A study of the relationship between C-type natriuretic peptide and pregnancy in sheep and red deer

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Bryony Alice McNeill

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C-type natriuretic peptide (CNP) is a signaling molecule with important roles in mammalian growth and vascular function. Unexpectedly high plasma CNP concentrations have been reported in late gestation ewes, suggesting a possible role during pregnancy. However, the temporal pattern of CNP in maternal circulation, site/s of production and function during pregnancy have not been identified. Consequently, the aim of this PhD project was to address these unexplored aspects of CNP biology in order to improve our understanding of CNP's role in pregnancy.

To characterise the temporal pattern of maternal circulating CNP during pregnancy, a longitudinal survey was conducted in two representative species of ruminant: sheep and red deer. Plasma concentrations of both CNP and a presumed bio-inactive fragment of its precursor (NTproCNP) increased in twin-bearing ewes at 40-50 days of gestation with peak values attained (CNP 31 ± 5 pmol l^{-1}, NTproCNP 270 ± 16 pmol l^{-1}) at about day 120. A similar temporal pattern was evident in red deer hinds. In pregnant ewes studied at day 120, there was a positive relationship between maternal CNP forms and fetal number ($P < 0.01$). Collectively, these results strongly implicated the uteroplacental unit as the major source of CNP during pregnancy. Therefore, studies were conducted to determine the relative contribution of uterine and placental tissues to circulating CNP concentration. The concentration of both CNP forms in the placenta exceeded that in intercaruncular uterine tissue throughout pregnancy ($P < 0.05$), with the highest concentrations measured in the fetal cotyledon. Immunohistochemistry revealed staining of CNP and NTproCNP around placental blood vessels and in trophoblast binucleate cells (BNC), identifying these cells as a probable source of maternal circulating CNP during pregnancy.

Having identified the source of CNP during pregnancy, further studies were undertaken to test the hypothesis that CNP production by the ovine uteroplacental unit is homeostatically regulated by nutritional status, using models of nutrient restriction (3-day fast) or nutrient abundance (caloric loading or mid-pregnancy shearing). Maternal circulating NTproCNP concentration, expressed as a percentage of pre-fasting concentration, was significantly elevated in ewes fasted in late pregnancy compared with controls (117 ± 5.1 % vs 107 ± 3.5 %, $P < 0.05$). In contrast, there was no effect of nutrient abundance on the concentration of either CNP form, suggesting that CNP production is selectively upregulated during situations of fetal restriction.

CNP's localisation to the BNC and high circulating concentrations in the pregnant ruminant suggest a novel endocrine role for the peptide during pregnancy, in addition to likely paracrine actions within the placental vasculature. The strong relationship between maternal circulating CNP and placental peptide production identified in this thesis makes the pregnant ewe a unique *in vivo* model for studying the regulation of CNP during pregnancy, and may reveal potential applications for this peptide as a marker of maternal and/or fetal health and wellbeing in both human medicine and animal production.

Keywords: C-type natriuretic peptide, CNP, NTproCNP, placenta, fetus, pregnancy, sheep, red deer, binucleate cell, caloric restriction, caloric loading, mid-pregnancy shearing

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Statement

The studies described in this thesis were conducted as part of an ongoing collaboration between Lincoln University and the University of Otago, Christchurch. Although I had a key involvement in the design and execution of all experiments and writing of this thesis, the significant contributions of my PhD supervisors and co-authors, various field and laboratory technicians and anonymous reviewers must be acknowledged. In all chapters, statistical analyses were performed under the direct supervision of a biometrician. My specific contribution to the individual studies is outlined at the beginning of each experimental chapter.

All procedures involving animals were approved by the Lincoln University Animal Ethics Committee (Chapters 4-6) or the Massey University Animal Ethics committee (Chapter 7). Collection of animal tissues for the immunohistochemistry performed at the University of Cambride (Chapter 5) was conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Publications arising from this thesis

Journal articles

McNeill, BA; Barrell, GK; Wooding, FBP; Prickett, TCR and Espiner, EA. (2011) The trophoblast binucleate cell is the source of maternal circulating C-type natriuretic peptide in ovine pregnancy. *Placenta,* **In press**

Prickett, TCR, McNeill, BA, Oliver, MH, Harding, JE, & Espiner, EA. (2010) Effect of cortisol on C-type natriuretic peptide (CNP) in ovine pregnancy: differential responses in fetal and placental tissues. *Pediatric Research* **68** 462-465

McNeill BA, Prickett TCR, Wellby M, Ridgway MJ, Espiner EA, Barrell GK (2010) Circulating levels of C-type natriuretic peptide (CNP) are strongly linked to pregnancy but not to liveweight changes in ruminants. *Proceedings of the New Zealand Society of Animal Production* **70** 13-18

McNeill BA, Barrell GK, Wellby M, Prickett TC, Yandle TG, Espiner EA. (2009) C-type natriuretic peptide (CNP) forms in pregnancy: maternal plasma profiles during ovine gestation correlate with placental and fetal maturation. *Endocrinology* **150** 4777-4783

Conference abstracts

McNeill BA, Barrell GK, Prickett TC, Espiner EA. (2010) Maternal circulating C-type natriuretic peptide (CNP) is produced by trophoblast binucleate cells during ovine pregnancy. *Poster presentation at the International Ruminant Reproduction Symposium, Anchorage Alaska, USA, September 2010*

Prickett TC, McNeill BA, Oliver MH, Harding JE, Espiner EA. (2010) Increased fetal cortisol reduces fetal but not maternal plasma NTproCNP in late ovine gestation. *Poster presentation at the 92nd annual meeting of the Endocrine Society, San Diego, California, USA, June 2010.*

McNeill BA, Wellby M, Prickett TC, Yandle TG, Espiner EA, Barrell GK. (2009) The fetal cotyledon is a major source of maternal circulating C-type natriuretic peptide in ovine

pregnancy. *Oral presentation at the NZSE/ESA combined meeting in Adelaide, Australia, August 2009.*

McNeill BA, Wellby M, Prickett TC , Harding JE, Yandle TG, Espiner EA, Barrell GK. (2009) The placenta is a major site of CNP synthesis during ovine pregnancy: differential contributions to maternal and fetal circulations. *Poster presentation at the 91st annual meeting of the Endocrine Society, Washington DC, USA, June 2009.*

McNeill BA, Barrell GK, Wellby M, Prickett TC, Yandle TG, Espiner EA. (2008) Sustained elevation of circulating maternal CNP forms in pregnant ewes and red deer hinds: a longitudinal study. *Oral presentation at Medsci, Queenstown, New Zealand, November 2008.*

McNeill BA, Barrell GK, Wellby M, Prickett TC, Yandle TG, Espiner EA. (2008) Maternal Plasma Concentrations of CNP Forms in Normal Ovine Pregnancy: Evidence of Sustained Production and Abrupt Fall One Week Pre Term. *Poster presentation at the 90th meeting of the Endocrine Society, San Francisco CA, USA, June 2008.*

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List of abbreviations

ANP	Atrial natriuretic peptide
BNC	Binucleate cell
BNP	Brain natriuretic peptide
β-OHB	β-hydroxybutyrate
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor-1
ir	Immunoreactive
IUGR	Intrauterine growth retardation
NO	Nitric oxide
NP	Natriuretic peptide
Nppc	CNP gene
NPR-A	Natriuretic peptide receptor A
NPR-B	Natriuretic peptide receptor B
NPR-C	Natriuretic peptide receptor C
NTproCNP	Amino-terminal pro C-type natriuretic peptide
oPL	Ovine placental lactogen
PAG	Pregnancy-associated glycoprotein
PBS	Phosphate buffered saline
proCNP	Pro C-type natriuretic peptide
REML	Restricted maximum-likelihood test
RIA	Radioimmunoassay
SEM	Standard error of the mean
TFA	T*rifluoroacetic acid*
UBF	Uterine blood flow
V_0	Void volume
vs	Versus
VSMC	Vascular smooth muscle cells

Chapter 1:
General introduction

C-type natriuretic peptide (CNP) is one of a number of structurally-related peptides belonging to the natriuretic peptide family. In contrast to the other natriuretic peptides, which are involved in salt and water homeostasis and cardiac health, the primary roles of CNP are believed to relate to vascular function and skeletal growth. A role in reproduction has also been proposed (reviewed by Walther & Stepan, 2004), as CNP and its receptor have been identified in a number of reproductive tissues in both male and female mammals, including the ovary, uterus, and testes.

In 2007, Prickett *et al.* reported unusually high plasma concentrations of CNP and the presumed bioinactive amino-terminal fragment released during processing of proCNP (NTproCNP) in late-gestation ewes, suggesting a possible role for CNP in pregnancy. The findings of this study also raised the hypothesis that CNP production during pregnancy may be homeostatically regulated by nutrition. However, nothing was known of CNP's involvement (if any) during early- and mid-pregnancy, or how these findings related to CNP activity during pregnancy in other ruminant species. Furthermore, the cellular site/s of CNP production and the factors controlling its regulation during pregnancy had not been determined. Consequently, this PhD project was initiated to address some of the important questions raised by the findings of Prickett *et al.* (2007), with the intention of furthering our understanding of the biology of CNP during pregnancy.

