Methods

3.3.1. Animals

Cycle stage was determined as outlined in Chapter 2 (section 2.2). To minimise the body mass range of the animals, cages of rats were chosen for daily oestrous monitoring in the order of heaviest mean cage body mass; 2 to 5 cages were monitored at a time.

Rats were monitored for a minimum of 2 complete cycles (often up to 4 cycles) to obtain sample groups at each stage of the oestrous cycle. A fed blood sample was taken the day before a required cycle stage was predicted in order to dissect animals at that predicted stage (proestrus n=12; oestrus n=11; metestrus n=9, diestrus n=11). A number of animals did not progress as predicted with their oestrous cycle (for example, with an additional day spent in diestrus), so there is a mis-match between the number of animals in

the fasted state cycle stages (as above) and the numbers obtained for the fed cycle stages (proestrus n=14; oestrus n=7; metestrus n=6, diestrus n=16).

3.3.2. Sample collection

Samples of fed and fasted plasma were taken as outlined in Chapter 2 (section 2.3.2). On culling, multiple measurements of the gut were made and gut samples were collected (section 2.3.4).

3.3.3. Measurements of food intake and body mass

In addition to weekly weighing, a sub-group of the animals undergoing oestrous monitoring was weighed daily to ascertain whether cycle stage influenced body mass, as described by Eckel *et al.*, (2000). Food intake per cage was estimated by weighing a full food hopper every Friday at 10.30 and weighing the hopper again every Monday at 10.30. This allowed an approximate measure of average food intake whilst the animals were minimally disturbed, for comparison with future breeding animals. Data collected for approximate food intake was then calculated for mean daily food intake per cage and divided by the number of rats in the cage. Body mass was measured both prior to fed blood sample withdrawal and culling.

3.3.4. Determination of cycle stage

Starting at 11.00 (lights off) rats were removed from the home cage, placed onto a towel with their heads covered and lightly restrained by hand to begin

oestrous monitoring. Vaginal lavage was carried out using a small volume (<0.5 ml) of distilled water in a sterile 3 ml plastic pipette, with care taken only to insert the fine tip of the pipette into the vagina. Water was expelled and collected approximately 3 times, or until the suspension became cloudy, before placing the solution into a double cavity microscope slide. Viewing of slides was aided using a black and white on-screen filter on a commercially available light microscope (Bresser 5201000 LCD microscope). The black and white on-screen filter allowed for clear viewing of cell morphology without need for fixation or staining, thus reducing analysis time.

Measurement of electrical resistance in the vagina was additionally used as a tool to help identify the stage of oestrus (section 2.2). However, even between replicate readings, measurements were not consistent enough to show any difference between cycle stages and were not used as part of this study.

3.3.5. Peptide assays

Radioimmunoassays to determine peptide concentrations of ghrelin, PYY and GLP-1 in plasma and tissue extracts were performed as detailed in Chapter 2 (section 2.5).

3.4. Results

3.4.1. Total ghrelin peptide analysis

3.4.1.1. Matched fed and fasted plasma comparison

For the full group, there was a non-significant trend (P=0.051) for fed plasma ghrelin concentrations (180.4 ± 6.66 ng/ml) to be higher than in the matched fasted plasma samples (174.1 ± 5.83 ng/ml). When data were split by cull day cycle stage, there was no difference between fed and fasted plasma ghrelin levels in the matched samples at any cycle stage. Fed and fasted plasma ghrelin concentrations positively correlated with each other (r=0.890, P<0.001; Figure 3.10).

3.4.1.2. Concentration change with cycle stage

Concentrations of ghrelin in fed acidified plasma were not significantly different between cycle stages (Table 3.1). Fasted acidified plasma ghrelin (Figure 3.1) showed a non-significant trend to be different between the cycle stages (P=0.056), being least concentrated during proestrus. Concentrations of ghrelin in stomach tissue extracts were similar between each cycle stage (Table 3.1).

Table 3.1. Ghrelin concentrations in fed plasma and in stomach tissue at each cycle stage
Stomach tissue concentrations expressed as µg per g of wet weight of tissue (WWT; number of rats in brackets).

	Fed plasma (ng/ml)	Stomach tissue (µg/g WWT)
Proestrus	202.3 ± 12.92 (14)	12.5 ± 0.65 (12)
Oestrus	173.1 ± 12.03 (7)	14.3 ± 1.20 (11)
Metestrus	187.4 ± 23.66 (6)	12.4 ± 1.09 (9)
Diestrus	160.6 ± 6.85 (15)	14.4 ± 1.84 (11)

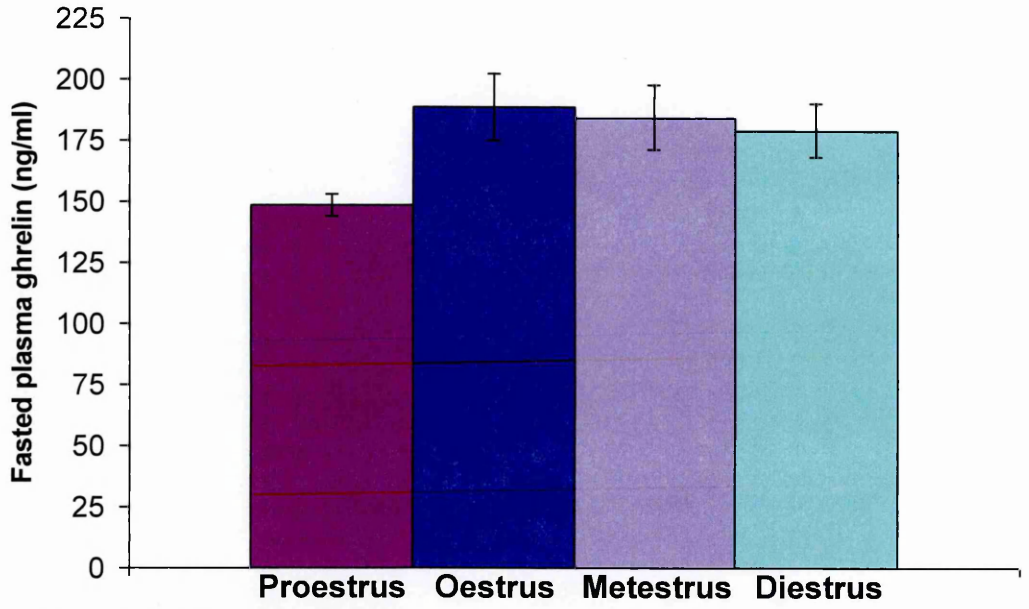

Figure 3.1. Ghrelin concentrations in fasted plasma at each cycle stage
(Proestrus, n=12; oestrus, n=11; metestrus, n=9; diestrus n=11).

3.4.2. Total peptide-YY (PYY) analysis

3.4.2.1. Matched fed and fasted plasma comparison

Matched fed and fasted plasma sample analysis for the whole group found that PYY concentrations were significantly ($t(41)=13.397$, $P<0.001$) higher in the fed state (453.6 ± 20.05 pg/ml) than the fasted state (177.6 ± 7.18 pg/ml).

There was no correlation between PYY concentrations in fed and fasted plasma.

3.4.2.2. Concentration change with cycle stage

The concentrations of PYY in fed acidified plasma were not significantly different with cycle stage. Fasted non-acidified plasma concentrations of PYY were also not significantly different between the groups (Table 3.2).

Table 3.2. PYY concentrations in fed and fasted plasma at each cycle stage (Number of rats in brackets).

	Fed plasma (pg/ml)	**Fasted plasma (pg/ml)**
Proestrus	496.8 ± 55.94 (14)	176.6 ± 10.64 (12)
Oestrus	456.0 ± 19.61 (7)	161.3 ± 5.90 (11)
Metestrus	444.8 ± 23.87 (6)	175.5 ± 6.51 (9)
Diestrus	415.5 ± 13.95 (15)	196.6 ± 24.21 (11)

Concentrations of PYY in the descending colon (Figure 3.2) did not significantly differ between the cycle stages due to large variations in concentration at each stage.

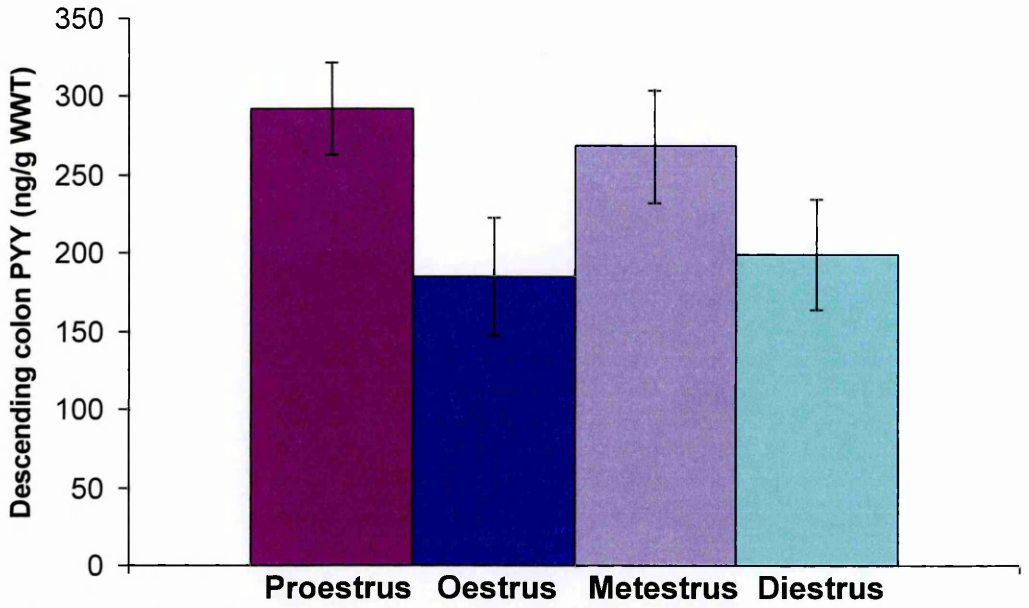

Figure 3.2. PYY concentrations in descending colon tissue at each cycle stage (Proestrus, n=12; oestrus, n=11; metestrus, n=9; diestrus, n=11).

3.4.3. Total glucagon-like peptide-1 (GLP-1) peptide analysis

3.4.3.1. Matched fed and fasted plasma comparison

GLP-1 concentrations in fed plasma (101.6 ± 13.15 pg/ml) were significantly ($t(41)=5.266$, $P<0.001$) higher than in fasted plasma (28.4 ± 15.17 pg/ml) in the whole group. Fed and fasted plasma GLP-1 concentrations did not correlate with each other.

3.4.3.2. Concentration change with cycle stage

GLP-1 concentrations in fed acidified plasma (Figure 3.3) were significantly (Kruskal-Wallis, $\chi^2=7.871$, 3 df, $P=0.049$) more concentrated in proestrus than in diestrus ($P=0.034$). Fasted non-acidified plasma concentrations of GLP-1 were similar at proestrus (27.9 ± 4.56 pg/ml), oestrus (29.1 ± 3.96 pg/ml), metestrus (23.8 ± 2.90 pg/ml) and diestrus (32.0 ± 6.22 pg/ml).

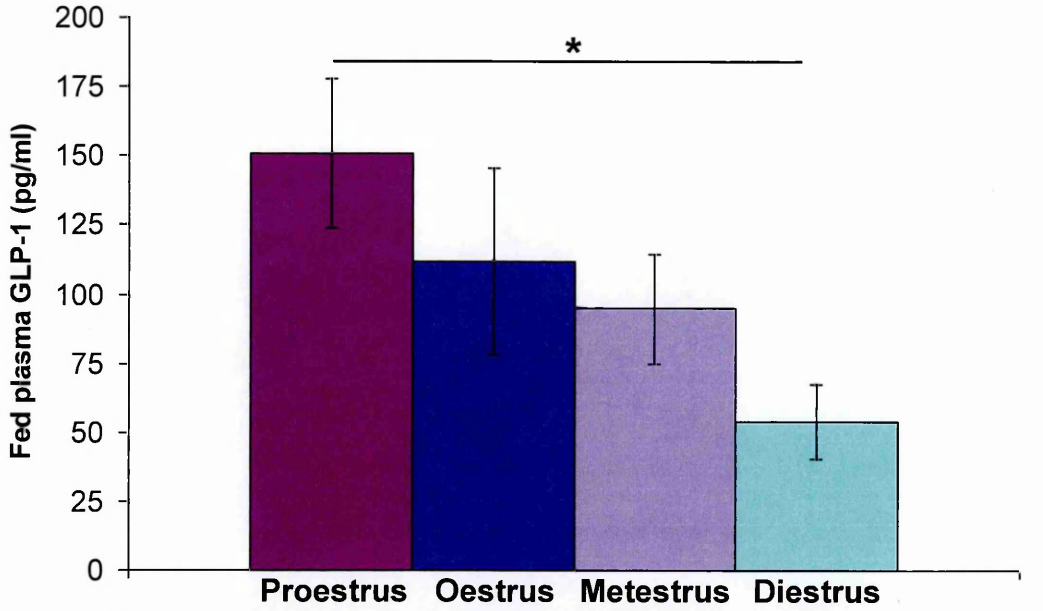

Figure 3.3. GLP-1 concentrations in fed plasma at each cycle stage
(Proestrus, n=14; oestrus, n=7; metestrus, n=6; diestrus, n=15; * P<0.05).

In descending colon tissue (Figure 3.4), GLP-1 was significantly more concentrated (F(3, 39)=3.921, P=0.015) at proestrus than during oestrus (P=0.045) and diestrus (P=0.029).

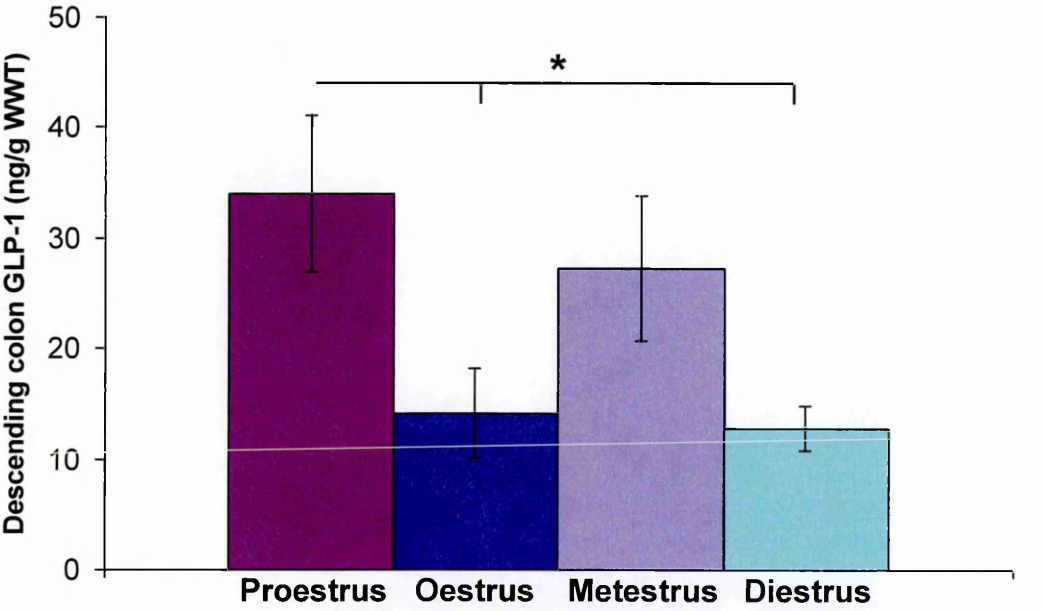

Figure 3.4. GLP-1 concentrations in descending colon tissue at each cycle stage
(Proestrus, n=12; oestrus, n=11; metestrus, n=9; diestrus, n=11; * P<0.05).

3.4.4. Relationships between total ghrelin, PYY and GLP-1 in all animals

Plasma concentrations of both fed (r=0.606, P<0.001) and fasted (r=0.514, P<0.001) ghrelin were significantly positively correlated with fed plasma GLP-1 concentrations (Figure 3.5).

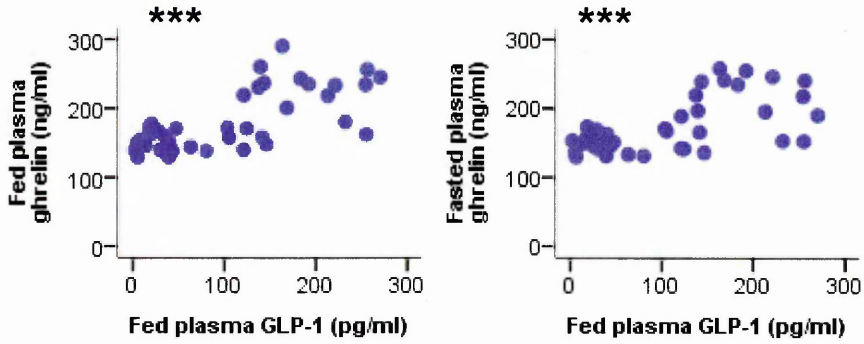

Figure 3.5. Fed and fasted plasma ghrelin significantly correlated with fed plasma GLP-1
(n=43 animals at various cycle stages; *** P<0.001).

There was also a significant positive correlation between the concentrations of PYY and GLP-1 in descending colon tissue (r=0.545, P<0.001; Figure 3.6).

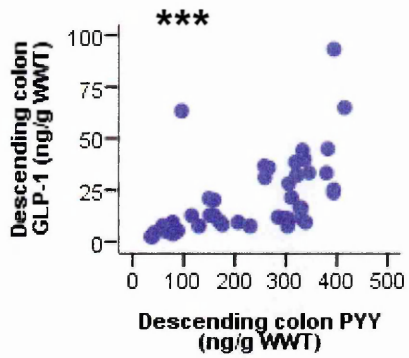

Figure 3.6. Descending colon PYY and GLP-1 significantly correlated with each other
(n=43 animals at various cycle stages; *** P<0.001).

3.4.5. Body and gut size of normally cycling, nulliparous female Wistar rats

At the time of culling, animals had a mean age of 38 ± 0.49 weeks (32 – 44 weeks) and a mean body mass of 269.2 ± 2.59 g (239.6 – 303.9 g). There

were no significant differences in age (P=0.180) or body mass (P=0.673) between the different groups. The mean daily food intake (see section 3.3.3) of all animals was calculated to be 15.5 ± 0.09 g over 26 weeks. Although fasted prior to dissection, a small amount of stomach contents were often present in these study animals (0.7 ± 0.06 g; 0.04 – 1.80 g), due to free access to water and ingestion of other non-food material.

Body mass did not correlate with age or body length, but significantly positively correlated with tissue wet weights of stomach (r=0.497, P=0.001; 1.6 ± 0.02 g; Figure 3.7 A) and small intestine (r=0.549, P<0.001; 7.0 ± 0.12 g; Figure 3.7 B). Mass of abdominal cavity white adipose tissue (15.1 ± 0.59 g; Figure 3.7 C) significantly positively correlated with body mass (r=0.656, P<0.001).

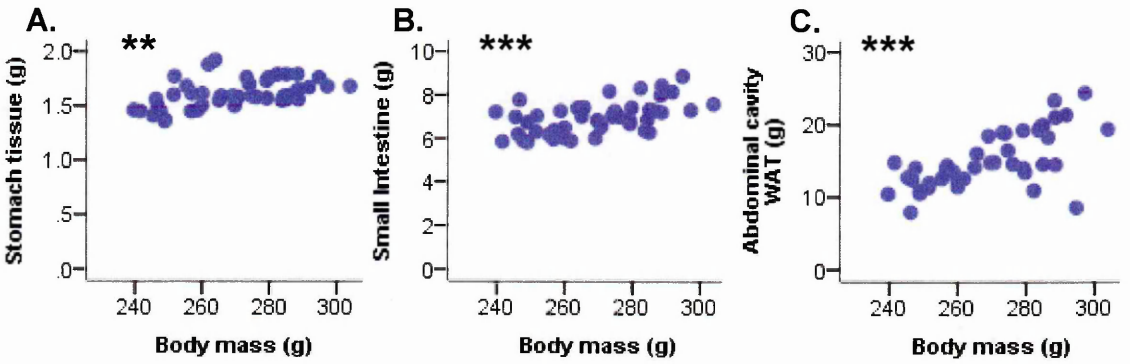

Figure 3.7. Correlations between body mass and gut tissue in normally cycling female rats
Body mass significantly correlated with **(A)** stomach tissue wet weight, **(B)** small intestine wet weight and **(C)** abdominal cavity white adipose tissue (WAT). (n=43 animals at various cycle stages; ** P<0.01; *** P<0.001).

3.4.5.1. Physical changes that occurred by cycle stage

As expected, no differences were found between gut measurements at each of the cycle stages but were established as a baseline to inform on the

magnitude of any changes to the gut during pregnancy and lactation in subsequent chapters.

Intact uterus mass was found to be significantly highest at proestrus ($F(3, 39)=7.998$, $P<0.001$), when the uterine cavity became fluid-filled (Figure 3.8 A). Fluid in the uterus was used as an additional confirmation of successful proestrus identification by vaginal lavage. When corrected for individual rat body mass, uteri remained significantly heavier at proestrus ($F(3, 39)=8.035$, $P<0.001$) than at all other cycle stages.

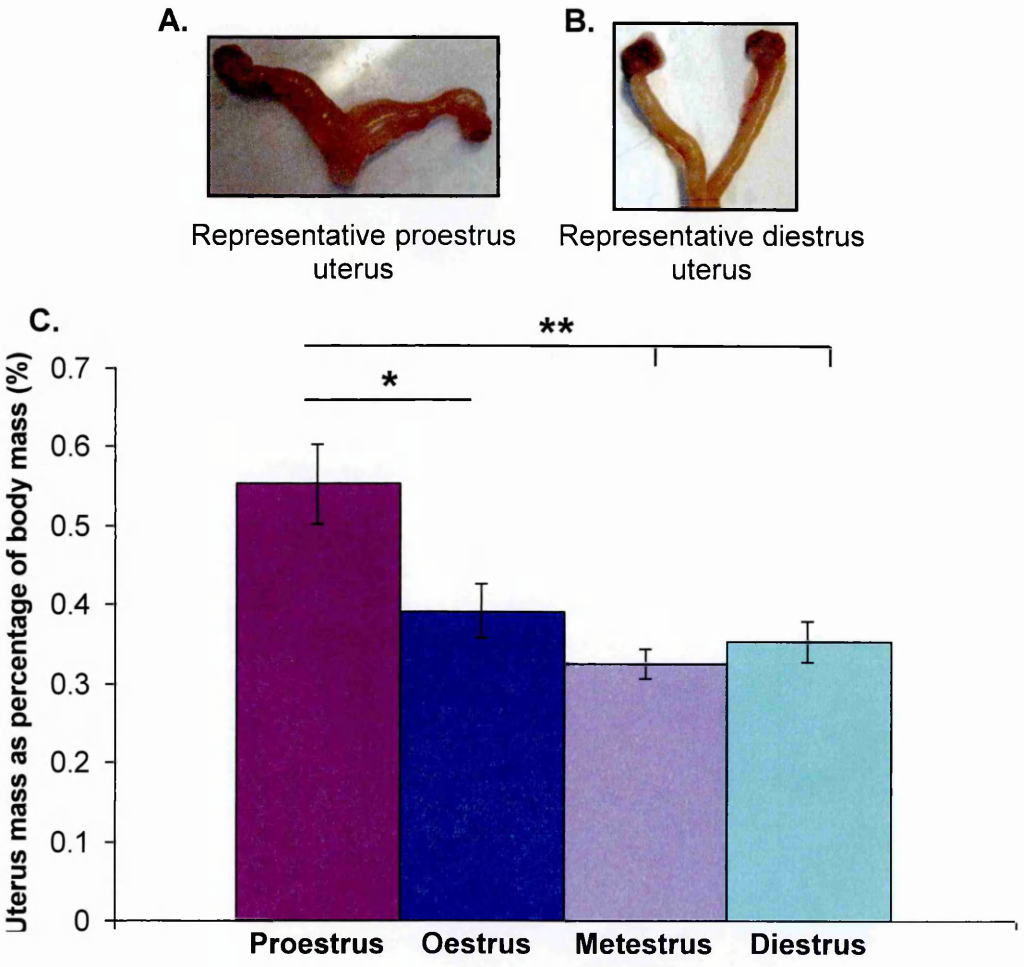

Figure 3.8. Uteri were significantly heavier at proestrus
Uteri (not to scale) became fluid filled at proestrus **(A)**, but not at other cycle stages **(B)**. **(C)** Intact uterus mass corrected for individual body mass (proestrus, n=12; oestrus, n=11; metestrus, n=9; diestrus, n=11; * $P<0.05$; ** $P<0.01$).

3.4.5.2. Body mass and food intake

The body mass of 12 animals was recorded daily at the time of oestrous monitoring. Where records existed and could be followed across multiple cycles, 4 animals showed no observable pattern in body mass change with cycle stage. In another 6 animals, the stages of metestrus and diestrus tended to coincide with an increase in body mass, but there were always exceptions between cycles. For example, 1 animal tended to be lighter during metestrus than at other cycle stages. A total of 4 animals tended to weigh less during oestrus.

Figure 3.9 shows that animals dissected at metestrus had significantly ($F(3, 39)=3.187$, $P=0.034$) more (fasted) stomach contents than those dissected at oestrus ($P=0.028$). Stomach contents data were analysed against the cycle stage of the preceding day as the rats were fasted from the beginning of the cull day (from 08.00; before lights off at 11.00), therefore analysis of stomach contents provided an indication of food consumption during the previous day/cycle stage. These results could therefore suggest that animals consumed the least between proestrus and oestrus and the most shortly after the end of oestrus leading into metestrus. Differences to the fasted stomach contents found between cycle stages could also indicate a different rate of gastric emptying, which was not measured.

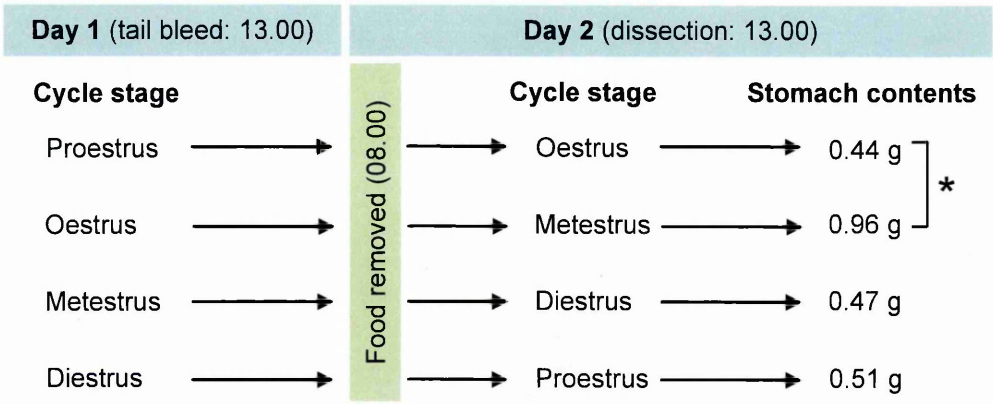

Figure 3.9. Stomach contents remaining after fasting between cycle stages (Cull cycle stages: proestrus, n=12; oestrus, n=11; metestrus, n=9; diestrus, n=11; * $P<0.05$).

3.4.6. Plasma ghrelin concentrations were affected by age

Further analysis revealed that plasma ghrelin concentrations were affected by age, with animals over the age of 36 weeks having a significantly reduced circulating ghrelin in both the fed ($F(1, 40)=101.77$, $P<0.001$) and the fasted (Kruskal-Wallis, $\chi^2=25.416$, 1 df, $P<0.001$) states (Figure 3.10 and Table 3.3). The older animals with less circulating ghrelin had a more significant correlation between fed and fasted concentrations ($r=0.619$, $P<0.001$) than the younger animals ($r=0.583$, $P=0.029$). Nearly half (48.3%) of the older rat group had a higher fasted plasma ghrelin than fed, as expected, however only 35.7% of the younger animals displayed this relationship. Stomach tissue ghrelin concentration was not significantly different between the age groups (Table 3.3).

Table 3.3. Ghrelin in plasma and in stomach tissue between two age groups
Stomach tissue concentrations expressed as µg per g of wet weight of tissue (WWT (number of rats in brackets).

	32 – 36 weeks of age	37 – 44 weeks of age
Fed plasma ghrelin (ng/ml)	231.5 ± 9.30 (14)	154.9 ± 2.77 (28)
Fasted plasma ghrelin (ng/ml)	220.9 ± 7.82 (14)	151.5 ± 2.45 (29)
Stomach ghrelin (µg/g WWT)	15.2 ± 1.58 (14)	12.6 ± 0.49 (29)

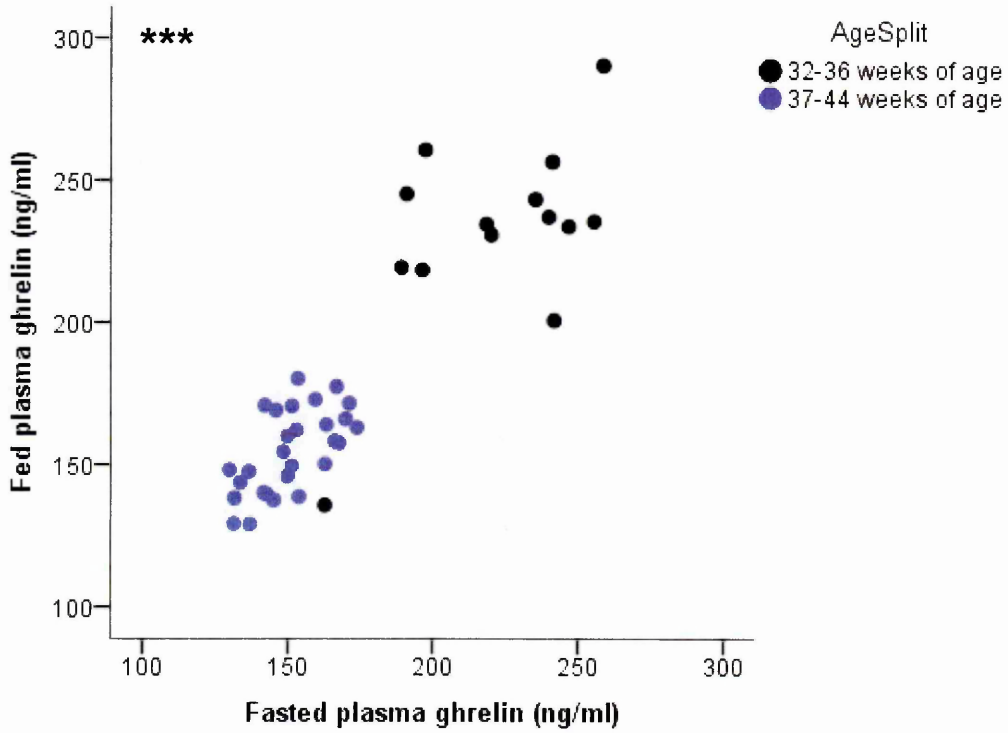

Figure 3.10. Age group differences in fed and fasted plasma concentrations of ghrelin
Fed (r= -0.730, P<0.001) and fasted (r= -0.744, P<0.001) plasma ghrelin concentrations significantly correlated with each other. (32-36 weeks, n=14; 37-44 weeks, n=29; *** P<0.001).

3.5. Discussion

This study aimed to elucidate the role of the gut hormones ghrelin, PYY and GLP-1 in reported changes to appetite during the oestrous cycle. This work was carried out prior to analysis of the changes in these hormones during pregnancy and lactation and is the first study to explore the concentrations of

both fed and fasted plasma and tissue concentrations of these peptides at each stage of the oestrous cycle.

The concentrations of total ghrelin did not change significantly with the stage of the cycle in either fed or fasted plasma or in stomach tissue extract. Ghrelin plasma concentrations were additionally found not to be significantly different between the fed and the fasted state at any cycle stage, which has not been reported previously. Plasma ghrelin concentrations were found to be significantly reduced after 36 weeks of age. PYY fed and fasted plasma and descending colon concentrations were also not significantly different between the cycle stages. Fed, but not fasted, plasma concentrations of GLP-1 changed significantly with cycle stage and were highest during proestrus. Descending colon GLP-1 was also significantly highest at proestrus, which may suggest increased satiety leading into oestrus.

3.5.1. Orexigenic gut hormone changes during the oestrous cycle

Although ghrelin concentrations did not significantly change with the stage of the cycle in either plasma or tissue, a number of findings were still of importance. For example, ghrelin concentrations were unexpectedly similar between fed and fasted plasma samples in this study, which is discussed below.

3.5.1.1. Plasma ghrelin concentrations were similar in the fed and fasted states

As described in Chapter 1, ghrelin is secreted from the stomach in response to a negative energy balance and stimulates hunger and initiates food intake, thus it is generally accepted and widely documented that ghrelin levels are higher in the fasted state (Cummings *et al.*, 2001, Stengel *et al.*, 2009, Toshinai *et al.*, 2001, Tschop *et al.*, 2000). In these animals, a significant difference between fed and fasted plasma ghrelin concentrations was not seen, although there was a trend for fed plasma to contain more ghrelin, which is the opposite of what was expected. The animals were adequately fasted, because PYY and GLP-1 levels were significantly lower in the fasted state than the fed state and act as an internal control. In addition, the animals lost body mass during the short fasting period which provided an additional confirmation of adequate fasting.

One explanation for not detecting a difference in ghrelin levels in fasted and fed plasma could be due to the time of day at which the samples were taken. In this study, animals were maintained under reverse lighting, while in many other studies reverse lighting has not been used. Thus the samples used in the present study could have been taken at more physiologically relevant times (i.e. during the active period) than in other studies. Furthermore, the vast majority of studies only take fasted plasma samples to analyse ghrelin concentrations, without taking the fed state into consideration at all, as one review clearly demonstrates (Stengel *et al.*, 2011). Some studies even fail to mention whether the animals were fed or fasted when sampling occurred (Hosoda *et al.*, 2000).

Another possible reason for the difference between the present and previously reported results is that acidification of plasma for ghrelin stability (Stengel *et al.*, 2009) preserved more of the peptide, making it more accurate and also less comparable to studies that have not used this same technique. However, Stengel *et al.* did report higher acyl and total ghrelin concentrations in plasma from 24 hour fasted than in *ad libitum* fed rats in samples analysed with and without RAPID methodology, so this does not seem to be a likely explanation, although a difference in results could be due to their use of male Sprague-Dawley rats compared to the present study using female Wistar rats. Another factor is that in previous work, separate animals were used for the fed and fasted samples (Stengel *et al.*, 2009), so the results from this present study may be different from those of others due to the use of matched samples.

3.5.1.2. Changes in ghrelin levels during the reproductive cycle

In this work, fasted plasma ghrelin concentrations showed a trend (P=0.056) to be lowest at proestrus. The possibility of a reduction in circulating ghrelin at proestrus could contribute to a decreased food intake between proestrus and oestrus and could be caused by the inhibitory effects of oestradiol on ghrelin, as previously detailed (reviewed by Butera, 2010).

In order to fully elucidate the actions of ghrelin during the reproductive cycle, further work needs to be carried out to analyse both the acyl and the desacyl forms of the ghrelin peptide in these samples. Although no significant differences were found in plasma or stomach tissue with cycle stage, it

remains possible that the ratio of the two different peptide forms will provide additional detail to this work, compared with the total amount of ghrelin peptide.

3.5.2. The effect of age on ghrelin concentrations

In this study, plasma ghrelin levels were found to be reduced in both the fed and fasted state after 36 weeks of age. One animal, aged 36 weeks, was the only exception and also had a low concentration of circulating ghrelin, which may be explained by this rat having the least amount of ghrelin peptide in her stomach tissue of all the study animals. It may be that this particular animal had naturally lower ghrelin levels than the others in this study, which is implied by reduced ghrelin concentrations in both plasma and the stomach, which is the main source of circulating ghrelin. Also of note was that the older animals had a much tighter correlation between their fed and fasted plasma ghrelin concentrations than the younger animals, although the implications of this are unknown at this time.

The optimal reproductive performance of laboratory rats is documented to begin declining from around 9 months of age (Niggeschulze and Kast, 1994, Suckow *et al.*, 2006), which is the age at which reduced ghrelin concentrations were measured here. Although these animals were not particularly old (>15 months of age before reproductive senescence), there is an association between a reduction in ghrelin concentrations and ageing, which has been linked to the condition known as anorexia of ageing. One study has reported a decline in plasma ghrelin with age in humans, leading to

reduced appetite and increased frailty, particularly in female participants (Serra-Prat *et al.*, 2010). However, others have reported differently, with one study documenting that total plasma ghrelin increased slightly with age in mice and that plasma acyl ghrelin also demonstrated this trend (Sun *et al.*, 2007). In agreement with this, another study found that calorie restriction in mice increased both total ghrelin levels in circulation and total ghrelin production in the stomach with age (Yang *et al.*, 2007).

In addition to the above findings, fewer of the younger animals had the expected relationship between fed and fasted ghrelin, with only 35.7%, compared with nearly half of the older rats, having a higher fasted ghrelin concentration. These findings remain to be explained, but could indicate a difference in the way that ghrelin regulates appetite between the younger and the older animals, or that ghrelin itself was being regulated differently.

Differences in reproductive hormones due to age could be related to the disparity found between ghrelin concentrations in the older and younger animals. Oestradiol has an inhibitory effect on endogenous ghrelin concentrations in circulation (reviewed by Asarian and Geary, 2006, Butera, 2010, Eckel, 2011), so declining levels of oestradiol with increasing age are unlikely to be responsible for the decrease in plasma ghrelin concentrations observed. One study in human females (Sowers *et al.*, 2008) has shown that plasma ghrelin levels increased during the peri-menopausal stage. Another study (Soni *et al.*, 2011) complemented these findings and demonstrated that women who discontinued hormone replacement therapy (oestrogen and

progesterone; HRT) showed increased plasma ghrelin levels compared with the group that continued HRT treatment.

In rats, oestrogen secreted from the gastric mucosa has been shown to induce stomach ghrelin expression (Sakata *et al.*, 2006), but a further study found that gastric oestrogen was not responsible for the fasted increase of ghrelin mRNA expression (Zhao *et al.*, 2008). Further investigation into oestrogen concentrations in the stomachs of these study animals may provide insights into whether reproductive ageing causes a decrease in gastric oestrogen, which could in turn cause a blunted ghrelin response to food intake. In this study, however, it is unlikely that reproductive age was a factor in the profound reduction of ghrelin levels. The differences were documented to occur from approximately 8 to 9 months of age, but reproductive senescence does not occur until between approximately 15 and 20 months of age in laboratory rats (Sengupta, 2013), which is when such large changes in ghrelin levels may be anticipated.

3.5.3. Anorexigenic gut hormone changes during the oestrous cycle

Neither fed nor fasted PYY concentration in the matched plasma samples fluctuated to a great extent across the cycle stages. Descending colon PYY concentrations also varied considerably within each group. A study in OVX non-human primates demonstrated that intracerebroventricular infusion of PYY consistently reduced daily food intake during oestrogen-only treatment for 2 weeks (Papadimitriou *et al.*, 2007). Following the 2 week oestrogen

treatment, the females were given a 2 week treatment of oestrogen plus progesterone, and food intake was only significantly reduced during week 1 of this treatment. These findings suggest that PYY-induced satiety may be affected by cycle stage, although further work would be necessary to confirm this.

Both fed plasma GLP-1 and descending colon GLP-1 were significantly more concentrated during proestrus. Like PYY plasma concentrations, GLP-1 plasma concentrations were also significantly reduced in the fasted state, which demonstrates that the animals were fasted sufficiently to produce a difference in these gut peptide levels. One study that quantified plasma GLP-1 concentrations during the menstrual cycles of lean and healthy women (Brennan *et al.*, 2009) found that plasma GLP-1 was significantly decreased during the follicular phase of the cycle after a glucose load, but baseline levels were not different between the follicular and luteal phases.

Significantly increased fed plasma and descending colon GLP-1 concentrations could have increased satiety in these animals between proestrus and oestrus and thus reduced food intake, which was indicated by reduced stomach contents at this time.

3.5.4. Food intake and body mass changes during the oestrous cycle

As the animals used in this study were group housed, daily food intake with cycle stage could not be monitored, but weighing stomach contents at

dissection provided an indication of food and water consumption. It is well established that oestradiol has a marked effect on food intake in females (reviewed by Butera, 2010, Eckel, 2004), causing a reduction in food intake at oestrus in rats that follows the peak in ovarian oestradiol secretion at proestrus. Mass of stomach contents in these study animals indicated that they consumed the least during the time between proestrus and oestrus and the most shortly after this, which is consistent with the literature (reviewed by Asarian and Geary, 2006, Butera, 2010, Eckel, 2011). The decreased body mass that has been reported to occur at oestrus (Eckel *et al.*, 2000) only presented in 4 out of the 12 rats examined in this current study, so was not deemed a useful addition to oestrous monitoring.

3.5.5. Oestrous monitoring technique

Despite oestrous monitoring being a widely implemented technique, there is little recent discussion on the best ways in which to determine cycle stage. For this study, vaginal lavage, alongside a record of the oestrus dance and subsequently the uterus mass, proved successful in determining cycle stage. The oestrus dance in these Wistar rats was an extremely reliable and non-invasive way to identify proestrus, yet this behavioural aspect of the oestrous cycle is not widely discussed in recent literature (Adler and Feder, 1981, Hardy, 1972). Some studies have specified that lordosis was used as a tool to monitor reproductive behaviour, for example the re-establishment of normal display of sexual receptivity (lordosis) after oestradiol treatment in OVX rats (Asarian and Geary, 2002). What the literature is lacking is study of the lordosis behaviour in conjunction with oestrous monitoring on ovary-intact

rats undergoing no treatment regimen to determine the exact timing of behavioural oestrus in comparison to the cytological appearance of oestrus. In these Wistar females, behavioural oestrus occurred during proestrus, as described by Adler and Feder (1981), but it is possible that different strains present differently. In addition, no literature currently exists detailing the use of the oestrus dance alone as a method of proestrus identification. If proestrus is the only desired stage for a study, for example when proestrus animals are used for controls, or for an accurate time-mating system, the oestrus dance alone could be implemented in Wistar rats under reverse lighting. Oestrous monitoring using only the oestrus dance was suitable for regularly 4 day cycling rats here and is predicted to reduce stress in these animals due to minimal intervention and it would dramatically reduce analysis time, thus greatly refining this methodology.

3.6. Conclusions

An indication of reduced food intake between proestrus and oestrus, provided by the reduced stomach contents in these animals, agrees with the findings of others (Clegg *et al.*, 2007, Eckel, 2004, Eckel *et al.*, 2000). Supporting the theory of increased satiety during oestrus were significantly increased fed plasma and descending colon GLP-1 concentrations during proestrus. Increased satiety from GLP-1 in both plasma and gut tissue was found to be in parallel with a trend for ghrelin to be lowest in fasted plasma at proestrus. Observed changes in these gut hormone concentrations at proestrus, leading to reduced fasted stomach contents during oestrus, could contribute to the reported reduction in food intake during oestrus.

3.7. Future work

A number of questions arise from this study. Why matched fed and fasted plasma ghrelin concentrations were not significantly different from each other remains to be established. These animals were adequately fed and fasted when the matched plasma samples were taken as both PYY and GLP-1 plasma concentrations were significantly higher in the fed plasma samples. Further study into the ratio of acyl ghrelin to desacyl ghrelin during the cycle would be an informative addition to this work. Why plasma ghrelin concentrations significantly decreased at 37 weeks of age in these animals would also require further study, although this finding does indicate a more appropriate age for Wistar females for future studies of this nature.

CHAPTER 4: GUT APPETITE HORMONES AND GUT GROWTH DURING PREGNANCY

4.1. Introduction

Pregnancy is a time of extensive maternal adaptation in order to facilitate foetal growth and development and also to build energy reserves in preparation to meet the demands of lactation. In rats, maternal body mass increases throughout pregnancy, starting from as early as day 3 post-conception (Cripps and Williams, 1975), partly resulting from increased accumulation of adipose tissue (Lopez-Luna *et al.*, 1991, Pujol *et al.*, 2006), which is stored ready to support lactation. One study concluded that the body mass increase in pregnant dams was reliant on increased food intake (Gualillo *et al.*, 2002). Maternal body mass of 30% food-restricted dams continued to decrease until day 16 of pregnancy, after which there was a slight body mass recovery, but not to pre-mating *ad libitum* fed body mass (Gualillo *et al.*, 2002). Hyperphagia during pregnancy in rodents has been reported to increase food intake by 20% during the first week of pregnancy (Crean and Rumsey, 1971, Trujillo *et al.*, 2011), peaking at between 50% (Crean and Rumsey, 1971) and 60% (Cripps and Williams, 1975) by the end of pregnancy compared to nulliparous controls. Modulation by hormones that regulate appetite is thought to be a key aspect of hyperphagia during pregnancy, and leptin has been the most widely researched appetite hormone in this context. As described in Chapter 1, maternal leptin resistance occurs as an adaptation to pregnancy (section 1.8.3). Leptin levels

increase in proportion to the adipose reserves accumulated during pregnancy, and leptin resistance develops from mid-gestation to support hyperphagia and allow further adipose accumulation.