The primary aim of this research project was to document the temporal pattern of CNP and NTproCNP in maternal circulation for the duration of pregnancy to determine the stage of pregnancy at which the increase in CNP occurred and relate this to important physiological events of pregnancy. In addition to studies in sheep, red deer were included to determine whether the previous findings in pregnant ewes are a common feature of ruminant pregnancy, or whether they may be unique to the ovine situation. The results of this longitudinal survey are presented in **Chapter 4**. Having characterised the pattern of CNP forms in maternal circulation throughout pregnancy, subsequent experiments were conducted to identify the source of CNP during pregnancy (**Chapter 5**) and to test the hypothesis that CNP production is homeostatically regulated by nutrition (**Chapters 6 and 7**), with an ultimate goal of understanding the role of this hormone during pregnancy. The major findings and important

contributions of these studies, as well as key areas remaining to be addressed are discussed in **Chapter 8**, with reference to the potential applications of this work both for human medicine and animal production.

Chapter 2:
Literature review

2.1 Introduction to the natriuretic peptides

2.1.1 ANP and BNP

In mammals, the natriuretic peptide (NP) family consists of at least three structurally-related peptides; atrial (ANP), B-type (BNP) and C-type (CNP) natriuretic peptide, although other NPs have been identified in lower vertebrates (Kawakoshi *et al.*, 2004). ANP and BNP are produced in the atrium and ventricle respectively, and their secretion is stimulated by atrial or ventricular wall stretch. These peptides act in an endocrine fashion to regulate blood pressure and volume homeostasis by promoting vasodilation, increasing fluid and electrolyte excretion (natriuresis) and inhibiting renin and aldosterone secretion (reviewed by Tremblay *et al.*, 2002). The natriuretic effects of ANP were first discovered when rats displayed a reduction in blood pressure and an increase in both urine quantity and electrolyte concentration after being given extracts of atrial myocardium (de Bold *et al.*, 1981). In health, circulating concentrations of ANP exceed those of BNP and CNP. A notable exception is in cases of congestive heart failure, in which circulating BNP concentrations increase dramatically. The biology of ANP and BNP has been described previously in numerous reviews (for example, Tremblay *et al.*, 2002; Potter *et al.*, 2006; Woodard & Rosado, 2008).

2.1.2 CNP

CNP was first identified following isolation from the porcine brain (Sudoh *et al.*, 1990) and, in mammals, is detectable in two forms; CNP-22 and CNP-53 (Figure 2-1). CNP-22 is the predominant form in the circulation whereas CNP-53 is more prevalent in tissues (Stingo *et al.*, 1992). Functionally, CNP is distinct from the other members of the NP family. For example, despite its name CNP does not stimulate natriuresis when administered to achieve physiological concentrations (Hunt *et al.*, 1994) and, unlike BNP, circulating CNP concentration is not elevated in congestive heart failure (Kalra *et al.*, 2001). Similarly, CNP's hypotensive effects are also smaller than those of the other natriuretic peptides (Sudoh *et al.*, 1990). Also, CNP's mode of action is unique within the NP family; acting primarily in a

paracrine/autocrine fashion in contrast to the classical endocrine actions of ANP and BNP (Scotland *et al.*, 2005).

All three NPs share a common 17 amino acid ring structure and amino-terminal 'tail' of varying length (Figure 2-1). The gene for CNP is located on a different chromosome from those of other NPs (Komatsu *et al.*, 2002) and is likely to be the ancestral molecule from which ANP and BNP are derived (Inoue *et al.*, 2003).

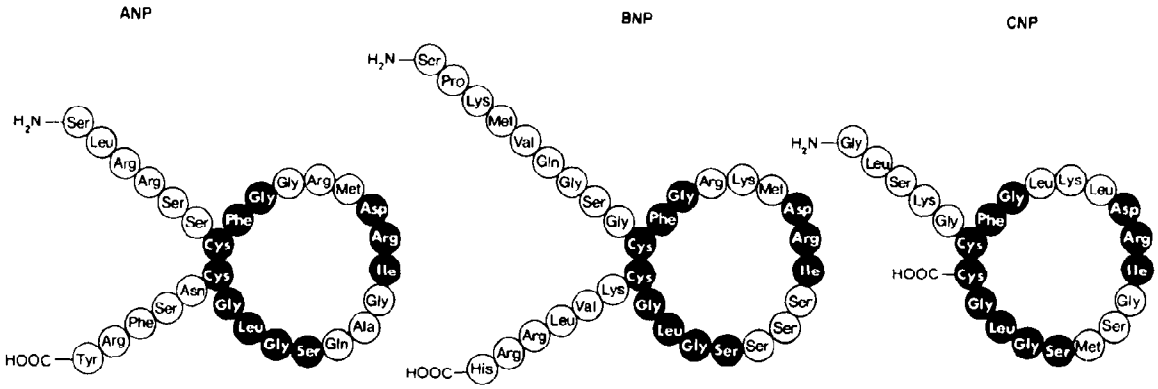

Figure 2-1.

The mature form of human ANP, BNP and CNP. Shaded amino acids are conserved among the three NPs. Figure reproduced from Yandle (1994) with permission from John Wiley and Sons.

2.1.3 Natriuretic peptide receptors

The biological effects of the NPs are achieved via interaction with the guanylyl cyclase receptors NPR-A or NPR-B and synthesis of the intracellular signalling molecule cyclic guanosine monophosphate (cGMP) (reviewed by Tremblay *et al.*, 2002). Binding of the natriuretic peptides to the extracellular binding domain of these transmembrane receptors results in activation of the particulate form of guanylyl cyclase, an enzyme involved in catalysing the production of cGMP (Tremblay *et al.*, 2002). In contrast to ANP and BNP, which interact with the NPR-A receptor, CNP binds preferentially to NPR-B, which is known also as ANP-B (Suga *et al.*, 1992), GC-B or NPR2 (Agoston *et al.*, 2006) receptor. To minimise confusion, the term NPR-B will be used throughout this review. The gene for this receptor, *NPR2*, has been identified in brain, pituitary gland, adrenal gland, kidney, lung, cartilage and various reproductive tissues (Olney *et al.*, 2006).

Also, another receptor known as the NPR-C functions to process circulating CNP (Madhani *et al.*, 2003), and is the most abundant NP receptor form (Scotland *et al.*, 2005). The physiological actions of NPR-C are achieved by inhibition of adenylyl cyclase activity and cAMP generation (reviewed by Anand-Srivastava, 2005). Also, NPR-C functions as a clearance receptor; modulating the bioavailability of the NPs (Scotland *et al.*, 2005). The structure of the three receptors is illustrated in Figure 2-2.

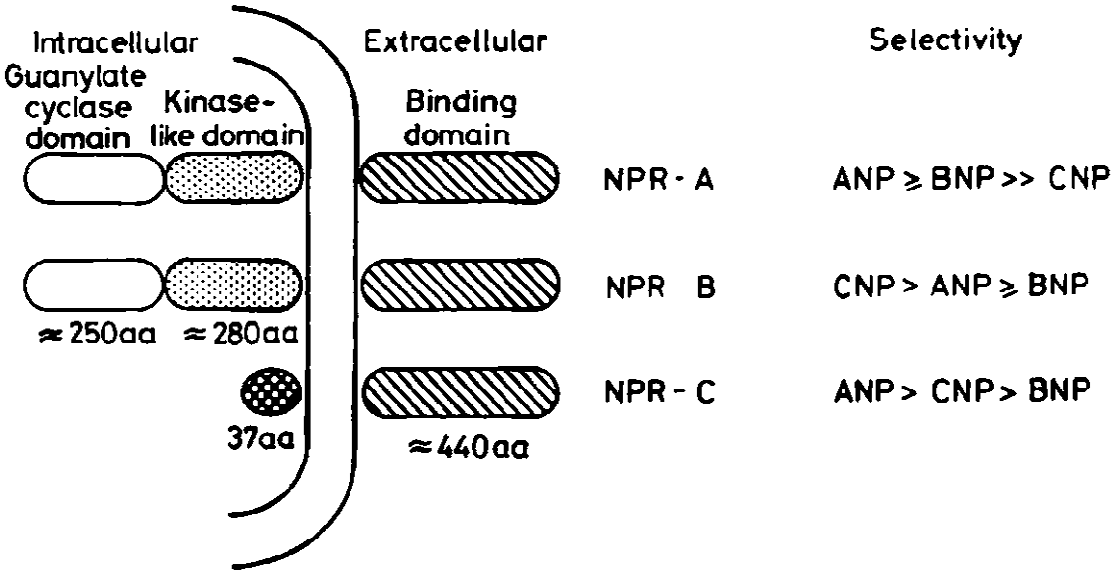

Figure 2-2.

Schematic diagram of the three natriuretic peptide receptors. From top to bottom: NPR-A, NR-B and NPR-C. The order of selectivity for the three receptor forms is shown to the right of the figure. Reproduced from Yandle (1994) with permission from John Wiley and Sons.

As well as interaction with NP receptors, CNP can also be processed by the enzyme neutral endopeptidase, a zinc metalloprotease (Walther *et al.*, 2004). This enzyme aids in the hydrolysis of internal peptide bonds in CNP, thus opening up the ring structure (Walther *et al.*, 2004). Neutral endopeptidase has been identified in a range of tissues including heart, kidney, lung and endothelium (reviewed by Scotland *et al.*, 2005).

2.1.4 N-terminal fragments

ANP, BNP and CNP are synthesised initially as preprohormones (Potter *et al.*, 2006), which are then processed intracellularly to form a biologically-active mature peptide as well as a biologically-inactive amino-terminal portion, known as NTproANP, NTproBNP and NTproCNP, respectively. These amino-terminal fragments circulate at much higher concentrations than the active peptide forms as they are not degraded by neutral endopeptidase or cleared from the circulation by NPR-C (Charles *et al.*, 2006). The extremely short half-life of CNP (2-4 minutes in sheep) has presented difficulties for measuring circulating concentrations of the peptide in blood samples (Prickett *et al.*, 2001). However, the extended half -life of NTproCNP provides an alternative and possibly more reliable measure of CNP activity *in vivo* (Prickett *et al.*, 2001).

2.1.5 The role of CNP

In contrast to ANP and BNP, with established roles in salt and water homeostasis, the functions of CNP are diverse, and have not been characterised to the same extent as the other NPs. To date, most research has focussed on CNP's activity within the vasculature, neural tissue, in the growing skeleton and in reproductive tissues.

2.1.5.1 CNP in the vasculature

CNP and NPR-B mRNA and protein have been identified in vascular smooth muscle cells (VSMCs) (Suga *et al.*, 1992; Woodard *et al.*, 2002), comprising the 'vascular natriuretic peptide system' (Suga *et al.*, 1993); a local NP system which involves paracrine/autocrine induction of vasodilation and growth inhibition of VSMCs by CNP (Suga *et al.*, 1993). The vasodilatory effects of CNP are achieved either by interaction with the NPR-B receptor, resulting in accumulation of cGMP (Furuya *et al.*, 1991) and relaxation of the vascular wall (Itoh *et al.*, 1998) or through interaction with NPR-C and activation of G protein-gated inwardly rectifying K+ channel (Chauhan *et al.*, 2003).The vasodilatory actions of CNP are more potent in venous compared with arterial vessels (Wei *et al.*, 1993).

Endothelial shear stress, when present at high levels, is a potent stimulator of CNP secretion in the vasculature (Okahara *et al.*, 1995; Chun *et al.*, 1997). Shear stress acts on the surface of blood vessels; in the direction of blood flow (Okahara *et al.*, 1995), and is detected by mechanoreceptors on cells of the vascular endothelium (Okahara *et al.*, 1995). In addition to regulating vascular tone and VSMC growth, CNP is likely also to be involved in angiogenesis

(Yamahara *et al.*, 2003). CNP's role in vascular health and disease has been reviewed previously (Ahluwalia & Hobbs, 2005; Scotland *et al.*, 2005).

2.1.5.2 CNP in nervous tissue

Very high concentrations of CNP and NPR-B have been identified in brain, spinal cord and cerebrospinal fluid (Togashi *et al.*, 1992; Potter *et al.*, 2006). The role of CNP within the nervous system has not been conclusively determined, but possible roles include regulating the synthesis and/or secretion of neuroendocrine hormones (Samson *et al.*, 1993) and neuronal development (DiCicco-Bloom *et al.*, 2004; Müller *et al.*, 2009).

2.1.5.3 CNP in skeletal tissue

In recent years, there has been significant interest in the importance of CNP in mammalian growth, particularly skeletal growth. The development of CNP knock-out mouse models, with impaired skeletal growth and pronounced dwarf phenotype, has been a strong driver of this research. Specifically, CNP plays a critical role in endochondral ossification; the process by which cartilaginous tissue becomes calcified and replaced by bone (Bartels *et al.*, 2004). In humans, a naturally-occurring mutation in the *NPPC* gene which codes for the NPR-B receptor has been identified in individuals with acromesomelic dysplasia: type Maroteaux, an autosomal recessive condition associated with a dwarf phenotype due to inappropriately formed growth plates (Bartels *et al.*, 2004). Conversely, overexpression of *NPPC* in humans results in marked skeletal overgrowth due to inappropriately high CNP production (Bocciardi *et al.*, 2007; Moncla *et al.*, 2007). The putative role of CNP in skeletal growth and development has been described in detail in published reviews (Komatsu *et al.*, 2002; Espiner *et al.*, 2007; Teixeira *et al.*, 2008).

2.1.5.4 CNP and the reproductive system

Elevated CNP gene expression has been identified in various reproductive tissues in both males and females in a number of mammalian species, indicating that a functional role for CNP in mammalian reproduction is likely. Modulation of CNP gene expression by oestradiol in the uterus (Acuff *et al.*, 1997; Jankowski *et al.*, 1997) and by luteinising hormone and gonadotrophin releasing hormone within the anterior pituitary gland (McArdle *et al.*, 1993; McArdle *et al.*, 1994), suggest that the circulating concentration and local production of the peptide may be regulated by reproductive hormones. A description of CNP mRNA expression and possible function in mammalian reproduction has been reviewed in detail by Walther and Stepan (2004).

2.1.5.5 CNP and fetal development

There is a relative paucity of data regarding the development of CNP signalling in the fetus, with the data published prior to 2004 summarised in a review by Walther and Stepan (2004). CNP signalling in the fetus appears to be functional by mid- to late-gestation with significant concentrations of CNP being detected in the circulation of fetal sheep (Prickett *et al.*, 2007) and humans (Stepan *et al.*, 2000), which is likely to be regulated independently from the maternal system (Prickett *et al.*, 2007).

The role of CNP in fetal development has not been determined conclusively. As discussed by Prickett *et al.* (2007), the skeletal effects of disrupted CNP signalling in gene knock-out models are generally not evident at birth, and in neonatal mice (12 hours post-delivery) CNP mRNA expression is strong in the brain but completely absent from the femur (Stepan *et al.*, 2000), indicating that CNP is unlikely to play an important role in fetal skeletal growth. Alternatively, CNP may have an important role in development of the fetal brain and peripheral nervous system. Expression of CNP and NPR-B mRNA is abundant in the embryonic mouse and rat brain (Cameron *et al.*, 1996; Müller *et al.*, 2009), and abnormalities in sensory neuron function have been reported in CNP knock-out mice. Furthermore, mitogenic effects of CNP on dorsal root ganglia (DiCicco-Bloom *et al.*, 2004) and strong temporal similarities in the expression of NPR-B and nestin, a marker of neural progenitor cell activity, indicate a possible role for CNP in neurogenesis (Müller *et al.*, 2009).

2.2 Pregnancy and placentation

Evolution of the eutherian (placental) mode of reproduction conferred significant evolutionary advantages, and has consequently become a highly successful reproductive strategy. In placental mammals, the developing embryo is physically contained and nourished by the pregnant female, providing protection from adverse conditions including dehydration, flooding, predation and temperature extremes (Blackburn, 1999). Pregnancy requires a coordinated communication between mother and fetus and is a time of many physiological changes. As the current study relates to CNP's role in ovine and cervine pregnancy, and because this may have implications for the human event, this review will focus on discussion of pregnancy in these species.

2.2.1 The placenta

The eutherian placenta is formed by the establishment of a connection between the extraembryonic membranes and the uterine epithelium (Senger, 2005) thus providing an effective means of nourishment for the developing fetus. Placentation in ruminants is characterised by the development of placentomes; discreet units where fetal placental tissue (cotyledon) attaches to caruncular regions on the maternal uterus to establish a point of contact between the developing conceptus and its dam, thus forming a placenta.

The number of placentomes in a pregnancy varies among ruminant species; ranging from 4-6 large placentomes in red deer (Wooding *et al.*, 2007) up to 100 in a normal ovine placenta (Ward *et al.*, 2006). Placentomes can be categorised as A-, B-, C- or D-type, depending on their morphology and proportion of maternal and fetal tissue (Vatnick *et al.*, 1991). The majority of pregnancy is dominated by A- and B-type placentomes, with a greater proportion of C- and D-type placentomes appearing only late in gestation (Ward *et al.*, 2006). A higher proportion of C- and D-type placentomes are also present with larger litter sizes and in cases of suboptimal intrauterine conditions in early pregnancy (Ward *et al.*, 2006). These placentomes are larger and believed to be more efficient than A- and B-types, and their increased abundance in compromised pregnancies may be an adaptive response to cope with reduced nutrient availability (Vonnahme *et al.*, 2006; Ward *et al.*, 2006).

The ruminant placenta was originally classified as syndesmochorial, as early studies reported partial destruction of the maternal uterine epithelium and direct contact between the trophectoderm and maternal connective tissue (reviewed by Wooding, 1992). However, an intact uterine epithelium has since been identified and the classification modified to epitheliochorial (Wooding, 1982). This type of placenta is characterised by a direct contact between the uterine epithelium and chorion with no destruction of the uterine epithelium (Wooding, 1992). Although this second classification may be more fitting, Wooding (1992) argues that, as the uterine epithelium is highly modified to form a fetal-maternal hybrid tissue, neither of these placental classifications is entirely appropriate for describing the placenta of ruminants.

In contrast, the human placenta is classified as haemochorial (Senger, 2005). This type of placenta is associated with invasive, or interstitial, implantation in which the conceptus becomes completely imbedded in the maternal endometrial tissue to become physically

separated from the luminal space (Senger, 2005). The deep invasion of maternal tissue by the human conceptus results in the establishment of a direct contact between the terminal villi of the chorionic syncytiotrophoblast and maternal blood (Johnson, 2007). In the non-invasive ruminant placenta, the conceptus remains in the uterine luminal compartment for the duration of pregnancy, also known as eccentric implantation (Johnson, 2007).

2.2.2 Trophoblast binucleate cells

A unique feature of ruminant pregnancy is the presence of trophoblast binucleate cells (BNC). These cells first appear at implantation (Wango *et al.*, 1990), making up approximately 20 % of fetal trophoblast cells and persist until the final week of pregnancy when their abundance sharply declines to around 8 % (Wooding, 1982). BNC are present in all species of ruminant examined thus far, including the mouse deer which belongs to the most primitive group of ruminant, the tragulidae (Wooding *et al.*, 2007). BNC provide a method of hormone delivery which allows transport of products from fetus to mother without activation of a maternal immune response. The formation, migration and control of BNC activity has been reviewed previously (Wooding, 1982; Lee *et al.*, 1995; Wooding *et al.*, 2007).