4.1.1. Orexigenic gut appetite hormone changes during pregnancy

Ghrelin levels have been shown to be suppressed by increased leptin levels (Ueno *et al.*, 2004), yet Taylor *et al.* (2009) demonstrated, in *ad libitum* fed pregnant rats, that total ghrelin concentrations in plasma and in some gut tissues were not suppressed by the increased leptin levels associated with mid-pregnancy.

Taylor *et al.* (2009) reported no significant change to plasma total ghrelin levels in rats during pregnancy. However, another study in rats reported that plasma total ghrelin decreased throughout pregnancy and was significantly decreased by days 10 and 15 of pregnancy, when rats are hyperphagic, with a recovery to control levels at day 20 (Shibata *et al.*, 2004). An additional study in *ad libitum* fed rats reported no significant change to plasma ghrelin between days 8 and 21 of pregnancy (Gualillo *et al.*, 2002), although it is unclear whether total, acyl or desacyl ghrelin was quantified in this study. Human females at 14 to 18 weeks of gestation did not have different fasted levels of plasma acyl ghrelin when compared to non-pregnant controls (Moshtaghi-Kashanian *et al.*, 2011) and a further human study found no difference in acyl ghrelin throughout the first, second and third trimesters (Valsamakis *et al.*, 2010). Another human study conversely found that serum

total ghrelin concentrations peaked at mid-gestation, after which serum ghrelin decreased until parturition (Fuglsang *et al.*, 2005).

As previously addressed (section 1.8.3.1), one difficulty in comparing measurements of ghrelin between studies is the measurement of different forms of the peptide. One human study measured both total and acyl ghrelin and reported that total ghrelin concentrations were lower at weeks 22 and 35 of pregnancy compared with postpartum values, whilst acyl ghrelin was found to be significantly reduced in these women (Tham *et al.*, 2009). It was calculated that acyl ghrelin only accounted for 10 to 18% of total ghrelin in pregnancy and 48 to 54% of total ghrelin in the postpartum period (Tham *et al.*, 2009). The authors suggest that pregnancy may affect the ability of ghrelin *O*-acyltransferase to acylate ghrelin, which may be caused by insulin resistance.

No difference has been reported in the concentrations of stomach tissue total ghrelin by Taylor *et al.* (2009), who studied days 4, 14 and 19 of pregnancy. Likewise, Shibata *et al.* (2004) found no change in stomach tissue total ghrelin levels in a study involving rats at days 5, 10, 15 and 20 of pregnancy.

4.1.2. Anorexigenic gut hormone changes during pregnancy

Despite previously identified interactions (section 1.6.4), such as attenuation of levels by ghrelin (Chelikani *et al.*, 2006), PYY and GLP-1 have been less well studied as gut hormones of interest during pregnancy. In one human

pregnancy study, there was no significant difference in plasma total PYY between the three trimesters of pregnancy (Valsamakis *et al.*, 2010). However in rats, Tovar *et al.* (2004) found plasma levels of total PYY to increase from day 16 of pregnancy and peak prior to birth. Taylor *et al.* (2009) also reported a gradual increase in plasma total PYY concentrations during pregnancy in rats. Additionally, gradually increasing PYY concentrations were found in descending, but not ascending, colon and in rectum tissue (Taylor *et al.*, 2009). Why PYY, a satiety hormone, appears to be elevated in both plasma and tissue despite pregnancy-associated hyperphagia remains to be established, but it may be reasonable to assume a degree of PYY-resistance during pregnancy, as is seen with leptin. Increased levels of PYY have previously been reported to increase body temperature in PYY transgenic male mice (Boey *et al.*, 2008), so measurement of body temperature in the current study was undertaken due to the expected increase in PYY levels.

4.1.2.1. Insulin resistance occurs during pregnancy

The incretin GLP-1 is involved in glycaemic control (Edwards *et al.*, 1999), and ensures appropriate pancreatic β-cell insulin secretion in response to circulating glucose (Schmidt *et al.*, 1985). It is established in the laboratory rat that insulin resistance develops in the latter stages of pregnancy due to reduced tissue sensitivity to insulin (Leturque *et al.*, 1984), similar to the response seen in humans (Vrachnis *et al.*, 2012).

During insulin-resistant states, such as in type 2 diabetes and in obesity, GLP-1 concentrations have been found to be decreased (Muscelli *et al.*, 2008, Toft-Nielsen *et al.*, 2001). However, one study reported that the active form of GLP-1 in plasma was significantly increased by the third trimester of human pregnancy, being higher than during both the first and second trimesters (Valsamakis *et al.*, 2010), combined with a significantly increased plasma insulin concentration. Leturque *et al.* (1984) studied the effect that late-stage pregnancy (19 days pregnant) had on insulin resistance in rats, and determined that blood glucose concentrations were lower, yet blood insulin concentrations were higher in the pregnant dams versus nulliparous matched controls. Taken together, these results suggest an altered response to blood glucose at day 19 of pregnancy in rats.

GLP-1 concentrations have been rarely explored in direct relation to gut L-cell GLP-1 secretion, yet a study using a non-obese mouse model of insulin resistance demonstrated the ability of chronically elevated insulin concentrations to impair L-cell secretion of GLP-1 (Lim *et al.*, 2009). Lim *et al.* (2009) also found that the insulin receptor was expressed in the L-cells of both their mouse model and in a human L-cell line. These findings highlight the importance of considering the possible changes to gut peptide levels when studying gut-secreted peptides, and not simply just exploring circulating changes.

4.1.3. Gut growth during pregnancy

Another key aspect of maternal adaptation during pregnancy involves structural changes to the gut, although most studies of gut size change during reproductive states have mainly focussed on lactation (Campbell and Fell, 1964, Datta *et al.*, 1995), and often exclusively on the small intestine (Burdett and Reek, 1979, Datta *et al.*, 1995).

Although Cripps and Williams (1975) documented that neither stomach wet nor dry tissue mass changed during pregnancy, Crean and Rumsey (1971) had reported differently. Stomach tissue wet weight increased steadily throughout pregnancy and was significant by day 14 of pregnancy, with hyperplasia of the gastric mucosa also evident by this stage (Crean and Rumsey, 1971). These authors suggested that hyperplasia of the gastric mucosa is unlikely to be due to increased food intake alone, and that something else must be influencing mucosal growth.

Cripps and Williams (1975) reported no change in the length or dry weight of the small intestine of pregnant rats, but the percentage of water, and therefore the wet weight, significantly increased compared to control tissue. However, another study reported significant increases to both small intestine wet weight and length (Burdett and Reek, 1979), with a 30 to 40% increase in mucosal epithelium mass greatly contributing to the overall wet weight. Colon tissue was also found to be significantly longer by day 21 of pregnancy (Cripps and Williams, 1975).

It is clear than the maternal adaptations to pregnancy are diverse and complex. The aim here was to further elucidate the possible roles of ghrelin, PYY and GLP-1 during pregnancy by investigating changes in both plasma and in gut tissues. Matched fed and fasted plasma PYY and GLP-1 levels during pregnancy have been measured for the first time. Comprehensive measurement of the gut was also performed, advancing the current literature of gut growth during pregnancy by examining multiple gut tissues. Measurement of the gut alongside both plasma and tissue peptide levels allows investigation into whether there is a relationship between these parameters. Although Taylor *et al.* (2009) documented changes in gut growth and ghrelin and PYY levels during pregnancy, the current study has looked at gut growth in more detail, with the addition of GLP-1 measurements and peptide analysis in both fed and fasted plasma. Ghrelin-immunoreactive (IR) cells in the stomach were also studied to provide a more complete study of changes to ghrelin during pregnancy. The study by Taylor *et al.* (2009) also statistically analysed the peptide levels and gut size of both pregnant and lactating dams as a single group, which may have masked some more subtle changes between the stages of pregnancy, due to more overt changes occurring during lactation. Analysis of pregnancy alone in the current study aims to provide more detail to the possible changes in peptide levels and gut size during pregnancy.

4.2. Objectives

Objectives for this part of the study were:

- to investigate concentration changes of the gut appetite hormones ghrelin, PYY and GLP-1 at defined stages of pregnancy in Wistar rats, by quantification of both plasma and gut tissue concentrations;
- to explore ghrelin endocrine cell density in stomach tissue to determine whether any changes to plasma or stomach ghrelin levels between mid-pregnancy and parturition could be due to a change in the cell density;
- to further elucidate reported changes to gut size during pregnancy;
- to determine whether a relationship exists between any changes in plasma or gut hormone concentrations and changes in gut size.

4.3. Methods

4.3.1. Animals

Female Wistar rats, bred in-house, were used at days 4, 12 and 18 of pregnancy (n=7 per group). Females were time-mated using the oestrus dance for accuracy (section 2.2.1); rats displaying a regular (every 4 days) dance were preferentially chosen. Day 4 pregnant rats were re-checked for the oestrus dance prior to culling. Vaginal lavage was performed to determine cycle stage (section 2.2) and to confirm that the pregnant animals had stopped cycling. Cycle stage was determined whilst rats were under

anaesthetic prior to the tail bleed and again the following day immediately after decapitation. Data from proestrus nulliparous controls were used for comparison purposes (n=6 unless otherwise stated). Where data are available, proestrus mean values have been added to figures using a dotted line.

4.3.2. Sample collection

Samples of gut tissue and fed and fasted plasma were taken, alongside multiple gut measurements, as outlined in Chapter 2 (section 2.3).

4.3.3. Measurements of food intake and body mass

Body mass was monitored daily in the early stages of pregnancy as a method to confirm conception. Food intake could not be determined because rats remained group housed until approximately 2 days before birth.

4.3.4. Peptide assays

Radioimmunoassays to determine peptide concentrations of ghrelin, PYY and GLP-1 in plasma and tissue extracts were performed as detailed in Chapter 2 (section 2.5).

4.3.5. Immunofluorescence

Immunofluorescence was performed and quantified in stomach (ghrelin) as described in Chapter 2 (section 2.6). For this aspect of the study, a mid-

pregnancy time point was chosen for comparison with parturition (d0L) to determine whether ghrelin cell density in the stomach changed over the course of pregnancy, in comparison to nulliparous proestrus controls.

4.4. Results

4.4.1. Total ghrelin peptide analysis

4.4.1.1. Concentration change with pregnancy stage

Fasted plasma ghrelin was significantly ($F(2, 18)=3.767$, $P=0.043$; Figure 4.1) more concentrated in day 4 pregnant dams (d4P) than in day 18 pregnant dams (d18P), but not day 12 pregnant dams (d12P). All pregnant dams had lower fasted plasma ghrelin than proestrus controls.

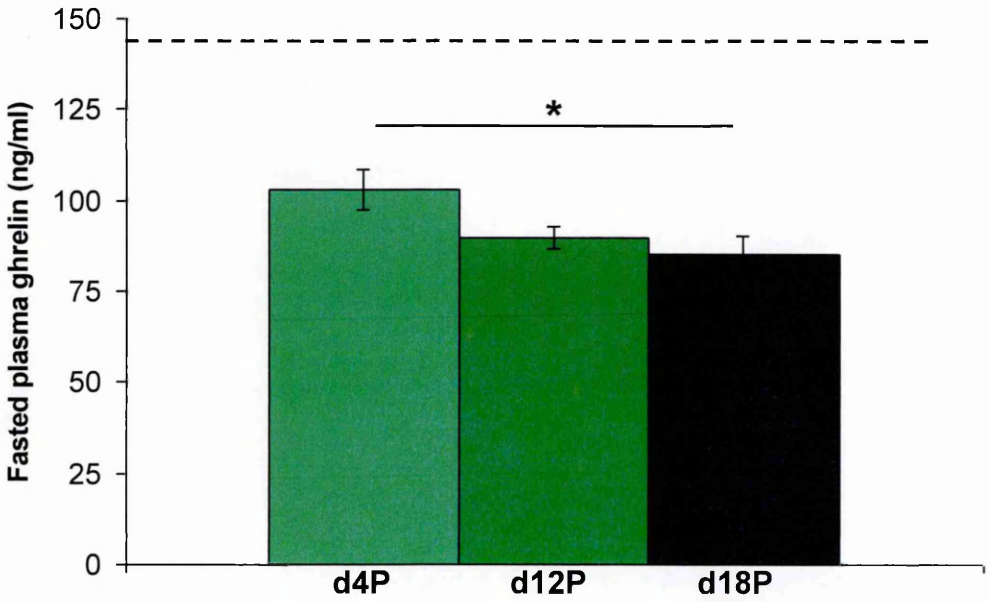

Figure 4.1. Ghrelin concentrations in fasted plasma during pregnancy
(n=7 per stage; * $P<0.05$). Dotted line shows approximate proestrus control mean.

Stomach tissue concentrations (Figure 4.2) of total ghrelin peptide did not significantly change with the stage of pregnancy but were reduced in

comparison to proestrus controls. Stomach ghrelin concentrations varied considerably within each dam group and there was no correlation between the concentrations of ghrelin in fasted plasma and stomach tissue.

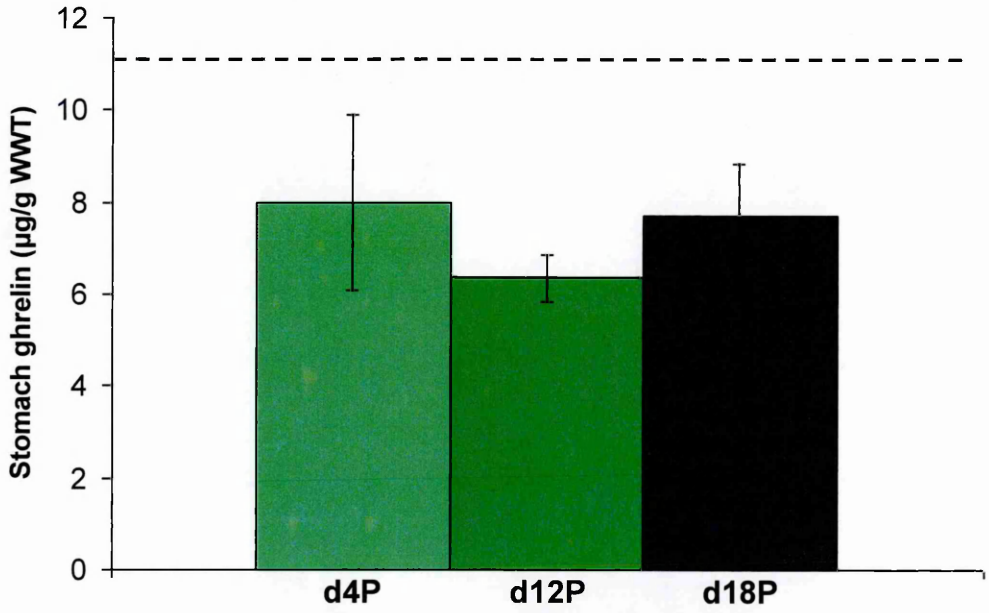

Figure 4.2. Ghrelin concentrations in stomach tissue during pregnancy (n=7 per stage). Dotted line shows approximate proestrus control mean.

4.4.2. Total peptide-YY (PYY) peptide analysis

4.4.2.1. Matched fed and fasted plasma comparison

Comparison of PYY concentration in the matched fed and fasted plasma samples from the whole group showed a significant difference between the two samples ($t(20)= -11.105$, $P<0.001$). Unexpectedly, the mean PYY concentration in fasted plasma (245.9 ± 16.03 pg/ml) was significantly higher than in fed plasma (71.9 ± 2.22 pg/ml), which is the opposite of what was found in Chapter 3 (likely due to method differences; see section 4.5.2).

4.4.2.2. Concentration change with pregnancy stage

Neither fed nor fasted plasma PYY were significantly different between the pregnant dam groups (Table 4.1). Fasted plasma PYY concentrations were all higher than in proestrus controls (172.0 ± 11.84 pg/ml).

Table 4.1. PYY concentrations in fed and fasted plasma during pregnancy (n=7 per stage).

	Fed plasma (pg/ml)	**Fasted plasma (pg/ml)**
d4P	74.9 ± 4.03 (53.1 – 85.8)	225.3 ± 12.90 (185.5 – 272.5)
d12P	67.7 ± 2.17 (58.6 – 75.0)	265.1 ± 37.74 (177.4 – 480.5)
d18P	73.1 ± 4.85 (59.1 – 99.5)	247.1 ± 29.10 (206.0 – 419.9)

Ascending colon (Figure 4.3 A) and descending colon (Figure 4.3 B) tissue concentrations of PYY also did not change with the stage of pregnancy, and concentrations varied considerably within each group. Ascending colon was not analysed for peptide concentrations in proestrus animals, but descending colon PYY concentrations in proestrus controls were 58% lower than in the d18P dams. PYY concentrations were not different between the ascending and descending colon in these pregnant dams, although in d12P dams there was a tendency (P=0.063) for the ascending colon to have a higher PYY content than in the descending colon.

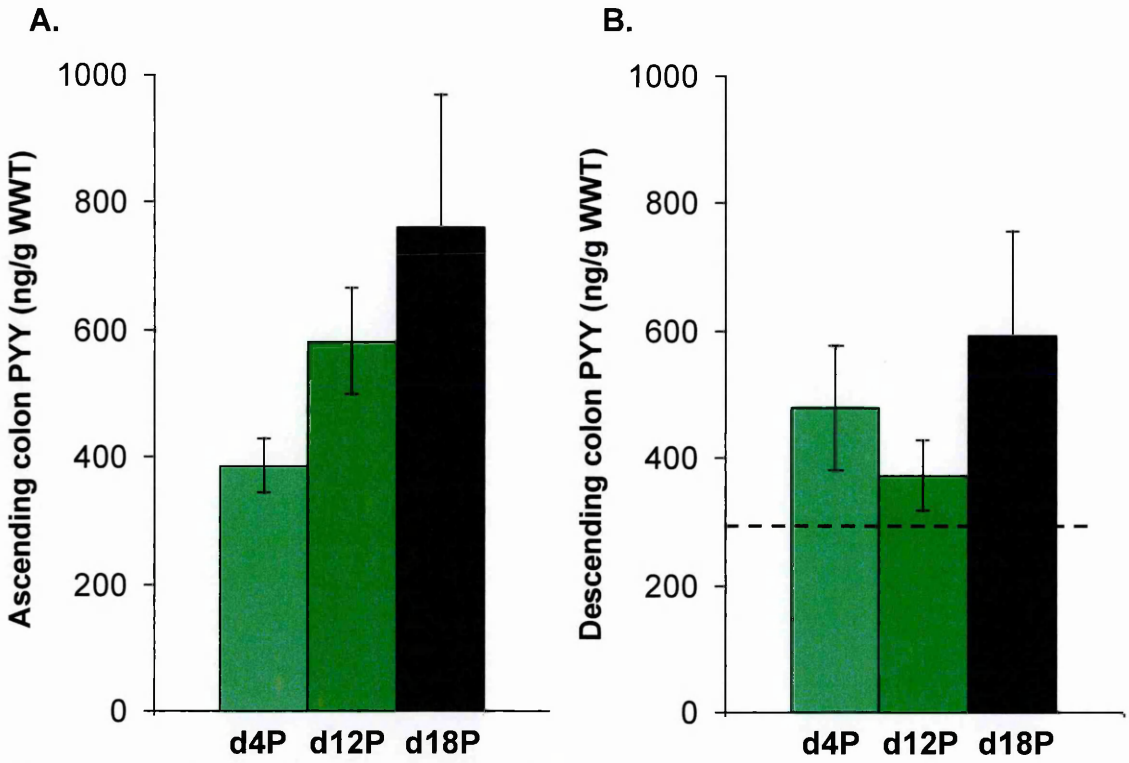

Figure 4.3. PYY concentrations in colon tissue during pregnancy
(A) ascending colon and **(B)** descending colon concentrations (n=7 per stage). Dotted line shows approximate proestrus control mean.

4.4.3. Total glucagon-like peptide-1 (GLP-1) peptide analysis

4.4.3.1. Matched fed and fasted plasma comparison

GLP-1 concentrations were 26 times higher ($t(20)=7.463$, $P<0.001$) in fed plasma (164.7 ± 21.4 pg/ml) than in paired fasted plasma (6.3 ± 1.13 pg/ml) for the whole group.

4.4.3.2. Concentration change with pregnancy stage

The concentration of GLP-1 in fed plasma was not significantly different between the sample groups (Figure 4.4) but was elevated 2 to 4 times during pregnancy compared to proestrus nulliparous controls.

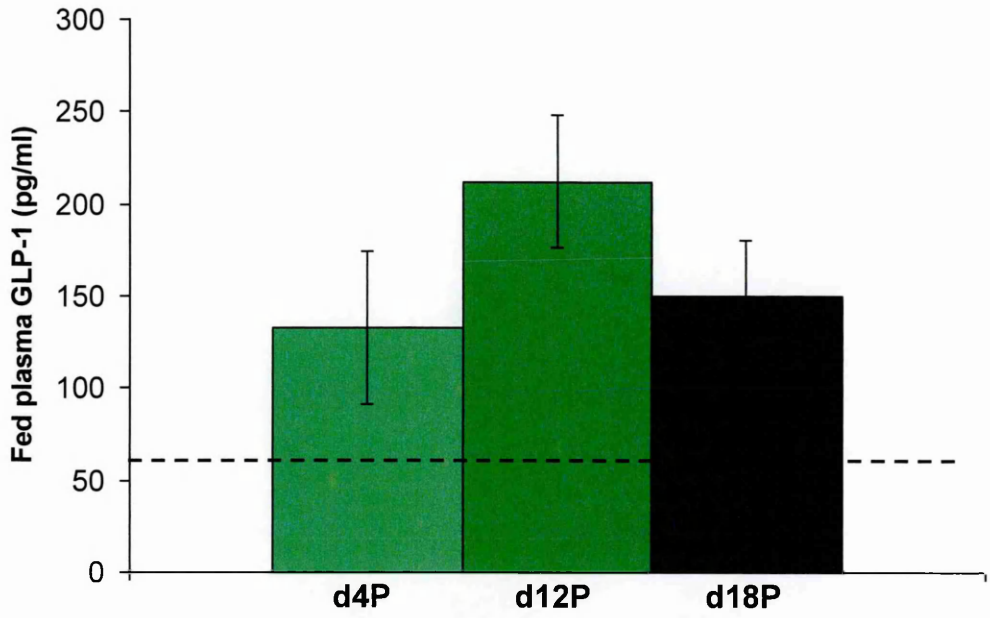

Figure 4.4. GLP-1 concentrations in fed plasma during pregnancy
(n=7 per stage). Dotted line shows approximate proestrus control mean.

Fasted plasma GLP-1 concentrations, however, were approximately 2 to 7 times lower at each stage of pregnancy than in the proestrus controls. Fasted plasma GLP-1 was significantly decreased with pregnancy ($F(2, 18)=3.664$, $P=0.046$), with a trend ($P=0.057$) for d18P dams to have less fasted plasma GLP-1 than d4P dams (Figure 4.5).

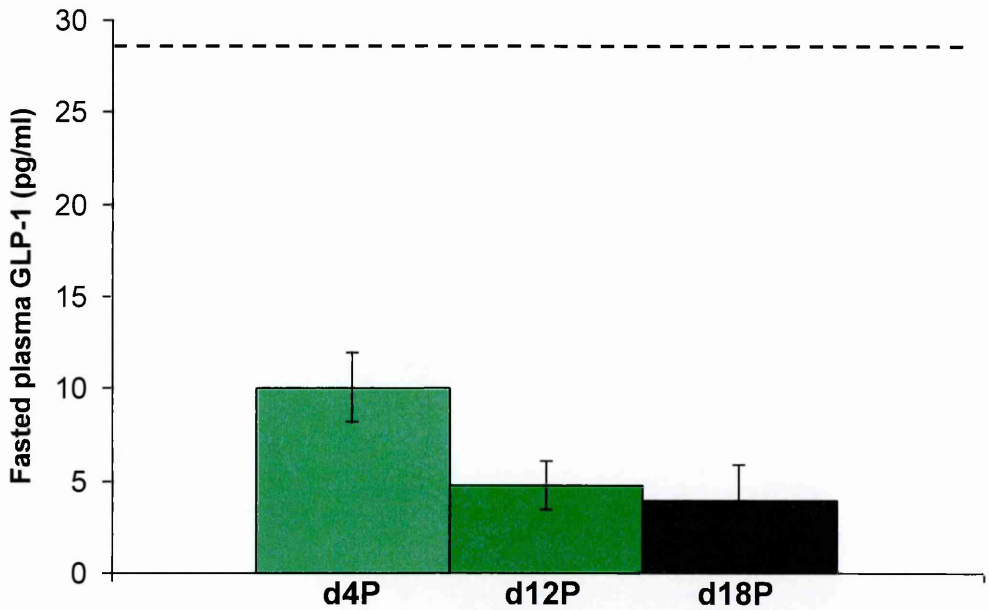

Figure 4.5. GLP-1 concentrations in fasted plasma during pregnancy
(n=7 per stage). Dotted line shows approximate proestrus control mean.

GLP-1 concentrations in ascending colon tissue were not homogeneous so the data were transformed by a log conversion. Log of ascending colon GLP-1 concentrations (Figure 4.6 A) was significantly different between the pregnant dams ($F(2, 18)=3.919$, $P=0.039$). D18P dams had twice the concentration of ascending colon GLP-1 than d12P dams, although this was not significant ($P=0.051$). Descending colon tissue concentrations of GLP-1 were not significantly different with pregnancy stage (Figure 4.6 B), but were approximately 2 to 4 times higher than in proestrus controls.

Ascending colon concentrations of GLP-1 were significantly higher than in descending colon tissue in d4P ($t(6)=5.224$, $P=0.002$), d12P ($t(6)=4.466$, $P=0.004$) and d18P ($t(6)=4.234$, $P=0.005$) dams.

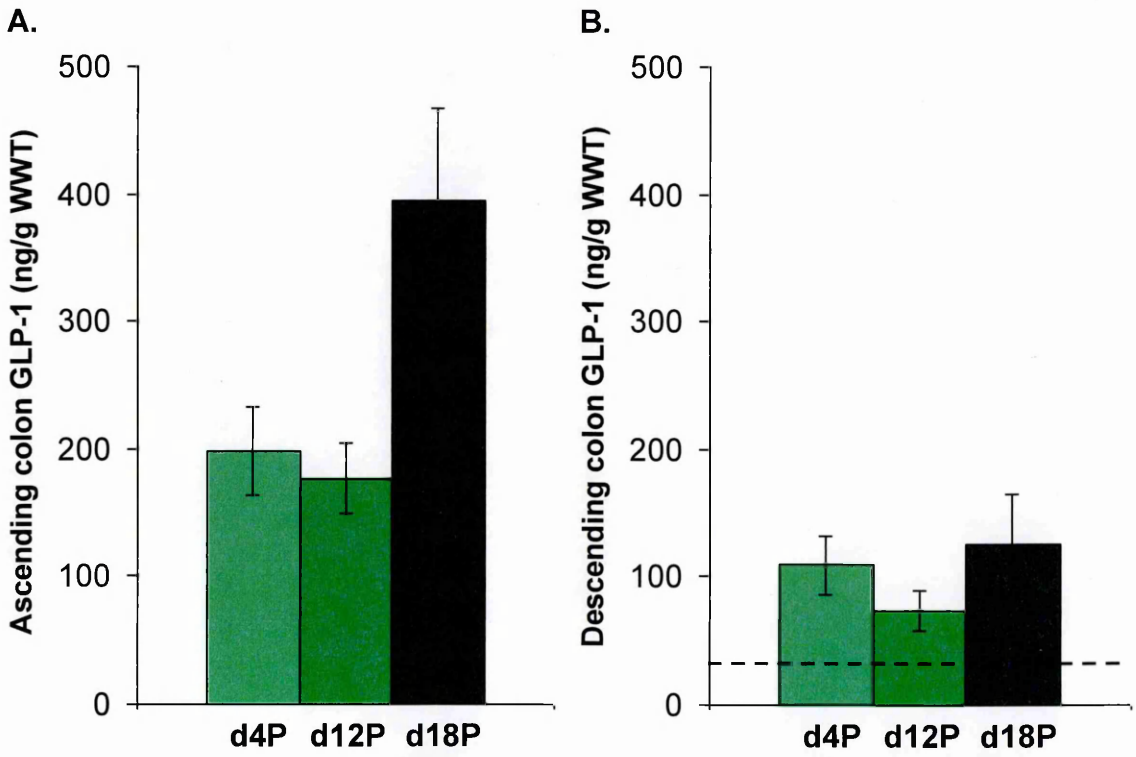

Figure 4.6. GLP-1 concentrations in colon tissue during pregnancy
(A) ascending colon and **(B)** descending colon concentrations (n=7 per stage). Dotted line shows approximate proestrus control mean.

4.4.4. Total ghrelin-immunoreactive enteroendocrine cells

Ghrelin-immunoreactive (IR) cells (Figure 4.7) in stomach tissue were found throughout the mucosa, predominantly so in the basal half of the mucosa, in all groups.

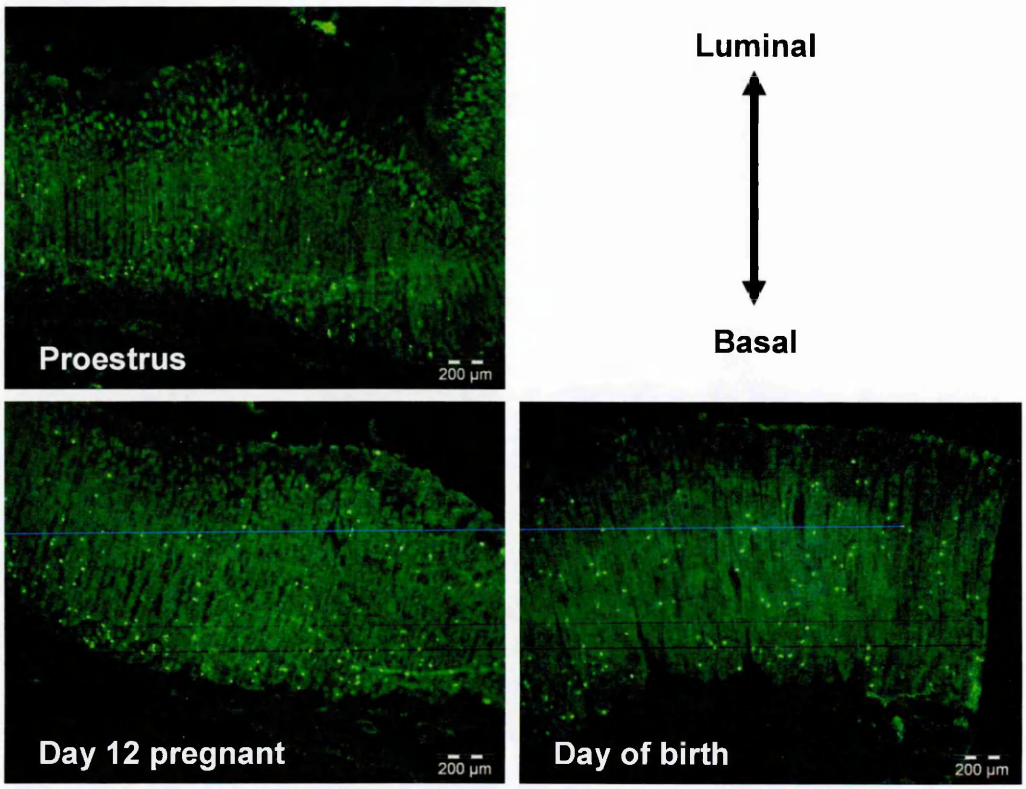

Figure 4.7. Ghrelin-immunoreactive cells in stomach tissue during different reproductive stages in female rats
Representative images of ghrelin immunoreactivity in stomach tissue of a proestrus nulliparous control rat, a day 12 pregnant dam and a dam on the day of birth. (Scale bars: 200 µm; tissue presented with luminal edge of mucosa at the top of each image).

Table 4.2. Stomach tissue measurements during different reproductive stages in female rats
Mean measurements of mucosal area and the mean maximum mucosal and muscle thickness of stomach tissue sections (n=6 proestrus, n=7 day 12 pregnant and day 0 lactating).

	Mucosal area (mm^2)	Mucosal thickness (mm)	Muscle thickness (mm)
Proestrus	11.2 ± 0.83 (8.7 – 14.5)	0.8 ± 0.06 (0.6 – 1.0)	0.6 ± 0.04 (0.5 – 0.8)
d12P	10.9 ± 0.50 (8.4 – 12.8)	0.8 ± 0.04 (0.7 – 1.0)	0.5 ± 0.03 (0.4 – 0.7)
d0L	11.2 ± 0.53 (9.0 – 13.2)	0.8 ± 0.03 (0.7 – 0.9)	0.5 ± 0.02 (0.5 – 0.6)

There was no difference in the mucosal area of the stomach sections analysed for proestrus controls, d12P and dams on the day of birth (d0L) (Table 4.2). The mean maximum mucosal and muscle thickness was also not different between the sample groups (Table 4.2). The ghrelin-IR cell density was significantly different between the sample groups ($F(2, 17)=29.735$, $P<0.001$) and increased significantly from the proestrus controls to pregnancy, and dams in the transition stage of parturition had a significantly higher ghrelin cell density than in both the other sample groups (Figure 4.8). Dams after parturition had more than double the ghrelin-IR cell density than proestrus controls.

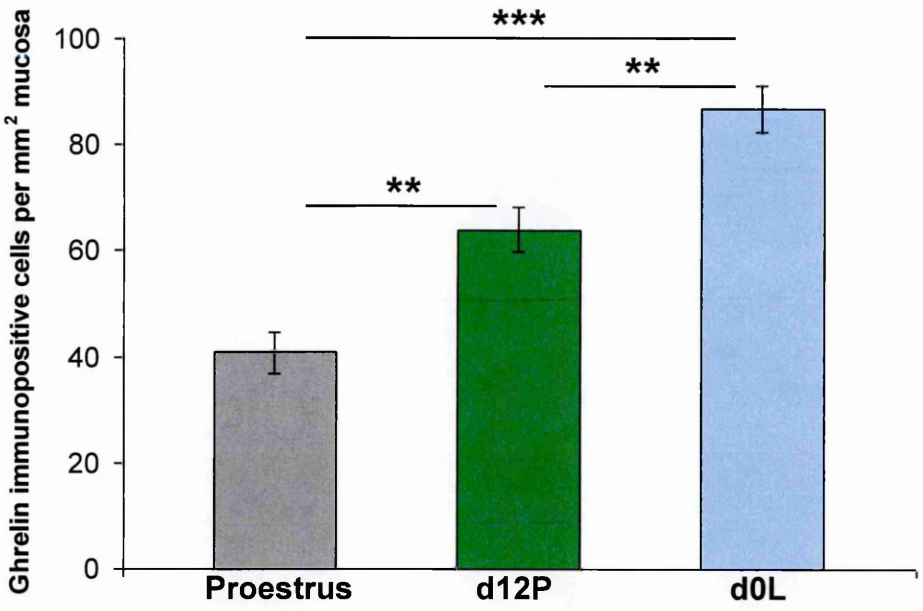

Figure 4.8. Ghrelin cell density during different reproductive stages in female rats (n= 6 proestrus controls; n=7 day 12 pregnant and day 0 lactating dams; ** $P<0.01$; *** $P<0.001$).

4.4.5. Changes to body and gut size during pregnancy

At the time of culling, the mean age of all animals used in this part of the project was 30 ± 0.68 weeks (24 – 36 weeks). Body mass of the d4P dams (Figure 4.9), recorded prior to the short period of fasting, was comparable

with proestrus controls. As expected, body mass significantly increased (F(2, 18)=12.565, P<0.001) with the advancing stages of pregnancy. Despite an observation that dams consumed less food and water in later stages of pregnancy, d18P dams lost significantly less body mass after fasting (F(2, 18)=6.904, P=0.006) than d12P dams (P=0.005; Table 4.3).

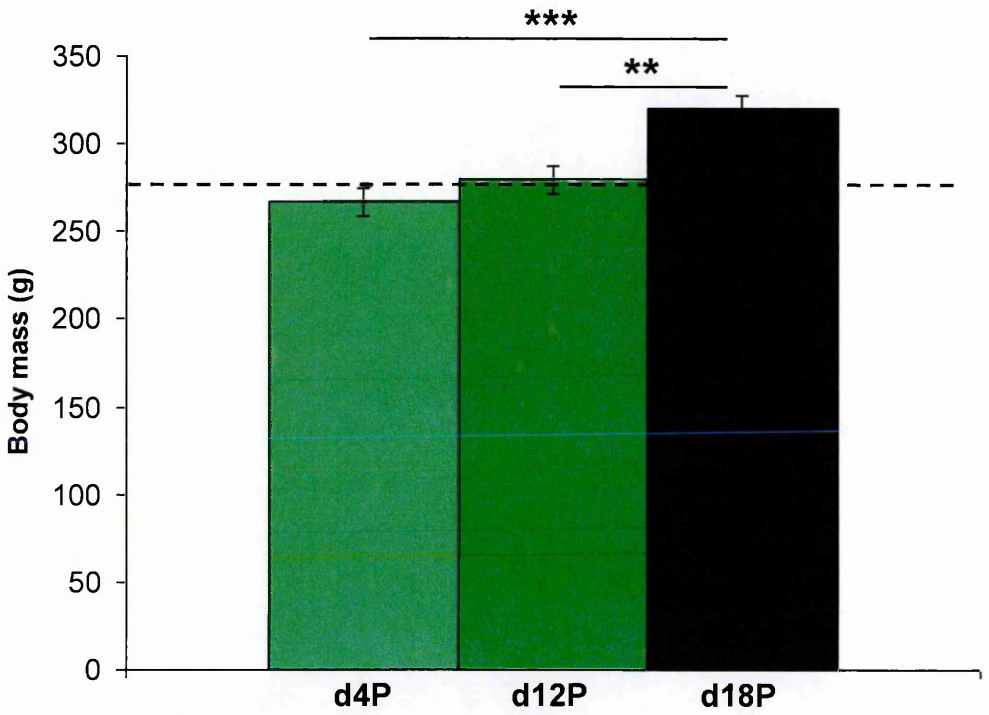

Figure 4.9. Fed state body mass during pregnancy
(n=7 per stage; ** P<0.01; *** P<0.001). Dotted line shows approximate proestrus control mean.

Table 4.3. The percentage of body mass lost during the fasting period in pregnant rats
(n=7 per stage; c>d, P<0.01).

	Body mass lost on fasting (%)
d4P	2.7 ± 0.33 (1.5 – 4.4)
d12P	3.9 ± 0.65 [c] (2.4 – 6.7)
d18P	0.6 ± 0.82 [d] (-1.3 – 6.7)

Body mass was closely monitored in d4P dams to confirm pregnancy. There was a significant change (t(6)= -0.106, P<0.001) between the pre-mating

body mass (253.9 ± 6.43 g) of the d4P group and their body mass at d3P (266.8 ± 7.58 g), both recorded prior to the fasting period.

Mass of abdominal cavity white adipose tissue (WAT) was significantly greater ($F(2, 18)=6.248$, $P=0.009$) in d18P dams than in d4P ($P=0.034$) and d12P ($P=0.010$) dams (Figure 4.10). Proestrus controls had less abdominal WAT than d18P dams.

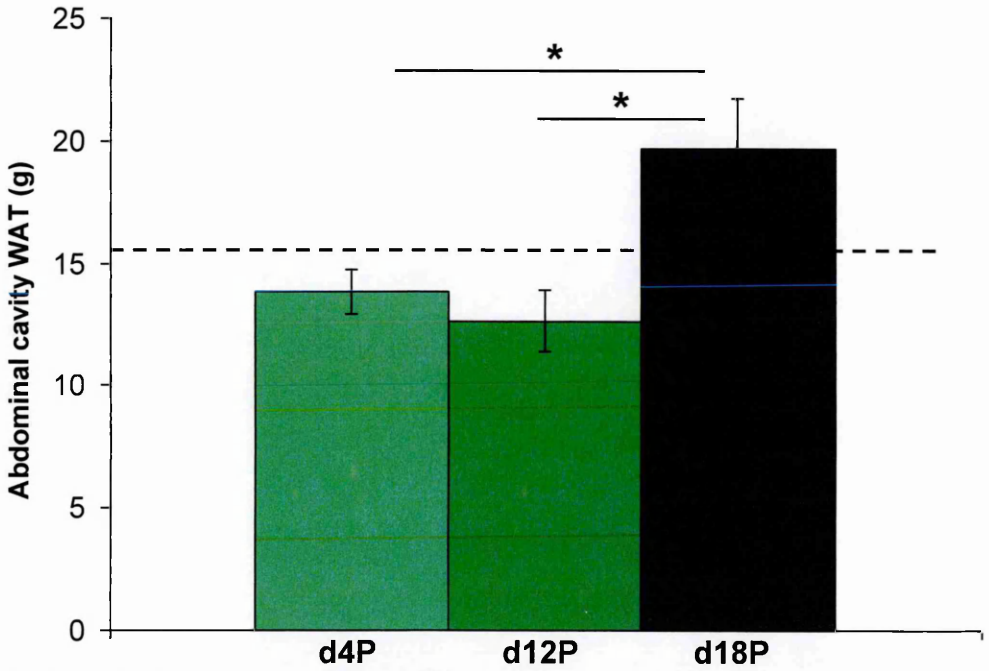

Figure 4.10. Abdominal cavity white adipose tissue (WAT) during pregnancy (n=7 per stage; * $P<0.05$). Dotted line shows approximate proestrus control mean.

Anaesthetised rectal temperature was significantly different between pregnancy stages ($F(2, 18)=9.065$, $P=0.002$) and d12P dams had a significantly higher temperature than both d4P ($P=0.017$) and d18P ($P=0.002$) dams (Figure 4.11).

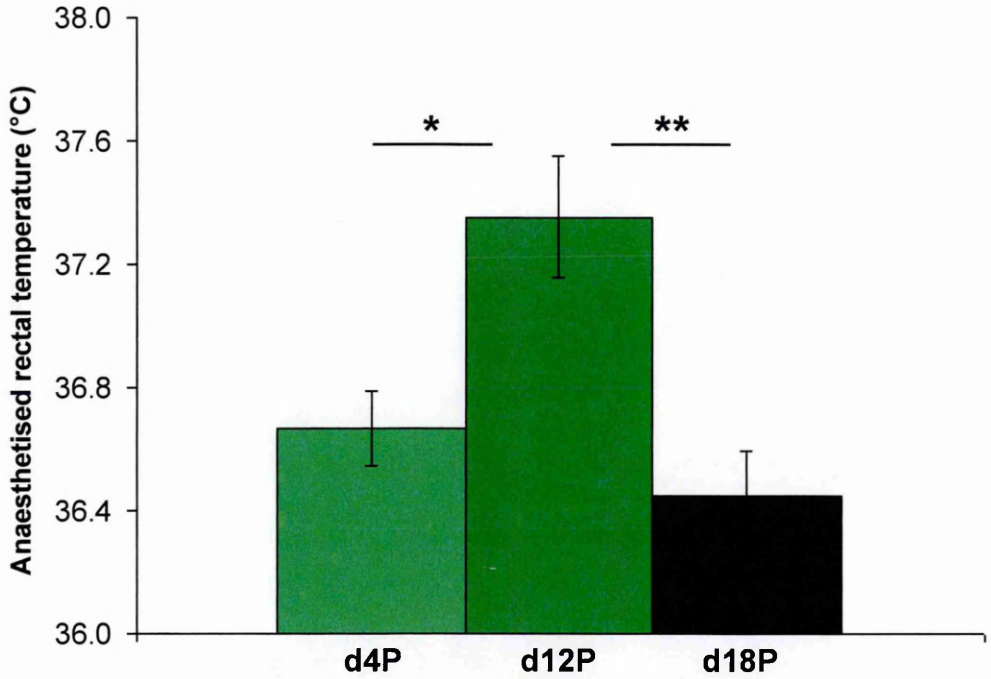

Figure 4.11. Anaesthetised rectal temperature during pregnancy
(n=7 per stage; * $P<0.05$; ** $P<0.01$).

Mean stomach tissue mass did not change throughout pregnancy (Figure 4.12) and was similar to proestrus controls.

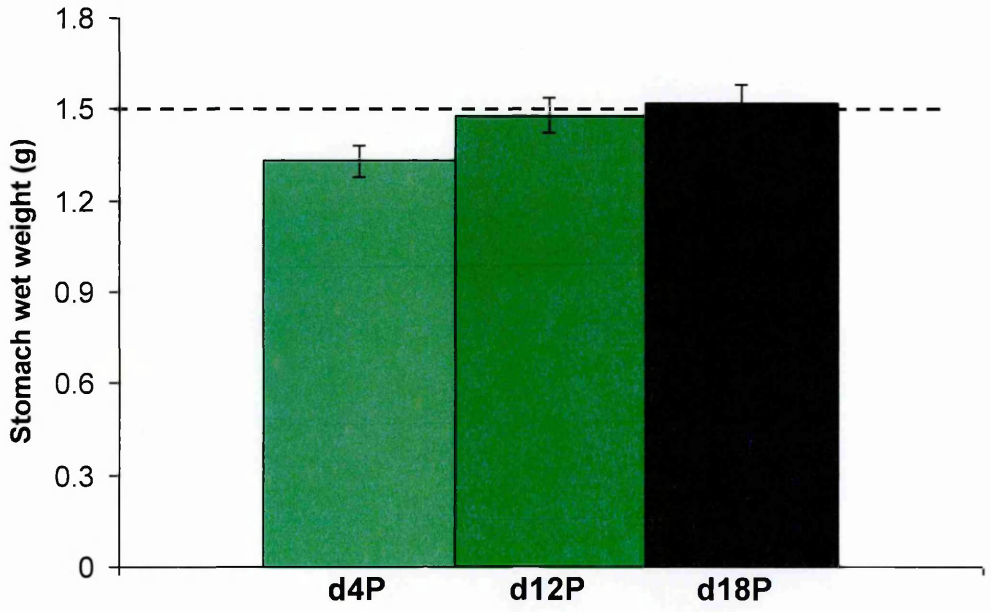

Figure 4.12. Stomach tissue wet weight during pregnancy
(n=7 per stage). Dotted line shows approximate proestrus control mean.

D18P dams had significantly heavier ($F(2, 18)=4.782$, $P=0.022$; Figure 4.13 A) and longer ($F(2, 18)=5.365$, $P=0.015$; Figure 4.13 B) small intestines than d4P dams. The small intestine of d18P dams also showed a trend ($P=0.051$) to be longer than in d12P dams. Small intestine lengths of d4P dams were comparable to those of proestrus controls, with no control data available for tissue mass.

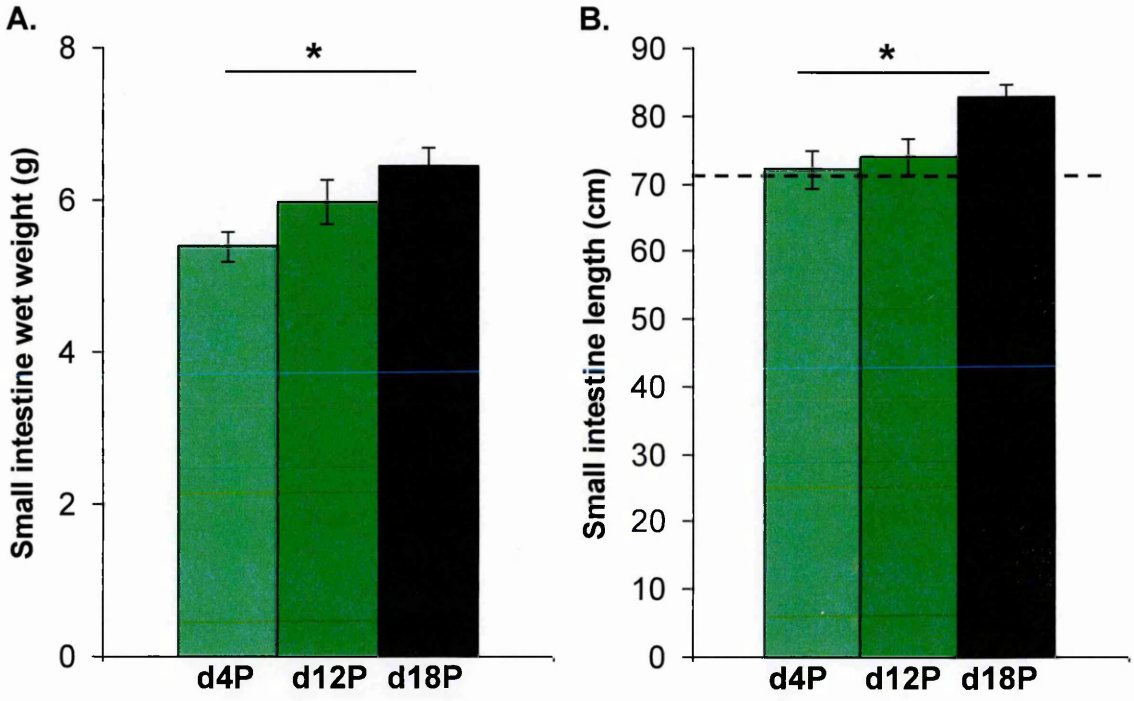

Figure 4.13. Small intestine size during pregnancy
(A) small intestine wet weight and **(B)** length (n=7 per stage; * $P<0.05$). Dotted line shows approximate proestrus control mean.

The wet weight of caecum tissue did not change significantly with the stage of pregnancy (Figure 4.14).

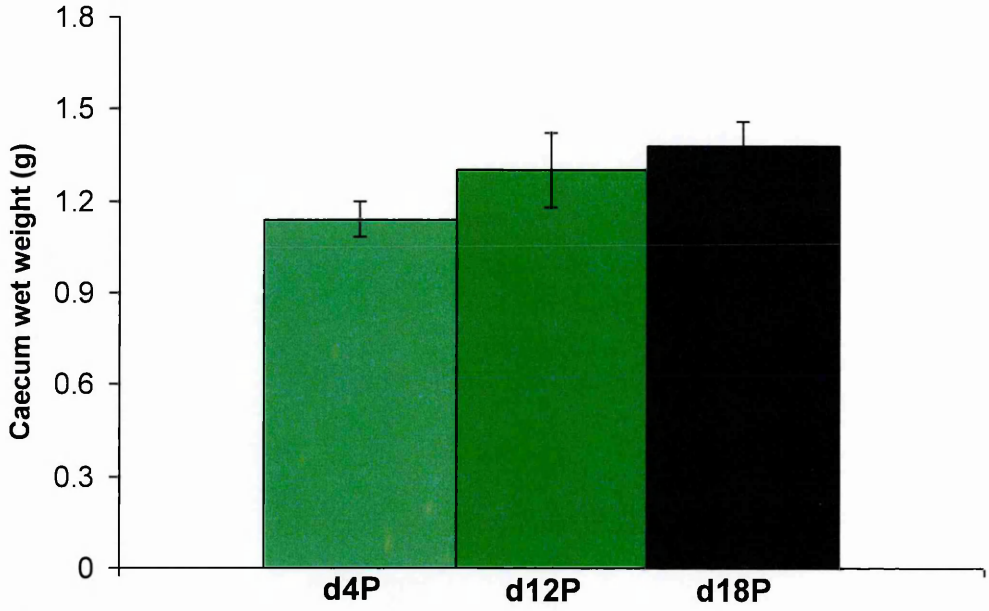

Figure 4.14. Caecum tissue wet weight during pregnancy
(n=7 per stage).

Large intestines of d18P dams were significantly heavier ($F(2, 18)=7.931$, $P=0.003$) than the d4P ($P=0.003$) and d12P ($P=0.046$) groups (Figure 4.15 A). Large intestines were significantly ($F(2, 18)=5.506$, $P=0.014$) shorter in d12P dams than in d4P ($P=0.044$) and d18P ($P=0.017$) dams (Figure 4.15 B). Proestrus controls had large intestine lengths (n=4) similar to the pregnant dams.

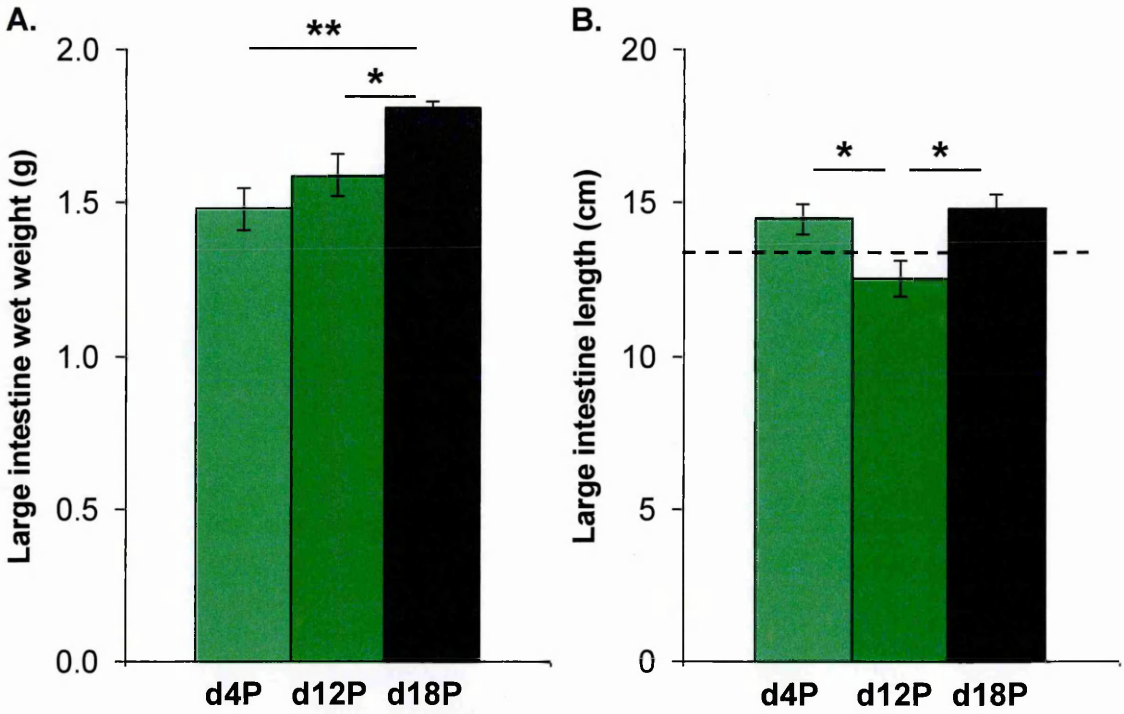

Figure 4.15. Large intestine size during pregnancy
(A) large intestine wet weight and **(B)** length during pregnancy (n=7 per stage; * $P<0.05$; ** $P<0.01$). Dotted line shows approximate proestrus control mean.

Gut circumferences were not significantly different between the groups in duodenum or descending colon tissue (Figure 4.16). However, ascending colon circumference was significantly larger ($F(2, 18)=3.953$, $P=0.038$) in d4P dams compared with d12P dams ($P=0.033$). Gut circumference measurements taken in proestrus controls show that only descending colon circumference was similar to the pregnant dams, with duodenum and ascending colon circumferences in proestrus controls being much smaller than in all of the pregnant dams.

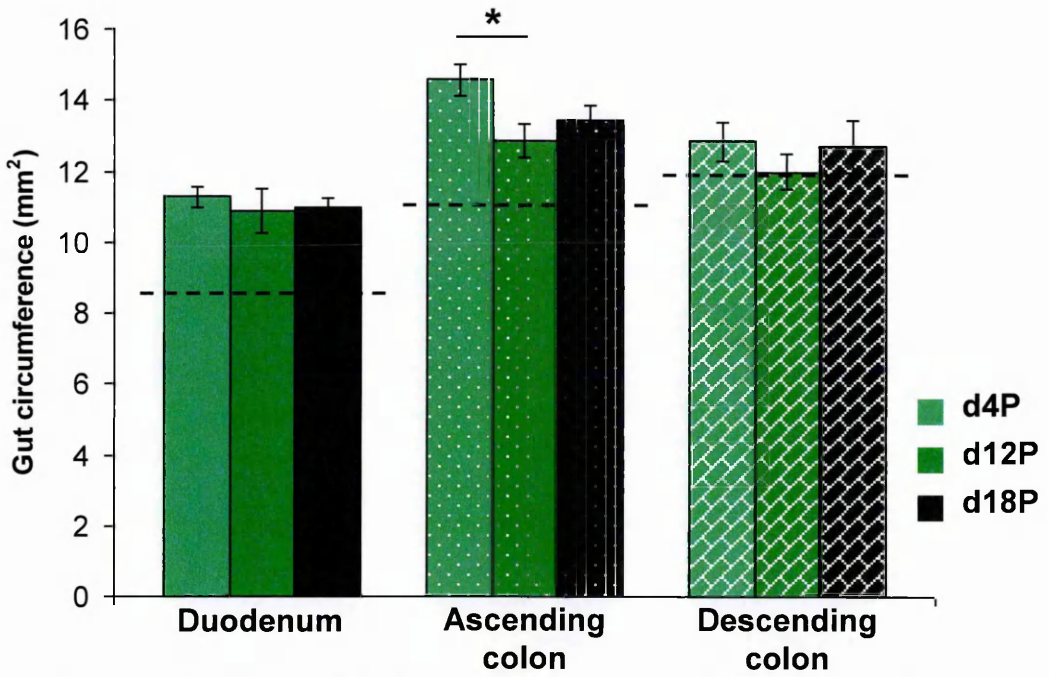

Figure 4.16. Circumferences of duodenum, ascending colon and descending colon during pregnancy
(n=7 per stage; * $P<0.05$). Dotted lines show approximate proestrus control means.

4.4.5.1. Mass of gut contents

The mass of gut contents from various gut locations (section 2.4.1) were all significantly different between the sample groups (Table 4.4). Significantly more stomach contents ($F(2, 18)=4.686$, $P=0.023$) were found in d12P dams than in d4P dams ($P=0.019$). More stomach contents were present at all stages of pregnancy compared to proestrus controls (0.5 ± 0.15 g). The pH of the stomach contents was not significantly different between the d4P (pH 4.0 ± 0.49), d12P (pH 3.8 ± 0.71) and d18P (pH 4.0 ± 0.36) dams.

The data for small intestine contents were log transformed for parametric statistical analysis. D18P dams had significantly ($F(2, 18)=10.310$, $P=0.001$) more small intestine contents than both d4P ($P=0.001$) and d12P ($P=0.018$) dams (Table 4.4).

D18P dams had significantly (F(2, 18)=4.827, P=0.021) more contents in their caecum than d12P dams (P=0.017) and the pH of the caecum contents (Table 4.4 and Figure 4.17) in d18P dams was significantly (F(2, 18)=8.592, P=0.002) more alkaline than in d12P dams (P=0.002).

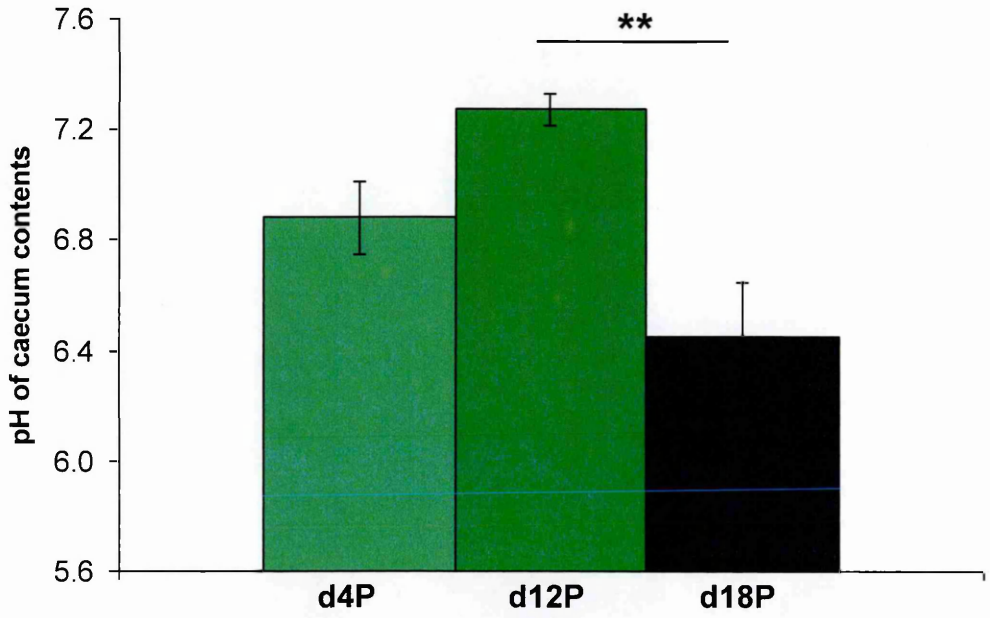

Figure 4.17. Caecum contents pH during pregnancy
(n=7 per stage; ** P<0.01).

D18P dams also had significantly (F(2, 18)=6.251, P=0.009) more contents in their large intestines than d12P dams (P=0.007).

Table 4.4. Gut contents mass during pregnancy
(n=7 per stage; a>b, P<0.001; c>d, P<0.01; e>f, P<0.05, within columns).

	Stomach contents (g)	Small intestine contents (g)	Caecum contents (g)	Large intestine contents (g)
d4P	1.2 ± 0.24 [f] (0.5 – 2.1)	1.9 ± 0.14 [b] (1.4 – 2.3)	3.3 ± 0.28 (2.0 – 4.2)	2.0 ± 0.23 (1.3 – 2.7)
d12P	2.2 ± 0.13 [e] (1.7 – 2.7)	2.3 ± 0.22 [f] (1.5 – 3.3)	2.8 ± 0.16 [f] (2.3 – 3.5)	1.1 ± 0.54 [d] (0.0 – 1.8)
d18P	1.6 ± 0.25 (0.8 – 2.5)	3.4 ± 0.37 [ae] (2.2 – 4.9)	4.2 ± 0.43 [e] (2.3 – 5.6)	2.5 ± 0.32 [c] (1.5 – 3.8)

4.5. Discussion

This study was the first to analyse the concentrations of ghrelin, PYY and GLP-1 during pregnancy in plasma and in gut tissues. This study also provided, for the first time, matched information about changes to GI tissue size alongside ghrelin, PYY and GLP-1 plasma and tissue concentrations.

The main findings of this study were that concentrations of ghrelin and GLP-1 were significantly different between the stages of pregnancy studied, in several of the samples analysed. Both fasted plasma ghrelin and GLP-1 concentrations significantly decreased during pregnancy. GLP-1 concentrations in ascending colon significantly increased during pregnancy. This suggests that there may be a change in the usual mechanisms of hormonal control of appetite, which increases during the late stages of pregnancy. This study also reported significant increases to small and large intestine tissue mass and length during pregnancy and further explored changes to gut size during pregnancy, as previously reported by others (Burdett and Reek, 1979, Crean and Rumsey, 1971, Cripps and Williams, 1975).

4.5.1. Orexigenic gut hormone changes during pregnancy

Ghrelin stimulates appetite and appetite is documented to increase by around 60% by late pregnancy in rats (Cripps and Williams, 1975), so one may expect ghrelin levels to increase during pregnancy to facilitate increased food consumption. In this study however, fasted plasma ghrelin

concentrations were found to significantly decrease between d4P and d18P, which is consistent with another rat study (Shibata *et al.*, 2004) and human studies (Fuglsang *et al.*, 2005, Tham *et al.*, 2009) discussed previously (section 4.1.1). Taylor *et al.* (2009) reported no significant change to plasma total ghrelin concentrations, although differences between pregnant dams may have been missed due to the statistical analysis of both pregnant and lactating dams together, whereas this study has examined them separately.

Determining the concentration of circulating leptin would also be of interest for further analysis of plasma ghrelin concentrations throughout pregnancy. The abdominal cavity white adipose tissue in the animals studied here increased significantly with pregnancy, so it may be reasonable to assume that leptin levels also increased in these animals as described in other rodent studies (Shibata *et al.*, 2004, Taylor *et al.*, 2009, Trujillo *et al.*, 2011), due to the established positive correlation between leptin and total adiposity (reviewed by Friedman, 2011, Ladyman *et al.*, 2010). Increased levels of leptin in pregnancy have also been implicated in a successful outcome of the pregnancy (French *et al.*, 2009). Previous studies have demonstrated a reciprocal relationship between ghrelin and leptin (section 1.8.3). One study, mentioned earlier, reported that ghrelin injection failed to induce feeding in male mice over-expressing the leptin gene in their hypothalamus (recombinant adeno-associated virus delivery) despite elevated plasma ghrelin levels, demonstrating that leptin can attenuate the feeding initiation effects of ghrelin (Ueno *et al.*, 2004). Another study demonstrated the ability of ghrelin to abolish leptin-induced satiety in rats (Shintani *et al.*, 2001).

These authors reported that whilst intracerebroventricular injection of leptin reduced food intake of male rats in a dose-dependent manner, co-injection of leptin with ghrelin increased food intake dose-dependently. Increased ghrelin mRNA in the stomach has also been observed after chronic leptin administration (Toshinai *et al.*, 2001). If leptin concentrations did increase in this study between each stage of pregnancy, a gradual reduction in plasma ghrelin concentrations could be due to suppression by increasing leptin levels. It is clear that more work is required to establish the relationships between leptin and ghrelin, but this was not a focus of this current research.

4.5.2. Anorexigenic gut hormone changes during pregnancy

GLP-1 levels are known to be decreased in insulin-resistant states, such as in diabetic and obese states (Lim *et al.*, 2009, Muscelli *et al.*, 2008, Toft-Nielsen *et al.*, 2001). A level of pregnancy-associated insulin resistance could therefore explain the significant difference in fasted plasma GLP-1 between the pregnant groups, with a trend for decreased fasted plasma GLP-1 between d4P and d18P.

In ascending colon tissue, GLP-1 was significantly different between the pregnant groups, with a non-significant trend to be higher in d18P dams compared with d12P dams. This is the opposite of GLP-1 concentrations in fasted plasma, which decreased with pregnancy duration. One group have shown (Lim *et al.*, 2009) that high concentrations of insulin can directly impair the ability of L-cells to secrete GLP-1 both *in vitro* and *in vivo*. For this

reason, one would expect that d18P dams with high insulin levels, presumed to be more insulin resistant than d4P dams, to have a low GLP-1 concentration in colon tissue in addition to low levels of circulating GLP-1. Why GLP-1 showed a trend to be more concentrated at d18P than d4P in ascending colon tissue remains to be seen. Due to the measurement of total GLP-1 in this study, this result cannot be due to an alteration in GLP-1 degradation once it reaches the circulation. One possibility could be a proliferation of the GLP-1 producing L-cell population as a result of gut growth, as d18P dams were found to have significantly heavier large intestine wet weights than at all other stages of pregnancy and significantly longer large intestines than d12P dams. Unfortunately data for the number of GLP-1-IR cells in the colon are not available to confirm whether gut growth increased the number of GLP-1 cells in the colon, which could in turn be responsible for increased tissue concentration of GLP-1.

PYY levels in fed and fasted plasma were not different during the stages of pregnancy studied, in agreement with Valsamakis *et al.* (2010), although other studies have reported that total PYY plasma concentrations increase with pregnancy (Taylor *et al.*, 2009, Tovar *et al.*, 2004).

Unexpectedly, fasted plasma PYY concentrations in this part of the study were higher than those of fed plasma PYY. Previous work (Chapter 3) found PYY to be highest in fed plasma. For the samples analysed in this chapter, all of the fed plasma was acidified and vacuum centrifuged (RAPID methodology, section 2.3.2.1) to be reconstituted when required, whereas for

the samples analysed in Chapter 3, acidified plasma was not dried down prior to analysis. It is possible that reconstituting dried-down fed acidified plasma in distilled water back to its pre-buffer volume was not sufficient to re-suspend all of the PYY into solution. If so, this discrepancy has prevented a direct comparison between the fed and fasted plasma PYY concentrations, however concentration changes between sample groups were still explored separately as all samples of fed plasma and all samples of fasted plasma were treated in the same way.

4.5.3. Enteroendocrine cell analysis

The density of ghrelin-IR cells was found to significantly increase as the dam physiology transitioned from pregnancy to parturition, despite there being no difference in stomach ghrelin concentration during pregnancy. It is possible that if additional samples had been taken daily approaching parturition, an increasing number of ghrelin-IR stomach cells leading up to parturition may have been seen. It could be hypothesised that an increase in stomach ghrelin-IR cells increases ghrelin production within the stomach, ready for release into the circulation around the time of birth to initiate the hyperphagia reported to occur in lactation (Cripps and Williams, 1975, Denis *et al.*, 2004).

4.5.4. Body and gut size change with pregnancy

Pregnant rats in this study were found to have significantly increased body mass by day 3 of pregnancy, which is in agreement with Cripps and Williams (1975). The mass of abdominal cavity white adipose tissue (WAT) had also

significantly increased by d18P compared with both d4P and d12P, matching what has been previously described about increased adipose tissue accumulation during pregnancy (Lopez-Luna *et al.*, 1991, Pujol *et al.*, 2006), facilitated by hyperphagia. Dams were group housed until near parturition and so an accurate measure of individual food intake was not possible, but an estimation was made using the mass of stomach contents that remained after a short period of fasting. D12P dams had significantly more fasted stomach contents than d4P dams, and all of the pregnant dams had more stomach contents remaining than proestrus controls. This finding could infer pregnancy-associated hyperphagia in the pregnant dams, which has been reported by others (Crean and Rumsey, 1971, Cripps and Williams, 1975, Trujillo *et al.*, 2011), with dams drinking more or eating bedding due to increased hunger.

Anaesthetised rectal temperature of the dams was significantly decreased at d4P and d18P in comparison with d12P dams. One study of 24 hour core temperature in rats found a significant decrease in body temperature from d15P onwards and that core temperature was lowest in d20P dams (Fewell, 1995). Fewell (1995) did not study dams prior to d10P, so it is not possible to compare those data with the significant increase in rectal temperature seen between d4P and d12P in the current study. However, a significant decrease in rectal temperature between d12P and d18P reported here agrees with the results of Fewell (1995). PYY transgenic male mice fed a high fat diet have been found to have significantly higher rectal temperatures than control mice on the same diet (Boey *et al.*, 2008). These authors have suggested that high

levels of PYY has a protective effect against obesity, due to a concomitant decrease in adiposity, and that PYY does so by affecting thermogenesis. However, in the present study, no significant changes in either circulating or colon PYY levels were noted, despite significant pregnancy-associated changes in rectal temperature. It is possible that the use of rats, females or the pregnant state is responsible for the differences seen between the current study and that of Boey *et al.* (2008).

No difference was found in either stomach or caecum tissue mass in this study, which is consistent with the study by Cripps and Williams (1975), although their data only included one pregnancy time point (d12P to d15P) in comparison to controls. Crean and Rumsey (1971), however, had reported differently, and found stomach mass steadily increased with pregnancy stage in Wistar females, which reached significance by d14P. The difference in stomach mass reported between the current study and that of Crean and Rumsey (1971) is likely due to method differences, as stomachs were fixed before weighing in their study.

In this work, it was found that both the wet weight and length of the small intestine were significantly increased between d4P and d18P, by 20% and 15% respectively. Here, it is suggested that the increase in small intestine size is an adaptation to increase the absorption of nutrients to support the dam in developing adequate adipose reserves (Lopez-Luna *et al.*, 1991, Pujol *et al.*, 2006) ready to support lactation. Cripps and Williams (1975), however, reported no change to small intestine length at mid-pregnancy

(d12P to d15P) in Sprague-Dawley dams compared with nulliparous controls. These authors did find small intestine wet weight (but not dry weight) to be significantly increased (19%) by parturition, which is similar to the difference reported here between early and late pregnancy. Another study (Burdett and Reek, 1979) reported a significant increase in small intestine length (13%) and wet weight (41%) by late pregnancy (d18P to d20P) in Sprague-Dawley dams compared to controls, but did not look at any other gut areas.

This chapter also reported that large intestine mass significantly increased with pregnancy, although length was similar between d4P and d18P dams. Cripps and Williams (1975) documented the opposite, with a significant increase in length, but not tissue mass, in mid-pregnancy compared to nulliparous controls. Both Cripps and Williams (1975) and Burdett and Reek (1979) measured intestine length vertically, allowing the tissue to hang under its own weight, which may have caused the tissue to stretch. This current study standardised measurement by free-floating the gut tissue in PBS to prevent tissue stretching. It is possible that the differences reported in colon length between this study and Cripps and Williams (1975) is due to this method difference.

This work has provided additional information to previously reported gut size changes in pregnancy by measurement of gut circumference, which has not previously been reported. Although neither duodenum nor descending colon circumferences were found to be different between the stages of pregnancy, ascending colon circumferences were significantly different between the

groups. This study has led to the suggestion that changes in circumference in different areas of the gastrointestinal tract may be an additional maternal mechanism to support pregnancy, increasing the capacity of the gut and possibly altering gut transit times in order to adapt to increasing nutrient requirements. Further study would be necessary to confirm this possibility. It was also interesting to note that d4P dam group tended to have the largest mean gut circumferences in the duodenum, ascending colon and descending colon compared with other pregnant dams and proestrus controls. These data could suggest an early maternal adaptation to increase gut capacity to accommodate increased food intake and possibly to optimise nutrient and water absorption prior to the occurrence of gut lengthening later on in pregnancy, which is expected to increase gut surface area.

Measurement of remaining gut contents offered an insight into gut transit times, because the dams were fasted for a short period prior to culling. Dams at d18P had significantly more contents in the small intestine than d4P dams, and significantly more contents in the caecum and large intestine than d12P dams. Thus, it is possible that gut transit was slower in the later stages of pregnancy, as has been documented by others (reviewed by Cullen and O'Donoghue, 2007).

4.5.5. Refinement of time-mating procedure

Implementing the oestrus dance to time-mate the pregnant dams was very successful and a distinct refinement of the time-mating procedure. Out of a total of 48 rats that were time-mated using only the oestrus dance, all 48

successfully conceived. This greatly reduced the number of animals required for a study relying on time-mated animals for the early stage of d4P. It is common for numerous dams to be time-mated for each study dam required, which allows for the possibility of dams not conceiving. A commonly used method for time-mating is to use a minimum of 5 females for each dam required, and proven breeders will nearly always be used (Barnett, 2007). For this study, only the number of nulliparous females required for pregnancy time-points were mated, which greatly reduced and refined common practice, without the need to disturb the dams by either oestrous monitoring or to check for the appearance of a vaginal plug for mating confirmation.

4.6. Conclusions

This study explored the interactions of both fed and fasted plasma and tissue concentrations of appetite hormones during pregnancy in Wistar rats, and further elucidated previously reported changes to dam GI physiology. This study is novel in the extensive number of gut areas examined for changes in size, mass and circumference at different stages of pregnancy and in the addition of GLP-1 measurements alongside ghrelin and PYY.

Fasted plasma GLP-1 and ghrelin were found to be significantly decreased in late pregnancy, whereas ascending colon concentration of GLP-1 was significantly increased. Why there was a difference between the changes in tissue and circulating levels of GLP-1 remains to be established, but it is possibly associated with an increase in GI size during pregnancy. Although fasted plasma ghrelin significantly decreased with the stage of pregnancy,

with no change in stomach tissue ghrelin concentration, the density of stomach ghrelin-IR cells was significantly increased between d12P and control dams. From this study, it is thought that an increase in the number of stomach ghrelin cells may be an important maternal adaptation prior to lactation, in readiness to help create a hyperphagic state after parturition to support lactation.

One aim of this study was to further explore when increases to gut size begin to occur in preparation to support lactation. Small and large intestine lengths and small intestine tissue mass increased steadily throughout the stages of pregnancy studied, reaching significance by d18P. Taken together, these results suggest that by d18P, the maternal gut is actively adapting to the pregnant state and is already hypertrophic, as is documented by others to occur during lactation (Burdett and Reek, 1979, Campbell and Fell, 1964, Crean and Rumsey, 1971, Cripps and Williams, 1975, Datta *et al.*, 1995).

4.7. Future work

Future work to quantify GLP-1-IR cells in the colon would aim to further investigate whether the increased GLP-1 concentration seen locally within the colon is due to an increased number of GLP-IR cells. One avenue for future analysis on samples from this study could be to determine the concentrations of acyl and desacyl ghrelin to determine whether the significant decrease found in plasma total ghrelin was initially driven by a decrease in ghrelin acylation by ghrelin *O*-acyltransferase as has been suggested by others (Tham *et al.*, 2009). Stomach tissue concentration of

ghrelin did not change significantly through pregnancy, which is in agreement with others (Shibata *et al.*, 2004, Taylor *et al.*, 2009).

In addition to the above, investigation of gut dry weights as well as gut wet weights may further clarify the process of gut growth during pregnancy. A difference was found between the current study and what was reported by Cripps and Williams (1975), in the increase in wet weight of colon tissue during pregnancy. Thus it may be worth replicating the study with the addition of dry gut weights to examine the process of maternal gut adaptations in further detail.

From this work, it is unclear whether a relationship exists between peptide concentrations and gut growth, as gut growth was only found to occur significantly by the last stage of pregnancy studied here. Further work studying additional late pregnancy time points may further elucidate this possible connection.

CHAPTER 5: GUT APPETITE HORMONES AND GUT GROWTH DURING LACTATION

5.1. Introduction

Multiple maternal changes take place in the early stages of lactation, enabling adaption to the physical demands of the lactation period. Body mass rapidly recovers after birth in rats, increasing beyond both control and early to mid pregnancy values. Cripps and Williams (1975) reported significant body mass gains from the third day of lactation in rats compared to nulliparous controls, with body mass remaining constant from day 12 of lactation. Maternal food intake during lactation in rats is reported to peak during the third week of lactation to approximately 250 to 300% that of nulliparous controls (Crean and Rumsey, 1971, Cripps and Williams, 1975, Denis *et al.*, 2004). Hyperphagia during lactation may be, in part, supported by a significant reduction of leptin levels, which is associated with a significantly decreased maternal adipose pad mass by day 15 of lactation compared to nulliparous controls (Woodside *et al.*, 2000). It has been documented that there is an approximate 60% loss of maternal whole body adipose tissue by day 16 of lactation in comparison with values obtained at day 2 of lactation, and this occurred on both a high and low protein maternal diet (Naismith *et al.*, 1982). As diet did not affect adipose tissue utilisation, this finding suggested that hyperphagia during lactation was not entirely under dietary control, implicating appetite-regulatory hormones.

There have been fewer studies documenting the role of gut appetite hormones during lactation compared to during pregnancy (Chapter 4). Many studies focus on the effects of maternal diet in the offspring, with metabolic programming during the early postnatal period being a key focus of this research area (see section 1.8.1 and additional reviews by Innis, 2011, Martin-Gronert and Ozanne, 2010, Singhal and Lanigan, 2007).

5.1.1. Orexigenic gut hormone changes during lactation

Shibata *et al.* (2004) documented that plasma total ghrelin levels were similar between dams at days 3 and 8 of lactation, but were significantly lower than at day 20 of pregnancy. These authors also reported that ghrelin mRNA levels in the hypothalamus tended to be decreased in lactation compared to during pregnancy, suggesting a systemic reduction in ghrelin levels after birth. In another rat study, plasma acyl ghrelin levels were found to be unchanged at days 5 and 15 of lactation compared to nulliparous controls (Suzuki *et al.*, 2014). Total ghrelin levels were measured by Taylor *et al.* (2009) in lactating rats and there was no difference between the groups in either plasma or stomach tissue levels. However in that study, stomach total ghrelin levels were significantly lower by day 25 of lactation (5 days after weaning) than they had been during pregnancy and in the nulliparous controls, which may suggest a post-lactation normalisation in appetite regulation.

In humans, Ilcol and Hizli (2007) found that serum total ghrelin levels were lowest at the onset of lactation (0 to 3 days postpartum) and rapidly

increased between days 4 and 14 of lactation before a gradual decrease throughout the remainder of the lactation period (180 days, study end). The same authors also measured acyl ghrelin levels in serum which, conversely to total ghrelin levels, were highest at the start of lactation before a significant decrease during the rest of lactation. A study in lactating sows similarly documented that plasma acyl ghrelin levels decreased steadily during lactation, and were significantly reduced compared with the acyl ghrelin peak at day 30 of an approximate 114 days of pregnancy (Govoni *et al.*, 2007). However, an additional human study found that acyl plasma ghrelin levels increased between days 1, 10 and 15 of lactation, but remained lower than non-lactating age-matched control women at each stage (Aydin *et al.*, 2006). The differences between these two human studies could be due to one study measuring acyl ghrelin levels in serum (Ilcol and Hizli, 2007) and the other in plasma (Aydin *et al.*, 2006), making direct comparisons difficult. In summary, ghrelin levels in rats have been found to decrease or remain unchanged, and in humans, levels appear to briefly peak early on and then decrease during lactation. Ghrelin levels appear to be lower during lactation than during pregnancy (Govoni *et al.*, 2007, Shibata *et al.*, 2004).

5.1.2. Anorexigenic gut hormone changes during lactation

There are limited data available on possible changes in the concentrations of maternal PYY during lactation, with no studies to date exploring circulating or gut tissue concentrations of GLP-1 during lactation. Taylor *et al.* (2009) reported significantly increased plasma total PYY levels at day 5 of lactation,

in rats when compared to nulliparous controls, which was paralleled by significantly increased descending colon and rectum tissue PYY levels, but no change to the levels in ascending colon tissue. Another study in lactating rats reported significantly increased plasma total PYY at day 5 of lactation, in conjunction with a significant increase in hypothalamic PYY mRNA at both days 5 and 15 of lactation compared to nulliparous controls (Suzuki *et al.*, 2014), so it seems possible that elevated PYY plays an important role in rats at day 5 of lactation.

5.1.3. Maternal gut growth during lactation

Chapter 4 reported findings similar to the work of others (Burdett and Reek, 1979, Crean and Rumsey, 1971, Cripps and Williams, 1975) in that there was significant gut growth by the later stages of pregnancy in rats. Growth of the maternal gut during the lactation period was once a focus of this earlier research, after observations that the small intestine in particular increases in size.

Significant increases in both stomach wet and dry weights have been documented to occur during lactation, peaking in late lactation in rats (Cripps and Williams, 1975, Taylor *et al.*, 2009) and in mice (Campbell and Fell, 1964). However, one study found stomach tissue mass only increased early in lactation, decreasing thereafter (Crean and Rumsey, 1971). Most studies have focussed on the small intestine, as alluded to earlier. Cripps and Williams (1975) found a 27% increase in small intestine length by late lactation in rats compared to controls and this increase in length was found to

be the main contributor to the significant increase in both the wet and dry weight of the same tissue. Examination of the anatomy of the small intestine revealed that by day 10 of lactation, there was a significant increase in both villus height (approximately 55%) and muscle layer thickness (approximately 75%) (Cripps and Williams, 1975). Datta *et al.* (1995) also reported significant increases to small intestine lengths and wet and dry weights in lactating rats, with Burdett and Reek (1979) finding that increased mass of the mucosal epithelium (140 to 150% increase) was the main contributor to increased small intestine wet weight. Taylor *et al.* (2009) investigated several lactation time-points in rats and found that at day 20 of lactation, the small intestine was significantly longer than all lactation groups except day 15 of lactation, showing a rapid increase in length from around mid lactation. A significantly increased surface area of the small intestine during lactation in rats has also been documented (Boyne *et al.*, 1966, Penzes and Regius, 1985). In mice, small intestine wet weight was found to increase by 79% compared to nulliparous controls (Campbell and Fell, 1964), showing that gut growth during lactation is not restricted to rats.

The distal gastrointestinal tract has also been found to significantly increase in size with lactation in rodents. Cripps and Williams (1975) reported significantly increased caecum wet weight of 127% and dry weight of 57% in day 21 lactating rats compared to nulliparous controls, with Taylor *et al.* (2009) finding a significant increase in caecum wet weight occurring from day 10 of lactation onwards. An additional study in mice found a 34% increase in caecum wet weight compared to controls (Campbell and Fell, 1964) showing

that the caecum may not increase in size in mice as considerably as is reported in rats. The colon has also been found to increase in both length and wet and dry weight with lactation (Cripps and Williams, 1975).

Gut growth has been investigated in relation to the hyperphagia of lactation, to investigate the hypothesis that gut growth occurs in response to increased food intake. In lactating rats on restricted diets, Campbell and Fell (1964) found that small intestines were dilated to the same degree, but showed only partial hypertrophic changes in comparison to *ad libitum* fed rats. These authors additionally found that the absorptive capacity of the small intestine did not differ between nulliparous and lactating rats showing varying levels of gut hypertrophy, hypothesising that changes to the small intestine size during lactation are proportionate to requirements. Datta *et al.* (1995) also reported that food restriction in lactating rats prevented small intestine hypertrophy.

Here, one aim was to further elucidate the possible roles of ghrelin, PYY and GLP-1 during lactation by investigating changes in both plasma and in gut tissues. Taylor *et al.* (2009) reported changes to both the gut peptides ghrelin and PYY and to gut growth during the lactation period. This current study included the analysis of GLP-1 levels in plasma and in gut tissue, and also aimed to update and advance documented changes to gut growth during lactation, with the inclusion of additional measurements, such as gut circumferences, aiming to provide more detail to potential changes and when they might occur. Measurement of the gut alongside both plasma and gut

tissue peptide levels aimed to explore whether any relationship may exist between the gut peptides and gut growth during lactation.

5.2. Objectives

Objectives for this part of the study were:

- to investigate whether levels of the gut appetite hormones ghrelin, PYY and GLP-1 change at defined stages of lactation in Wistar rats, by quantification of plasma and gut tissue concentrations;
- to further elucidate reported changes to gut size during lactation;
- to determine whether any relationship may exist between any changes in plasma or gut hormone concentrations and changes in gut size.

5.3. Methods

5.3.1. Animals

Female Wistar rats, bred in-house, were used for the time-points of days 0 (day of birth), 5, 10 and 25 of lactation (n=7 per group). All litters were standardised to 8 pups each by day 2 postpartum and pups remained with dams throughout the study. Dams used on the day of birth (d0L) had their litters standardised shortly after birth when a nest had been established and were sacrificed approximately 4 to 5 hours after birth. Data from proestrus nulliparous controls were used for comparison purposes (n=6 unless

otherwise stated). Where data are available, proestrus mean values have been added to figures using a dotted line.

5.3.2. Sample collection

Samples of gut tissue and fed and fasted blood were taken, alongside multiple gut measurements, as outlined in Chapter 2 (section 2.3).

5.3.3. Measurements of food intake and body mass

Rats were weighed a minimum of once a month throughout the study. Dams for lactation time-points were separated from their home cage approximately 20 days into pregnancy, allowing daily food intake monitoring by weighing the food hopper at the same time each day. In heavily pregnant dams, a sudden decrease in food intake coupled with a plateau in body mass gain was an indicator of imminent birth and both food intake and body mass were monitored closely for dams intended for the day 0 of lactation (d0L) sample group. Estimations of food intake usually ended at approximately day 10 to 15 postpartum, when the pups opened their eyes and began to eat small amounts of solid food.

5.3.4. Peptide assays

Radioimmunoassays to determine peptide concentrations of ghrelin, PYY and GLP-1 in plasma and tissue extracts were performed as previously detailed (section 2.5).

5.4. Results

5.4.1. Total ghrelin peptide analysis

5.4.1.1. Concentration change with lactation stage

The concentration of total ghrelin in fasted plasma samples significantly changed during lactation ($F(3, 24)=4.546$, $P=0.012$) and increased beyond proestrus control concentrations from day 10 of lactation (d10L). Day 25 lactating (d25L) dams had significantly more ghrelin in fasted plasma than day 0 lactating ($P=0.023$; d0L) and day 5 lactating ($P=0.017$; d5L) dams and had 20% more fasted plasma ghrelin than proestrus controls (Figure 5.1).