2.2.3 Relevance of animal models to human pregnancy

Due to the high risks and ethical constraints of studying human pregnancy, much of our knowledge regarding fetal and placental physiology has been gained from animal studies (Handwerger *et al.*, 1975). In particular, the pregnant ewe is one of the most widely studied models of pregnancy and parturition. There are considerable advantages of this model for the study of human pregnancy: the ewe has a long gestation period, is easy and inexpensive to house and both the ewe and her neonate are of comparable body weight to humans. The small litter size and relative maturity of the neonatal lamb may also provide a more relevant model than rodent species. Furthermore, the large size of the ewe and developing fetus allow for the easy collection of blood and tissue samples in sizable volumes, and allow for serial measurements.

However, despite the many similarities between ovine and human pregnancies there are some important differences between the two species. Sheep models have been used widely for the study of high-risk pregnancies, such as intrauterine growth retardation (IUGR) and preeclampsia. However, true preeclampsia occurs only in humans and apes, and the various ovine models designed to replicate this condition may involve different mechanisms to those

present in humans (Podjarny *et al.*, 1999). The placental structure and manner of implantation also show considerable variation between the two species (Barry & Anthony, 2008), and there are endocrine differences between the two species in normal pregnancy (Johnson, 2007). Consequently, although the sheep is an extremely useful tool in the study of human pregnancy, information gained from such animal models must be applied to the human situation with some caution.

2.3 Involvement of CNP in pregnancy

The function/s (if any) of CNP during mammalian pregnancy have only recently come under investigation, with results to date sometimes being inconclusive and often contradictory. However, a sufficient number of studies implicate a role for the peptide in pregnancy, and support the need for further research in this field. The remainder of this review will examine evidence for an involvement of CNP in pregnancy using the literature available at the commencement of this PhD programme.

In 1998 Itoh *et al.* reported a decrease in NP clearance receptor abundance in the uterine arteries of pregnant ewes (Itoh *et al.*, 1998). Together with the finding that the concentration of cGMP is significantly upregulated in plasma and urine during pregnancy (reviewed by Itoh *et al.*, 1999), these data may be indicative of an increase in CNP activity or synthesis in the pregnant female (Itoh *et al.*, 1998). Surprisingly however, when circulating CNP concentration was measured in pregnant women, no elevation was found compared with non-pregnant values (Stepan *et al.*, 1998). In contrast, a more recent study by Prickett *et al.* (2007) reported plasma CNP concentrations to be some 50 times higher in pregnant compared with non-pregnant ewes in late gestation, strongly supporting a role for CNP in ovine pregnancy.

Although the source of maternal circulating CNP during pregnancy has not been confirmed, there is evidence to suggest that the placenta may be a major contributor. Studies of CNP mRNA expression have revealed particularly high gene expression in murine (Cameron *et al.*, 1996) and human (Stepan *et al.*, 2001) placental tissue. Furthermore, maternal circulating NTproCNP concentration is directly related to placental weight in the pregnant ewe (Prickett *et al.*, 2007). Higher circulating CNP concentrations in twin- compared with single-bearing ewes is also consistent with a placental source, as total placental mass increases with fetal number (Prickett *et al.*, 2007).

The potent vasoactive properties of CNP and abundant gene expression around the blood vessels of the decidua basalis of the placenta suggest that the peptide may have an involvement in regulating blood flow and mediating the massive enlargement of blood vessels which occurs during pregnancy (Cameron *et al.*, 1996). In women with preeclampsia, circulating NTproCNP concentration is significantly elevated (Prickett *et al.*, 2004). In preeclampsia, vascular invasion of the uterus is insufficient, resulting in decreased blood flow to the placenta (DiFederico *et al.*, 1999), and elevated CNP secretion in these pregnancies may be an adaptive response to the impaired placental development and vascular pathology characteristic of the condition. Similarly, IUGR is another condition characterised by pathology of the placenta and inappropriate blood flow to the pregnant uterus, and, like preeclampsia, is associated with abnormal CNP expression in the uterus and placenta (Stepan *et al.*, 2002a).

In women with IUGR, maternal circulating CNP concentration was found not to differ from concentrations measured in normal pregnancies (Stepan *et al.*, 2002a). However, it must be noted that the elevation in plasma peptide concentration in women with preeclampsia reported by Prickett *et al.* (2004) was evident only in the case of NTproCNP, and not for CNP itself. Likewise, an earlier study by Stepan *et al.* (2002b) found no relationship between circulating maternal CNP concentrations and preeclampsia. Thus it is evident that NTproCNP concentration may be a more informative measurement of CNP's activity *in vivo*, and highlights the need for further studies characterising the activity of both CNP and NTproCNP in the placenta and maternal circulation in both normal and pathological pregnancies to reveal the full extent of CNP's involvement in pregnancy.

2.4 Rationale for thesis

The finding of extremely high circulating concentrations of CNP and NTproCNP in the pregnant ewe (Prickett *et al.*, 2007) suggests an important role in ovine pregnancy, and may reflect an entirely new function for this peptide. This finding, together with previous studies also raises a number of questions regarding CNP's regulation, relationship with health and disease status, and possible species-related differences. The very high plasma concentrations of CNP in ovine pregnancy has also challenged the conventional understanding of CNP's mode of action; strongly suggesting an endocrine role during pregnancy instead of, or in addition to, its established role as a paracrine/autocrine factor.

In keeping with the principles of comparative physiology, the pregnant ewe appears to be a unique model for the study of CNP biology, as it is the only situation identified to date in which CNP levels are significantly elevated in the healthy adult. This has implications for human health; for example, the elevated circulating oestradiol concentrations during pregnancy provide the opportunity to investigate CNP activity during periods of elevated steroid hormone concentration; potentially informative in assessing the possible effects of CNP on the cardiovascular and other systems during hormone-replacement therapy, for example in post-menopausal women (Acuff *et al.*, 1997).

During pregnancy, CNP has potential to be utilised as a marker of fetal and/or maternal health. The strong evidence suggesting a placental source for the peptide during pregnancy, and changes in CNP gene expression and plasma levels in pathological pregnancies open up the possibility that CNP may serve as a new diagnostic tool in indentifying pregnancies at risk of IUGR, preeclampsia or other conditions characterised by inappropriate placental function. Due to the biological importance and these potential practical applications, there is now a critical requirement for further research extending the findings of previous studies to clarify the source, maternal concentration and functional role of CNP during pregnancy.

Chapter 3:
General methods

This chapter outlines the field and laboratory procedures used throughout the studies described in this thesis. Any deviations from these methods are described in the methods section of individual experimental chapters.

3.1 Animal procedures

3.1.1 Collection of blood samples

Ewes were placed in groups of around 10 animals in outdoor yards and manually restrained for blood sampling, except for the study described in Chapter 6 in which ewes were restrained in individual indoor pens. Hinds were placed in groups of 4-6 in an indoor pen and manually restrained for blood sampling.

Blood collection from ewes and hinds was by jugular venipuncture into 10 ml evacuated blood tubes containing potassium ethylenediaminetetraacetate (EDTA) (natriuretic peptides, progesterone, oestradiol and β-hydroxybutyrate (β-OHB)) or sodium heparinate (insulin-like growth factor-1 (IGF-1), glucose and urea) as anticoagulant (Vacutainer, Franklin Lakes NJ, USA). Blood tubes were inverted several times to ensure complete mixing of the anticoagulant and then immediately placed on ice. Tubes were centrifuged at 800 g for 10 minutes at 4 °C, then plasma was transferred into polystyrene tubes and stored at -20 °C until required. Plasma was aliquotted into separate tubes for each analyte measured to reduce the number of freeze/thaw cycles. The time from collection until centrifugation did not exceed two hours.

Blood collection from fetal lambs was by cardiac puncture immediately post-mortem into a syringe. Fetal blood was then transferred from the syringe into evacuated blood tubes containing appropriate anticoagulant, mixed well and processed in an identical manner to that described for adult animals.

3.1.2 Measurement of live weight and body condition score

Live weight to ± 0.5 kg was measured by placing animals individually in an appropriate-sized holding crush fitted with electronic scales.

Body condition score was assessed by visual appraisal using a standard scale of 1 (emaciated) to 5 (obese). To ensure consistency, body condition score in ewes and red deer hinds was assessed by one person (M. Ridgway, Lincoln University) on all occasions throughout the studies described in this thesis.

3.2 Hormone assays

In all cases, the entire set of samples from an individual animal was processed in duplicate in a single assay to minimise any impact of interassay variation. Detailed methods for all assays are described below.

3.2.1 Natriuretic peptides

NP concentrations were determined by radioimmunoassay (RIA) performed at Christchurch Cardioendocrine Research Laboratory at the University of Otago, Christchurch, using in-house assay protocols.

Extraction of peptides from plasma

NPs were extracted from plasma samples using Sep-Pak C_{18} columns (Walters, Milford, MA, USA). The columns were prepared by the addition of 5 ml of 100 % methanol followed by 5 ml of 0.1 % trifluoroacetic acid (TFA). Plasma samples (1-4 ml) were then passed through the columns, followed by a further 5 ml of 0.1 % TFA. Peptides were eluted using 2 ml of 80 % isopropanol in 0.1 % TFA into polystyrene collection tubes containing 10 µl of 0.1 % Triton X-100 (addition of Triton to the collection tubes prevents binding of NPs to the tube). The eluant was collected, dried at 37 °C under an air stream and frozen at -20 °C.