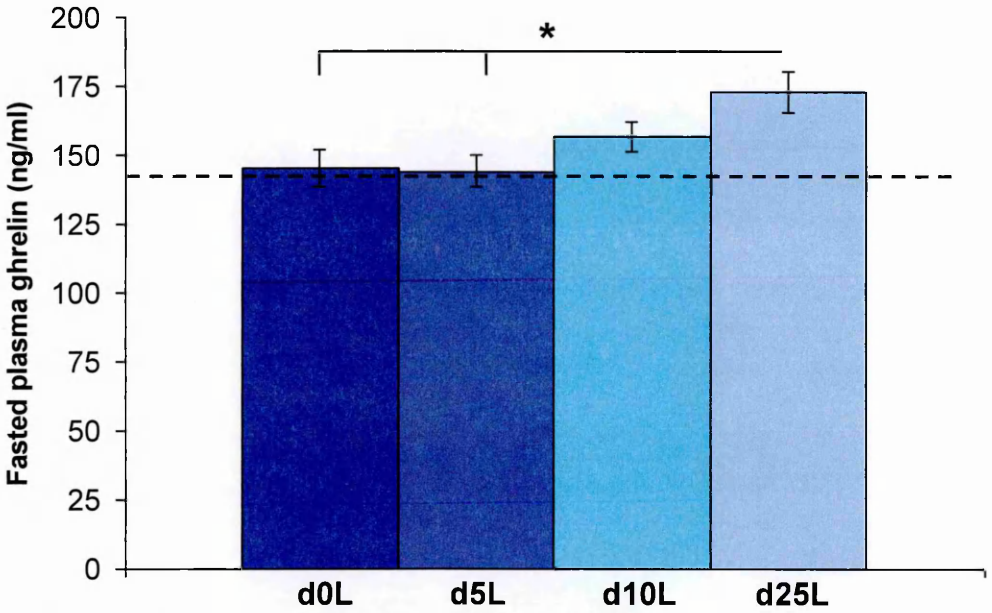

Figure 5.1. Ghrelin concentrations in fasted plasma during lactation
(* $P<0.05$; n=7 dams per stage). Dotted line shows approximate proestrus control mean.

Stomach tissue ghrelin concentration (Figure 5.2) was significantly (Kruskal-Wallis, $\chi^2=10.057$, 3 df, $P=0.018$) increased in d0L dams, with a significant decrease by d25L ($P=0.025$). Although d0L dams had the highest

concentration of ghrelin in their stomach tissue out of all of the lactating dams, this concentration was similar to those found in the proestrus controls.

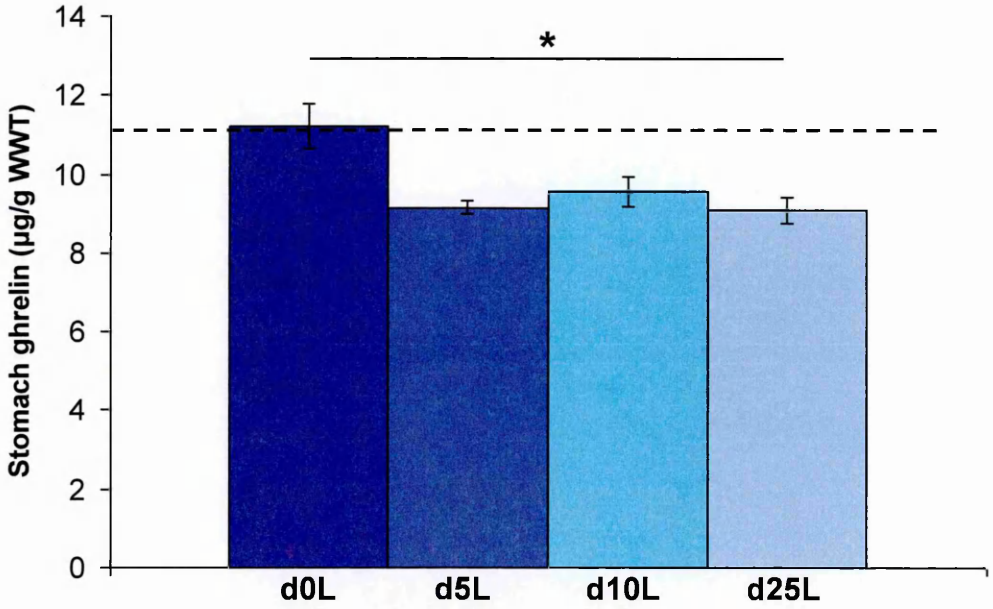

Figure 5.2. Ghrelin concentrations in stomach tissue during lactation
(* $P<0.05$; n=7 dams per stage, except d5L where n=5). Dotted line shows approximate proestrus control mean.

5.4.2. Total peptide-YY (PYY) peptide concentrations

5.4.2.1. Concentration change with lactation stage

Due to the differences in sample preparation between the fed and fasted plasma discussed in Chapter 4 (section 4.5.2), paired analysis was not carried out to compare PYY concentrations in the fed and fasted states. Neither fed nor fasted plasma concentrations of PYY were significantly different between the stages of lactation (Table 5.1). Fasted plasma PYY was 30 to 63% more concentrated during lactation than in the proestrus control animals (172.3 ± 11.84 pg/ml).

Table 5.1. PYY concentrations in fed and fasted plasma during lactation
(n=7 dams per stage, except fed plasma d25L (n=6)).

	Fed plasma PYY (pg/ml)	Fasted plasma PYY (pg/ml)
d0L	102.7 ± 3.22 (92.9 – 117.3)	223.3 ± 16.31 (187.4 – 311.5)
d5L	101.0 ± 4.90 (80.1 – 116.6)	247.1 ± 20.83 (179.5 – 322.1)
d10L	106.7 ± 2.77 (97.0 – 116.5)	280.5 ± 27.76 (181.8 – 395.4)
d25L	104.1 ± 3.68 (92.6 – 117.7)	239.1 ± 11.17 (180.9 – 270.9)

Ascending colon tissue concentration of PYY was significantly ($F(3, 22)=4.638$, $P=0.012$) higher at d5L than at d25L ($P=0.012$; Figure 5.3 A). Concentrations of descending colon PYY (Figure 5.3 B) varied considerably within each group. There was no significant difference between the concentrations of PYY in paired samples from ascending and descending colon tissue at any stage of lactation.

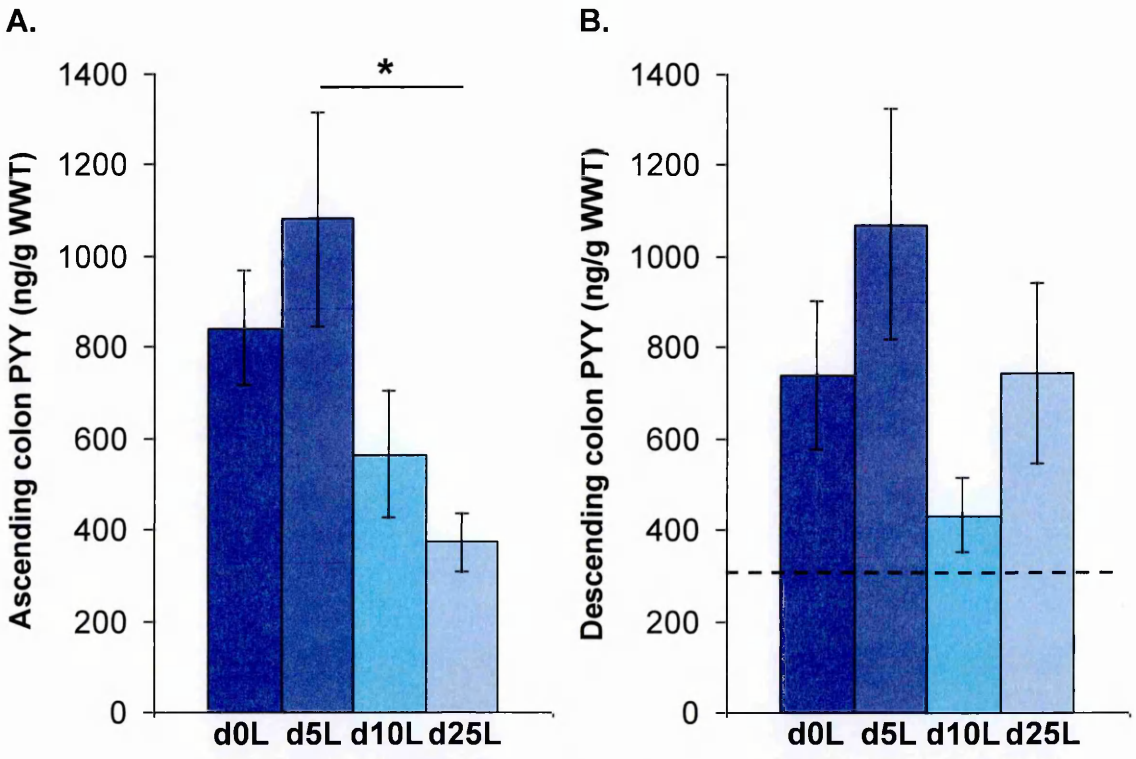

Figure 5.3. PYY concentrations in colon tissue during lactation
(A) ascending colon and **(B)** descending colon concentrations (* $P<0.05$; n=7 dams per stage, except d5L where n=5). Dotted line shows approximate proestrus control mean.

5.4.3. Total glucagon-like peptide-1 (GLP-1) peptide concentrations

5.4.3.1. Matched fed and fasted plasma comparison

Paired analysis of GLP-1 concentrations in fed and fasted plasma samples from the whole group showed that GLP-1 was 4.8 fold more concentrated ($t(24)=5.502$, $P<0.001$) in the fed state (249.4 ± 25.78 pg/ml) than in the fasted state (51.5 ± 17.77 pg/ml).

5.4.3.2. Concentration change with stage of lactation

All of the lactating dams had higher fed plasma GLP-1 than proestrus controls by 173 to 534%. Fed plasma GLP-1 was significantly ($F(3, 21)=5.505$, $P=0.006$) more concentrated in d0L dams than in both d10L ($P=0.010$) and d25L ($P=0.025$) dams (Figure 5.4).

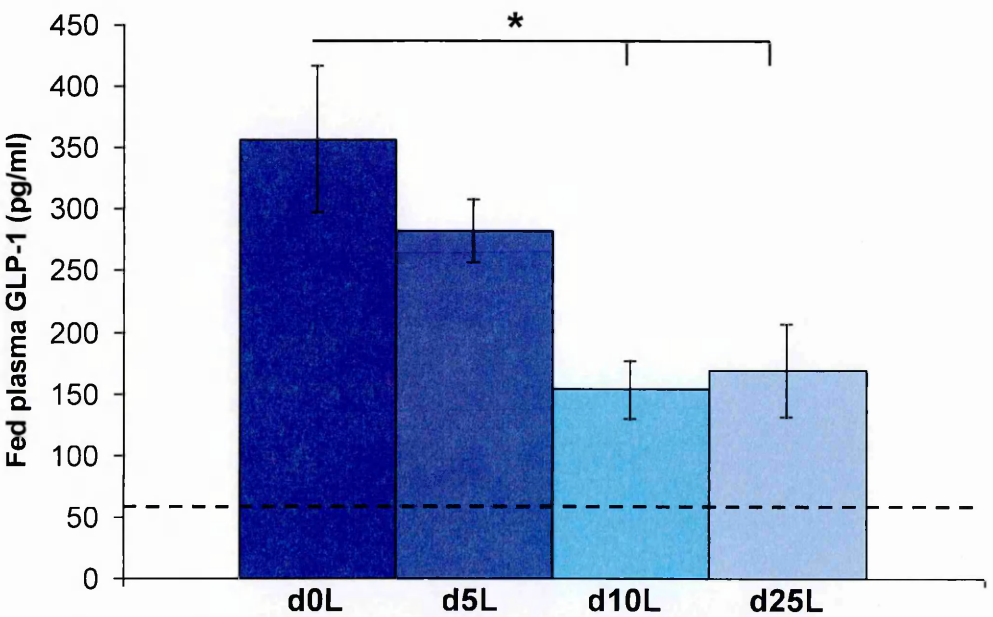

Figure 5.4. GLP-1 concentrations in fed plasma during lactation
(* $P<0.05$; n=7 dams per stage, except d10L where n=6 and d25L where n=5). Dotted line shows approximate proestrus control mean.

GLP-1 concentration in fasted plasma varied considerably within each sample group and was not significantly different between the dams (Figure 5.5).

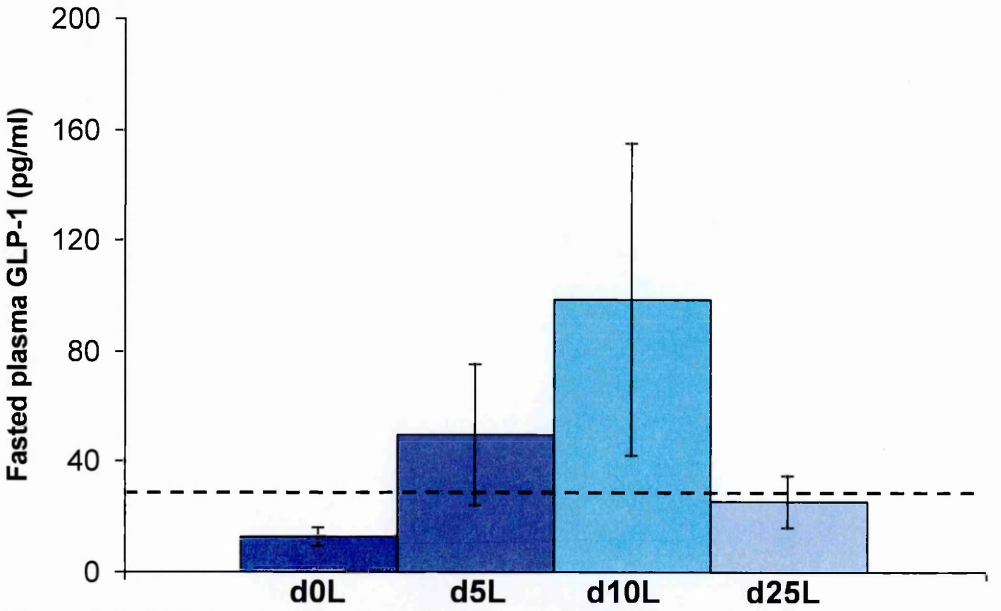

Figure 5.5. GLP-1 concentrations in fasted plasma during lactation (n=7 per stage). Dotted line shows approximate proestrus control mean.

Ascending colon GLP-1 concentrations (Figure 5.6 A) decreased as lactation progressed and were significantly ($F(3, 22)=4.164$, $P=0.018$) higher in d0L dams than in d25L dams ($P=0.016$). GLP-1 concentrations in descending colon tissue had to be log transformed for parametric statistical analysis. Log of descending colon GLP-1 was significantly different ($F(3, 22)=4.493$, $P=0.013$) between the dam groups, which were highest at d0L and d5L before a sharp decrease by d10L ($P=0.020$), with a significant decrease also found between d0L and d10L ($P=0.030$) dams (Figure 5.6 B). Descending colon GLP-1 concentrations appeared to be higher than in the proestrus controls at d0L and d5L.

Ascending colon GLP-1 concentrations were significantly higher than descending colon GLP-1 concentrations at d0L ($t(6)=3.651$, $P=0.011$) and d10L ($t(6)=4.369$, $P=0.005$), but not significantly higher at d5L and d25L.

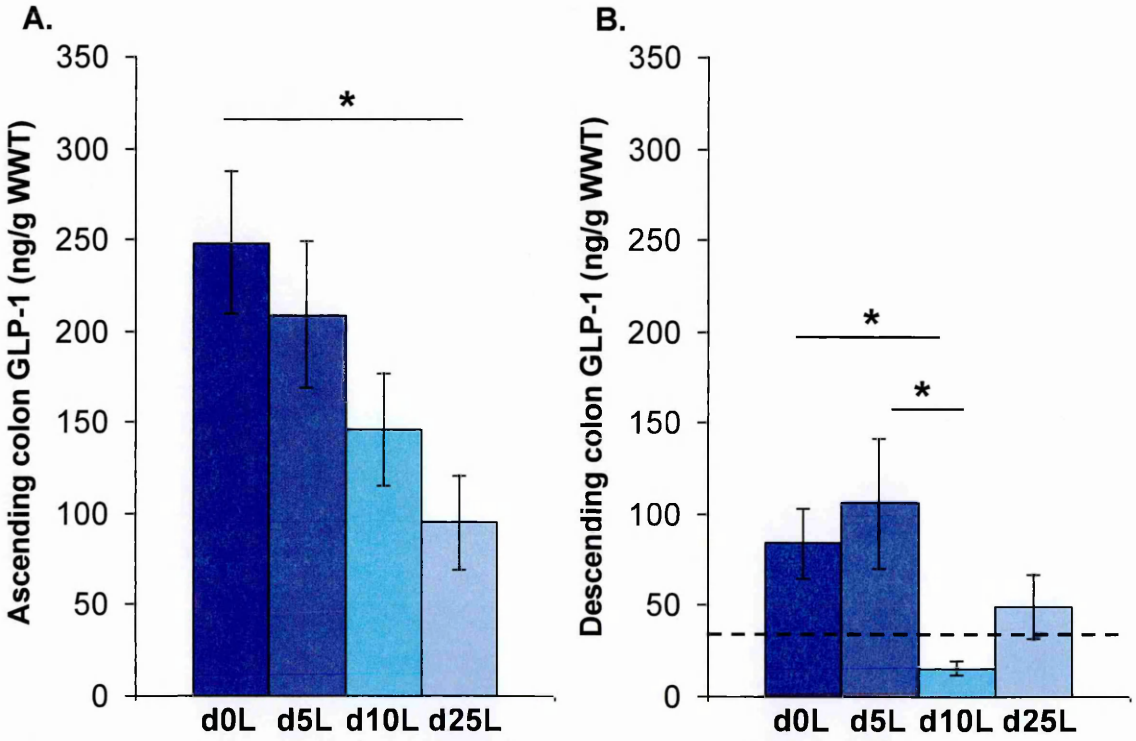

Figure 5.6. GLP-1 concentrations in colon tissue during lactation
(A) ascending colon and **(B)** descending colon concentrations (* $P<0.05$; n=7 dams per stage excluding d5L where n=5). Dotted line shows approximate proestrus control mean.

5.4.4. Changes to body and gut size during lactation

The mean age of the lactating dams was 28.5 ± 0.94 weeks (18 – 37 weeks) of age at the time of culling. Body mass of the animals prior to the tail bleed (fed state) was obviously highest in d0L dams, as this was a day before birth, and so these dams were excluded from fed state body mass analysis. In the fed state, d5L dams were significantly ($F(2, 18)=12.942$, $P<0.001$) lighter than d10L ($P<0.001$) and d25L ($P=0.003$) dams (Figure 5.7).

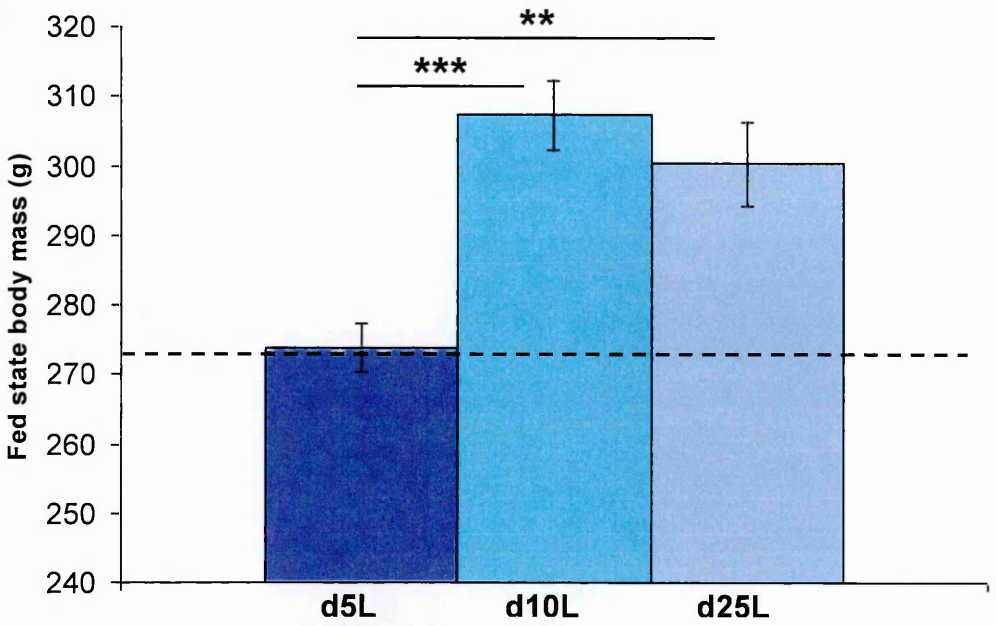

Figure 5.7. Fed state body mass during lactation
(n=7 per stage, with d0L excluded due to mass of pups; ** $P<0.01$; *** $P<0.001$). Dotted line shows approximate proestrus control mean.

Body mass recorded after the fasting period, however, was not significantly different between any of the groups, including d0L dams (Figure 5.8). D5L dams, in both the fed and the fasted state, had a similar body mass to the proestrus controls.

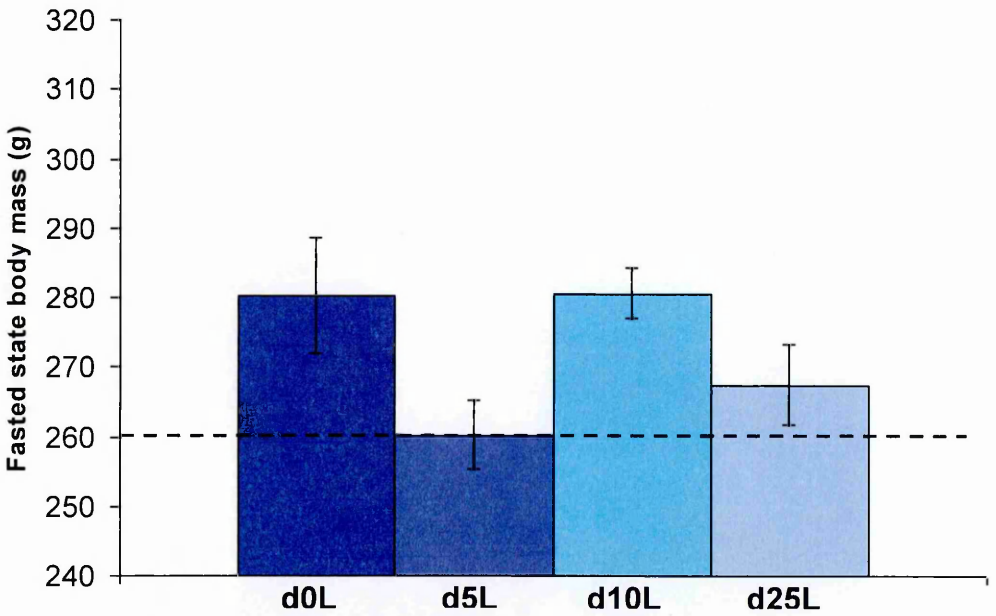

Figure 5.8. Fasted state body mass during lactation
(n=7 per stage). Dotted line shows approximate proestrus control mean.

The percentage of body mass lost during the fasting period (excluding d0L dams who gave birth during this time; Figure 5.9) was also significantly different between the dam groups ($F(2, 18)=6.086$, $P=0.010$) and those at d5L lost around the same mass as proestrus controls, which was significantly less body mass than the d25L ($P=0.008$) dams lost.

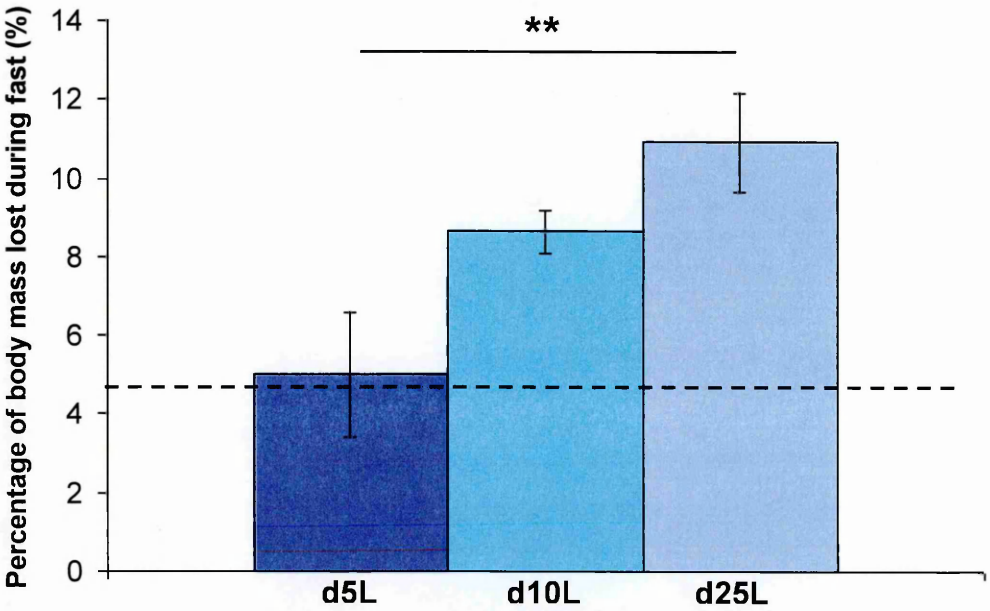

Figure 5.9. The percentage body mass lost after the fasting period in lactating rats (n=7 dams per stage; ** $P<0.01$). Dotted line shows approximate proestrus control mean.

Food intake of the dams was monitored in the days leading up to birth and into lactation. It was only possible to closely monitor daily food intake in a small number of rats, and 4 dams that were monitored for the most consecutive days are displayed in Figure 5.10. These 4 dams halved their food intake from 3 days peripartum (prior to birth; d3PP) to 1 day peripartum (d1PP). Food intake rapidly increased into the lactation period, doubling from d1L to d2L. Food intake continued to increase in the dams and was highest at d8L, when monitoring stopped. The d8L dams studied here consumed approximately 248% more than the mean daily food intake of all of the cycling animals (n=43) in Chapter 1.

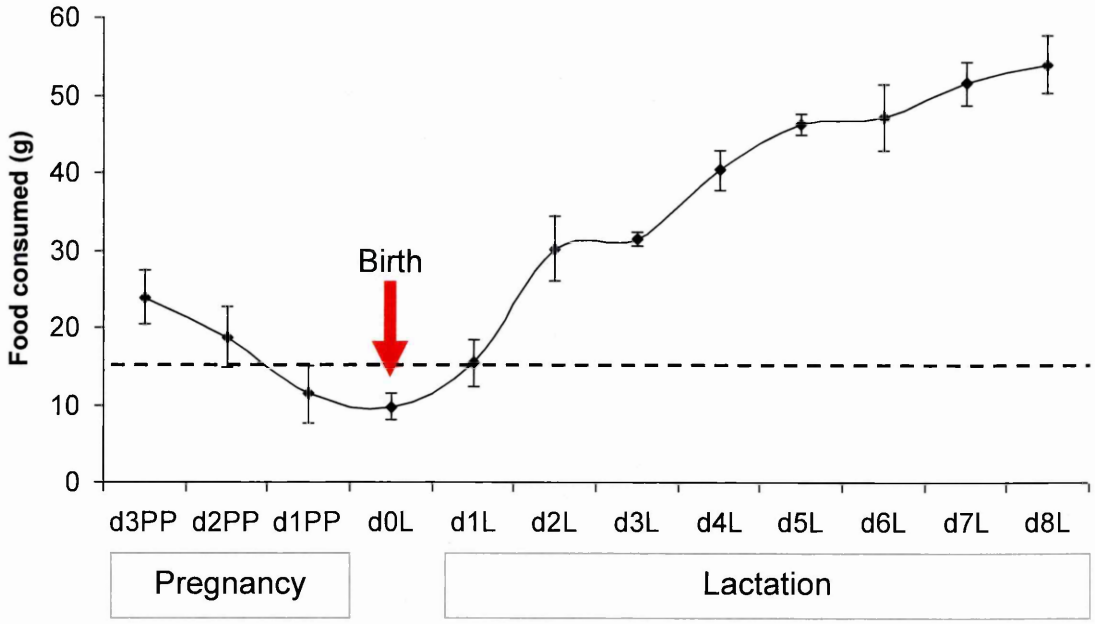

Figure 5.10. Food intake of dams leading up to birth and into lactation
The food intake of 4 dams monitored for 12 consecutive days through the peripartum (PP) period and into lactation (L). Dotted line shows approximate mean daily food intake of nulliparous cycling females (n=43).

Anaesthetised rectal temperature was not significantly different between the groups, with d0L (36.7 ± 0.06 °C), d5L (37.0 ± 0.23 °C), d10L (37.2 ± 0.18 °C) and d25L (37.3 ± 0.24 °C) dams all having similar temperatures.

The mass of abdominal cavity white adipose tissue (WAT) was significantly ($F(3, 24)=13.899$, $P<0.001$) heaviest in d0L dams when compared with d5L ($P=0.005$), d10L ($P<0.001$) and d25L ($P<0.001$) dams (Figure 5.11). Dams at d25L had less than half of the abdominal cavity WAT mass compared to nulliparous proestrus controls.

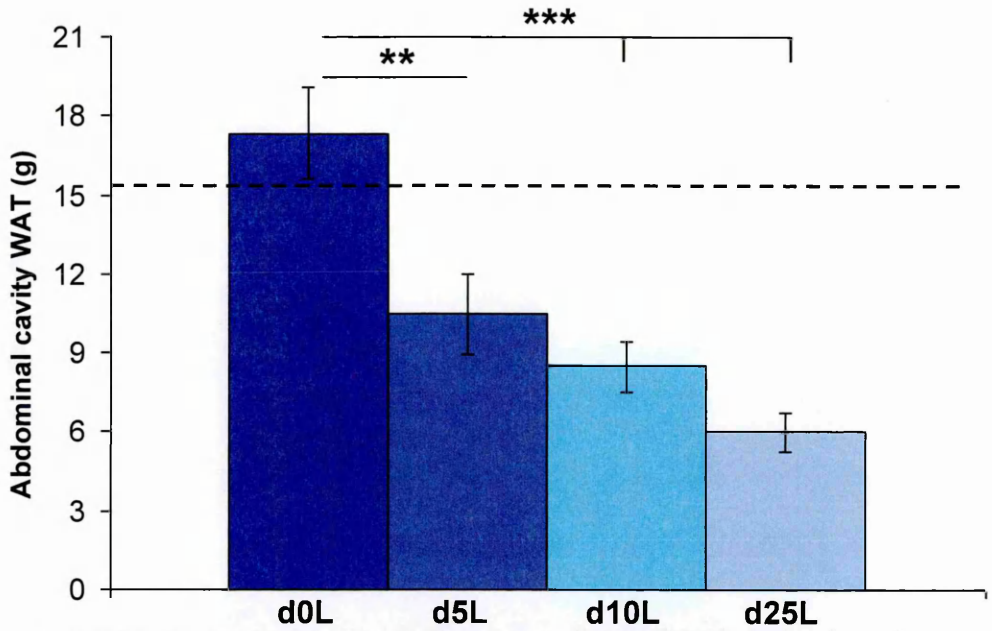

Figure 5.11. Abdominal cavity white adipose tissue (WAT) during lactation
(** P<0.01; *** P<0.001; n=7 dams per stage). Dotted line shows approximate proestrus control mean.

Stomach tissue wet weight significantly increased in late lactation (Kruskal-Wallis, χ^2=15.015, 3 df, P=0.002), with d25L dams having significantly heavier stomachs than d0L (P=0.018) and d5L (P=0.002) dams (Figure 5.12). The stomach tissue wet weight at d25L was approximately 31% heavier than in proestrus controls.

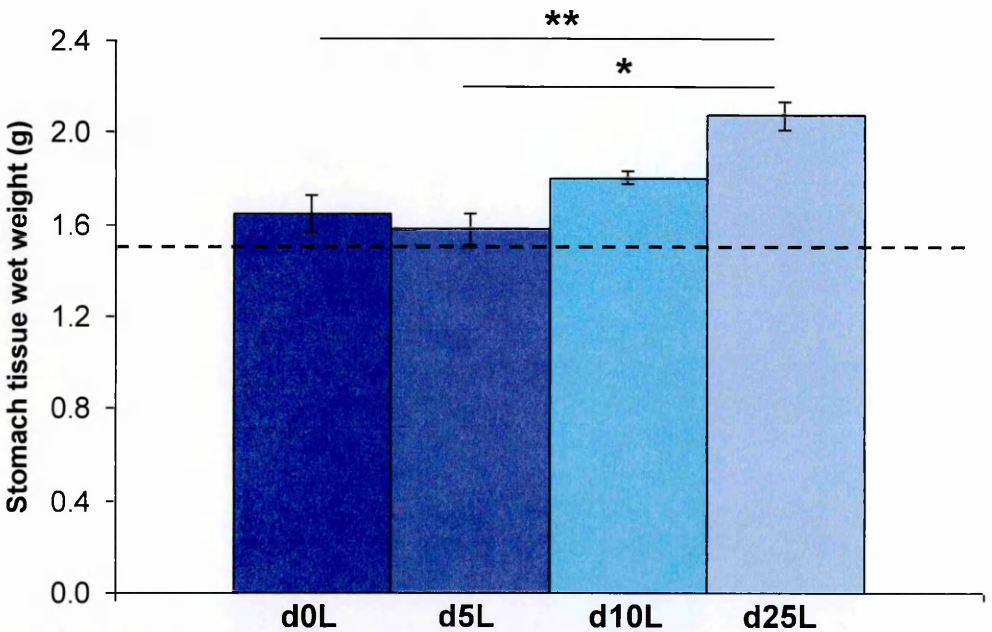

Figure 5.12. Stomach tissue wet weight during lactation
(* P<0.05; ** P<0.01; n=7 dams per stage, except d25L where n=6). Dotted line shows approximate proestrus control mean.

Small intestine wet weight (Figure 5.13 A) was significantly heavier ($F(2, 18)=39.220$, $P<0.001$) in d10L dams than in d0L ($P<0.001$) and d5L ($P<0.001$) dams. Additionally, d5L dams had significantly heavier small intestines than d0L ($P=0.022$) dams. Due to differences in sample collection (see section 2.4.1), small intestine wet weight is not available for comparison with the d25L dam group or the proestrus controls. The small intestine also significantly increased in length in later lactation ($F(3, 24)=17.944$, $P<0.001$), being significantly longer in both d25L and d10L dams than in d0L ($P<0.001$) and d5L ($P<0.001$) dams (Figure 5.13 B). D10L dams had approximately 48% longer small intestines than proestrus controls and d0L and d5L dams had similar small intestine lengths to controls.

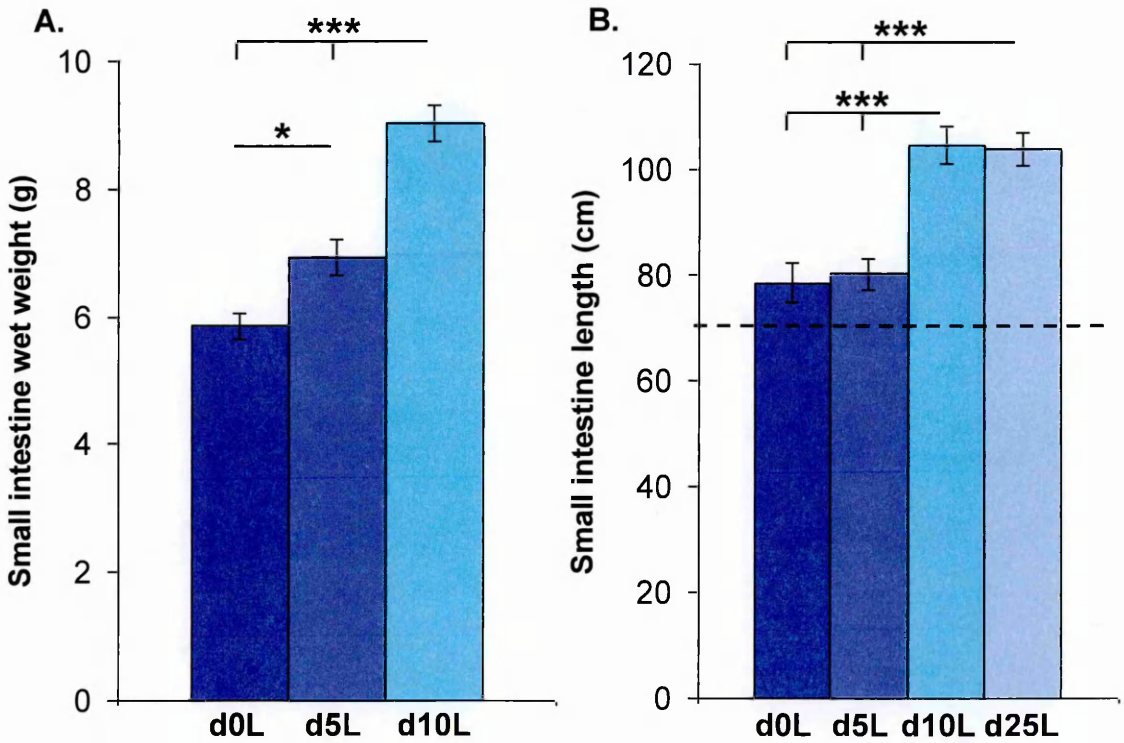

Figure 5.13. Small intestine size during lactation
(A) small intestine wet weight and **(B)** length during lactation (n=7 per stage; * $P<0.05$; *** $P<0.001$).

Caecum tissue (log transformed) was significantly heavier ($F(3, 24)=40.888$, $P<0.001$) in both the d10L and d25L dams than in the d0L ($P<0.001$) and d5L ($P<0.001$) dams (Figure 5.14).

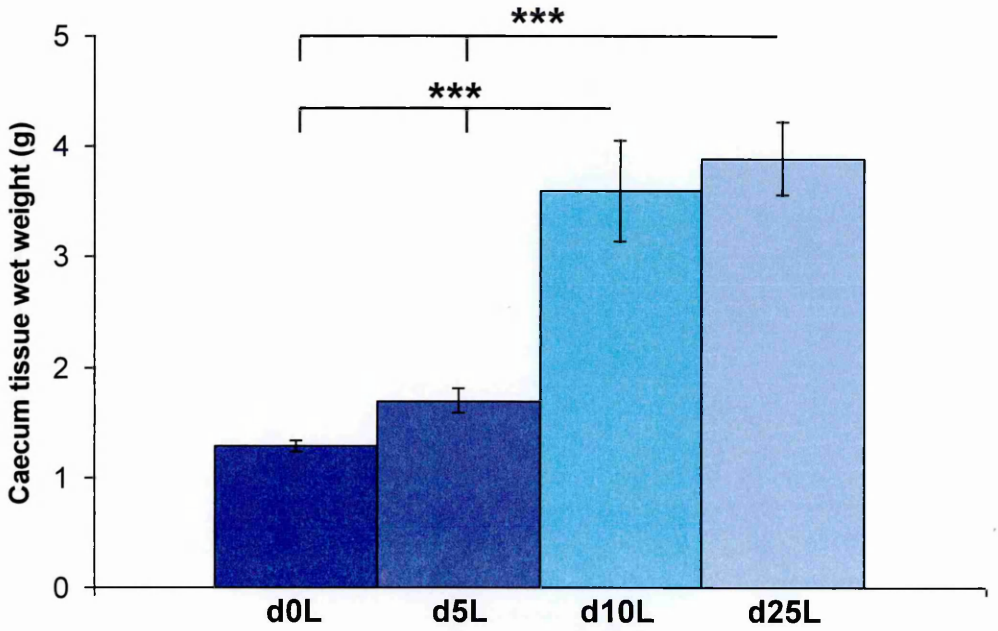

Figure 5.14. Caecum tissue wet weight during lactation
(*** $P<0.001$; n=7 dams per stage).

Large intestine wet weight (Figure 5.15 A) significantly increased in late lactation ($F(3, 19)=19.322$, $P<0.001$). Dams at the end of the lactation period (d25L) had significantly heavier large intestine tissue (log transformed) than d0L ($P<0.001$), d5L ($P<0.001$) and d10L ($P=0.001$) dams. Dams also had significantly shorter ($F(3, 24)=15.519$, $P<0.001$) large intestines at d0L ($P<0.001$) and d5L ($P=0.001$) compared to d25L dams, with d0L dams also having shorter large intestines than d10L ($P=0.004$) dams (Figure 5.15 B). D5L dams had similar large intestine lengths to the proestrus controls.

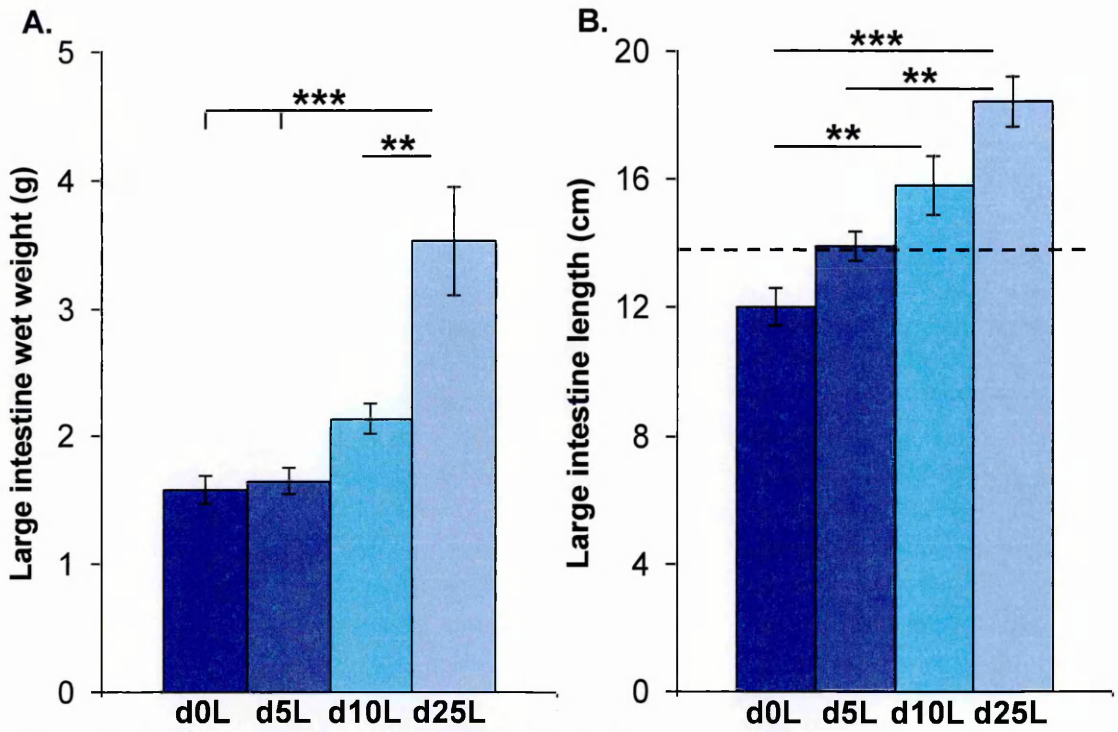

Figure 5.15. Large intestine wet weight during lactation
(A) large intestine wet weight and **(B)** length during lactation. Wet weight: n=4 d0L; n=7 d5L and d10L; n=5 d25L. Length: n=7dams per stage ** $P<0.01$; *** $P<0.001$). Dotted line shows approximate proestrus control mean.

All gut circumferences were significantly different between the sample groups (Figure 5.16).

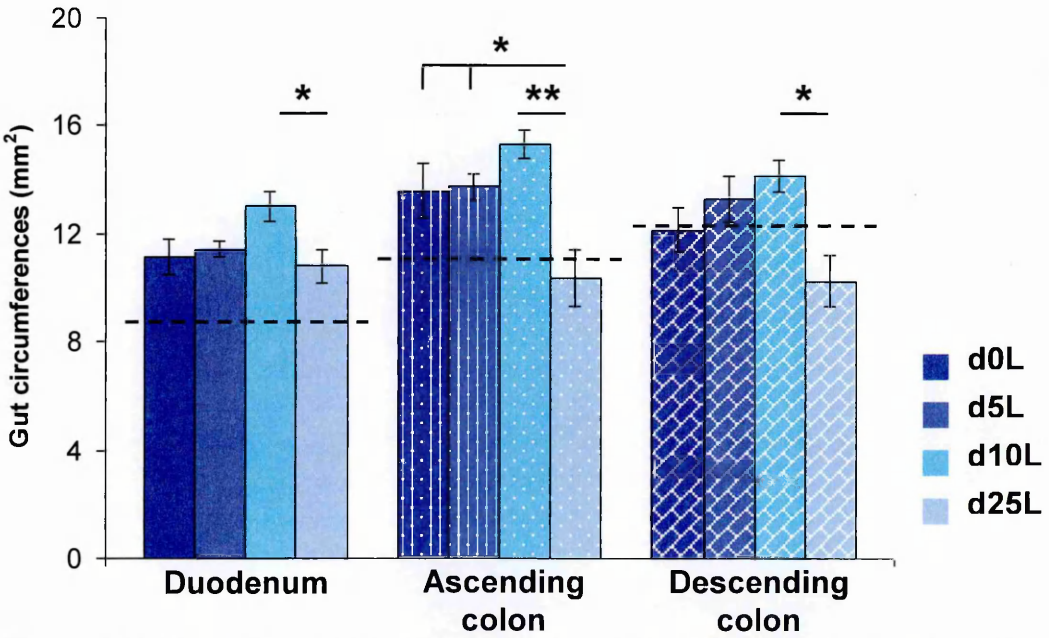

Figure 5.16. Circumferences of duodenum, ascending colon and descending colon during lactation
(n=7 per stage; * $P<0.05$; ** $P<0.01$). Dotted lines show approximate proestrus control mean in each tissue.

Duodenum circumference ($F(3, 24)=3.052$, $P=0.048$) was significantly smaller in d25L than in d10L ($P=0.046$) dams and all lactating dams appeared to have larger duodenum circumferences than proestrus controls. Ascending colon circumference ($F(3, 24)=6.506$, $P=0.002$) was significantly increased in d0L ($P=0.045$), d5L ($P=0.035$) and d10L ($P=0.001$) dams compared to d25L dams, which had circumferences similar to proestrus controls. Circumference of descending colon ($F(3, 24)=4.346$, $P=0.014$) was also significantly smaller in d25L than in d10L ($P=0.012$) dams, with proestrus control values most similar to d0L dams.

5.4.4.1. Mass of gut contents

Neither the mass nor the pH of the stomach contents (Table 5.2) changed significantly with the stage of lactation, although more stomach contents were present at each stage of lactation than in proestrus controls (0.5 ± 0.15 g).

Table 5.2. Stomach contents wet weight and pH during lactation
(n=7 dams per stage, except stomach contents wet weight d25L (n=6) and pH d0L (n=6) and d25L (n=5)).

	Stomach contents (g)	Stomach contents pH
d0L	2.6 ± 0.79 (0.3 – 6.3)	4.3 ± 0.51 (1.9 – 5.4)
d5L	2.0 ± 0.40 (0.6 – 3.8)	3.2 ± 0.50 (1.9 – 5.4)
d10L	1.8 ± 0.23 (0.7 – 2.4)	3.4 ± 0.34 (2.5 – 5.1)
d25L	1.0 ± 0.13 (0.5 – 1.4)	4.4 ± 0.39 (3.0 – 5.3)

No data are available for the mass of small intestine contents in d25L dams due to differences in sample collection. Small intestine contents (Table 5.4) were significantly ($F(2, 18)=16.540$, $P<0.001$) heavier in d10L dams than in

d0L (P<0.001) and d5L (P=0.011) dams. Caecum contents of d10L dams were significantly (Kruskal-Wallis, χ^2=14.462, 3 df, P=0.002) heavier than in d0L dams (P=0.001; Table 5.3). The pH of caecum contents was also significantly more acidic (F(3, 24)=4.458, P=0.013) in d10L dams than in d0L (P=0.020) and d5L (P=0.029) dams (Table 5.3). The mass of the contents from the large intestine was not significantly different between the groups (Table 5.4).

Table 5.3. Caecum contents wet weight and pH during lactation
(n=7 dams per stage; c>d, P<0.01; e>f, P<0.05, within columns).

	Caecum contents (g)	Caecum contents pH
d0L	2.6 ± 0.25 [d] (1.7 – 3.3)	6.6 ± 0.07 [f] (6.3 – 6.8)
d5L	5.6 ± 1.02 (2.3 – 9.0)	6.6 ± 0.09 [f] (6.3 – 6.8)
d10L	7.8 ± 0.31 [c] (6.8 – 8.8)	6.2 ± 0.12 [e] (6.0 – 6.8)
d25L	5.6 ± 0.65 (2.9 – 8.0)	6.3 ± 0.11 (6.0 – 6.9)

Table 5.4. Small and large intestine contents during lactation
(n=7 dams per stage, except large intestine contents d0L (n=4) and d25L (n=5); a>b, P<0.001; e>f, P<0.05, within columns).

	Small intestine contents (g)	Large intestine contents (g)
d0L	2.0 ± 0.33 [b] (1.0 – 3.2)	1.0 ± 0.11 (0.7 – 1.3)
d5L	4.4 ± 0.92 [f] (1.3 – 7.5)	2.7 ± 0.23 (2.0 – 3.8)
d10L	7.7 ± 0.76 [ae] (4.5 – 10.6)	3.1 ± 0.65 (0.5 – 4.8)
d25L	No data	3.2 ± 0.97 (0.3 – 6.2)

5.5. Discussion

This chapter aimed to investigate reported changes to the levels of the gut peptides ghrelin, PYY and GLP-1 during lactation in rats, studying matched plasma and tissue samples for the first time. This chapter also aimed to further elucidate reported gut growth during lactation, and whether this may occur in relation to possible changes in the corresponding gut peptide levels.

Fasted plasma ghrelin was significantly increased in late lactation, however stomach tissue ghrelin concentrations were significantly decreased after d0L. Both fed plasma GLP-1 and ascending colon GLP-1 significantly decreased between early and late lactation, with ascending colon PYY being significantly more concentrated at d5L than at d25L. Descending colon GLP-1 levels were significantly decreased at d10L compared with earlier in lactation. All tissue wet weights and lengths measured significantly increased by late lactation, with caecum and small intestine wet weights and small intestine length increasing significantly between d5L and d10L after PYY and GLP-1 levels appeared to be maximal in the ascending and descending colon respectively.

5.5.1. Orexigenic gut hormone changes during lactation

Total ghrelin concentration in fasted plasma significantly increased from early lactation to d25L in this study, and d25L dams had 20% higher fasted plasma ghrelin than proestrus controls. Taylor *et al.* (2009) also measured total ghrelin plasma concentrations, although reported no significant difference

between similar stages of lactation in rat fasted plasma. Shibata *et al.* (2004) reported no significant changes to fed plasma total ghrelin in rats between d5L and d15L. One study that measured total ghrelin levels during lactation in humans found that serum total ghrelin was lowest at the onset of lactation, increased rapidly for the first 14 days of lactation and steadily decreased thereafter for 180 days (Ilcol and Hizli, 2007). These authors also measured acyl ghrelin serum levels in the same participants and documented the opposite findings, in that acyl ghrelin was highest at lactation onset and then significantly decreased at each time point. The remainder of the studies of ghrelin during lactation have measured acyl ghrelin levels only, which is the 'active' portion of the peptide (see section 1.6.1.1). In rats, acyl ghrelin levels in plasma were not found to change in d5L and d15L rats compared to nulliparous controls (Suzuki *et al.*, 2014) and likewise no difference has been documented between early (d7L) and late (d21L) lactation in sows (Govoni *et al.*, 2007). Another study in lactating women found that acyl ghrelin levels in plasma increased from d1L to d10L and d15L but remained less concentrated than the non-pregnant control women at all stages (Aydin *et al.*, 2006). The collective results of others appear variable, which is not helped by the measurement of different forms of the ghrelin peptide. Although Taylor *et al.* (2009) reported no changes in total ghrelin plasma concentrations at similar stages of lactation, this may be due to the statistical analysis also including pregnant groups, as described earlier.

Whilst there was an increase in fasted plasma ghrelin in later lactation, stomach tissue ghrelin levels were significantly decreased at d25L compared

with d0L levels, which were comparable to proestrus control levels. Only one other study has previously documented stomach tissue total ghrelin concentrations during lactation. Taylor *et al.* (2009) reported a significant decrease in stomach total ghrelin levels in d25L dams compared to proestrus controls. However, pups were weaned 5 days prior to maternal sampling at d25L, so this result is not comparable to the currently reported findings.

The significant increase in plasma ghrelin levels in the present study could be related to the significant decrease in abdominal cavity white adipose tissue (WAT) at the late stages of lactation, as described in more detail later (section 5.5.3). In lactating Wistar rats, others have reported a concomitant significant decrease in adiposity and circulating leptin levels (Taylor *et al.*, 2009, Woodside *et al.*, 2000), which is logical due to leptin levels being directly proportionate to whole body adiposity (reviewed by Friedman, 2011, Ladyman *et al.*, 2010). The orexigenic effects of ghrelin have been documented to be suppressed when leptin levels are high in the circulation, by injection of ghrelin in male mice over-expressing leptin (Ueno *et al.*, 2004). So, it is possible that the increase in plasma ghrelin levels seen here by late lactation may have occurred due to a reduction in adiposity and thus of leptin levels. A lactation-associated reduction in leptin levels is also a possible explanation for the significant reduction in stomach tissue ghrelin levels between d0L and d25L dams. If leptin restraint of ghrelin occurred in the circulation early in lactation, it is possible that the stomach up-regulated production of ghrelin as a compensatory mechanism to increase circulating ghrelin to increase food intake by promoting hyperphagia. This requirement

would reduce as leptin levels reduce and ghrelin levels become unrestrained in the circulation; this could explain a significant reduction in stomach tissue ghrelin whilst there is a significant increase in plasma ghrelin. It is also possible that an increase in stomach ghrelin levels at d0L was a typical physiological response in order to initiate lactation-associated hyperphagia. Measurement of leptin levels in plasma would be of interest for future work to further explore the relationship between leptin and ghrelin during lactation. It would also be of interest to quantify the acyl and desacyl forms of ghrelin in these samples, as Ilcol *et al.* (2007) reported differences between total and acyl ghrelin levels in the same samples, indicating that there may be differences in the regulation of the different ghrelin forms during lactation.

5.5.2. Anorexigenic gut hormone changes during lactation

As introduced earlier, there are limited data available on the possible changes of PYY and no data on GLP-1 levels during lactation. Neither fed nor fasted plasma total PYY were different between the stages of lactation in this study, although fasted plasma PYY was 63% higher than proestrus control values at d10L. Suzuki *et al.* (2014) reported significantly higher fed plasma PYY levels at d5L and d15L compared to nulliparous control rats and PYY levels were highest at d5L. Taylor *et al.* (2009) also documented maximal fasted plasma PYY levels at d5L compared to all other pregnant, lactating and nulliparous controls studied. The findings of those two studies indicate that, for measurement of plasma PYY levels, the feeding status of lactating dams may not be important, as the PYY peak reported by Suzuki *et*

al. (2014) was also documented by Taylor *et al.* (2009) even after a 12 hour fasting period, which would normally be expected to reduce PYY to baseline levels.

Ascending colon concentrations of PYY were found to peak in d5L dams, with a decrease by d10L, which reached significance (a 65% decrease) by d25L. Descending colon PYY was not significantly different between the stages of lactation studied. Taylor *et al.* (2009) reported no difference in ascending colon concentrations between similar stages of lactation in rats, however did document a significant d5L PYY peak in descending colon and an increase in PYY from early to mid lactation in rectum tissue. The differences reported between PYY levels in ascending and descending colon tissues in this study and their study may arise from differences in statistical analysis. Taylor *et al.* (2009) analysed lactating dams alongside pregnant and control animals, which may result in some of the more subtle differences between lactating groups being missed, particularly as there appeared to be a lot of variation in the concentrations between the groups. However, what seems consistent between these studies is that PYY levels in colon tissue appeared to be highest at d5L and decreased from d5L to late lactation.

In addition to the above, Suzuki *et al.* (2014) have also reported a significant increase in arcuate nucleus PYY mRNA at both stages of lactation studied (d5L and d15L) in comparison with nulliparous control rats, suggesting that a lactation-associated increase of PYY in rats also occurs at the hypothalamic level.

There are no other studies that have explored the concentrations of GLP-1 in lactating dams; the focus has mainly been on insulin resistance during pregnancy (see Chapter 4). In this study, there was a significant decrease in fed plasma GLP-1 from d0L to d10L and d25L towards the levels seen in proestrus controls, which may represent a normalisation of maternal GLP-1 into lactation. Ascending colon GLP-1 levels decreased throughout lactation, with concentrations at d25L being significantly lower than in d0L dams. Between d5L and d10L, there was a significant reduction (85%) in GLP-1 concentration in descending colon. Why the highest levels of both PYY and GLP-1 appeared to occur early in lactation remains to be established, as food intake was 199% higher by d5L compared to nulliparous controls. The accepted role of PYY and GLP-1 is to rise in response to food intake and signal fullness, causing a reduction in appetite (section 1.6).

5.5.3. Changes in food intake, body size and body temperature during lactation

In this study, the fed state body mass of lactating dams recovered to values similar to proestrus controls by d5L, significantly increased by d10L and then remained constant until d25L. These findings are in line with previous studies. Cripps and Williams (1975) reported an increase in maternal body mass from d3L to d12L, which then remained constant until d18L and Crean and Rumsey (1971) documented a significant increase in body mass at d7L, d14L and d21L in comparison with nulliparous controls. In the fasted state, there was no significant difference in body mass between the dams, which may suggest that body mass was influenced by short term food intake and

may be linked with lactation-associated hyperphagia. Dams in the present study became more susceptible to losing body mass after the short period of fasting at d25L. As solid food was removed from the cage for the fasting period, this result may be due to increased pup persistence to feed from their mothers, with 25 day old pups potentially feeding more than the younger pups. This finding could also indicate that the dams were consuming less during the light period, with appetite levels normalising.

The dams studied for food intake demonstrated a rapid increase in food intake after birth, consistent with the reported hyperphagia of lactation (Crean and Rumsey, 1971, Cripps and Williams, 1975, Denis *et al.*, 2004). After a decrease leading up to birth, food intake recovered to control values the day after birth and doubled after a further 24 hours. By d8L (end of sampling), food intake was 248% higher than in controls, which is consistent with the findings of others, where food intake has been documented to increase by 250 to 300% by late lactation (Crean and Rumsey, 1971, Cripps and Williams, 1975, Denis *et al.*, 2004). Dams at all stages of lactation also had more stomach contents remaining after the fasting period compared to proestrus controls, which could indicate more food intake during the light period prior to food removal, or be due to the ingestion of more water and/or bedding material after the diet was removed.

As food intake significantly increased, abdominal cavity WAT significantly decreased in the dams. Naismith *et al.* (1982) reported a significant reduction in whole body adiposity by d16L compared to d2L. Another study also

documented a decrease, although not significant, in maternal adipose pad mass from d5L through to d25L (Woodside *et al.*, 2000). In agreement, the present study found that abdominal cavity WAT was significantly increased at d0L compared to all other stages of lactation, which is consistent with the hypothesis that rodents utilise adipose reserves as a key energy source to support lactation (Naismith *et al.*, 1982).

In this study, there were no significant differences in anaesthetised rectal temperature between the lactating dams, which is in agreement with results from a previous study in rats between days 2 and 10 of lactation (Fewell, 1995). As previously described (section 4.5.4), increased PYY levels have been associated with increased thermogenesis and decreased adiposity in obese PYY transgenic male mice (Boey *et al.*, 2008). It is possible that the up-regulation of thermogenesis by PYY requires high PYY levels in the circulation, which were not found in the present study. Boey *et al.* (2008) suggested that increased serum PYY in their animals may have affected thyroid hormones which, in turn, increased thermogenesis. It is also possible that there are sex differences in this proposed mechanism, and/or that the physiology of lactation changes this potential action of PYY.

5.5.4. Increased gut size during lactation

Stomach tissue wet weights were significantly increased by d25L in this study. Cripps and Williams (1975) studied rats at d21L and reported a 50% increase in stomach wet weight compared with nulliparous controls, which was greater than reported in this study between d25L and proestrus controls

(31%). Additionally, Taylor *et al.* (2009) documented a significant difference in stomach wet weights between all lactating rat groups and the proestrus controls. It is possible that due to the proestrus control rats in the present study being slightly older than would have been optimal for comparison purposes (see section 3.5.2), the difference between their stomach tissue mass and those of the slightly younger lactating dams was reduced in comparison to other studies, although neither of the above studies report rat age. Further work using age matched controls would have to be carried out to verify this possibility. Another study in Wistar rats only documented an increase in stomach mass from d7L to d14L, with a decrease by d21L, but this is likely due to methodology differences as the authors fixed stomachs before weighing them (Crean and Rumsey, 1971).

As described earlier, most studies of gut growth during lactation in rodents have focussed on the small intestine. This study reported a significant increase in both the wet weight and the length of the small intestine in the later stages of lactation. Compared with proestrus control values, the small intestines of d10L dams increased in length by approximately 48%. Without control values available due to method differences, small intestine wet weight increased by approximately 34% between d0L and d10L dams. In their rats, Cripps and Williams (1975) reported a 27% increase in small intestine length between d21L dams and nulliparous controls and Datta *et al.* (1995) documented a 28% increase at the same stage of lactation. For small intestine wet weight, those separate authors reported a 107% increase and an 84% increase respectively. In comparison to these two studies, it is

unclear why there was a more modest increase in wet weight, yet a clear increase in length of small intestines in the present study. However, Taylor *et al.* (2009) also reported much larger growth than those studies, with a mean small intestine length in d20L rats of 151 cm, which is approximately 45% longer than found in this study at a comparable stage of lactation. As described in Chapter 2, complete standardisation of gut length measurements using the calcium channel blocker nicardipine was not possible for this study, and could be one avenue for future work to fully elucidate the growth seen in this tissue during lactation.

Caecum wet weight was also found to significantly increase during lactation in this study, and the major increase occurred between d5L and d10L (112%) with a 200% increase by the end of the lactation period. Previously, a 127% increase in caecum wet weight has been documented between d21L and nulliparous controls (Cripps and Williams, 1975). Taylor *et al.* (2009) documented a significantly increased caecum wet weight from d10L, where it was significantly different from d<1L and d5L dams, which is in agreement with what is reported in the current study.

In addition to the increase in size seen in the small intestine, both large intestine lengths and wet weights were significantly increased at d25L in comparison to all other lactation groups studied. Large intestines were 33% longer in d25L dams compared to proestrus controls. Only one other study has documented changes to length and wet weight of the colon in lactating rats, and that study documented a 23% increased colon length (Cripps and

Williams, 1975), which is similar to what is reported here. Cripps and Williams (1975) also reported a 73% increase in colon wet weight in d21L dams compared with controls. Due to differences in measurement (described in section 2.4.1), it was not possible to compare proestrus control intestine wet weights to the dams in this chapter, however, large intestine wet weight increased by 119% from d0L to d25L. As outlined earlier, to be completely certain of large intestine length measurements during lactation, further work with maximally relaxed intestinal tissue would have to be done.

Gut circumferences of lactating rats have been measured here for the first time. In the duodenum, ascending and descending colon tissues, there was a non-significant increase in circumference leading up to d10L, with a significant decrease in circumference from d10L to d25L. As previously discussed, intestinal tissue was heaviest and longest in the d25L dams. Taylor *et al.* (2009) documented decreased gut size 5 days after weaning in their rats, so it is hypothesised here that by the end of the lactation period, when the gut is no longer required to adapt to increased food intake, the gut circumferences become smaller prior to further gut hypotrophy. Chapter 6 will go on to explore effects on maternal gut size after different postpartum outcomes.

5.5.4.1. Peptide changes in the large intestine may be related to gut growth

Reported here are high levels of PYY in ascending colon tissue at d5L, which are significantly higher than levels found at d25L. Others have additionally

reported a significantly increased level of PYY in plasma, descending colon and rectum tissues and in the hypothalamus in d5L dams compared to nulliparous controls (Suzuki *et al.*, 2014, Taylor *et al.*, 2009). From d5L, caecum wet weight and small intestine wet weight and length significantly increased into later lactation. Caecum wet weight increased by 112% between d5L and d10L and small intestine wet weight increased significantly by 30% between d5L and d10L dams and was 31% longer between the same groups. In other gut tissue (stomach, large intestine) the increase in wet weight and length was more gradual between the stages, all peaking in late lactation.

PYY has previously been linked with gut growth. Gomez *et al.* (1995) reported that PYY mRNA was highest in the colon during postnatal development in rat pups and peaked between day 7 and 13 of age. These authors treated 7 day old male and female rat pups with 3 times daily intraperitoneal injections of PYY for 14 days and reported a significant increase in small intestine wet weight, total DNA content and total protein content compared with vehicle-treated control litter mates, and the effect was most profound in the males. There were no trophic effects seen in the colon of either male or female pups. In adult female mice, the same group reported the ability of PYY to stimulate growth of both proximal and distal small and large intestinal tissue in a dose-dependant manner. The small intestine was most affected by PYY injection, with significant increases to wet weight, DNA content and protein content at many of the PYY concentrations tested, whereas the colon wet weight and DNA content only increased with higher

PYY doses. Their study only included previously optimised PYY doses and reported that PYY treatment did not significantly affect body or organ masses and that there were no changes to the normal histology of the gut (Gomez *et al.*, 1995). Collectively, their results suggest that PYY is a direct cause of gut growth and that this may be both age and sex dependant.

Tovar *et al.* (2004) found that a significant pregnancy-associated rise in PYY levels was completely suppressed in dams on 30% food restriction. Both Campbell and Fell (1964) and Datta *et al.* (1995) have studied the effects of food restriction in lactating rats, as detailed earlier (section 5.1.3), and have reported that food restriction prevents small intestine hypertrophy, but does not affect its absorptive capacity. It would be of interest to reproduce these studies and investigate the effect of food restriction on PYY levels and gut growth during lactation in rats. It may be that PYY levels are suppressed during food restriction to facilitate increased food intake, but if gut growth during lactation is a result of increased PYY then there may be no lactation-associated gut growth seen in food restricted lactating rats.

GLP-2 is co-secreted from gut L-cells (Mojsov *et al.*, 1986) alongside GLP-1 and PYY and has also been implicated in gut growth. It has been documented that GLP-2, but not GLP-1, can cause small intestine hypertrophy by subcutaneous injection of the peptide, twice daily, into male mice (Drucker *et al.*, 1996). A time course experiment demonstrated the ability of GLP-2 to increase small intestine wet weight and mucosal villus height after just 4 days of GLP-2 treatment, and these results were

comparable to those seen in mice with tumours positive for GLP-2 (Drucker *et al.*, 1996). Subsequent work by this group showed that GLP-2-induced small intestine hypertrophy increased the capacity for nutrient absorption in male mice (Brubaker *et al.*, 1997). A case study of a male patient with a neuroendocrine tumour immunopositive for PYY, GLP-1 and GLP-2 reported that the tumour was responsible for an increased level of these hormones in the circulation and also marked small intestine hypertrophy (Byrne *et al.*, 2001). Ghatei *et al.* (2001) reported that in male rats receiving total parenteral nutrition (TPN), which causes gut hypotrophy, both GLP-1 and GLP-2 infusion (separately) through a jugular catheter increased stomach, small intestine, caecum and colon wet weights, although the result was most marked in the rats receiving GLP-2 doses. GLP-2 administration to TPN rats was able to increase small intestine size beyond that of the orally fed controls, leading to a suggestion by the authors that gut secretion of GLP-2 may be key in the control of intestinal hypertrophy, keeping hypertrophy proportionate to food intake (Ghatei *et al.*, 2001).

It is evident from these studies that PYY and GLP-2 can cause substantial intestinal hypertrophy, with possible small increases in gut size caused by GLP-1. Due to its co-secretion with GLP-1 and PYY from intestinal L-cells, it may be reasonable to predict that GLP-2 levels also increase in early lactation. Future work to measure GLP-2 during lactation in relation to gut growth would be necessary to determine whether this occurs. It would also be of interest to further elucidate the role of the L-cell in gut hypertrophy.

5.6. Conclusions

This chapter focussed on changes to the levels of ghrelin, PYY and GLP-1 in matched plasma and gut tissue in lactating Wistar rats, and further explored documented changes to gastrointestinal growth. This work is novel in the study of GLP-1 levels in lactating dams and is the first to measure all three of the aforementioned gut peptides in conjunction with the study of lactation-associated gut growth. Key findings included a significant increase in fasted plasma ghrelin with significantly decreased fed plasma GLP-1 in late lactation, coupled with significantly increased food intake during lactation. However, stomach tissue ghrelin significantly decreased from d0L. Ascending colon PYY and descending colon GLP-1 levels were significantly increased in d5L dams compared with later stages of lactation, and it is suggested that this could be a signal that resulted in a significant increase in gut growth from mid-lactation.

5.7. Future work

To progress this work, additional peptides could be measured. As discussed earlier, leptin has the potential to alter ghrelin levels and so this would be one avenue for future work in order to further elucidate the possible causes of ghrelin changes during lactation. Quantifying the acyl portion of ghrelin may also provide more information to the changes and regulation of ghrelin levels during lactation, with previous work by others (Ilcol and Hizli, 2007) indicating that acyl ghrelin levels may change differently to total ghrelin levels. In addition to circulating and tissue levels of the peptides, hypothalamic peptide levels would be of interest to measure in future, as Suzuki *et al.* (2014)

reported similar changes to PYY levels in the hypothalamus and circulation. It is also possible that receptor levels change in the hypothalamus during lactation (Suzuki *et al.*, 2014) to adapt to the large increase in food intake and investigation of this possibility would be another interesting addition to this work.

As discussed in detail earlier, an extension of the work by others (Campbell and Fell, 1964, Datta *et al.*, 1995, Tovar *et al.*, 2004) on gut growth and its relation to food intake would also be of interest for future studies. Using pair-fed rats, it would be possible to explore changes to gut growth when lactating dams have their food intake restricted to control levels. This kind of experiment would aim to further the understanding of the relationship between gut peptides and gut growth, whilst food intake is controlled for.

CHAPTER 6: CHANGES IN MATERNAL GUT APPETITE HORMONES AND GUT GROWTH DUE TO LACTATION LITTER SIZE

6.1. Introduction

The work described in the previous two chapters showed that changes in maternal gut peptides and gut size occur during pregnancy (Chapter 4) and lactation (Chapter 5). Here, the possibility that the changes seen during lactation are influenced by the size of the litters being suckled was investigated. Such additional postpartum changes may occur to aid maternal adaptation to feeding different litter sizes.

As described in Chapter 1 (section 1.8.2), data suggest that maternal changes that occur to support pregnancy and lactation may persist into later life in humans, and may then influence the risk of developing obesity and subsequent obesity-related illness, such as type 2 diabetes. A 20 year prospective study reported that a longer lactation period was associated with a decreased risk of developing the metabolic syndrome in women with and without a history of gestational diabetes, presumably partly due to reduced maternal adiposity as a result of lactation (Gunderson *et al.*, 2010). Studies have also begun to explore levels of ghrelin and PYY in women with different durations of breastfeeding. One study has reported a significantly decreased level of fasted ghrelin in 4 to 5 week postpartum women compared to never-pregnant controls, with the authors suggesting that ghrelin may play a role in

the regulation of postpartum maternal body mass (Larson-Meyer *et al.*, 2010). Another study conducted in women 3 years postpartum reported that a longer lactation period resulted in increased levels of both total ghrelin and PYY in fasted plasma and concluded that this may contribute to reducing the risk of later life metabolic diseases (Stuebe *et al.*, 2011).

Work described in this chapter is the first to explore whether additional maternal lactation-associated changes to gut peptides and gut size occur as a result of litter size at the end of a complete lactation period in rats.

6.2. Objectives

Objectives for this part of the study were to:

- determine whether dams feeding different litter sizes have different levels of ghrelin, PYY and GLP-1 in either gut tissue or in circulation;
- establish whether the gut growth of dams observed during the lactation period is changed by the number of pups being suckled.

6.3. Methods

6.3.1. Animals

Two groups of dams had their litters manipulated to 4 pups (n=7 dams) or 12 pups (n=6 dams) by cross-fostering. Cross-fostering was achieved by distraction of the dam whilst her litter was manipulated. Groups of dams were always mated at the same time in order to obtain multiple births on the same

day to aid this work. Only pups of the same age were cross-fostered, preferably between litters of sister dams, with minimal intervention.

Samples were taken from the dams at day 25 of lactation (d25L) and the data were compared with those obtained in Chapter 4 from dams with litters standardised to 8 pups. Data from proestrus nulliparous controls were also used for comparison purposes (n=6 unless otherwise stated). Where data are available, proestrus mean values have been added to figures using a dotted line.

6.3.2. Sample collection

Blood and gut tissue was collected and measured from dams as described in Chapter 2 (section 2.3).

6.3.3. Peptide assays

Radioimmunoassays on plasma and tissue extracts were performed for total ghrelin, PYY and GLP-1 as described in Chapter 2 (section 2.5).

6.4. Results

6.4.1. Total ghrelin peptide analysis

6.4.1.1. Concentration change with lactation litter size

Fed plasma ghrelin concentration (Figure 6.1) was significantly different between the dam groups ($F(2, 9)=8.412$, $P=0.009$). Dams feeding 12 pups had significantly reduced fed plasma ghrelin compared to the dams feeding 8 ($P=0.007$) pups.

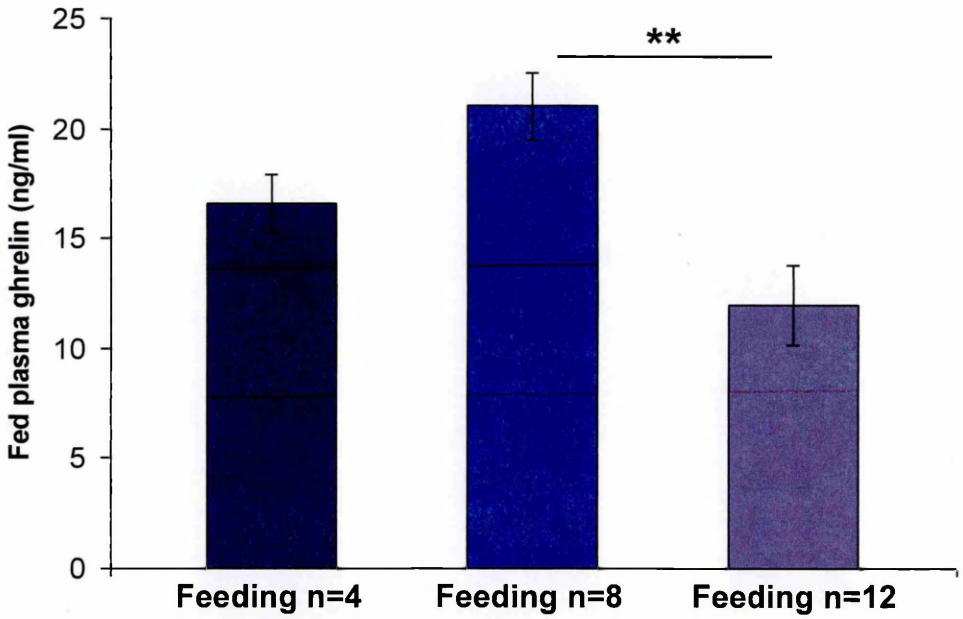

Figure 6.1. Ghrelin concentrations in fed plasma of dams with different lactation litter sizes
(n=4 dams per group; ** $P<0.01$).

Maternal concentrations of ghrelin in fasted plasma (Figure 6.2) did not change with the number of pups they were feeding. Concentrations of ghrelin in fed and fasted plasma could not be directly compared due to sample preparation differences (see section 2.5.3). The concentrations of ghrelin in stomach tissue extracts (Figure 6.3) were significantly ($F(2, 17)=8.882$,

P=0.002) higher in dams feeding 12 pups compared with the dams feeding the control litter size of 8 pups (*P*=0.002). Dams feeding 4 pups had similar stomach ghrelin concentrations to proestrus controls.

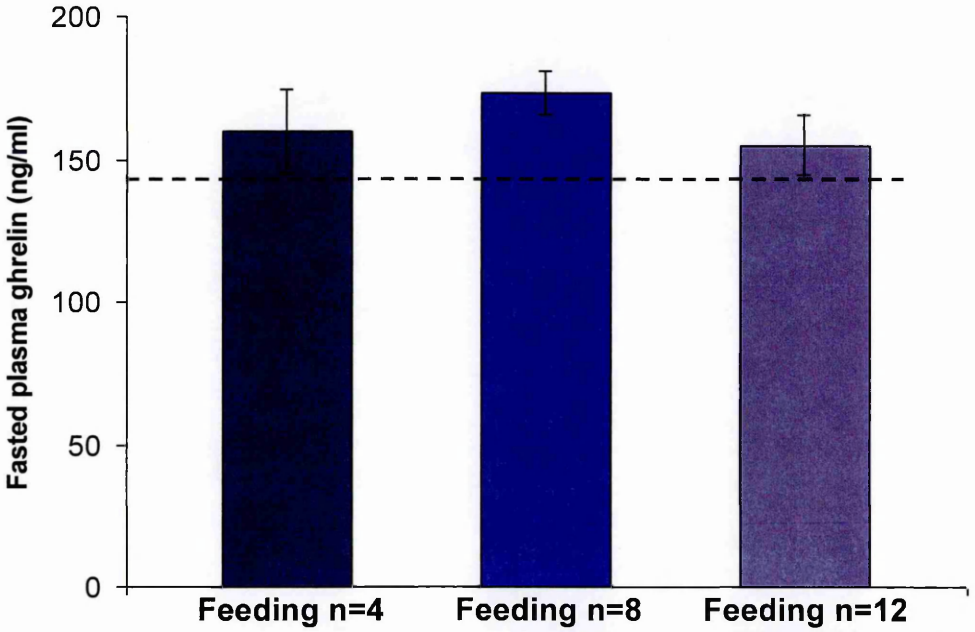

Figure 6.2. Ghrelin concentrations in fasted plasma of dams with different lactation litter sizes
(n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups). Dotted line shows approximate proestrus control mean.

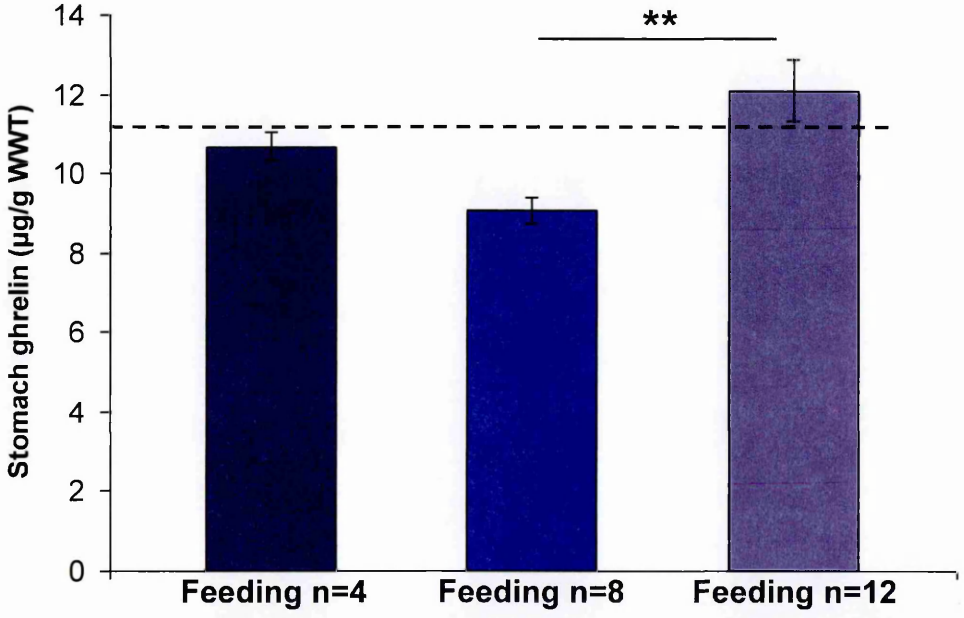

Figure 6.3. Ghrelin concentrations in stomach tissue of dams with different lactation litter sizes
(n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups; ** *P*<0.01). Dotted line shows approximate proestrus control mean.

6.4.2. Total peptide-YY (PYY) peptide analysis

6.4.2.1. Concentration change with lactation litter size

Neither fed nor fasted plasma PYY changed significantly in the dams feeding different litter sizes and there were a number of outliers (Table 6.1). All of the dams had higher levels of PYY in fasted plasma than the proestrus controls (172.0 ± 11.84 pg/ml).

Table 6.1. PYY concentrations in fed and fasted plasma of dams with different lactation litter sizes
(n=7 dams feeding 4 and 8 pups, except fed plasma where n=6 dams feeding 8 pups; n=6 dams feeding 12 pups).

	Fed plasma (pg/ml)	Fasted plasma (pg/ml)
Feeding n=4	104.5 ± 4.58 (89.1 – 117.7)	227.0 ± 8.25 (201.1 – 266.0)
Feeding n=8	104.1 ± 3.68 (92.6 – 117.7)	239.1 ± 11.17 (180.9 – 270.9)
Feeding n=12	115.9 ± 11.19 (91.4 – 116.8)	316.9 ± 93.55 (192.2 – 780.2)

Ascending colon PYY concentrations were also not significantly different between the dams (Figure 6.4 A). However, descending colon PYY, log transformed, was significantly ($F(2, 17)=4.437$, $P=0.028$) higher in dams with the control litter size of 8 pups than in dams feeding 4 ($P=0.045$) pups (Figure 6.4 B). All dams had more descending colon PYY than proestrus controls.

There was no significant different between the concentration of PYY in the ascending colon and the descending colon regions in any of the dams.

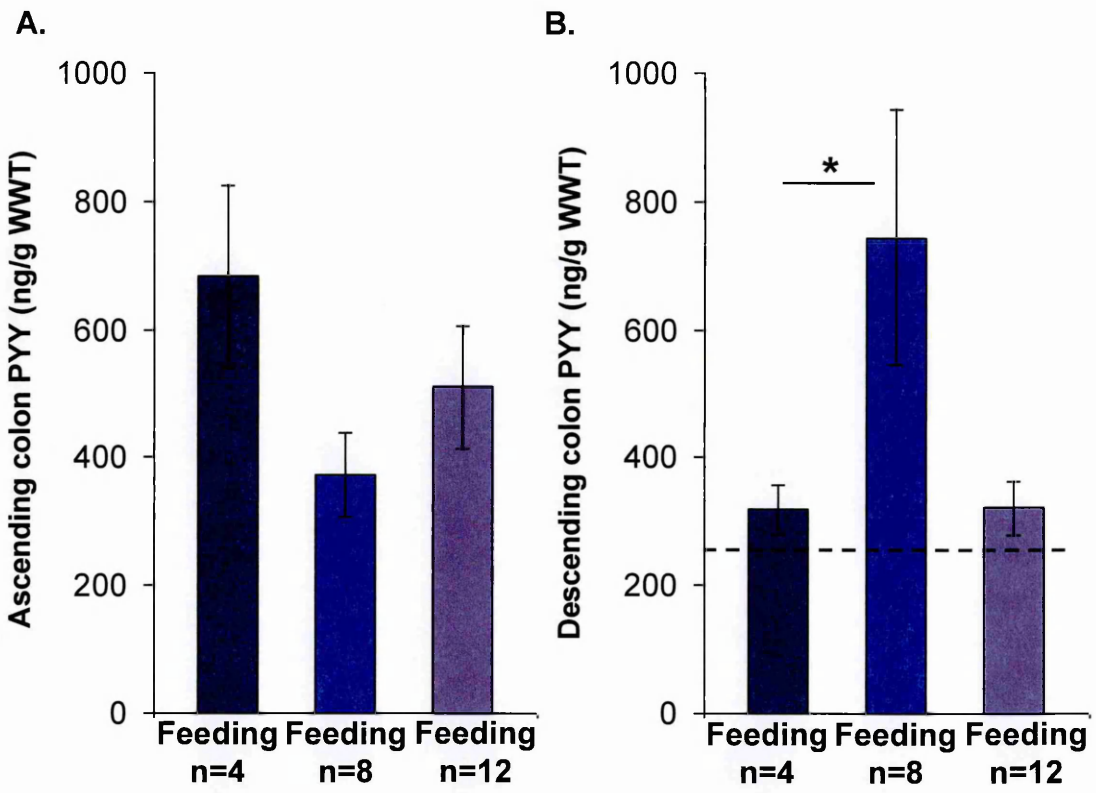

Figure 6.4. PYY concentrations in colon tissue of dams with different lactation litter sizes
(A) ascending and **(B)** descending colon concentrations (n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups; * $P<0.05$). Dotted line in (B) shows approximate proestrus control mean.

6.4.3. Total (GLP-1) peptide analysis

6.4.3.1. Matched fed and fasted plasma comparison

Paired analysis of plasma samples found that GLP-1 was significantly more concentrated ($t(16)= 5.241$, $P<0.001$) in fed (328.9 ± 52.98 pg/ml) plasma than in fasted (44.1 ± 9.56 pg/ml) plasma, as expected.

6.4.3.2. Concentration change with litter size

There were no significant differences between the concentrations of plasma GLP-1 in any of the groups (Table 6.2). In fed plasma, GLP-1 concentration was considerably higher in the lactating dams than in the proestrus controls

(56.2 ± 25.66 pg/ml), yet fasted plasma GLP-1 levels were similar to the proestrus controls (28.9 ± 8.60 pg/ml).

Table 6.2. GLP-1 concentrations in fed and fasted plasma of dams with different lactation litter sizes
(n=7 dams feeding 4 pups; fed plasma: n=5 dams feeding 8 and 12 pups; fasted plasma: n=7 dams feeding 8 pups and n=6 dams feeding 12 pups).

	Fed plasma (ng/ml)	Fasted plasma (ng/ml)
Feeding n=4	499.8 ± 98.83 (166.5 – 879.7)	47.5 ± 15.51 (12.6 – 132.8)
Feeding n=8	168.8 ± 37.60 (78.8 – 259.1)	25.6 ± 9.20 (3.0 – 70.2)
Feeding n=12	319.8 ± 68.57 (180.3 – 562.2	45.6 ± 19.95 (6.0 – 126.0)

There was no difference in either ascending or descending colon GLP-1 concentration between the groups (Figure 6.5). Dams feeding 4 ($t(6)=3.909$, $P=0.008$) and 12 ($t(5)=4.837$, $P=0.005$), but not 8 ($P=0.267$) pups, had a significantly higher concentration of GLP-1 in their ascending colon than the descending colon.

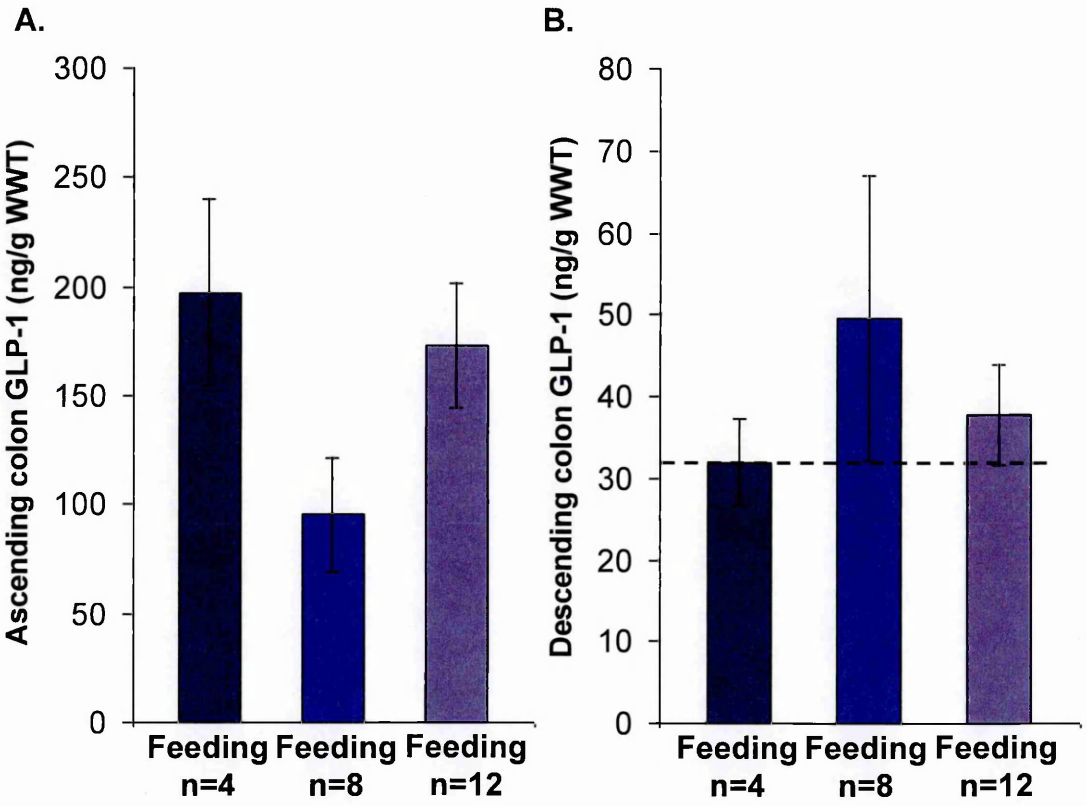

Figure 6.5. GLP-1 concentrations in colon tissue of dams feeding different lactation litter sizes
(A) ascending and **(B)** descending colon concentrations (n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups). Dotted line shows approximate proestrus control mean. (Note y axes scale difference).