Extraction of peptides from uteroplacental tissues

Samples of frozen uterine and placental tissue (approximately 1 g) were finely diced on a chilled plastic chopping board, weighed and gently boiled for 5 minutes in 10 ml distilled water containing 10 µl Triton X-100. After boiling, the tissue suspension was cooled by immediately placing on ice. After cooling, 610 µl of glacial acetic acid was added and the tissue suspension homogenised (4 x 20 second bursts at 24,000 rpm) using an Ultra-Turrax homogeniser (IKA-Labortecknik, Staufen, Germany). The tissue homogenates were then

centrifuged (3000 *g*, 4 °C, 30 minutes), extracted over SepPac C_{18} columns and assayed in an identical manner to that for plasma samples.

CNP

CNP was assayed as previously described (Yandle *et al.*, 1993; Prickett *et al.*, 2007), with minor changes: 50 µl standard (human proCNP(82-103)) or sample extract was preincubated with 50 µl of a commercial primary rabbit antiserum raised against proCNP (82-103) (Phoenix Pharmaceuticals Inc., Belbont, CA, USA. Catalogue #RAB–014–03) and diluted to 1:3000, then incubated for 22-24 hours prior to the addition of 50 µl of tracer (^{125}I labelled Tyr^{0}-proCNP(82-103)) containing 1500 cpm. Within- and between-assay coefficients of variation were 6.6 % and 8.6 % respectively at 1.1 pmol l^{-1}. The detection limit and EC50 for this assay was 0.4 and 6.1 pmol l^{-1} respectively.

NTproCNP

NTproCNP was measured as previously described (Prickett *et al.*, 2001; Prickett *et al.*, 2007) with the following alterations: 100 µl standard or sample extract was preincubated with 50 µl primary rabbit antiserum raised against NTproCNP (1-15) (J39, diluted 1:6000) then incubated for 22-24 hours prior to addition of 50 µl of tracer(^{125}I labelled proCNP(1-15)Tyr^{16}) containing 1500 cpm. Following a further 22-24 hour incubation period, bound and free labelled antigen were separated by addition of 500 µl of solid phase secondary antibody (5 % v/v Donkey anti-Rabbit Sac-cell (IDS Ltd, UK) diluted in assay buffer containing 5 % w/v dextran). Peptide standards were made from synthetic human proCNP(1-19) taking into account the purity data supplied (Chiron Technologies Pty Ltd, Australia). Within- and between-assay coefficients of variation were 7.5 % and 7.9 % respectively at 24 pmol l^{-1}. The detection limit and EC50 for this assay was 1.3 and 58 pmol l^{-1} respectively.

ANP

ANP was measured as described previously (Yandle *et al.*, 1986), with the following modifications: 50 µl standard/sample was preincubated with 50 µl of primary rabbit antiserum diluted to 1:10000, to which 50 µl of tracer was added after a 24 hour incubation period. Bound and free labelled antigen were separated by addition of 500 µl of solid phase secondary antibody (5 % v/v Donkey anti-Rabbit Sac-cell (IDS Ltd, UK) diluted in assay buffer containing 2 % polyethylene glycol). After 15 minutes incubation at room temperature, tubes were centrifuged at 4 °C for 15 minutes, and radioactivity of the pellet counted

following aspiration of the supernatant. The detection limit and EC50 for this assay was 10.5 and 31 pmol l^{-1} respectively.

NTproBNP

NTproBNP was measured by RIA as described previously (Pemberton *et al.*, 1998) with the following alterations: 50 µl standard/sample was preincubated with 50 µl of primary rabbit antiserum raised against synthetic ovine proBNP and diluted to 1:3000, to which 50 µl of tracer was added after a 24 hour incubation period. Bound and free labelled antigen were separated by addition of 500 µl of solid phase secondary antibody (5 % v/v Donkey anti-Rabbit Sac-cell (IDS Ltd, UK) diluted in assay buffer containing 2 % polyethylene glycol). After 30 minutes incubation at room temperature, tubes were centrifuged at 4 °C for 15 minutes, and the pellet counted following aspiration of the supernatant. The detection limit and EC50 for this assay was 9.7 and 65 pmol l^{-1} respectively.

3.2.2 Other hormone assays

Progesterone

Plasma progesterone concentration was measured in triplicate 50 µl aliquots by enzyme-linked immunosorbent assay (ELISA) as described by Anderson and Barrell (1998). Within- and between-assay coefficients of variation were 6.1 % and 14.9 % respectively.

Oestradiol

Plasma oestradiol concentration was measured in a commercial laboratory (Endolab, Canterbury District Health Board, Christchurch, New Zealand) by RIA using a commercial kit (E2 – Estradiol-2; Sorin Biomedica, Saluggia, Italy). Within- and between-assay coefficients of variation were 9.7 % and 20 % respectively at 41 pmol l^{-1}. The detection limit and EC50 for this assay was 4.5 and 130 pmol l^{-1} respectively.

β-Hydroxybutyrate

Plasma β-OHB concentration was measured in a commercial laboratory (Gribbles Veterinary Pathology, Christchurch, New Zealand) on a Hitachi Modular analyser using an enzymatic method based on the change in absorbance which occurs following oxidation of

D-3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase, and concomitant reduction of NAD+ to NADH (Randox Ranbut D-3-Hydroxybutyrate Manual, Randox Laboratories Ltd., County Antrim, United Kingdom).

Glucose

Plasma glucose concentration was measured photometrically by a commercial laboratory (Lincoln University, Lincoln, New Zealand) using a Roche Hitachi Cobas Mira Plus analyser.

Urea

Plasma glucose concentration was measured by a commercial laboratory (Lincoln University, Lincoln, New Zealand) using a kinetic UV assay performed on a Roche Hitachi Cobas Mira Plus analyser.

Insulin-like growth factor-1

IGF-1concentration in fetal plasma was measured by a commercial laboratory (Endolab, Canterbury District Health Board, Christchurch, New Zealand) by RIA. Briefly, 100 µl of plasma extract (previously extracted using hydrochloric acid and ethanol) or human IGF-1 standards (Novoenzymes GroPep, receptor grade) were incubated with anti-human IGF-1 antiserum (1:6000; Novoenzymes GroPep) together with an excess insulin-like growth factor-2 (IGF-2) (Novoenzymes GroPep, animal media grade) overnight at 4 ºC. 100 µl ^{125}I-labelled IGF-1 (Novoenzymes GroPep, receptor grade) was added and samples incubated overnight at 4 ºC. γ globulin was added, and bound and free radioactivity separated using polyethylene glycol followed by centrifugation for 15 minutes at 4000 rpm at 4 ºC. The inter-assay CV for this assay was 10 %.

3.3 Size-exclusion high performance liquid chromatography (HPLC)

Placental tissue samples were extracted in an identical manner as for NP RIA. The extracts were then resuspended in 20 % acetonitrile containing 0.1 % TFA and subjected to size exclusion HPLC using a G3000 (Toyo Soda, Tokyo, Japan, 7.5 x 600 mm) column equilibrated with 20 % acetonitrile in 0.1 % TFA at a 0.5 ml/minute flow rate. Eluted fractions were collected at one minute intervals into test-tubes containing 10 µl 0.1 % Triton X-100. The HPLC fractions were dried at 37 °C under an air stream and then assayed for CNP as described for plasma samples.

Chapter 4:
Maternal circulating CNP forms during pregnancy

Declaration

The majority of the content of this chapter has been published in the journal articles McNeill *et al.* (2009a) and McNeill *et al.* (2010). I was involved in all aspects of the study design and animal procedures. With the exception of the oestradiol assay, all hormone assays were conducted by me. The writing of the published manuscripts which form this chapter included contributions from co-authors, and benefited from the comments and suggestions of anonymous reviewers.

4.1 Abstract

Circulating concentrations of CNP are elevated in late-gestation ewes but the time course of these changes and relation to fetal maturation are unknown. Maternal plasma concentrations of CNP and NTproCNP were monitored at weekly intervals from preconception to 3 weeks postpartum in ewes with twins (n = 8), and in non-pregnant ewes (n = 8) studied similarly. In contrast to low and stable values in non-pregnant ewes (CNP 0.75 ± 0.08, NTproCNP 22 ±2 pmol l^{-1}), in pregnant ewes CNP forms increased abruptly at 40-50 days of gestation and rose progressively to peak values (CNP 31 ± 5 pmol l^{-1}, NTproCNP 270 ± 16 pmol l^{-1}) at about day 120. Approximately 7 days pre partum, the concentration of both CNP forms fell precipitously to preconception values immediately postpartum. An identical study of red deer hinds carrying singletons (n = 8) showed similar patterns though the peak maternal concentration of both CNP (2.14 ± 0.2 pmol l^{-1}) and NTproCNP (132 ± 9.5 pmol l^{-1}) was much lower. In a separate study of pregnant ewes, maternal plasma concentrations of CNP forms at day 120 were found to be positively related to fetal number - consistent with a major contribution from the utero-placental unit. These findings - the first report of maternal circulating CNP forms throughout pregnancy in any species - in showing an abrupt rise coinciding with placentation, and sharp decline within days of parturition, support a significant role of CNP in placental and fetal maturation in ruminants.

4.2 Keywords

C-type natriuretic peptide, NTproCNP, pregnancy, placenta, fetus, sheep, red deer

4.3 Introduction

CNP, a member of the NP family of structurally related peptides, is a paracrine/autocrine factor with important roles in cardiovascular homeostasis and skeletal development. CNP and its receptor, NPR-B, have also been identified in uterine and placental tissues and in both male and female gonads (reviewed by Walther & Stepan, 2004). Moreover in mice, uterine CNP mRNA expression is stimulated by oestradiol (Acuff *et al.*, 1997) and CNP concentration in ovarian tissues fluctuates with the oestrous cycle (Jankowski *et al.*, 1997). These findings suggest that CNP may be of particular importance in female reproductive biology.

Recent studies by Prickett *et al.* (2007) show that maternal circulating concentrations of CNP are some 50-fold higher in late gestation ewes when compared with non-pregnant control sheep. Plasma concentrations of the (presumably) biologically inactive form of CNP, NTproCNP, were also elevated at least 10-fold. A major contribution by utero-placental tissues to these high systemic concentrations was suggested by high veno-arterial CNP gradients across the gravid uterus (Prickett *et al.*, 2007), and significant correlation between maternal NTproCNP with placental weight (Prickett *et al.*, 2007). These findings in sheep contrast with those in humans where evidence of strong CNP gene expression in placental and uterine tissues (Walther & Stepan, 2004) is not reportedly reflected by elevation in plasma CNP concentration in healthy women during pregnancy (Walther & Stepan, 2004).

Postulating that in sheep the time course of maternal plasma concentration of CNP forms during pregnancy may provide insights to placental and fetal maturation, the aim of the current study was to conduct a longitudinal survey, from preconception to parturition, of maternal plasma CNP forms in healthy ewes and to compare the findings to normal non-pregnant ewes studied under identical conditions. A parallel study was conducted in red deer to establish whether the hormone profile in sheep may be common to ruminants or is unique to ovine pregnancy. Additionally, the hypothesis that CNP secretion has a positive relationship with fetal number was tested by measuring maternal circulating CNP and NTproCNP concentration in late gestation ewes with single-, twin- and triplet-pregnancies.

4.4 Methods

4.4.1 Longitudinal survey

Fifty-three Coopworth, or Coopworth x South-Hampshire cross ewes (*Ovis aries*) and fifteen red deer hinds (*Cervus elaphus*) of mixed age were randomly selected from the Lincoln University Research Farm for this study. Animals were grazed outdoors at the Lincoln University Research Farm, Canterbury, New Zealand, with supplementary feed supplied during winter. Synchronisation of the oestrous cycle was achieved using intravaginal progesterone-releasing devices ('Eazi-breed' type G CIDRs. Pfizer New Zealand Ltd., Auckland) in accordance with published protocols for sheep (Wheaton *et al.*, 1993) and deer (Rhodes *et al.*, 2003). On the day of mating, ewes were randomly allocated into two groups and run with two crayon-harnessed entire rams (pregnant group, n = 32) or with a crayon-harnessed vasectomised ram (control group n = 21). Hinds were also randomly allocated into two groups and run either with an entire red deer stag (pregnant group, n = 10) or as hinds only (control group, n = 5). Pregnant and non-pregnant animals were reunited five weeks later and run together for the remainder of the study. Confirmation of pregnancy was determined by ultrasonography in early gestation and subsequent birth of a lamb or deer calf. As conception dates were estimates, results are expressed in relation to actual parturition date.

Blood samples were collected weekly beginning one week preconception until three weeks postpartum (and throughout the same time period from non-pregnant ewes and hinds) for measurement of plasma CNP and NTproCNP concentration (ewes and red deer hinds). Plasma concentration of oestradiol and progesterone was also measured in pregnant ewes. Plasma concentration of ANP and NTproBNP was measured in a randomly-selected subsample of pregnant (n = 4) and non-pregnant (n = 4) ewes. Non-fasting live weights were recorded fortnightly.

4.4.2 Effect of number of fetuses

Mixed-age natural-mated Suffolk, Perendale or Corriedale ewes from the Lincoln University Research Farm were examined by transabdominal ultrasonography on approximately day 94 of pregnancy. Ewes were marked according to the number of fetuses detected by the scanning procedure. At approximately day 120 of pregnancy, ewes with a single-, twin- or triplet-pregnancy (20 of each category) were selected at random and a single blood sample was collected from each of the 60 animals.

4.4.3 Statistical analyses

Statistical analyses were performed using Systat version 7.0 (SPSS Inc., Chicago Il, USA). A P value of ≤ 0.05 was considered statistically significant. Changes in CNP and NTproCNP during pregnancy were determined using one-way repeated measures ANOVA with time as the independent variable. Two-way repeated measures ANOVA was used to determine changes in ANP and NTproBNP concentration over time, and to identify differences between pregnant and non-pregnant animals. Differences in hormone concentration with number of fetuses were determined by one-way ANOVA. Data were log-transformed where appropriate to meet ANOVA assumptions. Bonferroni *post hoc* analyses were performed for both the longitudinal and number of fetuses studies.

4.5 Results

An excess number of ewes were included in the longitudinal study to allow for animals failing to become pregnant, death or illness of mother and/or neonates, and differing number of fetuses. Because single fetuses were under represented, their number being insufficient to allow statistical evaluation, the study groups were confined to healthy twin-bearing ewes with normal pregnancy and delivery, and healthy non-pregnant control ewes. From each of these two groups, 8 animals were chosen at random for completion of hormone profiles (Figure 4-1). Likewise, hormone profiles are presented for 8 healthy single-bearing red deer hinds and 4 non-pregnant control hinds (Figure 4-2). One red deer calf presented for delivery in a breech position and birth assistance was required, but this did not appear to have an adverse effect on either the calf or its dam, or on results presented here.

4.5.1 Live weight and birth weights of lambs and fawns

All animals used in the longitudinal study had weight gain profiles and birth weights consistent with normal pregnancy for red deer and Coopworth sheep on this farm (data not shown).

4.5.2 Maternal circulating hormone concentration throughout pregnancy

4.5.2.1 CNP and NTproCNP

Plasma concentrations of CNP and NTproCNP increased significantly during pregnancy in ewes ($P < 0.01$, Figure 4-1) and hinds ($P < 0.01$, Figure 4-2). One week preconception, plasma CNP concentration was 0.98 ± 0.06 and 1.03 ± 0.03 pmol l^{-1} in ewes and hinds, respectively. Plasma NTproCNP concentration was 27.2 ± 1.07 (sheep) and 16.2 ± 1.18 (deer) pmol l^{-1}. In pregnant ewes, circulating concentrations of CNP and NTproCNP became significantly elevated above pre-pregnancy values by 115 and 110 days before parturition, respectively (ovine gestation length is approximately 145 days) ($P < 0.001$ CNP; $P = 0.04$ NTproCNP). Peak plasma concentration of CNP (31 ± 5 pmol l^{-1}) and NTproCNP (270 ± 16 pmol l^{-1}) was observed at around 30 days before parturition, followed by a significant decline in the concentration of both peptide forms (7 days before parturition; CNP 36 % of peak, $P < 0.001$; NTproCNP 71 % of peak, $P = 0.006$). A similar pattern was observed in pregnant hinds, although the magnitude of the rise in concentration was much smaller. Plasma NTproCNP concentration became significantly elevated ($P < 0.001$) above pre-pregnancy levels by 171 days before parturition (cervine gestation length is approximately 233 days), whereas the increase in CNP concentration was not significant until 94 days before parturition ($P < 0.05$). Peak plasma NTproCNP concentration (132 ± 9.5 pmol l^{-1}) was recorded 38 days before parturition, whereas the peak CNP concentration (2.14 ± 0.2 pmol l^{-1}) did not occur until 10 days before parturition. Concentrations of both CNP forms appeared to decline in the final week of cervine pregnancy, although this did not reach statistical significance. In both species, circulating CNP and NTproCNP concentrations returned to pre-pregnancy values immediately after parturition (P = NS).

In non-pregnant ewes there was a small but significant change in plasma concentration of CNP ($P = 0.014$) and NTproCNP ($P < 0.001$) during the study period (Figure 4-1). There were no significant changes in the concentration of either peptide form in non-pregnant hinds during the study (P = NS, Figure 4-2).

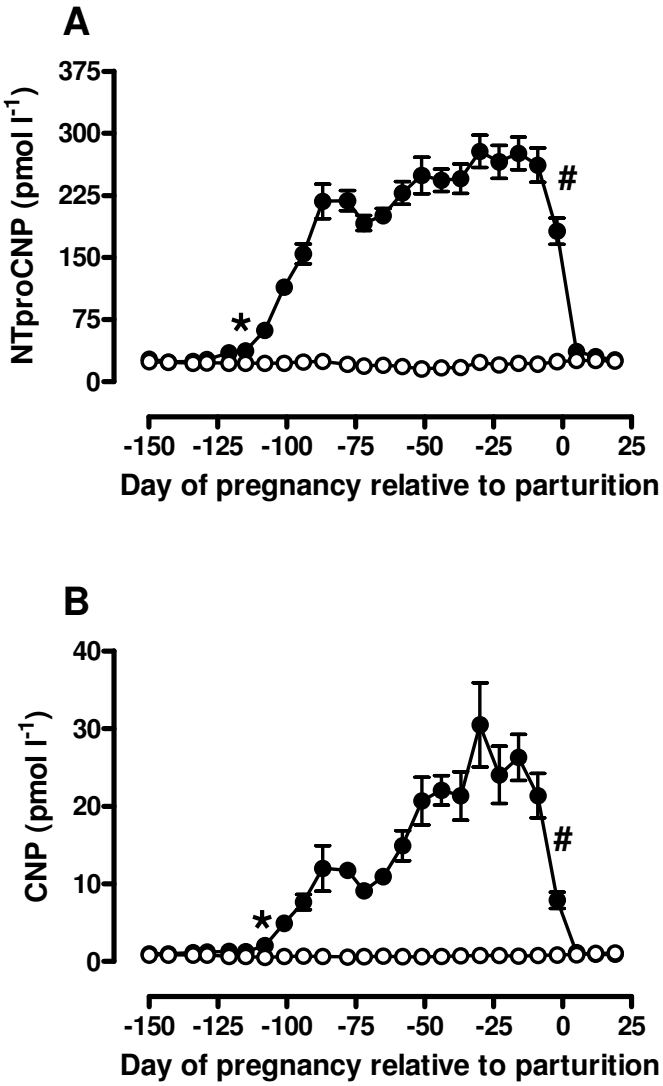

Figure 4-1.