6.4.4. Changes to body and gut size in dams feeding different litter sizes

The mean age of all dams was 31.6 ± 1.26 weeks (24 – 39 weeks) of age at the time of culling. Body mass recorded before and after the short fasting period was not significantly different between the groups, and all of the dams lost a similar percentage of their body mass during fasting (Table 6.3).

Table 6.3. Fed and fasted state body mass and the percentage of body mass lost during the fasting period of dams with different lactation litter sizes
(n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups).

	Fed state body mass (g)	Fasted state body mass (g)	Body mass lost on fasting (%)
Feeding n=4	294.4 ± 5.34 (281.1 – 319.1)	268.7 ± 5.02 (250.1 – 292.0)	8.7 ± 1.01 (3.6 – 12.3)
Feeding n=8	300.3 ± 5.96 (275.4 – 317.3)	267.5 ± 5.85 (239.9 – 284.3)	10.9 ± 1.25 (6.9 – 16.5)
Feeding n=12	306.2 ± 11.63 (264.9 – 346.3)	275.8 ± 10.32 (241.6 – 304.2)	9.8 ± 0.92 (8.0 – 13.2)

The mass of abdominal cavity white adipose tissue (WAT) was similar between the dams feeding different litter sizes (Figure 6.6), but was approximately 50% less in comparison to proestrus control animals. Anaesthetised rectal temperature was also not different between the dams (Figure 6.7).

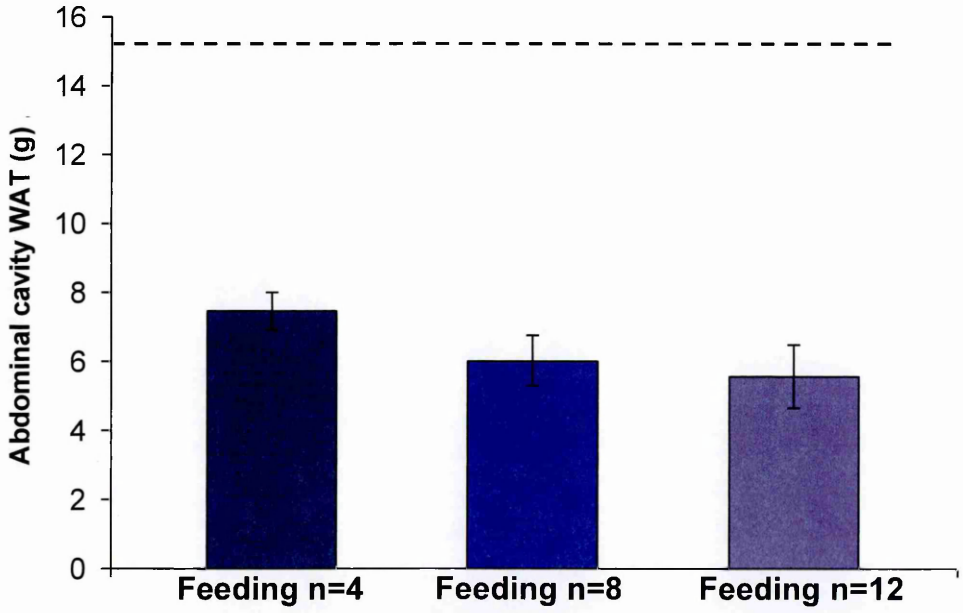

Figure 6.6. Abdominal cavity white adipose tissue (WAT) of dams with different lactation litter sizes
(n=7 dams feeding 4 pups; n=4 dams feeding 8 pups; n=6 dams feeding 12 pups). Dotted line shows approximate proestrus control mean.

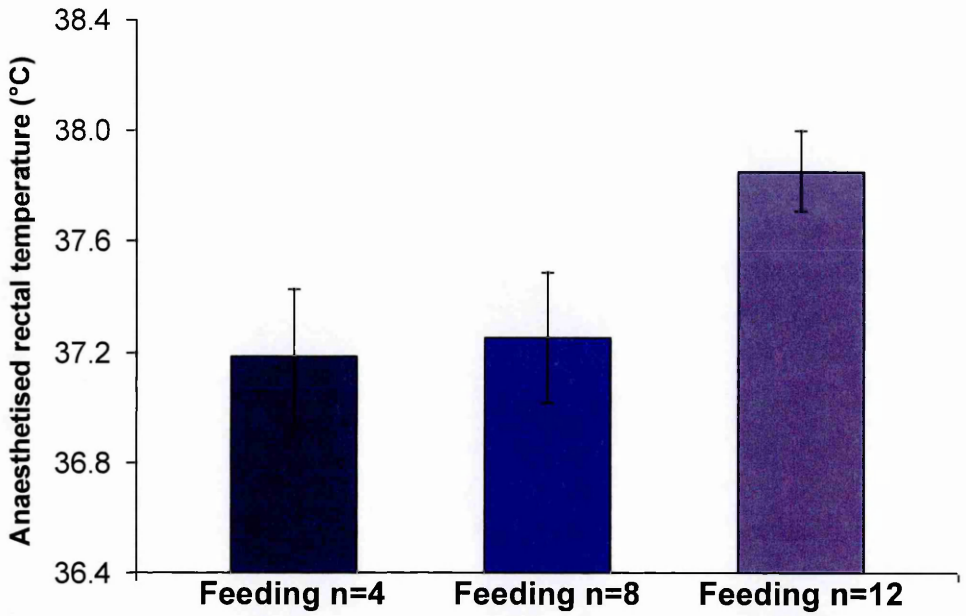

Figure 6.7. Anaesthetised rectal temperature of dams with different lactation litter sizes
(n=7 dams feeding 4 pups; n=4 dams feeding 8 pups; n=6 dams feeding 12 pups).

The wet weight of stomach tissue (Figure 6.8) was not significantly different between the dam groups but was 20 to 40% increased compared to proestrus controls.

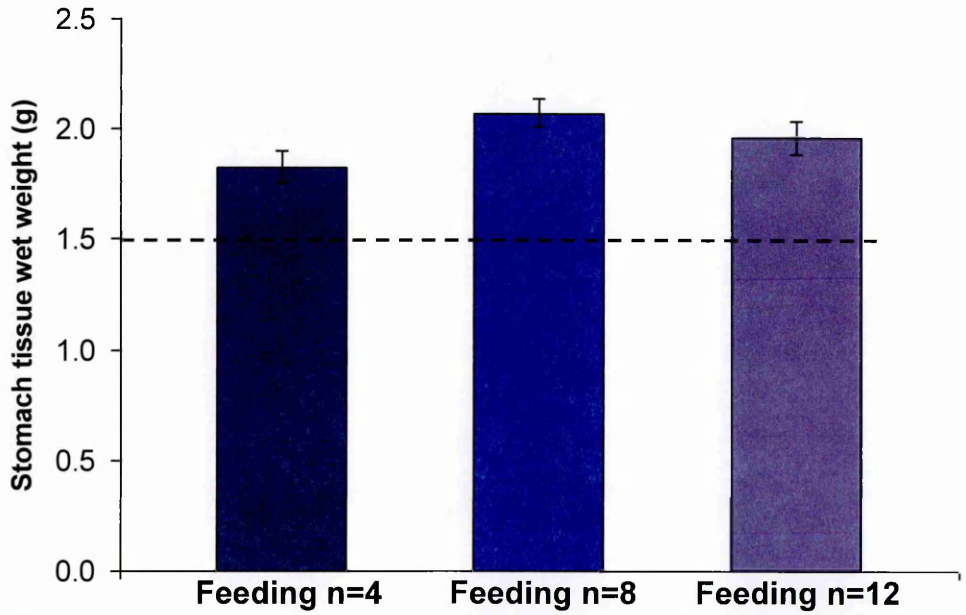

Figure 6.8. Stomach tissue wet weight of dams feeding different lactation litter sizes
(n=7 dams feeding 4 pups; n=6 dams feeding 8 and 12 pups). Dotted line shows approximate proestrus control mean.

Small intestines were significantly lighter ($t(11)= -2.596$, $P=0.025$) in dams feeding 4 pups compared with dams feeding 12 pups (Figure 6.9 A). Due to differences in sample collection, the wet weight of small intestine tissue is not available for the dam group feeding the control litter size of 8 pups or proestrus controls (see section 2.4.1). Small intestines were also significantly shorter ($F(2, 17)=6.222$, $P=0.009$) in dams feeding 4 pups than in dams feeding 8 pups ($P=0.007$; Figure 6.9 B). All of the dams had small intestines that were, on average, 1.3 times longer than in the proestrus controls.

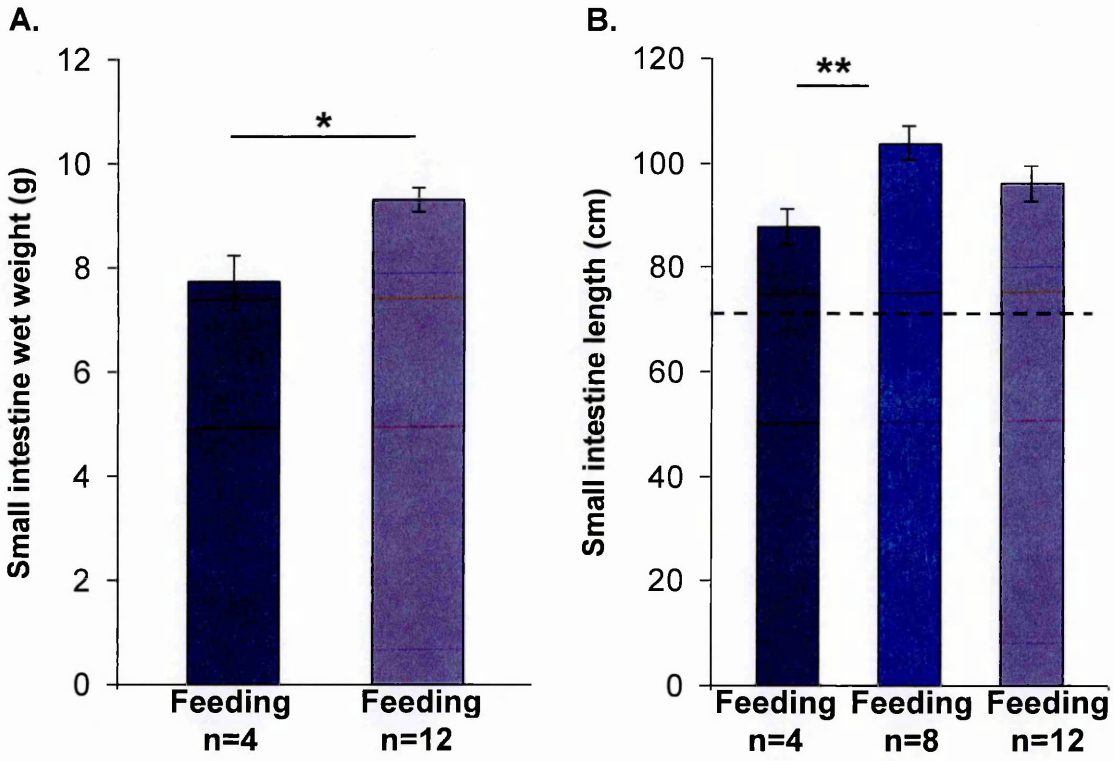

Figure 6.9. Small intestine size of dams with different lactation litter sizes
(A) small intestine wet weight and **(B)** length (n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups; * $P<0.05$; ** $P<0.01$). Dotted line shows approximate proestrus control mean.

Log transformation of caecum wet weight showed a significant difference between the groups ($F(2, 16)=31.512$, $P<0.001$), with dams feeding the control litter size having significantly heavier caecum tissue than dams

feeding the small (P<0.001) and large (P=0.001) litter sizes. Caecum wet weight was also significantly lighter in dams feeding small litters than those feeding large litters (P=0.036; Figure 6.10).

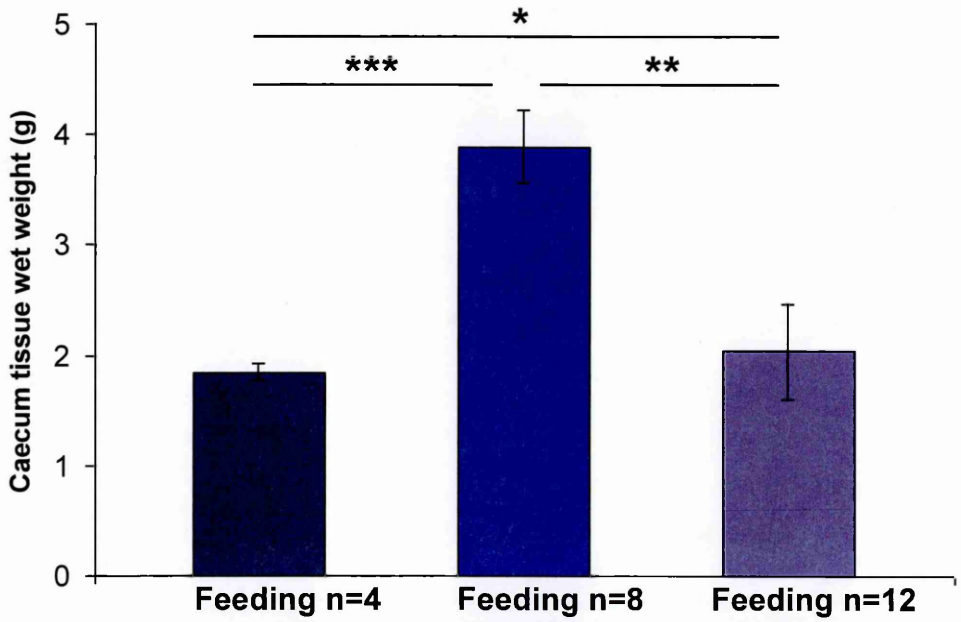

Figure 6.10. Caecum tissue wet weight of dams with different lactation litter sizes (n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups; * P<0.05; ** P<0.01; *** P<0.001).

Large intestine tissue (Figure 6.11 A) was significantly heavier (Kruskal-Wallis, χ^2=13.111, 2 df, P=0.001) in dams feeding 8 pups than in dams feeding 4 pups (P=0.001). The large intestine was significantly longer (F(2, 17)=10.042, P=0.001) in dams feeding 8 pups when compared with those feeding 4 (P=0.001) and 12 (P=0.039) pups (Figure 6.11 B). The large intestine length of proestrus controls (n=4) was similar to the dams feeding litters of 4 pups.

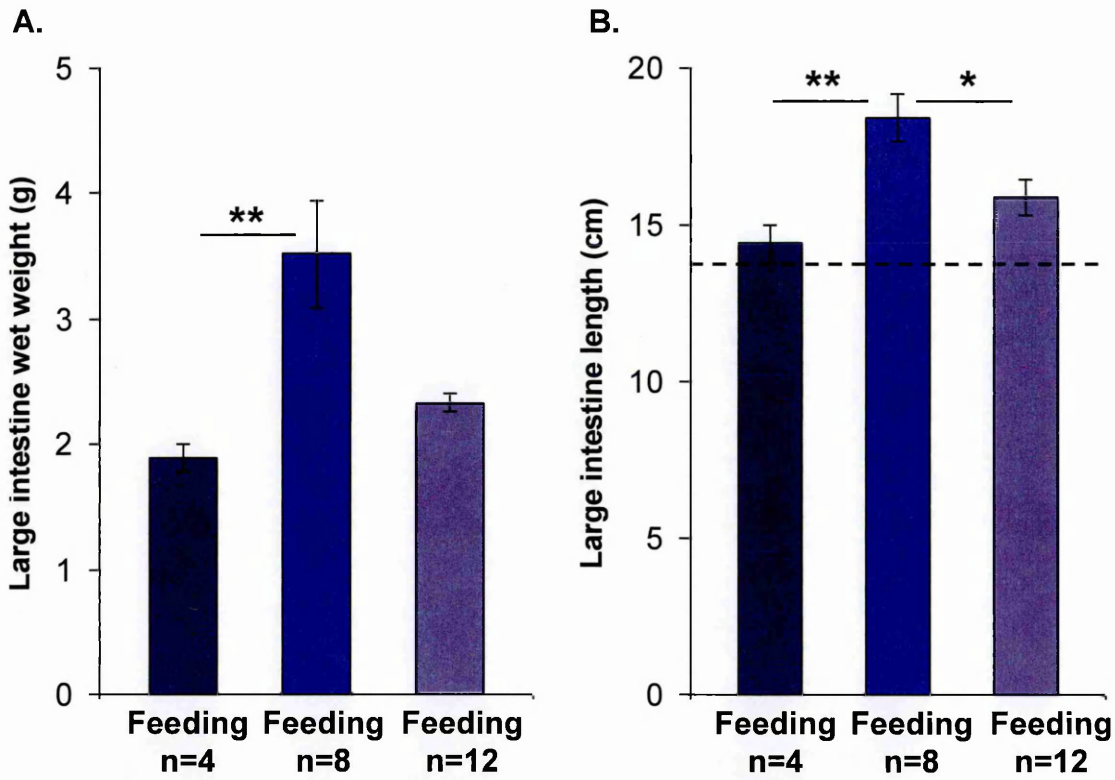

Figure 6.11. Large intestine size of dams with different lactation litter sizes
(A) large intestine wet weight and **(B)** length (n=7 dams feeding 4 and 8 pups, except wet weight, where n=5 dams feeding 8 pups; n=6 dams feeding 12 pups; * $P<0.05$; ** $P<0.01$). Dotted line shows approximate proestrus control mean.

Duodenum ($F(2, 17)=6.961$, $P=0.006$), ascending colon ($F(2, 17)=9.576$, $P=0.002$) and descending colon ($F(2, 17)=9.998$, $P=0.001$) circumferences were significantly different between the dam groups (Figure 6.12). Duodenum circumference was significantly smaller in dams feeding 8 pups compared with dams feeding 12 pups ($P=0.005$). In both ascending and descending colon, circumferences were significantly smaller in dams feeding 8 pups compared with both dams feeding 4 and 12 pups.

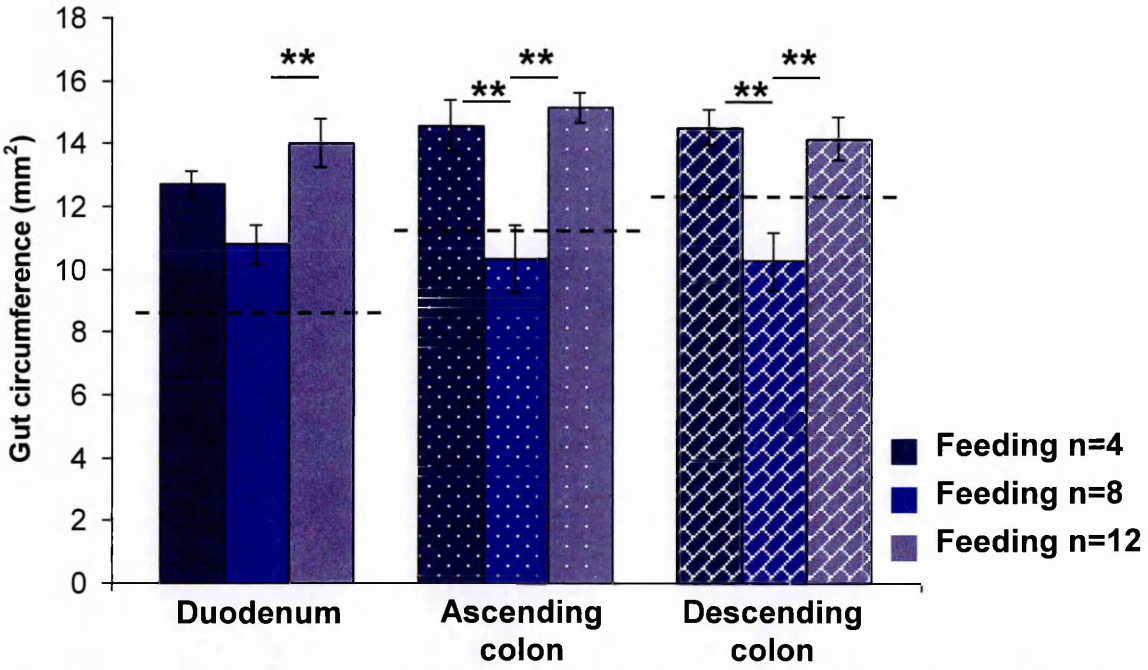

Figure 6.12. Circumferences of duodenum, ascending and descending colon of dams with different lactation litter sizes
(n=7 dams feeding 4 and 8 pups; n=6 dams feeding 12 pups; ** $P<0.01$). Dotted lines show approximate proestrus control mean.

6.4.4.1. Mass of gut contents

The mass and pH (Table 6.4) of fasted stomach contents in the dams did not change significantly with litter size.

Table 6.4. Stomach contents wet weight and pH of dams with different lactation litter sizes
(n=5 dams feeding 4 and 8 pups; n=4 dams feeding 12 pups).

	Stomach contents (g)	Stomach contents pH
Feeding n=4	0.9 ± 0.15 (0.4 – 1.5)	3.3 ± 0.25 (2.4 – 3.9)
Feeding n=8	1.0 ± 0.13 (0.5 – 1.4)	4.5 ± 0.39 (3.0 – 5.3)
Feeding n=12	0.9 ± 0.15 (0.4 – 1.4)	3.8 ± 0.54 (2.3 – 4.8)

The mass of caecum contents were significantly ($F(2, 16)=7.921$, $P=0.004$) heavier in dams feeding the largest litter size than in those feeding 4 ($P=0.004$) and 8 ($P=0.020$) pups (Figure 6.13). However, the pH of caecum

contents was not significantly different between the dams feeding small (6.8 ± 0.15 pH), control (6.3 ± 0.11 pH) and large litter (6.8 ± 0.15 pH) sizes.

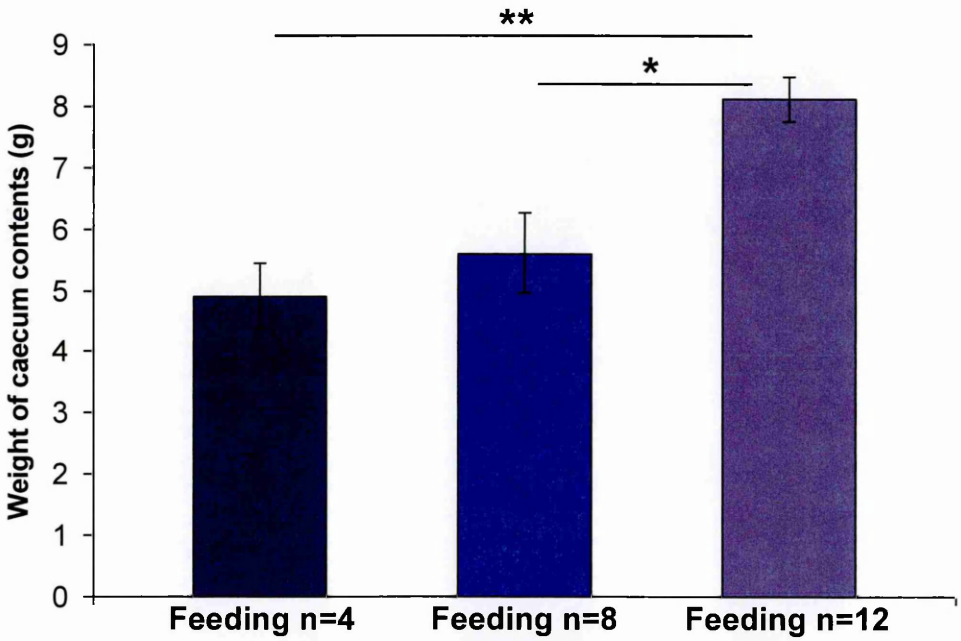

Figure 6.13. Caecum contents wet weight of dams with different lactation litter sizes (n=7 dams feeding 4 and 8 pups; n=5 dams feeding 12 pups; * $P<0.05$; ** $P<0.01$).

Small and large intestine contents were not different between the dam groups (Table 6.5).

Table 6.5. Small and large intestine contents of dams with different lactation litter sizes
(n= 7 dams feeding 4 pups; n=6 (stomach) and n=5 (large intestine) dams feeding 8 pups; n=6 dams feeding 12 pups).

	Small intestine contents (g)	Large intestine contents (g)
Feeding n=4	2.8 ± 0.51 (1.8 – 5.7)	3.4 ± 0.98 (0.0 – 7.7)
Feeding n=8	No data	3.2 ± 0.97 (0.3 – 6.2)
Feeding n=12	4.2 ± 0.84 (2.2 – 6.8)	2.4 ± 0.92 (0.2 – 6.6)

6.5. Discussion

The work described in this chapter was intended to extend that of the previous chapter, which established changes to gut hormone levels and gut size at different stages of lactation. Here, the aim was to determine whether the changes in ghrelin, PYY and GLP-1 levels and gut size during lactation are influenced by the number of pups the dams were feeding.

Ghrelin in fed plasma was significantly reduced and stomach tissue ghrelin was significantly increased in dams feeding 12 pups compared to dams feeding the control litter size of 8 pups. Although there were no changes in plasma PYY levels, PYY in descending colon was significantly reduced in dams feeding 4 pups compared to those feeding 8 pups. No significant differences were found in GLP-1 levels in any of the samples. No differences were evident in body mass, temperature or abdominal white adipose tissue (WAT), but there were changes in some gut tissues. The small intestine was significantly lighter in dams feeding 4 pups compared to those feeding 12, and significantly shorter in dams feeding 4 than those feeding 8 pups. The wet weight of caecum tissue was significantly lightest in dams feeding the smallest litter size. The large intestine was also significantly lighter in dams feeding 4 pups than those feeding 8, and was significantly longer in dams feeding 8 pups than the other dams. Despite not having the longest or heaviest gut, dams feeding 12 pups had significantly larger gut circumferences than the dams feeding 8 pups.

6.5.1. Orexigenic gut hormone changes resulting from lactation litter size

There was no difference in fasted plasma ghrelin levels, but in fed plasma, ghrelin levels were significantly lower in dams feeding 12 pups compared with dams feeding 8 pups. It seems reasonable to speculate that the dams feeding the most pups would eat more and/or more frequently, which may explain why they had significantly reduced plasma ghrelin in the fed state when they may have been expected to have increased plasma ghrelin to increase food intake. Stomach tissue ghrelin was significantly increased in the same dams after the short period of fasting, which is suggestive of more stored ready for release, with potential to increase food intake most in the dams feeding the most pups.

One question that arose from the results described in Chapter 3 was whether the rats were adequately fasted, due to no significant difference being found between fed and fasted plasma ghrelin levels in the matched samples (section 3.5.1.1). Although absolute concentration differences cannot be explored here with a paired t-test (discussed in section 2.5.3), the results in this chapter suggest that fasting may have standardised ghrelin levels between the dams due to a significant difference found in fed, but not fasted plasma ghrelin levels. However, fed plasma ghrelin was only quantified in 4 dams, to match with their pups analysed in the next chapter, so this result would need to be replicated using a greater number of dams. In the fasted state, significantly increased stomach tissue ghrelin in the absence of any increase in plasma ghrelin in the dams feeding 12 pups may also suggest

that the fasting length was adequate in these animals. The fasting period was for a long enough period to cause increased ghrelin production by the stomach, before a more chronic starvation pattern presented, where one may additionally expect increased ghrelin levels in the plasma to increase food intake.

6.5.2. Anorexigenic gut hormone changes resulting from lactation litter size

Despite there being no differences in the levels of PYY in the circulation between the dams, descending colon levels were significantly higher in dams feeding 8 pups compared to those feeding 4 pups. Dams feeding 12 pups also had reduced descending colon PYY levels in comparison to dams feeding 8 pups, but this difference was not significant. It is possible that the increased level of descending colon PYY in dams feeding 8 pups is linked with increased gut size in this group compared to the other dams; this will be discussed later (section 6.5.3).

There were no significant changes in GLP-1 levels in either plasma or gut tissue between the dams feeding different litter sizes. This finding may indicate that feeding different litter sizes did not have a great effect on glucose regulation in the dams because GLP-1 levels are known to change for instance, in insulin-resistant states (Lim *et al.*, 2009, Muscelli *et al.*, 2008, Toft-Nielsen *et al.*, 2001). Similar to the data reported in the previous chapter, all of the dams here had fed plasma GLP-1 levels that were 200 to 789% elevated compared to proestrus controls, which reduced after fasting to just

above control levels (11 to 64% increase). This finding may suggest that GLP-1 was increased appropriately in the fed state in response to food intake. Assuming that food intake in these dams was considerably higher than in nulliparous controls, as has previously been reported during late lactation (Crean and Rumsey, 1971, Cripps and Williams, 1975, Denis *et al.*, 2004), these dams may not have been as sensitive to the satiating effects of increased levels of GLP-1, which may suggest a level of resistance to it, as is seen during pregnancy with resistance to increased leptin levels (Friedman, 2011, Ladyman *et al.*, 2010).

6.5.3. Maternal gut size changes resulting from lactation litter size

No changes were found in body mass, abdominal cavity WAT or anaesthetised rectal temperature between the dam groups. One study reported no significant differences in total abdominal adiposity in women with different lactation durations, but did report a significant difference in visceral adiposity (McClure *et al.*, 2012). It is therefore possible that adiposity may have been altered in different areas of the body in the study dams, and this possibility could be one avenue for future study.

The work described in the previous chapter established that significant gut growth occurred from mid-lactation onwards and measurements were highest at day 25 of lactation, which is the stage at which the dams in this chapter were sacrificed. There was no significant difference in the wet weight of the

stomach tissue, although all dams had heavier stomachs than proestrus controls, which is consistent with the findings from the previous chapter.

The wet weight of the small intestine was significantly reduced in the dams feeding 4 pups compared to dams feeding 12 pups, but there were no data for the dams feeding control litter sizes of 8 pups. The length of the small intestine was significantly reduced in the dams feeding 4 pups compared to those feeding 8 pups but was, surprisingly, similar in length in the dams feeding litters of 12 pups. Only one other study has explored the effects that halving litter size has on maternal small intestines. Datta *et al.* (1995) reported that dams with a restricted litter size of 4 pups had small intestines that were not significantly different in length or wet weight than the lactating dam group with control litter sizes of 8 pups. It is possible that the difference between the present study and that of Datta *et al.* (1995) is a rat strain difference or due to method differences, as these authors standardised length measurement differently to this study by stretching the tissue.

Empty caecum mass was significantly reduced in dams feeding 4 pups compared to the other dams, however, dams feeding 8 pups had significantly increased caecum mass compared to those feeding 12 pups. Again, this finding was surprising as it indicates significant tissue hypertrophy in the dams feeding 8 pups compared to dams feeding 12 pups and one may expect gut size to increase in proportion to the increased demand of suckling more pups. Why dams feeding 8 pups had significantly increased gut size compared to dams feeding 12 pups remains to be established. Although the

dams feeding 12 pups had significantly lighter empty caecums compared to the dams feeding 8 pups, they had 45% more contents in their caecums. This, combined with significantly increased stomach tissue ghrelin, may indicate that the dams feeding the large litter sizes had a more profound hyperphagia and consumed more food prior to the fasting period. Future work would have to determine dam food intake, independently of any diet that the pups may consume, to confirm this suggestion.

In addition to having larger small intestines and caecums, dams feeding 8 pups had heavier and longer large intestines compared to dams feeding 4 pups and longer large intestines than dams feeding 12 pups. The decreased small intestine, caecum and large intestine size in the dams feeding 4 pups may represent a reduced requirement to physically adapt to lactation, as the metabolic drain would be reduced in comparison to that of dams feeding more pups. However, it was the dams feeding 8 pups, not those feeding 12, that had the most increased gut measurements, when it was hypothesised that the gut would increase in size in relation to the number of pups the dam was feeding. It is possible that the dams feeding 12 pups were simply too energy deficient to be able to support gut growth as an adaptation to lactation. An increased level of gut growth in the dams feeding 8 pups could be related to a significantly increased level of descending colon PYY in the same animals. The possibility of colonic PYY being involved in increased gut growth during the latter stages of lactation was discussed in detail in Chapter 5 (section 5.5.4.1), with the work of Gomez *et al.* (1995) suggesting that increased PYY is a direct cause of gut growth.

As previously discussed (section 5.5.4.1), studies in rats have shown that food restriction prevents small intestine hypertrophy in lactating rats (Campbell and Fell, 1964, Datta *et al.*, 1995). It may be reasonable to assume that although not food restricted, the dams feeding 12 pups were probably less able than the other dams to match food intake and nutrient absorption to their nutritional requirement and may therefore have been more energy deficient. If this was the case, energy deficiency could prevent increased gut growth in dams feeding 12 pups in a similar way as is reported to occur in food restricted dams; the dams feeding litter sizes of 8 pups may have more of an energy reserve to enable support of gut growth. Measurement of gut circumferences provides support for this possibility. The dams feeding 12 pups had significantly increased circumferences in the duodenum, ascending and descending colon compared to the dams feeding 8 pups. Increasing gut capacity appears to be a key adaptation during lactation and it could be that the dams feeding 12 pups have achieved this by increasing gut circumference without increasing gut length, which could be a less energetically costly adaptation. Also in support of this hypothesis was a larger ascending and descending colon circumference in dams feeding 4 pups compared to dams feeding 8 pups. Dams feeding the small litter sizes also had reduced gut growth in comparison to those feeding 8 pups. In their case, it may be that whilst they have reduced requirement to optimise nutrient absorption compared to dams suckling more pups, they may have similar requirements to excrete additional waste and thus have increased colon circumference compared to the dams feeding 8 pups that are more able to accommodate their waste material with longer gut tissue. Future

study to establish the cause of changes in gut circumference would have to be undertaken to further this work, microscopically examining and measuring each layer of the gut. Gut circumference could be changed by thickening or thinning of the muscle and/or the epithelial layers. Determining exactly what changed the gut circumference would further the understanding of maternal gut changes as an adaptation to lactation.

6.6. Conclusions

This study has, for the first time, explored the effects that suckling different litter sizes had on gut peptide levels and gut size in rat dams. Dams feeding 4 pups had significantly smaller small intestine, caecum and large intestine tissues compared with dams feeding 8 pups and it is suggested that this was due to a reduced suckling demand. It is likely that significantly increased descending colon PYY in dams with 8 pups is linked with increased gut size in these dams. Despite no increase in gut length or wet weight, dams feeding 12 pups had significantly increased gut circumferences, which may represent an alternative adaptation to lactation that requires less available energy. Energy insufficiency is thought to be the reason why dams feeding 12 pups did not have increased gut size compared to the dams feeding 8 pups. This work additionally highlights the importance of standardising litter size very early in lactation when studying the dams, as unseen changes to the metabolic status and gut size of the dam may occur early in lactation. It is possible that similar changes to gut size may occur in humans during the lactation period, and that duration of lactation, or a multiple birth, or even overlap, may additionally affect gut growth.

6.7. Future work

It would be of great interest to quantify the food intake of dams feeding different litter sizes, to see whether it is increased in proportion to the number of pups in the litter. This would require special cages that kept the pups separated from the cage diet but not their mothers. Examination of adiposity in different areas of the body may also provide additional information to the results from this study.

Replicating this experiment, but sacrificing dams at time-points throughout lactation would also be a direction for future study. Determining exactly when the differences become evident between the dams feeding different litter sizes may provide useful information for other studies on timings of litter manipulation, for instance. To further elucidate the possible reasons for increased gut growth during lactation, analysis of gut transit times in the dams would aim to determine whether changes to gut circumference may be a mechanism to alter gut transit time in the absence of gut growth. Such work could be supported by additional groups of dams, with different litter sizes, that are food restricted to differing degrees to test the possibility that gut size is reduced in the dams feeding 12 pups compared to those feeding 8 pups due to energy insufficiency. It would also be of interest to explore whether the structural adaptations found in dams feeding 8 pups remain after the lactation period and whether this could contribute to, or refine, adaptation to future pregnancies and lactation periods.

CHAPTER 7: CHANGES IN OFFSPRING GUT APPETITE HORMONES AND STOMACH SIZE DUE TO LACTATION LITTER SIZE

7.1. Introduction

Having established that differences occurred in maternal gut peptides and gut size when dams were feeding different lactation litter sizes, this chapter presents data on their male and female offspring. Chapter 1 (section 1.8.1) reviewed some of the studies that have established the adverse effects that maternal under- or over-nutrition has on the health of their offspring, but this was not a focus of this chapter. Here, dams were fed *ad libitum* on a standard diet with the only difference being lactation litter size. The effects of being raised in different lactation litter sizes were studied in both the levels of gut appetite hormones and in the body and gut size of male and female offspring at weaning age, without any other manipulation.

7.1.1. Body size is affected by lactation litter size

A number of studies have examined the effects that litter size has on the physical size of pups, with studies often only reporting results for males. One study (Widdowson and McCance, 1963) explored the effects that small (n=3, male pups only) and large (15 to 20, males and females) litter sizes had on body size in male rat pups. Pups suckled in large litters had reduced body mass in comparison to pups from the small litters, a difference that continued past 40 weeks of age, which demonstrated that the effects of litter size on

pups during lactation affects body mass into adulthood. Body length was also increased in rats from small litter sizes compared to those from large litters when measured at 6, 9 and 12 weeks of age. A later study also reported significantly increased body mass in male rat pups from small litters (n=3 to 4) compared to controls (n=12 per litter) throughout their study (until 8 months of age), however found no body mass difference between pups from large (n=20 to 24) and control litter sizes into adulthood, suggesting that pups from large litter sizes may not be as affected by litter size differences as those from small litters (Plagemann *et al.*, 1992). A further study by this group (Plagemann *et al.*, 1999) found that differences in body mass were evident from 8 days of age. By 21 days of age (weaning) in that study, pups from small litters (n=3) were significantly heavier and longer than controls, with body mass relative to body length also having significantly increased.

Results from the above studies suggest that male rat pups from small litters have increased body size than those from large litters, and that this difference may persist into adulthood. This change may lead to increased susceptibility to obesity later in life.

It has been reported that male rats from small litters (n=4) had significantly increased body adipose content than rats from large litters (n=16) at both weaning and 12 weeks of age (Balonan and Sheng, 2000). Indeed, another study documented that male rats at weaning had 3 times greater total adipose mass and double the percentage adiposity when from small litters (n=4) compared to control litters (n=10 to 12) (Schmidt *et al.*, 2001), which

was in agreement with an earlier study (Faust *et al.*, 1980). Faust *et al.* (1980) reported that male rats from the small litters (n=4, male pups only) weighed significantly more and had significantly increased adiposity than male rats from large litters (n=20, mostly male pups) at weaning and at 11 months of age. When fed a HFD from 6 months of age, all rats in that study gained in both body mass and percentage adiposity, but there was no significant difference by litter size and all pups had similar plasma insulin levels. However, the wet weight of adipose tissue from the major adipose depots (epididymal, retroperitoneal and subcutaneous adipose tissue) was significantly increased in male rats from small litter sizes (Faust *et al.*, 1980). Collectively, these data suggest that male rats from small litter sizes may be more likely to have increased body mass and adiposity into adulthood than those from larger litter sizes, with increased susceptibility to becoming overweight.

Adding to the data on pup adiposity as a result of litter size is a study that reported a significantly increased rate of whole body adipose mass accumulation throughout each day of the lactation period in pups (males and females) from small litters (n=4) compared to pups from control (n=10) and large (n=16) litters (Fiorotto *et al.*, 1991).

7.1.2. Other pup changes due to lactation litter size

Some studies have additionally reported metabolic changes in pups as a result of their lactation litter size. Faust *et al.* (1980) reported no change in insulin concentrations between pups from small and large litter sizes at 11

months of age, however others have documented differences in younger rats. In two studies, Plagemann *et al.* found that plasma insulin concentrations were significantly increased in male pups from small litters in comparison to pups from the control litter sizes at 15 and 21 days old (Plagemann *et al.*, 1999, Plagemann *et al.*, 1992). Balonan and Sheng (2000) similarly reported that plasma insulin levels were significantly reduced in male rat pups from large litters (n=16) than in control (n=10) and small (n=4) litters at weaning, but that this difference was not seen at 12 weeks of age. Plagemann *et al.* (1999) and Schmidt *et al.* (2001) additionally reported that plasma leptin concentrations were significantly different between pups from the litter size groups, positively correlating with body mass.

Further data from Plagemann *et al.* (1992) additionally showed that the larger male rats suckled in small litters had significantly higher systolic blood pressure than rats from control and large litters. Glucose regulation of the males raised in small litters was also more affected after a small dose of the pancreatic β-cell-toxic drug streptozotocin in adulthood, which the authors suggest demonstrated an increased susceptibility to the development of diabetes compared to the rats suckled in control litter sizes.

7.1.3. Potential differences in appetite regulation due to lactation litter size

Analysis of the orexigenic hypothalamic peptide neuropeptide-Y (NPY) in both the arcuate nucleus (ARC) and paraventricular nucleus (PVN) found significantly increased NPY levels and significantly more NPY-positive

neurons in the ARC of pups from large litters (n=18) compared to control litters (n=10), with no difference in pups from small litters (n=3) compared to controls (Plagemann *et al.*, 1999). These authors suggest that no change in hypothalamic NPY in rats from small litters compared to controls, despite hyperleptinaemia and hyperinsulinaemia, which would usually cause a down-regulation of NPY, demonstrated a potential resistance that is similar to that seen in obese adult rats, thus inferring a susceptibility to an obese phenotype in male rat pups from small litters.

It is clear from previous work in this area that pups are affected by their lactation litter size, both in body size and, most importantly, metabolically. It is possible that the effects outlined here may persist into adulthood. The current study aims to add to this research by establishing whether the appetite hormones ghrelin, PYY and GLP-1 in the circulation and relevant gut tissues are affected by altering the lactation litter size. Analysis of both male and female pups additionally aims to determine whether there are sex differences to the body size changes reported by others, as the vast majority of studies only report findings from male offspring.

This type of study has the potential to change the way future laboratory studies on, for example, obesity are carried out, because results on appetite hormone concentrations in adult animals from these studies may be biased by the simple fact that they came from different sized litters during lactation. Breeding study animals in-house may be the optimal way to obtain animals for such studies, to ensure that peri- and postnatal growth is controlled for.

7.2. Objectives

Objectives for this chapter were to:

- determine whether male and female pups from different lactation litter sizes have different levels of ghrelin, PYY and GLP-1 in either gut tissue or circulation at weaning age;
- examine the body size of male and female pups at the end of the suckling period to establish how lactation litter size affects pup growth and whether this is sex specific.

7.3. Methods

7.3.1. Animals

For each dam used at day 25 of lactation (d25L), 2 male and 2 female pups (where possible) were dissected alongside the dam. As described in Chapter 6 (section 6.3.1), dams had their lactation litter size adjusted to either 4 pups (n=7; small litters), 8 pups (n=7; control litters) or 12 pups (n=6; large litters) by minimal cross-fostering.

7.3.2. Sample collection

Gut tissue was collected from pups as described in Chapter 2 (section 2.3). Due to time restrictions, only measurements of body mass, body length and stomach tissue wet weight were possible (section 2.4). 1 male and 1 female pup from each litter were used to bank fixed tissue for future experiments and the second male and female pup had gut tissue frozen for peptide extraction.

Trunk blood was obtained from all pups and classed as a fed sample because pups were kept with dams throughout dam fasting.

7.3.3. Peptide assays

Radioimmunoassays on pup plasma and tissue extracts were performed as described in Chapter 2 (section 2.5). Plasma and gut tissue from 8 pups for each litter size sample group (1 male and 1 female pup per litter) were analysed for ghrelin, PYY and GLP-1.

7.4. Results

7.4.1. Total ghrelin peptide analysis

Fed plasma ghrelin was not significantly different between pups from different sized litters for the whole group (Figure 7.1) or when split by sex (Table 7.1).