Mean (± SEM) plasma (A) NTproCNP and (B) CNP concentration in twin-bearing pregnant (closed circles) and non-pregnant (open circles) ewes (n = 8). Day of pregnancy refers to pregnant animals. *, Significant difference from pre-pregnancy baseline; #, significant change in mean concentration in the final 2 weeks of gestation. Parturition occurred at day 0.

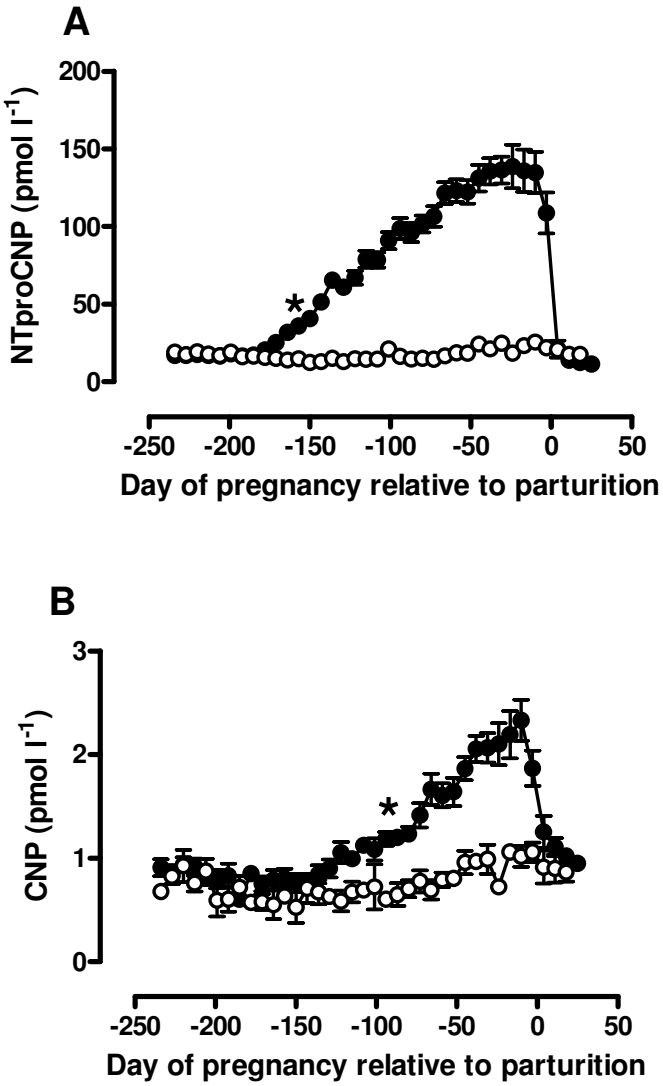

Figure 4-2.

Mean (± SEM) plasma (A) NTproCNP and (B) CNP concentration in pregnant (n = 8) (closed circles) and non-pregnant (n = 4) (open circles) red deer hinds. Day of pregnancy refers to pregnant animals. *, Significant difference from pre-pregnancy baseline. Parturition occurred at day 0.

4.5.2.2 ANP and NTproBNP

There was no change in either ANP or NTproBNP concentration in maternal plasma during ovine pregnancy (P = NS, Figure 4-3), nor was there any difference in plasma concentration of these peptides between pregnant and non-pregnant ewes at any stage (P = NS).

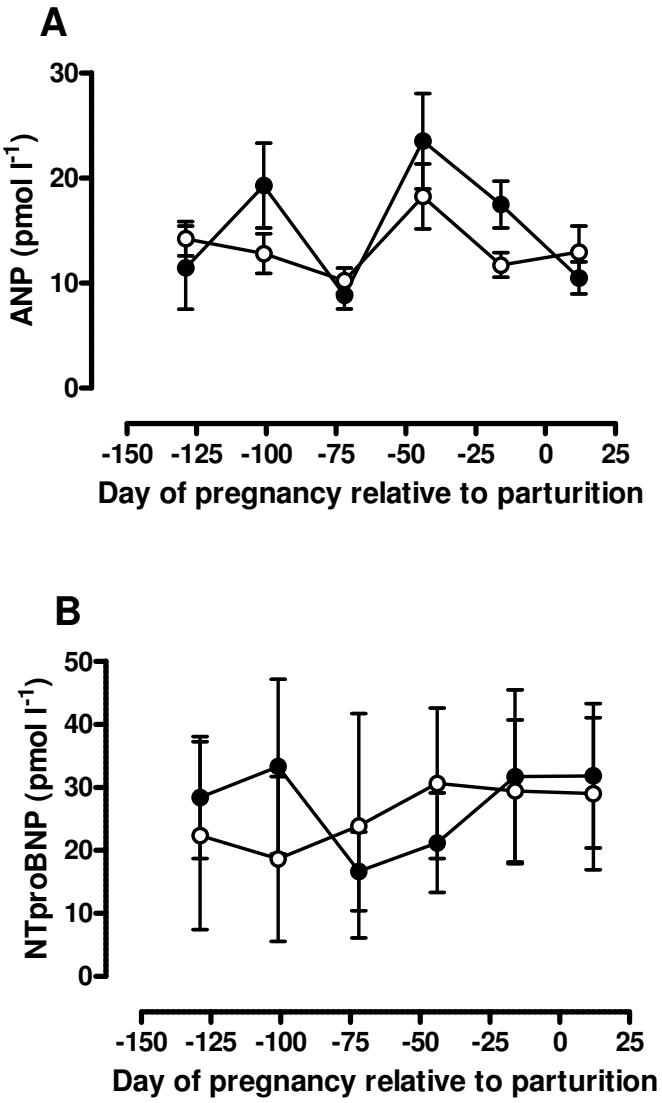

Figure 4-3.

Mean (± SEM) plasma (A) ANP and (B) NTproBNP concentration in twin-bearing pregnant (closed circles) and non-pregnant (open circles) ewes (n = 4). Day of pregnancy refers to pregnant animals. Parturition occurred at day 0.

4.5.2.3 Oestradiol and progesterone

Mean plasma oestradiol concentration declined steadily during the 1st trimester (Figure 4-4). Thereafter, the values fluctuated but there was a marked elevation during the final week of pregnancy before falling sharply at parturition.

An increase in mean plasma progesterone concentration commenced soon after mid-gestation peaking approximately 23 days before parturition, following which the mean concentration

declined progressively (Figure 4-4). After parturition both oestradiol and progesterone were barely detectable in the maternal venous plasma.

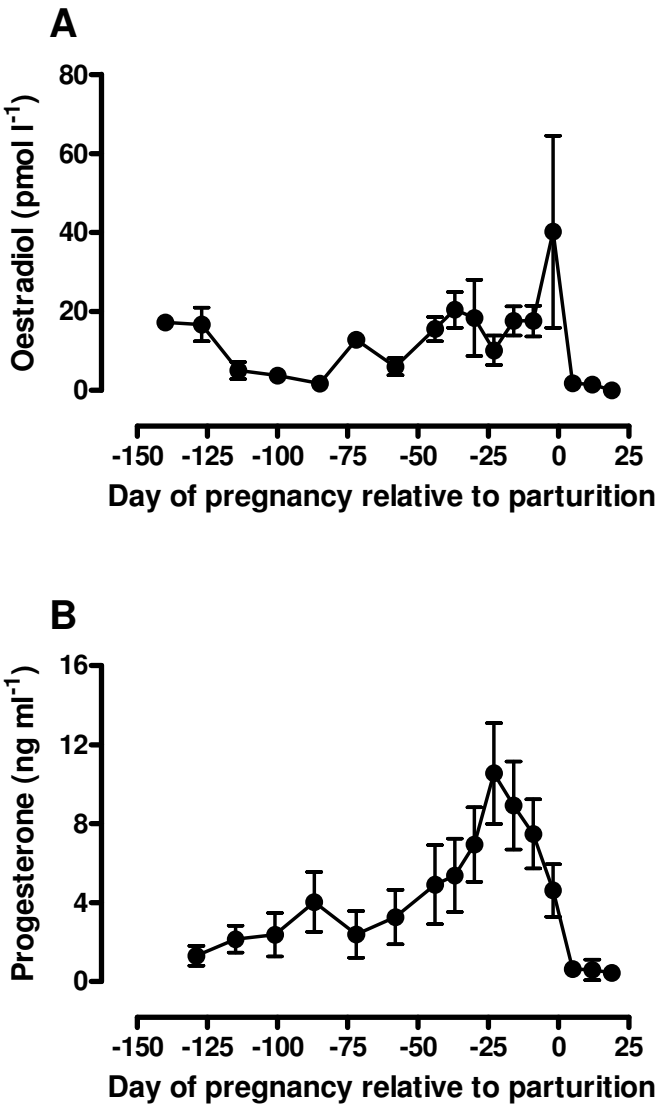

Figure 4-4.

Mean (± SEM) plasma (A) oestradiol and (B) progesterone concentration in twin-bearing pregnant ewes (n = 8) throughout the course of gestation. Parturition occurred at day 0.

4.5.3 Effect of fetal number

The number of fetuses had a significant positive effect on the maternal concentration of circulating CNP forms, with the highest concentrations measured in triplet-bearing ewes (CNP $P < 0.001$; NTproCNP $P < 0.001$) (Figure 4-5).