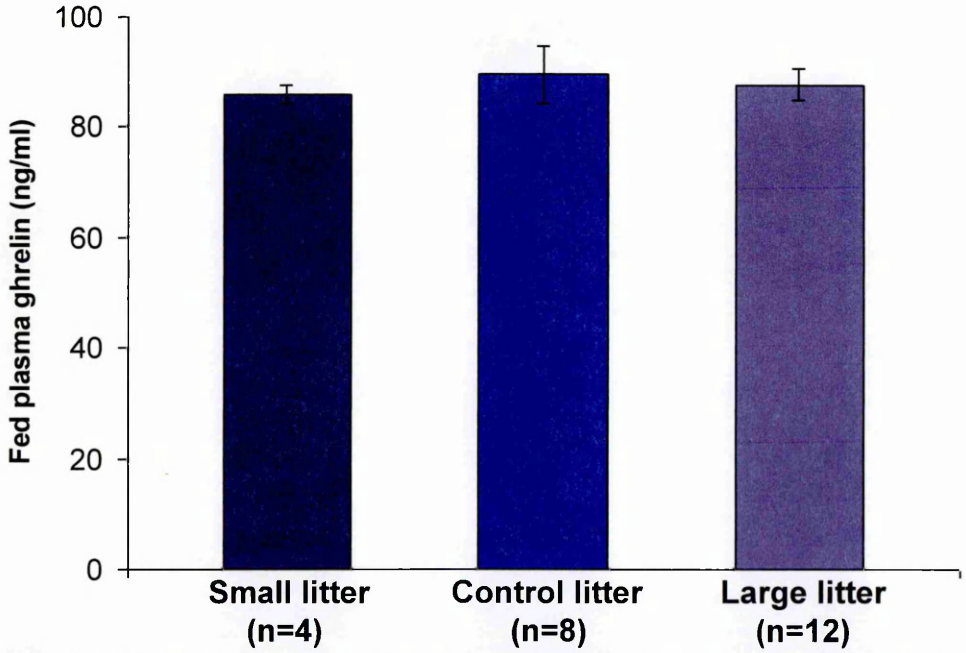

Figure 7.1. Ghrelin concentrations in fed plasma of pups from different lactation litter sizes
(n=8 pups per group).

Table 7.1. Ghrelin concentrations in fed plasma of male and female pups from different lactation litter sizes
(n=4 pups per sex per group).

	Fed plasma ghrelin (ng/ml)	
	Male pups	Female pups
Small litter (n=4)	86.6 ± 1.64 (82.6 – 89.6)	85.1 ± 2.82 (79.7 – 92.7)
Control litter (n=8)	90.8 ± 6.57 (78.3 – 106.7)	88.2 ± 9.09 69.0 – 108.6)
Large litter (n=12)	90.4 ± 3.93 (82.6 – 97.9)	85.0 ± 4.53 (72.7 – 93.0)

Stomach tissue ghrelin concentrations were also not different between the pups (Figure 7.2; Table 7.2).

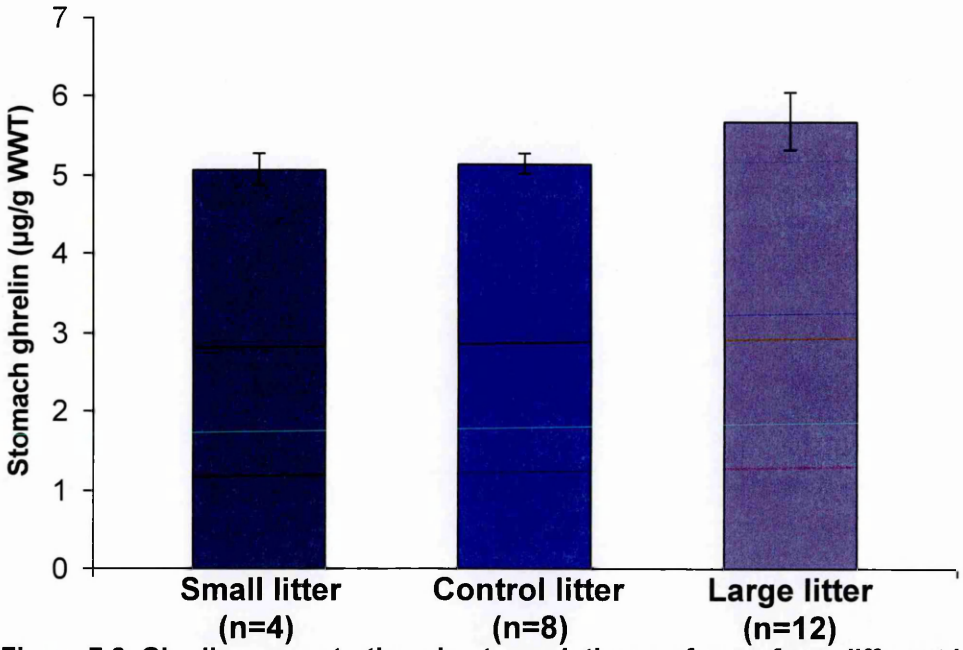

Figure 7.2. Ghrelin concentrations in stomach tissue of pups from different lactation litter sizes
(n=8 pups per group).

Table 7.2. Ghrelin concentration in stomach tissue of male and female pups from different lactation litter sizes
(n=4 pups per sex per group).

	Stomach tissue ghrelin (µg/g WWT)	
	Male pups	Female pups
Small litter (n=4)	5.2 ± 0.31 (4.4 – 6.0)	4.9 ± 0.27 (4.4 – 5.5)
Control litter (n=8)	5.1 ± 0.21 (4.6 – 5.6)	5.2 ± 0.18 (4.7 – 5.4)
Large litter (n=12)	5.8 ± 0.57 (4.6 – 7.2)	5.6 ± 0.52 (4.4 – 6.9)

7.4.2. Total peptide-YY (PYY) peptide analysis

Fed plasma PYY was not significantly different between the pups from litters of different sizes either analysed as a whole group (Figure 7.3) or split by sex (Table 7.3).

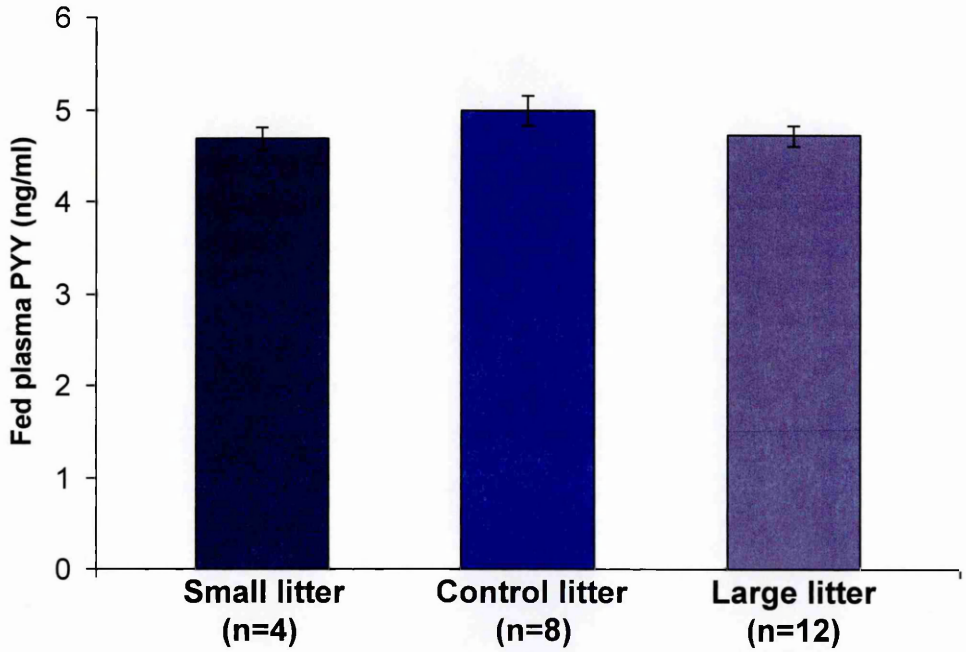

Figure 7.3. PYY concentrations in fed plasma of pups from different lactation litter sizes
(n=8 pups per group).

Table 7.3. PYY concentrations in fed plasma of male and female pups from different lactation litter sizes
(n=4 pups per sex per group).

	Fed plasma PYY (ng/ml)	
	Male pups	Female pups
Small litter (n=4)	4.7 ± 0.19 (4.2 – 5.1)	4.7 ± 0.20 (4.1 – 5.1)
Control litter (n=8)	4.9 ± 0.15 (4.6 – 5.2)	5.1 ± 0.31 (4.6 – 5.9)
Large litter (n=12)	4.6 ± 0.14 (4.3 – 5.0)	4.8 ± 0.16 (4.5 – 5.2)

Ascending colon PYY concentration was also not different between the pups from lactation litters of different sizes when data were analysed either for the whole groups (Figure 7.4) or when the data were split by the sex of the pups (Table 7.4).

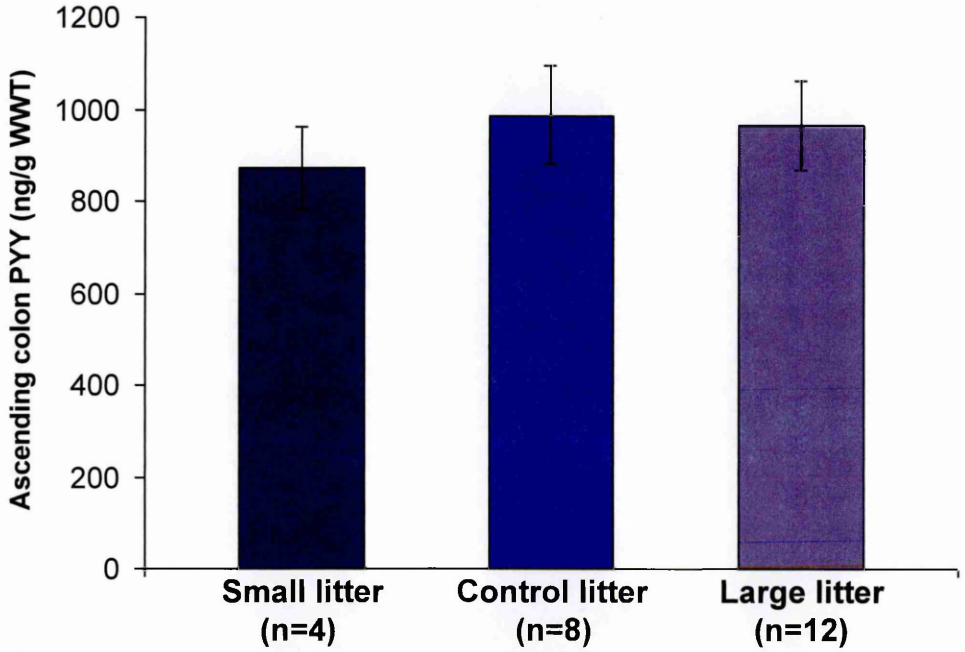

Figure 7.4. PYY concentrations in ascending colon tissue of pups from different lactation litter sizes
(n=8 pups per group).

Table 7.4. PYY concentrations in ascending colon tissue of male and female pups from different lactation litter sizes
(n=4 pups per sex per group).

	Ascending colon tissue PYY (ng/g WWT)	
	Male pups	Female pups
Small litter (n=4)	915.0 ± 124.25 (686.0 – 1263.2)	832.1 ± 144.81 (539.3 – 1224.4)
Control litter (n=8)	870.2 ± 181.57 (329.7 – 1104.0)	1105.1 ± 106.25 (877.0 – 1288.5)
Large litter (n=12)	906.2 ± 172.08 (411.4 – 1193.7)	1023.9 ± 112.45 (777.9 – 1249.4)

PYY in descending colon tissue was significantly different between the pups from litters of different sizes ($F(2, 21)=5.580$, $P=0.011$) and was significantly less concentrated in pups from the large litter size compared to pups from the small ($P=0.047$) and control ($P=0.013$) litter sizes (Figure 7.5).

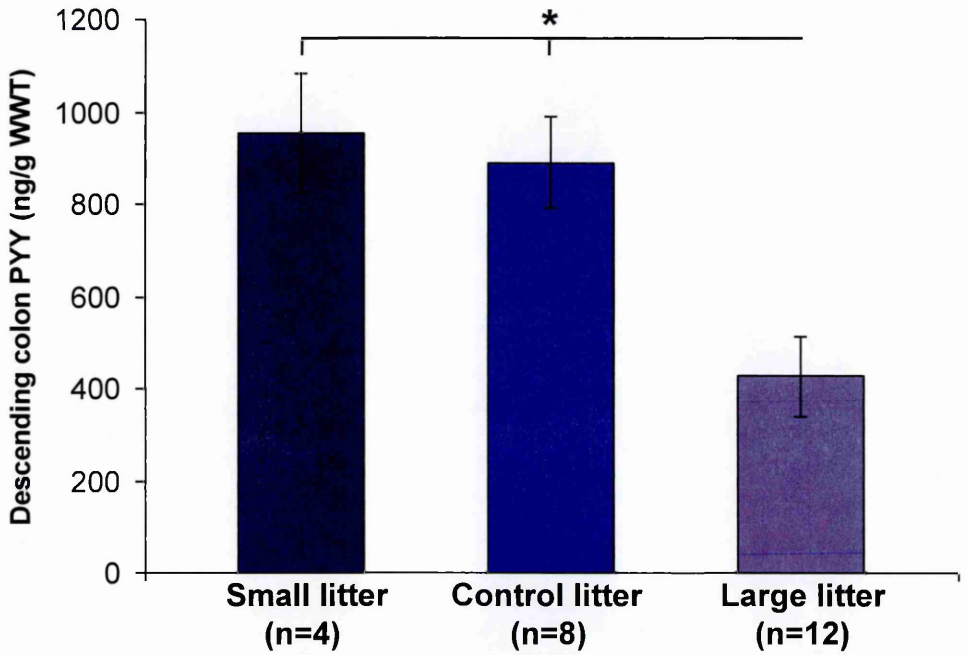

Figure 7.5. PYY concentrations in descending colon tissue of pups from different lactation litter sizes
(n=8 pups per group; * $P<0.05$).

When the data were split to analyse the difference between the sexes, only the male pups ($F(2, 9)=7.222$, $P=0.013$; Figure 7.6 A) had significantly reduced descending colon PYY concentrations when from the largest litter size, with no difference found between the females ($P=0.244$; Figure 7.6 B).

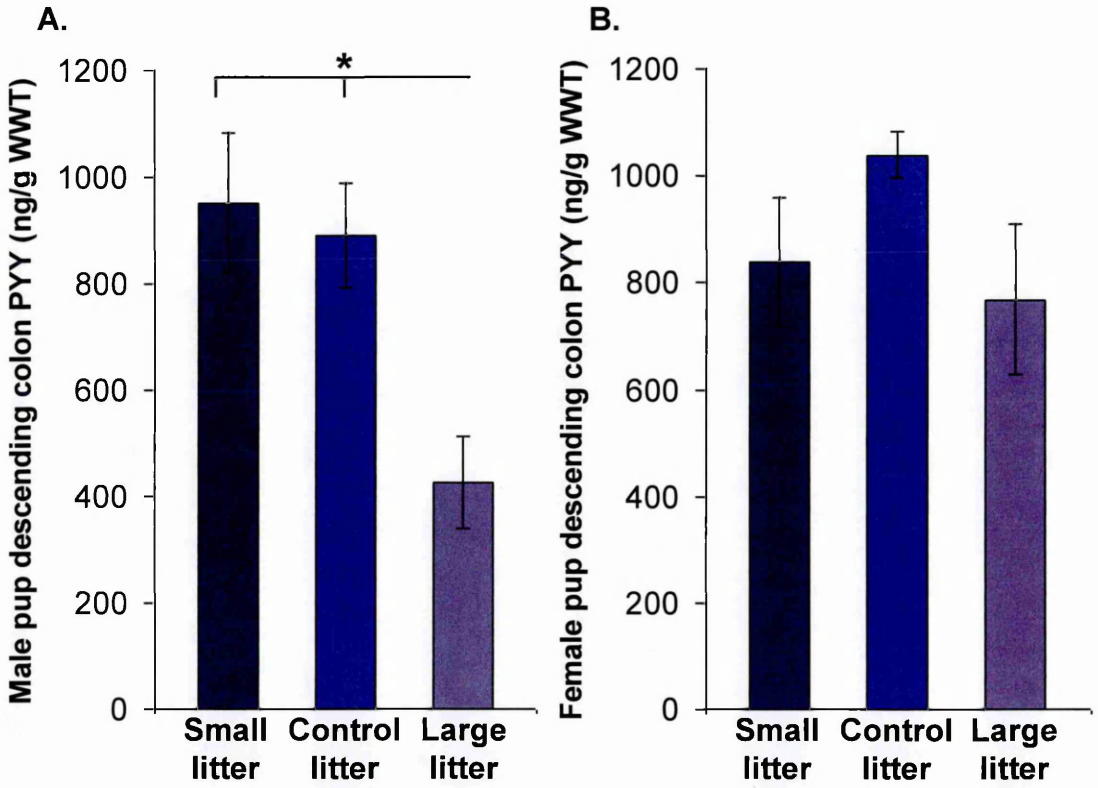

Figure 7.6. PYY concentrations in descending colon tissue of male and female pups from different lactation litter sizes
(A) male; **(B)** female (n=4 pups per group; * $P<0.05$).

7.4.3. Total glucagon-like peptide-1 (GLP-1) peptide analysis

Fed plasma concentrations of GLP-1 were very low in all samples and were below the detection limit of the radioimmunoassay (3 pmol/l) despite an extraction process (recommended by kit protocol) that was adapted to double the concentration of GLP-1 obtained.

Concentrations of GLP-1 in ascending colon tissue were significantly ($F(2, 21)=5.034$, $P=0.016$) reduced in pups from the smallest litter size compared to pups from control ($P=0.024$) and large ($P=0.043$) litters (Figure 7.7).

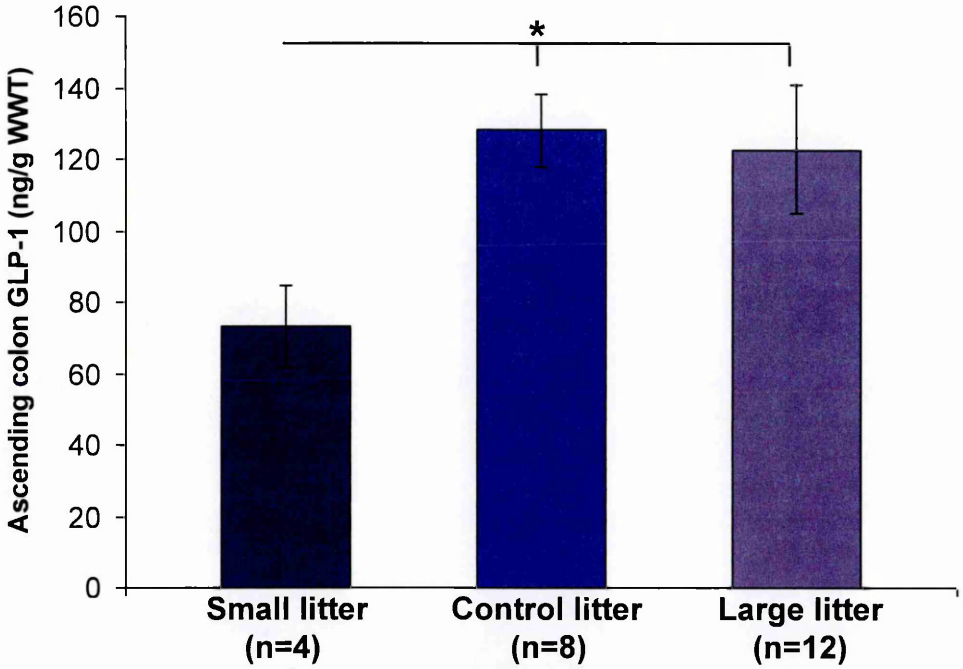

Figure 7.7. GLP-1 concentrations in ascending colon tissue of pups from different lactation litter sizes
(n=8 pups per group; * $P<0.05$).

No significant difference remained in ascending colon GLP-1 concentrations when the data were split by pup sex, although pups from the small litters still had numerically lowest concentrations (Table 7.5).

Table 7.5. GLP-1 concentrations in ascending colon tissue of male and female pups from different lactation litter sizes
(n=4 pups per sex per group).

	Ascending colon tissue GLP-1 (ng/g WWT)	
	Male pups	Female pups
Small litter (n=4)	64.2 ± 18.41 (13.0 – 94.2)	82.2 ± 13.99 (47.9 – 114.7)
Control litter (n=8)	137.8 ± 15.15 (104.9 – 164.4)	118.4 ± 12.95 (90.0 – 143.1)
Large litter (n=12)	109.5 ± 30.20 (49.6 – 184.9)	136.1 ± 21.69 (91.1 – 177.9)

Descending colon GLP-1 concentrations for all of the pup groups were not significantly different (Figure 7.8). In the males, descending colon GLP-1 was significantly reduced ($F(2, 9)=4.351$, $P=0.048$; Figure 7.9 A) in pups from

large litters compared to pups from small litters (P=0.045), with no differences and more variation between the females (Figure 7.9 B).

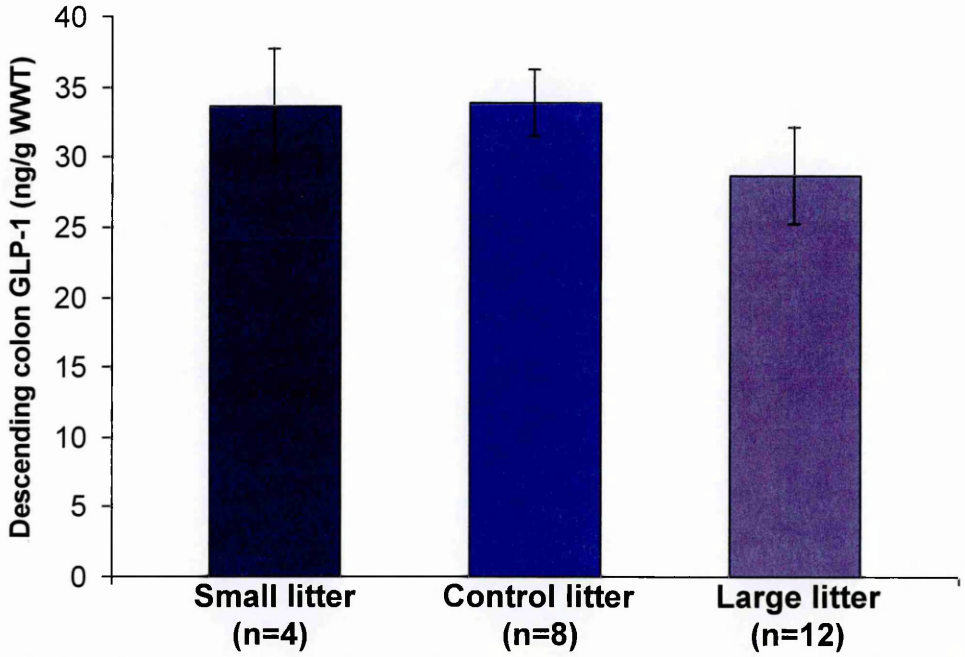

Figure 7.8. GLP-1 concentrations in descending colon tissue of pups from different lactation litter sizes
(n=8 pups per group).

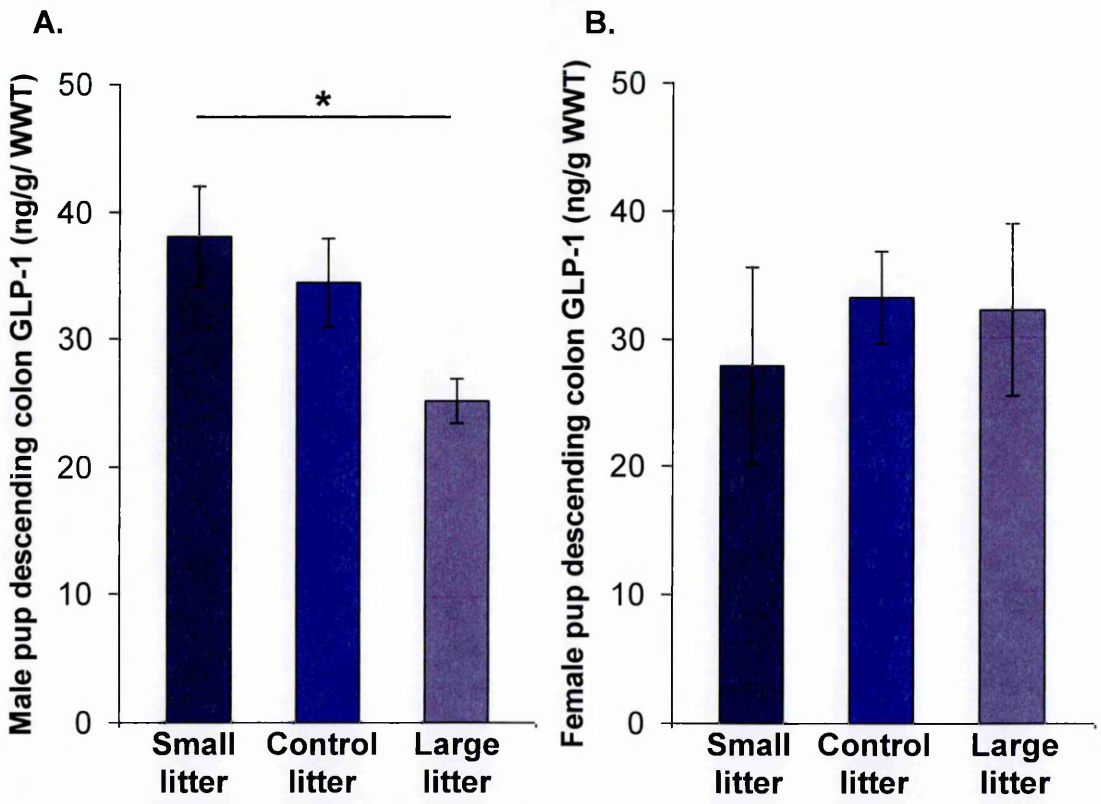

Figure 7.9. GLP-1 concentrations in descending colon tissue of male and female pups from different sized lactation litters
(A) male; **(B)** female pups (n=4 pups per group; * P<0.05).

7.4.4. Body size change in pups from different lactation litter sizes

Body mass of all of the pups (Figure 7.10) was significantly different ($F(2, 81)=50.659$, $P<0.001$), being highest in pups from the small litter size of 4 pups compared to the other groups.

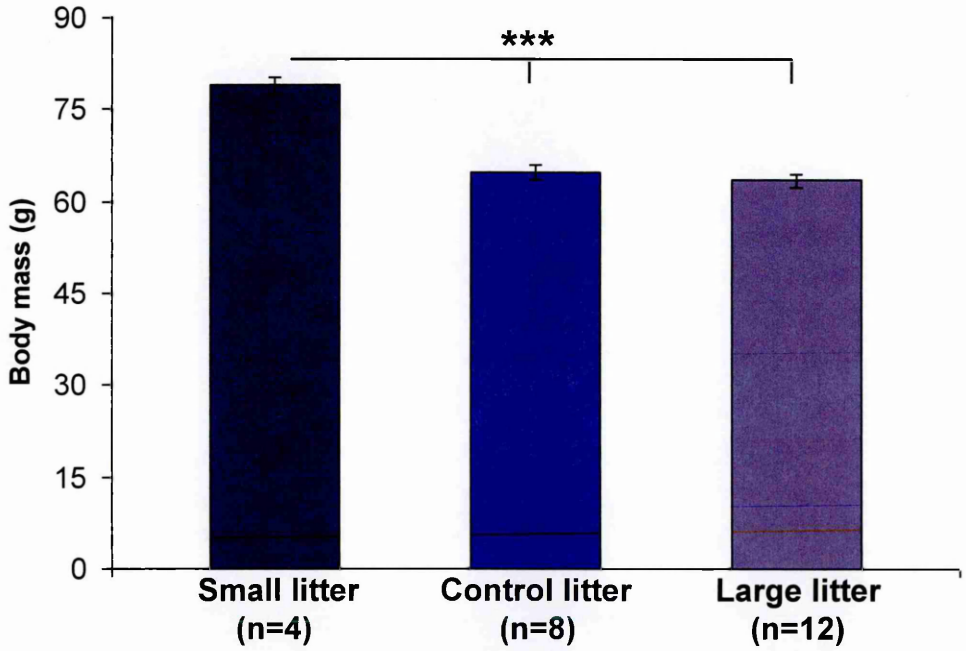

Figure 7.10. Body mass of all pups from different lactation litter sizes (n=28 pups per group; *** $P<0.001$).

The body mass of both the male ($F(2, 39)=32.523$, $P<0.001$) and female ($F(2, 39)=21.080$, $P<0.001$) pups was equally affected by litter size (Figure 7.11).

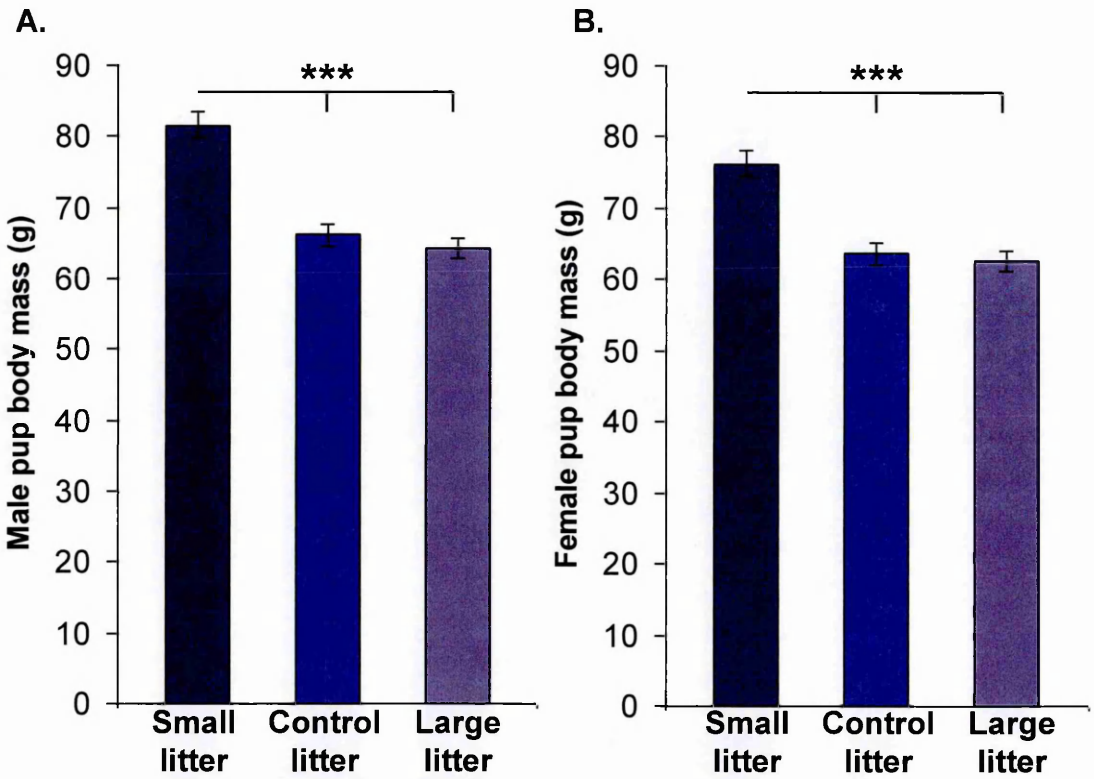

Figure 7.11. Body mass of male and female pups from different lactation litter sizes **(A)** male; **(B)** female (n=14 pups per group; *** $P<0.001$).

Pups from large litters were significantly shorter ($F(2, 81)=19.414$, $P<0.001$) compared to other litter size groups (Figure 7.12 and Figure 7.14).

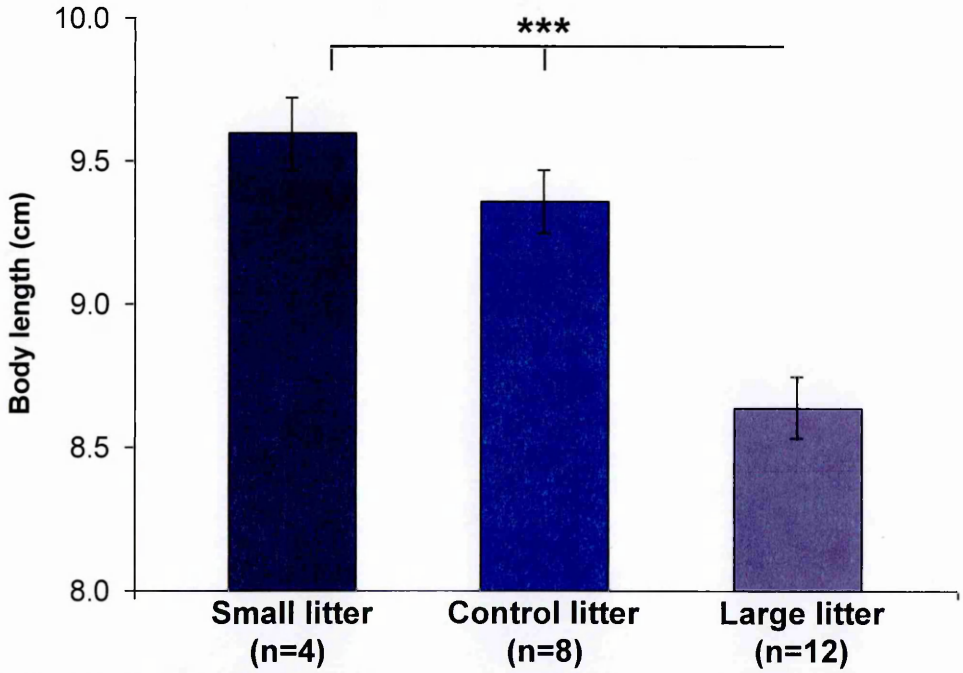

Figure 7.12. Body length of all pups from different lactation litter sizes (n=28 pups per group; *** $P<0.001$).

Similar to body mass, the body length of both male (Kruskal-Wallis, χ^2=14.490, 2 df, P=0.001) and female (F(2, 39)=10.910, P<0.001) pups appeared to be similarly affected by litter size (Figure 7.13 and Figure 7.14).

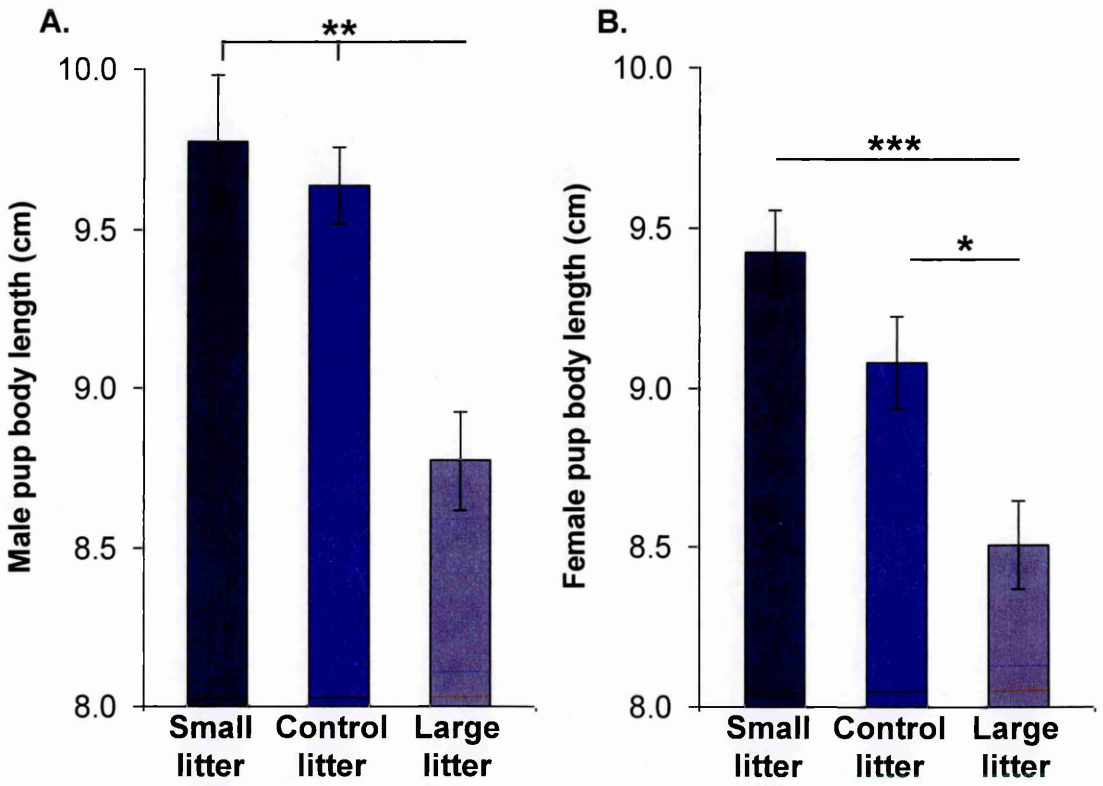

Figure 7.13. Body length of male and female pups from different lactation litter sizes **(A)** male; **(B)** female (n=14 pups per stage; * P<0.05; ** P<0.01; *** P<0.001).

The largest difference in both the mean body mass and mean body length were found between pups from the small and large litters. In males, the difference in body mass between the small and large litters (17.4 g) was larger than the difference between female pups from the small and large litters (13.6 g). Likewise, the difference in mean body length of males from small and large litters (1.0 cm) was larger than in females from the same litter sizes (0.9 cm).

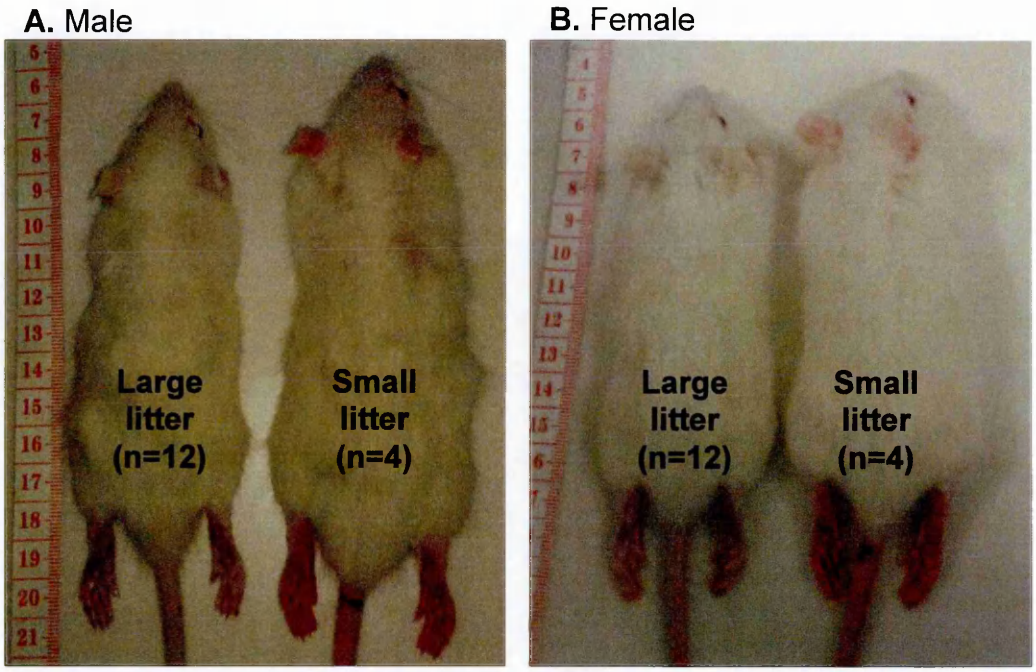

Figure 7.14. A representative image showing body sizes of pups from different lactation litter sizes
(A) males and **(B)** females from large (left) and small (right) litters.

7.4.5. Stomach tissue size in pups from different lactation litter sizes

Stomach tissue wet weight (Figure 7.15) was significantly different between the pups ($F(2, 80)=27.319$, $P<0.001$), significantly decreasing as litter size increased.

When from a small lactation litter size, both male ($F(2, 38)=17.030$, $P<0.001$) and female ($F(2, 39)=14.524$, $P<0.001$) pups had significantly increased stomach tissue wet weight (Figure 7.16).

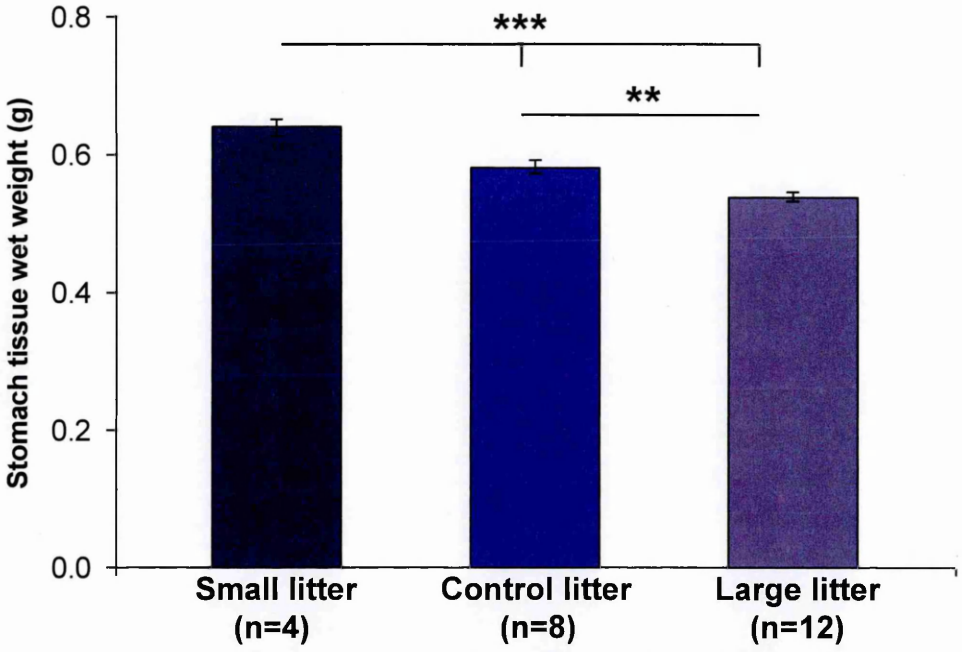

Figure 7.15. Stomach tissue wet weight of all pups from different lactation litter sizes (n=28 pups from small and large litters; n=27 pups from control litters; ** $P<0.01$; *** $P<0.001$).

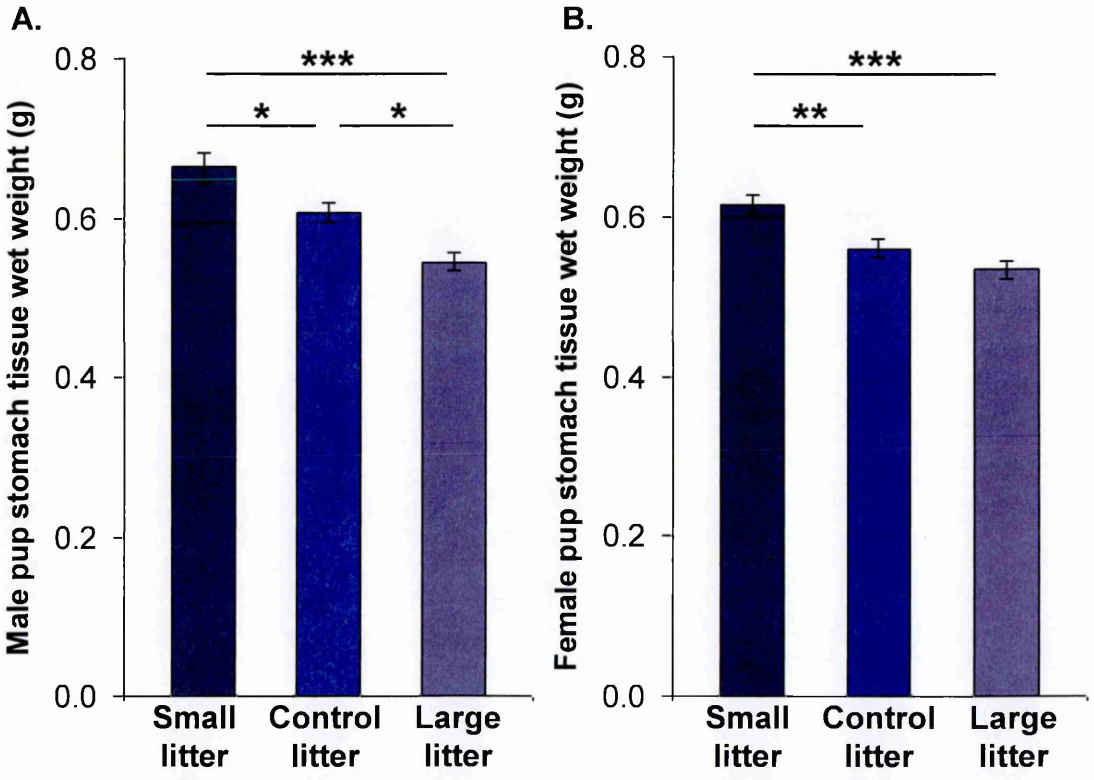

Figure 7.16. Stomach tissue wet weight of male and female pups from different lactation litter sizes

(A) male; **(B)** female (n=14 pups per stage, except control litter males where n=13; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

The mass of stomach contents was not different between the pups from different litter sizes, either for the whole group or when split by pup sex (Table 7.6).

Table 7.6. Stomach contents of male and female pups from different lactation litter sizes
(n=14 pups per stage, except control litter males where n=13).

	Stomach contents (g)	
	Male pups	Female pups
Small litter (n=4)	0.5 ± 0.08 (0.5 – 0.8)	0.6 ± 0.06 (0.5 – 0.7)
Control litter (n=8)	0.5 ± 0.08 (0.5 – 0.7)	0.5 ± 0.05 (0.5 – 0.6)
Large litter (n=12)	0.7 ± 0.17 (0.5 – 0.6)	0.6 ± 0.12 (0.5 – 0.6)

7.5. Discussion

This study has been the first to examine the effects on gut appetite hormones at weaning age in both male and female pups raised in litters of different sizes. Concentrations of ghrelin, PYY and GLP-1 were quantified in both plasma and gut tissue to establish whether appetite regulation was altered as a result of being in different litter sizes during the suckling period. Measurements of body and stomach tissue size provided additional information on the physical changes that occurred between the pups for comparison with the studies of others.

There were no changes in ghrelin or PYY in fed plasma between pups in litters of different sizes, with plasma concentrations of GLP-1 being too low to quantify using the radioimmunoassay kit. Stomach tissue ghrelin and ascending colon PYY concentrations were not different between the pups

from different litter sizes. Descending colon PYY was significantly reduced in pups from large litter sizes analysed as a group, but when split by sex, this was only found to occur in the male pups. Conversely, ascending colon GLP-1 was significantly reduced in the entire group of pups from small litter sizes, but no differences were found when males and females were separately analysed. GLP-1 in descending colon was also only significantly different in the males, and was reduced in pups from large litters. Both body mass and body length were significantly reduced in all pups from large litters. Stomach tissue was also significantly lighter in all pups from large litters.

7.5.1. Gut peptide changes in pups from different lactation litter sizes

Although there were no differences in either plasma or stomach tissue ghrelin concentrations between the pups, there was evidence of an altered satiety profile. Plasma concentrations of PYY were not different, and plasma GLP-1 could not be analysed using the same technique, but significant colon tissue changes in these two satiety hormones were found, which may suggest that more of these hormones were being synthesised by the gut L-cells. Due to the pups remaining with their mothers until culling, without any solid diet available, it is possible that variable fed states were seen between pups from differently sized litters and was representative of how much they were fed by their mothers, which may affect plasma levels of all of the appetite hormones.

7.5.1.1. Changes in gut satiety hormones were only found in male offspring

PYY levels in the ascending colon were similar between the pups, but descending colon concentrations were significantly different. When all of the pups were analysed together, those from the large litter sizes had significantly reduced descending colon PYY, however this only remained in the males when the data were split by pup sex. Males from the large litters had significantly less descending colon PYY compared to males from both the small and control litters, and had 45% less PYY in this tissue than their female littermates, which could suggest a reduced level of satiety in the males. Concentrations of descending colon PYY between male and female pups from the small and control litter sizes were much more similar, which suggests that coming from a large litter size may affect future PYY levels or regulation in males. This may result in male pups from larger than average litter sizes having reduced satiety in order to achieve catch-up growth through increased nutrient intake, in a similar way as has been described in human growth-restricted offspring (Ounsted and Sleigh, 1975). As described earlier, Plagemann *et al.* (1999) found increased hypothalamic NPY in male pups from large litters compared to pups from control litters, which could indicate increased appetite, perhaps to aid increased nutrient intake. Supporting this possibility is the observation that pups from large litter sizes were significantly smaller than other pups, which will be discussed in more detail below (section 7.5.2).

GLP-1 concentrations in ascending colon tissue were significantly reduced in pups from the smallest sized litters compared to those in both the control and

large sized litters. However, when the data were split by pup sex, this difference was no longer seen in either sex. It is possible that adding more male and female pups to the individual data sets would reveal a sex difference, as a large range of concentrations were measured in these samples.

The opposite trend was found in descending colon GLP-1 levels in comparison to the ascending colon levels. No significant difference was found between the pups from different litter sizes, however when analysed separately, the male pups from large litters had significantly reduced descending colon GLP-1 compared to males from small litters. The female pups had very large concentration variations between the groups, which masked overall pup differences when analysed alongside the males.

7.5.2. Body size and stomach mass were increased in pups from small lactation litters

Body mass of pups from the control and large litter sizes were similar to each other, but pups from small litters were significantly heavier at weaning. Both the male and female pups had similar body masses. Body length followed a similar pattern between the pups, being significantly increased in pups from small litter sizes, however the female pups from small and control litter sizes appeared to be shorter than their male littermates. The reduced body length of the pups from control compared to large litter sizes was more significant in the males than in the females, suggesting that the growth of males could have been most affected by the increased litter size. The results presented

here on increased body size are in line with the studies described earlier, which found that body mass, length and adiposity of pups from small litters was significantly increased compared to other litter size groups studied (Balonan and Sheng, 2000, Faust *et al.*, 1980, Fiorotto *et al.*, 1991, Plagemann *et al.*, 1999, Plagemann *et al.*, 1992, Schmidt *et al.*, 2001, Widdowson and McCance, 1963), although mainly male offspring have been reported on in previous studies.

Stomach tissue wet weight was similar between male and female littermates and was significantly decreased in pups from large litters compared to pups from control litters. Additionally in male pups, there was a significant reduction in stomach mass between control and large litters, which was not present in the females, again suggesting that males may be more affected by litter size. Although the stomach tissue changed in size, there was no difference in ghrelin levels, suggesting that stomach tissue size is not necessarily related to ghrelin levels in pups. Future work to quantify the number of ghrelin-IR cells in the stomach tissue of these pups would aim to establish whether there are any changes to ghrelin cell number as a result of increased stomach size.

7.6. Conclusions

Decreased satiety was indicated by decreased levels of PYY and GLP-1 in the descending colon of male pups from large litter sizes. As suggested earlier, this could be a mechanism to increase food intake to increase body size, which is significantly reduced in these animals. No such effect was seen

in female offspring, suggesting that males and females are affected differently, even before sexual maturation.

This work additionally shows the importance of standardising litter size as early as possible, as coming from a different lactation litter size can affect both body size and appetite hormone profile and the differences outlined in this chapter may become evident at an earlier age. For Wistar females, litter sizes of 12 are not uncommon and all of the dams had 12 nipples, so the findings described here are not a result of dams being unnaturally metabolically compromised.

7.7. Future work

There are a number of questions that arise from this study that would require further work to answer. For instance, it would be of interest to study the levels of the gut appetite hormones in the hypothalamus, and whether levels of their receptors are altered here due to being suckled in different litter sizes. As described earlier (section 7.1.3), Plagemann *et al.* (1999) suggested that the absence of an appropriate change to the levels of hypothalamic NPY showed potential for the development of an obese phenotype in male pups from small litters, so it is possible that other changes occur in the appetite-regulatory centres of the hypothalamus.

Detailed gut measurements of the pups from different litter sizes would also be of interest in the future. Stomach tissue mass was significantly decreased in pups from large litters, and it is hypothesised that the rest of the gut would

be reduced to a similar extent, possibly linked with overall body size. The pups from the small litters that had the largest bodies and stomach tissue also had the most PYY in descending colon tissue. It is possible that this increased PYY is linked to increased gut size (discussed in detail in section 5.5.4.1), which is why detailed analysis of gut size between the pup groups would add to this work. Different levels of PYY and GLP-1 in colon tissue could be due to changes in the number of cells secreting these peptides, and so quantification of these cells would aim to further elucidate the significant reduction in PYY and GLP-1 in male pups from large litter sizes.

Another addition to this area of research could be to include analysis of gut hormones and gut size in pups from different lactation litter sizes at different stages of development to determine at what age the previously described changes occur. A continuation of that study could be to investigate whether changes that occurred due to being suckled in different litter sizes persist into later life, alongside analysis of food intake and perhaps food preference into adulthood.

CHAPTER 8: GENERAL DISCUSSION

8.1. Introduction

Obesity and its associated comorbidities are known to adversely affect the health of pregnant and lactating women and can developmentally and epigenetically programme their offspring to be more susceptible to metabolic disorders and obesity in later life (section 1.8). Further elucidating how appetite regulation by gut peptide hormones is altered during these times may allow better interventions and treatment to improve mother and offspring health but prior to this, investigation of normal appetite regulation during the reproductive cycle is paramount. The majority of studies exploring gut hormones and appetite are performed in males, due to known changes in steroid hormones and the fluctuating appetite of mammals during the reproductive cycle presenting additional experimental factors to control for. Some preliminary studies during the reproductive cycle, pregnancy and lactation have looked at gut appetite hormones, but more detailed work is necessary to elucidate the mechanisms influencing substantial appetite changes during these times and has formed the focus for studies described in this thesis.

Studies that have explored appetite during the female reproductive cycle have established that a reduction in appetite leading up to ovulation is facilitated by high levels of oestradiol (reviewed by Asarian and Geary, 2006) and that high levels of oestrogens have an inhibitory effect on the orexigenic

gut hormone ghrelin, supporting this reduction in appetite (reviewed by Butera, 2010). Studies exploring changes in ghrelin during pregnancy and lactation are more contradictory, with both rodent and human studies often presenting differing findings (section 1.7). Fewer studies have investigated the potential roles that gut satiety hormones may have in appetite changes during the reproductive cycle, pregnancy and lactation. PYY may be most effective at reducing appetite when there are high levels of oestradiol (Papadimitriou *et al.*, 2007, Parker *et al.*, 1996), which may support the reduction in appetite around ovulation. In rats, studies agree that PYY levels increase throughout pregnancy and into early lactation (Suzuki *et al.*, 2014, Taylor *et al.*, 2009, Tovar *et al.*, 2004), although this contradicts PYY's peripheral role as a satiety hormone, due to the large increase in food intake seen at these times. GLP-1 levels have generally been overlooked with regards to appetite regulation, with the main research focus on its role as an incretin (discussed in section 1.8.3.3) and thus until now, there have been no studies on GLP-1 levels during a complete reproductive cycle, pregnancy or lactation. In addition to the above, many studies often only consider circulating levels of gut hormones, with limited information available on possible concentration changes in gut tissue.

The main aims of this work were therefore to study the plasma and gut tissue levels of the total peptide forms of ghrelin, PYY and GLP-1 at each stage of the rat oestrous cycle (Chapter 3) and at defined stages of pregnancy (Chapter 4) and lactation (Chapter 5). As gut growth was observed by others in lactating rodents many decades ago (described in section 5.1.3), this work

has been repeated and extended with more detailed gut measurements taken from pregnant and lactating dams to determine whether changes in gut size may be related to changes in gut hormone levels. Chapter 6 addressed whether changes in maternal gut hormone levels and gut size were affected by the lactation litter size of the dams, leading into Chapter 7, which studied changes that had occurred in male and female pups from these different litter sizes by weaning age.

8.2. Gut appetite hormone changes during the reproductive cycle, pregnancy and lactation

As described in detail in Chapter 1 (section 1.7.1), decreased food intake is seen in rats during oestrus due to an earlier peak in ovarian oestradiol secretion at proestrus. A key finding from Chapter 3 was that concentrations of GLP-1 in fed plasma were significantly increased at proestrus, which could indicate increased satiety in these animals. This suggestion was supported by the findings of significantly decreased fasted stomach contents in the rats shortly after the onset of oestrus and a non-significant decrease in fasted plasma ghrelin levels during proestrus. Increased GLP-1 and a potential decrease in ghrelin levels during proestrus would favour an anorexigenic tone between proestrus and oestrus. Thus, the results from Chapter 3 suggest a potential explanation contributing towards how food intake is decreased around ovulation by gut appetite hormones.

Although the hyperphagia of pregnancy and lactation is well established (described in Chapters 4 and 5), the potential role of the gut appetite

hormones during these times was largely uncharacterised. This study has identified a significant decrease in fasted plasma ghrelin levels by late pregnancy, coupled with a decline in food intake leading up to birth. On the day of birth, approximately 5 hours after birthing began, there was a peak in stomach tissue ghrelin concentrations, which may act as a feeding initiator at this stage. Fasted plasma ghrelin was lowest in early lactation and significantly increased in late lactation. In Chapter 6, dams with large lactation litters had significantly increased stomach tissue ghrelin compared to dams feeding the control litter size. These combined data on ghrelin levels during pregnancy and lactation support its role as an orexigenic gut hormone, with high plasma levels at the end of lactation when food intake has been reported to be maximal and high stomach tissue levels when there was a greater requirement to initiate and sustain feeding. A significant reduction of GLP-1 levels in fed plasma and in both PYY and GLP-1 levels in ascending colon tissue were seen in late lactation, which could also support the marked hyperphagia documented to occur in late lactation by a reduction in satiety.

8.3. Gut size changes during pregnancy and lactation

The detailed gut measurements described here have added to the body of knowledge of how rat GI organs and tract change in size and capacity during pregnancy and lactation. The vast majority of studies of maternal gut size changes have focussed on lactation, with less information available on changes occurring during pregnancy. Here, it was hypothesised that reported gut size changes in lactating dams may begin to develop during pregnancy, which was confirmed in the small and large intestine with significant changes

in their lengths and wet weights. These measurements increased further and peaked in late lactation, where stomach and caecum tissue wet weights were additionally significantly increased. Dams feeding small litters had significantly reduced small and large intestine lengths and wet weights in comparison to dams feeding the control litters, which may demonstrate the occurrence of gut growth in proportion to the metabolic demand of lactation. These results suggest that maternal gut growth starts during pregnancy and continues during lactation to increase the absorptive area of the gut, further optimising nutrient uptake in relation to lactation demand.

Although dams at day 25 of lactation had significantly increased intestine lengths and wet weights, they had significantly reduced circumferences in duodenum, ascending and descending colon tissue. It is thought that a reduction in gut circumference may be linked to a small decrease in maternal demand by this stage, with pups feeding on solid food and a decrease in milk yield reported from day 21 of lactation in Wistar rats (Knight *et al.*, 1984). Other evidence in support of this suggestion is that dams feeding 12 pups had significantly increased gut circumferences in the absence of significant length or wet weight increases in comparison to dams feeding 8 pups. In Chapter 6 the possibility is discussed that the dams feeding 12 pups were too energy deficient to structurally increase their gut length, with significantly reduced gut measurements compared to the dams feeding control litter sizes of 8 pups (section 6.5.3). An increase in gut circumference may represent a less energetically costly adaptation in the dams feeding 12 pups in order to increase gut capacity to physically support the hyperphagia of lactation.

Assuming human gut size increases by a similar mechanism to support pregnancy and the lactation period, an important aspect in terms of human health would be to know whether changes to the gut persist long after the end of pregnancy and/or lactation. An increase in rat stomach size by the day of birth occurred with a significantly increased number of cells immunopositive for ghrelin compared to proestrus controls and in mid-pregnancy in the present study (section 4.5.3). It is possible that permanent changes in appetite regulation may occur as a result of pregnancy, which could then be further altered by lactation demands and in humans, by variable lactation length.

Another question that arises from the finding of increased gut size, and the possibility of growth persisting indefinitely, is whether this changes the adaptations to future pregnancies, which may in turn make optimal postpartum weight management different in women who have had single or multiple births. Although the dams in Chapter 6 with different sized litters during lactation appeared the same externally, with similar body sizes, there were a number of changes internally to gut size and gut appetite hormones, as described above. Preliminary work with a group of multiparous dams that were used to set up the breeding colony provided an insight into what occurs in the later postpartum period. Multiparous dams gave birth to two litters, and after the 25 day lactation period of the second litter, dams were left to normalise for a period of two weeks prior to dissection. The multiparous dams were found to have a significantly reduced gut size in comparison to primiparous dams feeding the control litter size at day 25 of lactation

(appendix 2). These data suggest that gut hypotrophy in rats may occur after the pup stimulus is removed and food intake reduces back to control levels. Further support was provided by another preliminary test group of dams who were pregnant but did not lactate due to loss of their litter soon after birth. These pregnant/no lactation dams, also dissected 25 days postpartum, did not show gut hypertrophy, again suggesting that increased food intake during lactation, in combination with the pup suckling stimulus, may be responsible for gut hypertrophy and, in their absence, gut growth does not occur. It is unknown whether intestinal growth during pregnancy and lactation increases the number of ECs secreting appetite-regulatory hormones and it remains possible that EC density in the small and large intestine increases in line with gut growth, as was reported here in stomach tissue with ghrelin-IR cells (section 4.5.3). It may be reasonable to assume that similar gut growth is seen in human females, which could change the current advice on controlling body mass during pregnancy and lactation to equally include advice about food quantity and meal frequency in order to control potential gut size modifications and EC changes.

The results described in Chapter 5 revealed a possible link between significant gut growth by late lactation and paradoxical high levels of PYY in gut tissue during late pregnancy and early lactation (section 5.5.4.1). Gomez *et al.* (1995) reported that intraperitoneal injection of PYY into adult female mice significantly increased gut growth and that growth of the small intestine was the most significant. That finding is comparable to what has been reported here, with a 44% increase in length and a 67% increase in wet

weight of the small intestine between early pregnancy and late lactation. A longer lactation period has been associated with increased circulating levels of PYY in humans at 3 years postpartum (Stuebe *et al.*, 2011) and it is possible that PYY is increased due to an increase in gut growth associated with the duration of lactation.

In humans, it may be that the nearest possible direct comparison that can be made between increased circulating PYY and gut growth is post-gastric bypass surgery (section 1.5). It is possible that chronically elevated PYY levels post-gastric surgery may be, in part, due to an adaption to regenerate gut tissue to overcome malabsorption after the surgery has shortened the amount of intestine to which nutrients are exposed. This suggestion has parallels with that outlined above for this study, in that gut hypertrophy during nutritionally uncompromised pregnancy and lactation may occur as a mechanism to increase gut size in order to optimise nutrient uptake. To confirm, human studies would have to evaluate gut length before and immediately after gastric bypass surgery, perhaps by magnetic resonance imaging (MRI), and then again several years later in order to evaluate whether increased levels of PYY are correlated with an increase in gut size. Indeed, one study demonstrated that Roux-en-Y gastric bypass (RYGB) resulted in increased circulating levels of PYY and GLP-2 in diet-induced obese male rats and in obese female humans (le Roux *et al.*, 2010). In that study, RYGB male rats had a significantly increased number of intestinal cell crypt mitoses and significantly more cells in S-phase of the cell cycle, which the authors suggest indicates increased crypt cell proliferation. In addition to

this, intestinal thickening was macroscopically noted in the RYGB rats compared with the control group. These authors suggest that a similar increase in gut size in humans, years after gastric bypass, could be responsible for a limit to body mass loss in the long term (see section 1.5).

It is possible that PYY may play a more important role in preparing the gut for digestion and absorption of optimal amounts of nutrition than as a true satiety signal, which may explain its increase after RYGB to potentially overcome malabsorption caused by the procedure by partially restoring gut size. Similarly, one study has challenged ghrelin's role as a true hunger signal, instead suggesting that its role is more likely to be in the signalling of energy availability, increasing as a result of a negative energy balance rather than to directly stimulate appetite (Borer *et al.*, 2009). As it is difficult to fully separate the precise roles of each individual gut hormone in response to hunger, appetite and energy homeostasis, more in depth study is required to say with certainty whether PYY is raised to create a pause in hunger signalling or to prepare the gut for dealing with quantities of food sensed by the upper GI tract. As described previously (section 5.5.4.1), food restriction in lactating dams prevents gut hypertrophy and in pregnant dams, food restriction suppresses PYY levels to non-pregnant levels (Campbell and Fell, 1964; Datta *et al.*, 1995, Tovar *et al.*, 2004), which supports the possibility of PYY's role as a gut modifier.

Another implication of significantly elevated levels of PYY in early lactation is that appetite may actually be increased as a result. Described in section

1.6.2.1 is a review (Ballantyne, 2006) of a number of studies that have demonstrated an injection of PYY directly into the brain can cause a more profound hyperphagia than is seen with injection of its orexigenic family member NPY. The review author suggests that it is the full peptide form PYY_{1-36} that is causing an orexigenic effect, whereas the 'active' form PYY_{3-36} induces satiety by binding to different Y receptor subtypes. The paradoxical PYY increase reported to occur here during early lactation may actually be supporting hyperphagia by acting in this manner, rather than acting as a satiety hormone. Further work to establish any changes in circulating peptide forms and PYY and Y receptor levels in the brain would have to be done to confirm this suggestion.

8.4. Changes in body mass and adiposity as a result of pregnancy and lactation

A further health implication in humans is retention of increased body mass long after lactation has ended (see section 1.8.2). Preliminary analysis of a pregnancy/no lactation group (see above) showed that these dams had significantly increased abdominal white adipose tissue (WAT) compared to dams that lactated for 25 days (appendix 2). This may be expected, due to the accumulation of adipose reserves ready to support lactation; no lactation would require limited use of these adipose reserves and may have health implications for women who cannot or who choose not to breastfeed. In addition to this preliminary finding, the multiparous group of dams used for setting up the breeding colony (see above) also had significantly increased abdominal WAT, two weeks after normalisation, in comparison to the dams at

the end of the lactation period (appendix 2). These findings could indicate that there is rapid accumulation of adipose tissue after pups are removed, possibly with some time taken for appetite to return to control levels, or that multiple births with or without lactation leads to a progressive accumulation of adipose tissue. The latter suggestion has implications for human health and may affect the ability of women to maintain an appropriate body mass and/or level of adiposity after multiple pregnancies, and thus may require different interventions for successful postpartum body mass loss, particularly if gut size and gut ECs have additionally been altered (see section 8.3).

8.5. Gut appetite hormones in pups from different lactation litter sizes

Human studies have explored the health implications of being born small for gestational age (SGA) compared to those born appropriate for gestational age (AGA). It is thought that the accelerated catch-up growth shown in SGA babies may lead to an increased risk of altered glucose metabolism and obesity in later life. At 6 years of age, children who were SGA have been found to have significantly increased visceral adiposity, without body mass differences, compared to AGA children, and they also had significantly higher fasted insulin levels (Ibanez *et al.*, 2008). Differences in glucose regulation have been found to occur very early in the life of SGA babies and one study documented a significant occurrence of hypoglycaemia in SGA compared to AGA babies, which was most significant during the first 12 hours of life (Mazumder *et al.*, 2012).

In an attempt to further elucidate the possible mechanisms behind increased adiposity and increased obesity risk in adults born SGA, a recent study analysed the genotype of SGA babies and documented that genes associated with obesity played a smaller role in relation to body mass index in SGA babies compared to AGA babies at 3.5, 7 and 11 years of age (Han *et al.*, 2013). These authors suggest that this finding indicates that accelerated postnatal growth and subsequent increased risk of obesity in SGA babies may be largely governed by other non-genetic and environmental factors.

For the first time, gut hormone levels were examined in both male and female littermates from small, control and large lactation litter sizes at weaning age (Chapter 7). No differences were found in ghrelin levels between the pups, but levels of the satiety hormones PYY and GLP-1 were found to be altered in colon tissue. In descending colon, PYY and GLP-1 concentrations were significantly lower in male, but not female, pups from the large litter size. Physically, the pups fed in the large litter size were significantly shorter and lighter and had significantly lighter stomach tissue. It was thus suggested that the significantly smaller male pups may have had significantly reduced satiety as a mechanism to achieve catch-up growth into adulthood. It is unclear whether females may show similar adaptations but at a later stage. This study demonstrated that differences between males and females were evident even before sexual maturation.

A number of studies follow-up SGA babies a couple of years into life, but one study has explored the health of SGA babies at 20 years of age. Those authors reported that at a mean age of 20.6 years, height, body mass and head circumference were significantly lower in men and women who were SGA (Leger *et al.*, 1997). Results of an oral glucose tolerance test demonstrated that plasma glucose levels at 30 minutes and serum insulin levels up to 120 minutes were significantly higher in adults who were SGA. Furthermore a sex difference was noted, with significantly higher fasted baseline insulin levels in SGA women but not in SGA men compared to AGA controls, which, combined with the findings of Chapter 7, may indicate that females are affected later and in different ways to males.

The findings of the present study may suggest that a difference in appetite regulation may occur simply as a result of changing the early postnatal environment, by postpartum manipulation of litter size. One may expect offspring that were growth restricted *in utero* during key stages of development could be more at risk of changes in glucose regulation and adiposity. Studies of SGA and even large for gestational age (LGA) offspring next need to establish levels of appetite hormones to further elucidate adulthood obesity risks and whether early alterations in appetite regulation may be partly responsible for these increased risks. Further work could then explore whether manipulating litter sizes, or modifying lactation duration in humans, can modify the risks of being born SGA or LGA.

8.6. Limitations of study

There were a number of limitations to this study that would need to be addressed in future studies. In Chapter 3, it was found that plasma total ghrelin levels in both the fed and the fasted state were significantly reduced in rats after 36 weeks of age, with only one exception (section 3.5.2). It remains unclear why this occurred, but it is possible that there was an early decline in reproductive efficacy by this age in the rats (Niggeschulze and Kast, 1994, Suckow *et al.*, 2006), which could in turn alter the way in which ghrelin is regulated, or how ghrelin regulates appetite, between younger and older rats. The age of these animals also meant that the proestrus controls used for comparison purposes for subsequent chapters were not age-matched, which would have been desirable, particularly after the finding of altered plasma ghrelin concentrations with age.

Another key limitation of this study was that the rats had to be group housed due to the number of study animals required, thus daily individual food intake could not be accurately measured. It was possible to use fasted stomach contents as an indication of food consumed leading into the beginning of the fasting period. The findings of Chapter 3 demonstrated that fasted stomach contents may be an accurate representation of food consumed, as results were in agreement with others in that cycling rats consumed the least leading up to oestrus (section 1.7.1).

At the beginning of the study, one aim was to compare gut hormone levels between fed and fasted plasma samples from each individual animal. Due to

a limitation in available funds and the findings of Chapter 3, it was decided to exclude analysis of ghrelin in fed plasma as fed concentrations were similar to fasted plasma ghrelin concentrations in the cycling rats (section 3.5.1.1), and made more fed sample available for PYY and GLP-1 analysis.

Another issue was that the volume of plasma obtained from the tail bleed was limited, even after the sample was diluted in buffer and acidified for optimal peptide preservation (see section 2.3.2.1), thus the decision was made from Chapter 4 onwards to follow the RAPID methodology described by Stengel *et al.* (2009), which involved drying down acidified plasma using a vacuum centrifuge in order to concentrate the sample and improve peptide yield, which was especially important for GLP-1 analysis. Replicating this sample preparation, however, appeared to reduce the peptide yield in this study. When comparing the concentration of PYY in reconstituted fed acidified plasma with concentrations in non-acidified non-dried down fasted plasma, PYY levels were significantly reduced in the fed rather than the fasted plasma (section 4.5.2), which was not found in Chapter 3 when acidified fed and non-acidified fasted plasma samples were not dried down. It was also not possible to directly compare fed and fasted plasma ghrelin levels for dams in Chapter 6, as it was unclear whether the reduced levels of ghrelin in the fed plasma were a true reflection of peptide concentration, or whether they were artificially reduced as was seen with PYY concentrations.

One limitation to how informative the analysis of the fed plasma samples can be is that there was no measure of precisely how much food the animals had

consumed. The lighting schedule and timing of sample collection (figure 2.4) was designed to control for food intake as an influencing factor as much as possible, but it may be necessary in the future to explore this design further. A preliminary test could be done using multiple timed plasma samples to demonstrate when levels of PYY and GLP-1 peak after lights out at 11.00. The results of such a study, done using a large enough number of animals, would allow a sampling window to be set where the majority of animals show peak levels of PYY and GLP-1. Having a clearly defined sampling window for fed plasma analysis of PYY and GLP-1 would allow more confidence to be placed in the findings of the study because it is thought that this would reduce the likelihood of some animals being 'more fed' than others.

8.7. Future directions

The data described in this thesis have provided key insights into appetite regulation by the gut hormones ghrelin, PYY and GLP-1, as well as additional physiological changes that occur during pregnancy and lactation, such as increased gut size. There are a number of ways in which this research could be progressed, some of which are described below and involve samples that were banked as part of this study.

To replicate (in the dark phase) or provide an explanation for the unexpected finding that there was no difference between fed and fasted plasma levels of ghrelin (section 3.5.1.1), despite an appropriate fasting response from PYY and GLP-1, more study is required. A longer fasting period could be attempted in normally cycling females to see whether this result still remains.

Section 2.3.1 outlines that many studies of appetite hormones standardise their measurements by fasting for 24 hours and sometimes even longer. It was important for this work that fasting time was reduced, as comparisons were to be made between reproductively cycling females and pregnant and lactating dams. Pregnant dams in particular could not have been subjected to a prolonged period of fasting. A future study in cycling females only could determine whether ghrelin levels are changed by increasing fasting length.

Additionally, more sensitive methods of analysis, such as LC/MS-MS, would allow a fed and fasted state plasma hormone comparison with additional quantification of the different forms of each peptide, as was intended at the onset of the study. Despite sample collection for the analysis of different peptide forms, unfortunately this was not possible. Using a technique such as LC/MS-MS would also have the advantages of allowing gut hormone analysis using a smaller volume of plasma.

Another way in which gut peptide measurements in this study could be made more robust is to repeat the experiments using an AccuSampler, which could take small repeated blood samples daily. During such a fast-moving oestrous cycle, for example, hourly peptide measurements at each cycle stage would provide additional information about appetite changes during the cycle that would otherwise be overlooked. A study such as that could also use the AccuSampler to directly compare reproductive hormone levels with appetite hormone levels at any given time.

As outlined earlier (section 8.6), one of the key limitations to this work was the inability to measure individual rat daily food intakes throughout the study. The most ideal way to overcome this issue would be the use of metabolic cages that could accurately measure both fluid and food consumed. Another use for metabolic cages would be to replicate and extend the work of Eckel *et al.* (2000) in determining activity levels to provide another insight into body mass regulation during the reproductive cycle, pregnancy and lactation. If this work were to be implemented in the future, the metabolic cages would have to keep pups separate from the cage diet so that accurate dam food intake could be established. In future studies, another way in which the limitation of not being able to measure food intake could be taken into account is to explore markers of energy intake and metabolism using the blood samples. Levels of glucose and insulin could be quantified to further clarify the energy status of the rats at the sampling times and would be of most interest to measure in the day 18 pregnant dams, which were presumed insulin resistant. If the effects of certain diets given to the rats during pregnancy and lactation were to be explored in a prospective study, measurement of circulating macronutrients could be considered.

To further elucidate the process of gut growth in pregnancy and lactation, additional experiments could be carried out, such as inclusion of dry gut masses to examine the process of maternal gut adaptations in further detail (section 4.7). Pair-feeding pregnant and lactating dams to control levels would also be of interest in more closely exploring the link between increased food intake and the increase seen in gut size. It is thought that restricting

food intake to control levels in pregnant and lactating dams would alter gut size changes from what have been presented here, and information from an experiment like this would provide a more robust link between gut peptides and gut growth to be examined in more detail. In addition, measurement of gut transit times in pregnant and lactating dams would increase the understanding behind the changes in gut size and the role of gut peptides during these times.

Continued analysis of banked gut samples for cells immunopositive for ghrelin, PYY and GLP-1 would add to this work, and it is possible that there may be changes in the properties or density of other gut cells, such as enterocytes (section 1.4) during different reproductive stages. It was established that ghrelin cell density in the stomach significantly increased from proestrus controls to pregnant rats and even further in rats shortly after parturition. Unfortunately staining did not work in the colon tissue for PYY and GLP-1 (see section 2.6.5), which would have provided additional detail about the role of these peptides during pregnancy and lactation. The work of subsequent chapters would also be made more robust with the addition of gut cell counts to determine whether intestinal lengthening increases the number of ECs positive for gut hormones and whether this is further changed in dams feeding different litter sizes and in their pups. A question remains as to whether possible changes in EC density are retained in mothers into the future and permanently change appetite regulation, and the same question is asked concerning their offspring. Does early life nutrition affect gut EC

density during childhood and affect appetite regulation and nutrient utilisation into adulthood?

For the animals included in this study, samples of hypothalamus, stomach, ascending and descending colon were taken and prepared for mRNA extraction and subsequent quantification using RNAlater Solution (Life Technologies). This approach would provide a more in depth understanding of how ghrelin, PYY and GLP-1 may be up- or down-regulated during different reproductive states by investigation of the synthesis of these gut hormones in the areas of the gut where their concentration is reported to be maximal. When collecting hypothalamic tissue, the main aim was to quantify any changes that may occur to hypothalamic neuropeptides and receptors for gut appetite hormones, as this would provide evidence of central appetite regulation during different reproductive states. Future work should aim to localise and quantify levels of the neuropeptides in the arcuate nucleus of the hypothalamus (samples banked) and other brain regions (e.g. paraventricular nucleus) to determine what changes in appetite regulation may occur at this level during pregnancy and lactation. Recent work by Suzuki *et al.* (2014) reported that mRNA levels of orexigenic agouti-related protein (AgRP) were significantly increased and that anorexigenic pro-opiomelanocortin (POMC) was significantly decreased in the arcuate nuclei of lactating rats. These findings suggest central mechanisms by which hyperphagia during lactation is facilitated.

More detailed information could be obtained by quantifying receptor levels in gut and hypothalamic tissues alongside their ligands to further clarify some of the reported changes occurring during pregnancy and lactation. Samples could be analysed in a number of ways, such as staining for the desired receptors with subsequent image analysis or RNA could be quantified using polymerase chain reaction (PCR). Such work in the later postpartum period could explore whether changes at the hypothalamic level persist and could contribute to future changes in appetite and body mass regulation in both rats and humans. With all of this information, it would be easier to establish a difference between synthesis (gut peptide mRNA analysis), storage (gut peptide analysis) and release (plasma peptide analysis) of the gut hormones from tissues and would be a valuable and very interesting addition to this work.

As outlined in a number of the results chapters, the measurement of leptin would be interesting in future studies of this nature, not only due to the changing adiposity and leptin resistance that is associated with pregnancy and lactation but because of the relationship that leptin may have with ghrelin (see section 1.8.3). Finally, a clear expansion of this study would be to include the analysis of additional gut appetite hormones to further elucidate the mechanisms behind changes in appetite and food intake during the reproductive cycle, pregnancy and lactation.

8.8. Conclusions

In conclusion, the results from the studies presented in this thesis have further elucidated the control of appetite by ghrelin, PYY and GLP-1 in female rats during their reproductive cycle, pregnancy and lactation, with additional work focussing on both maternal and offspring adaptations to a decreased or increased litter size. Studies here have also examined changes in gut size during pregnancy and lactation and have built on existing knowledge surrounding lactation-associated gut hypertrophy and found that these changes are initiated during pregnancy. The main findings in this thesis were:

- Fasting did not significantly increase ghrelin concentrations in plasma at any stage of the reproductive cycle.
- Circulating ghrelin levels were significantly reduced after 36 weeks of age in normally cycling rats.
- GLP-1 significantly increased at proestrus supporting a decreased appetite leading into oestrus, which was further corroborated by significantly reduced food intake, as indicated by fasted stomach contents, and a trend for ghrelin to be decreased at proestrus.
- The density of ghrelin-positive stomach cells was significantly increased by parturition, alongside stomach tissue ghrelin concentration.
- Levels of PYY and GLP-1 were paradoxically highest during late pregnancy and early lactation when food intake was high, which may be linked with significant gut growth from mid-lactation.

- By late lactation, when nutritional requirement and food intake were high, ghrelin levels were significantly increased and both PYY and GLP-1 levels were decreased.

- Maternal gut size increased from late pregnancy onwards and appeared to be proportional to maternal demand.

- Male, but not female, pups from large litters were smaller and have decreased descending colon levels of PYY and GLP-1 at weaning.

From the findings reported in this thesis, it is possible that maternal changes in appetite regulation and in gut size that occur during pregnancy and lactation may continue long after the end of lactation and be further altered by lactation duration and parity. Increased postpartum PYY in humans (Stuebe *et al.*, 2011) may be associated with an increase in gut size, proportional to lactation duration. Maternal obesity and insulin resistance may additionally alter these adaptations to pregnancy and lactation, which in turn could affect the health of their offspring. It is clear that more work is required to further elucidate the appetite-regulatory system during these times, which may lead to more effective preventative measures and treatments to avoid health complications in both mothers and their offspring.

CHAPTER 9: REFERENCES

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CHAPTER 10: APPENDICES

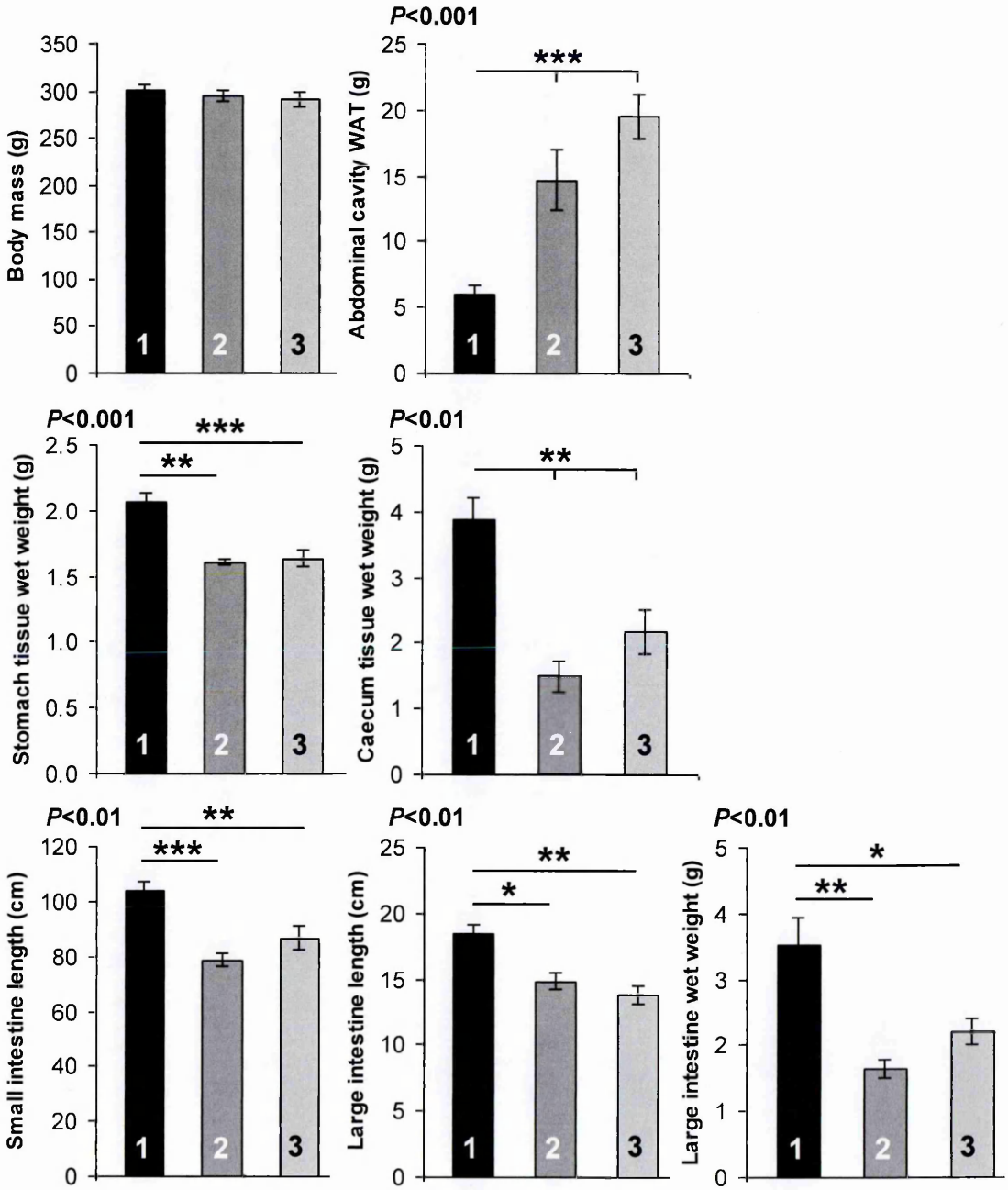
Appendix 1. Selected ion flow tube mass spectrometry (SIFT-MS) of caecum contents In d12P dams, acetonitrile (t(7.574)=3.438, P=0.010) and isoprene levels (t(12)=2.346, P=0.037) (in parts per trillion (ppt)) were significantly higher than in d10L dams. Proestrus controls used as a non-statistical comparison. (Precursor ion used: H3O+).

	Acetonitrile (ppt)	Isoprene (ppt)
Proestrus (n=2)	2.7 ± 0.98 (1.7 – 3.7)	1.3 ± 0.70 (0.6 – 2.0)
d12P (n=7)	5.5 ± 1.22 (0.6 – 9.2)	3.1 ± 0.52 (1.3 – 4.8)
d10L (n=7)	1.0 ± 0.45 (0.3 – 3.6)	1.6 ± 0.38 (0.1 – 3.0)

These findings may indicate that there could be quantifiable changes to the gut microbiota between pregnancy and lactation, although the group sizes are small for this study. Potential future work could aim to establish differences in contents from different gut locations, urine and faeces to further elucidate the maternal changes that occur to support pregnancy and lactation.

Appendix 2. Changes in maternal body and gut size resulting from different postpartum outcomes

Despite being of similar body mass, control primiparous dams at day 25 of lactation **(1)** had significantly reduced abdominal cavity white adipose tissue (WAT) and increased gut size compared to **(2)** dams who gave birth but did not lactate (dissected 25 days postpartum) and **(3)** dams who had two litters, with a recovery period of two weeks prior to dissection. (Group 1 dams: n=7, except for stomach mass where n=6 and large intestine mass where n=5. Group 2 dams: n=4, except for large intestine mass where n=3. Group 3 dams: n=8, except for large intestine mass where n=7. * $P<0.05$; ** $P<0.01$; *** $P<0.001$).



Appendix 3. Product suppliers

Abbott - Abbott House, Vanwall Business Park, Vanwall Road, Maidenhead, Berkshire, SL6 4XE

Abcam - 330 Cambridge Science Park, Cambridge, CB4 0FL

Acorus Therapeutics Ltd - Office Village, Chester Business Park, Chester, CH4 9QZ

Agar Scientific - Elektron Technology UK Ltd, Unit 7, M11 Business Link, Parsonage Lane, Stansted, Essex, CM24 8GF

Bayer UK/Ireland - Bayer House, Strawberry Hill, Newbury, Berkshire, RG14 1JA

Fisher Scientific UK Ltd - Bishop Meadow Road, Loughborough, LE11 5RG

Hanna Intruments - Eden Way, Pages Industrial Park, Leighton Buzzard, Bedfordshire, LU7 4AD

Harlan Laboratories UK - Shaws Farm, Station Road, Blackthorn, Bicester, Oxfordshire, OX25 1TP

Life Technologies Ltd - 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF

Millipore UK Ltd - Suite 3 & 5 Building 6, Croxley Green Business Park, Watford, Hertfordshire, WD18 8YH

Phoenix Pharmaceuticals, inc. - UK distributer: Phoenix Europe GmbH, Viktoriastrasse 3-5, D-76133 Karlsruhe, Germany

Santa Cruz Biotechnology, inc. - UK distributer: Insight Biotechnology Ltd, PO Box 520, Wembley, HA9 7YN

Sigma-Aldrich Company Ltd - The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT

Smith & Nephew Medical Ltd - Healthcare House, 101 Hessle Road, Hull, HU3 2BN

TekLab - 9 Dorothy Terrace, Sacriston, County Durham, DH7 6LG

Vector Laboratories Ltd - 3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS

Vet-Tech Solutions Ltd - Unit 17, Daneside Business Park Congleton, Cheshire, CW12 1UN

VetXX Ltd - The Malthouse, Mill Lane, Scotsgrove, Thame, Oxfordshire, OX9 3RP