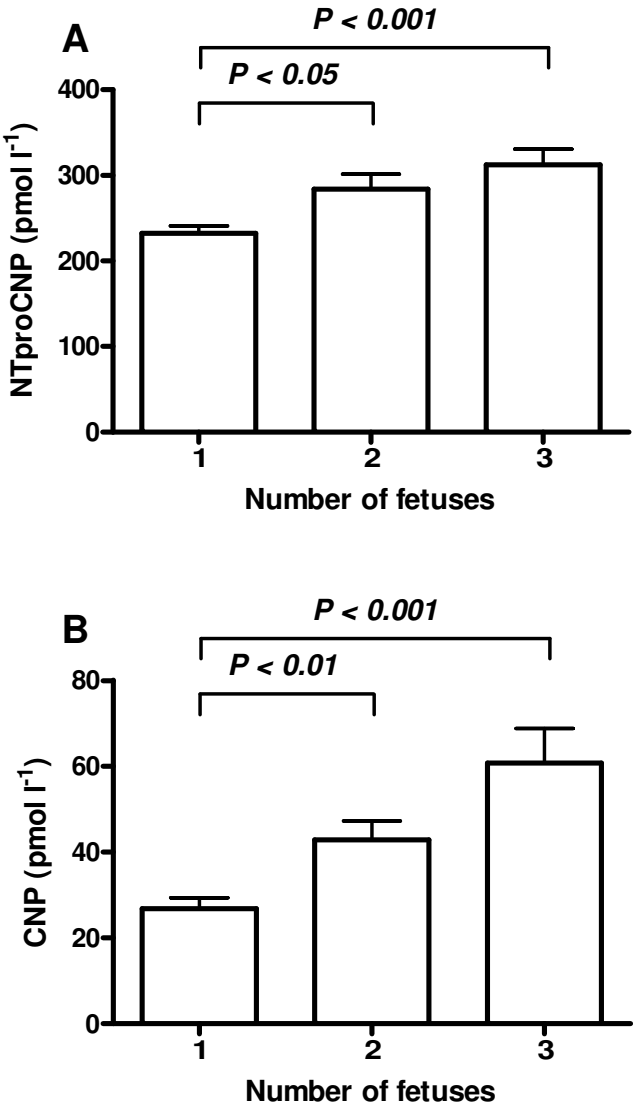

Figure 4-5.

Mean (± SEM) plasma concentration of (A) NTproCNP and (B) CNP at around day 120 of gestation in ewes carrying one (n = 23), two (n = 20), or three (n = 17) fetuses.

4.6 Discussion

This chapter describes the first longitudinal study of maternal circulating CNP forms throughout pregnancy in any species. In confirming previous findings of greatly raised concentrations in late gestation in the ewe (Prickett *et al.*, 2007), the present results extend our knowledge of CNP's participation in ruminant pregnancy by showing an abrupt increase coinciding with placentation followed by a progressive rise until near-term in two ruminant species, sheep and red deer. In addition they show a direct relationship between maternal concentration of CNP forms and fetal number, and a marked decline in maternal

concentrations during the final week of pregnancy. These observations strongly support a role for CNP in placental and fetal maturation and preparation for parturition and serve as a basis for future *in vivo* studies of CNP's functional role.

In both ewes bearing twins and hinds with a single fetus, venous plasma NTproCNP concentration increased abruptly towards the end of the 1st trimester - in strong contrast to the much lower and unchanging concentration of NTproCNP in non-pregnant adult control animals. In ewes, the initial rate of increase in plasma NTproCNP concentration was greater than that of CNP but increases in both forms slowed some 90-60 days before parturition (at a gestational age of 55-85 days). Of interest, similar patterns of change are observed in plasma nitric oxide (NO) (Vonnahme *et al.*, 2005) and may be associated with reduced oestrogen production during the same period (Carnegie & Robertson, 1978). After mid-gestation, the rate of increase in maternal CNP concentration exceeded that of NTproCNP. Whether these differential changes in CNP forms in ewes relate to reduced activity of degradative pathways, including NPR-C (which is downregulated in uterine arteries in late gestation) (Itoh *et al.*, 1998), neutral endopeptidase or possibly associated with the phase of rapid fetal growth is unclear. Although CNP synthesis is high in the fetus, any contribution of the fetus itself to maternal levels is unlikely as the fetal lamb plasma concentration of CNP (3.8 ± 0.2 pmol l^{-1}) is much lower than maternal levels and appears to be regulated independently (Prickett *et al.*, 2007).

The dramatic changes in circulating CNP forms were in stark contrast to ANP and NTproBNP, which remained low for the duration of pregnancy and showed no difference from non-pregnant controls. An increase in BNP has been reported previously in maternal plasma during normal human pregnancy (Franz *et al.*, 2009; Hameed *et al.*, 2009), with the rise attributed to volume overload as a consequence of the marked increase in maternal blood volume during pregnancy (Hameed *et al.*, 2009). Reports of elevated maternal ANP concentration have been reported in human pregnancy, although this reported rise may be an artefact of inappropriate study design and an increase in ANP during pregnancy remains to be demonstrated conclusively (Reviewed by Steegers, 1991). Similarly, previous reports of ANP and BNP concentration in the pregnant ewe have been inconclusive (Cheung *et al.*, 1987) or show little or no change during the course of pregnancy (Lubbers & Eghtesady, 2007). The results of the current study are supported by these earlier studies and suggest that these peptides do not play an important role in ovine pregnancy.

The present study was not designed to determine the mechanisms underlying the dramatic changes observed in maternal CNP concentrations, nor their functional consequences. Nonetheless, the time sequence and close correlation of plasma concentration of CNP forms with fetal number strongly substantiate previous findings (Prickett *et al.*, 2007) and indicate that uteroplacental tissues are an important source. Of note, the initial increase in maternal NTproCNP concentration in ovine pregnancy corresponds to the phase of maximum placental growth and invasion of the maternal stroma (Ehrhardt & Bell, 1995). In mice, there is abundant CNP expression closely associated with the maternal blood vessels of the placenta (Cameron *et al.*, 1996) - possibly initiated by increases in shear stress (Zhang *et al.*, 1999). This localisation suggests a role for the hormone in angiogenesis (Doi *et al.*, 1997; Yamahara *et al.*, 2003) and vasodilation (Suga *et al.*, 1993). Indeed, later in gestation it seems likely that both the local (auto/paracrine) actions within placenta and uterus (Acuff *et al.*, 1997; Walther & Stepan, 2004), as well as endocrine contributions from high circulating CNP concentrations (Charles *et al.*, 1995), all contribute to the massive increase in uteroplacental blood flow associated with the pregnant state (Reynolds & Redmer, 1995; Itoh *et al.*, 1998).

In this context, increased oestrogen production is known to be important in maintaining vasodilation (Rosenfeld *et al.*, 1996). The effects of oestrogens are mediated in part by NO but the changes in this pathway are considered to be insufficient to account for the increases in uterine blood flow observed in late gestation (Rosenfeld *et al.*, 1996). Oestradiol increases CNP gene expression in the murine uterus (Acuff *et al.*, 1997) and also stimulates plasma CNP forms in adult (non-pregnant) ewes (Espiner *et al.*, 2008). Together these findings raise the possibility that some of oestradiol's actions on uterine blood flow may be mediated by CNP. While this may be true after mid-gestation, it is notable that the initial increases in NTproCNP occur during a period of declining plasma oestradiol concentration, making it unlikely that oestradiol is the main driver of CNP synthesis in pregnancy. Clearly separating uterine from placental sources of CNP will be an important future objective.

A striking observation in the present study is the precipitous decline in the plasma concentration of both CNP forms in ewes during the final week of pregnancy, with a similar downward trend evident in deer. The time sequence of these changes coincides with the period of rapid hypothalamus-pituitary-adrenal activation in the fetus (Barnes *et al.*, 1978). The rise in fetal plasma cortisol concentration in lambs commences around 15 days before delivery (Barnes *et al.*, 1978) and is associated with progressive induction of placental

17α-hydroxylase, and fall in maternal plasma progesterone as observed here. Conceivably the upsurge in fetal cortisol secretion in the final 3-5 days (Barnes *et al.*, 1978) may be sufficient at the placental tissue level to down regulate CNP expression. Glucocorticoids can rapidly inhibit CNP synthesis *in vivo* (Prickett *et al.*, 2005; Prickett *et al.*, 2008a), and the possibility that similar inhibition can occur at the much lower concentrations of glucocorticoids attained near term awaits further study. In any event, the current findings suggest that inhibiting CNP once the fetus is mature may be an important step in preparing for parturition - for example by reducing vasodilation (Suga *et al.*, 1993) and potential blood loss associated with placental separation.

In this study of ruminant pregnancy, sheep and deer exhibited similar profiles in circulating maternal CNP forms - both species showing a steeper rise in CNP concentration after mid-gestation. However, plasma CNP concentration - similar in the 2 species prior to conception - was much lower in pregnant deer. Thus the peak CNP concentration in deer was approximately 2-fold preconception values compared with the 30-fold elevation in ewes. Presumably these differences reflect enhanced degradation rates of CNP in pregnant deer - possibly by placental proteases such as neutral endopeptidase (Kikkawa *et al.*, 2002) - since the amino acid sequence of CNP-22 is likely to be identical in deer and sheep. On the other hand, the lower concentration of peak NTproCNP measured in deer (some 100 pmol l^{-1} lower than that recorded from single-bearing ewes in late gestation) could be based on differences in amino acid sequence in proCNP 1-50. Although both proteins eluted during HPLC at the expected position of the 5 KDa protein (Prickett *et al.*, 2003), precise quantitation of deer and sheep NTproCNP requires knowledge of the sequence in deer and use of the appropriate standards in RIA.

Of note, plasma concentrations of CNP and NTproCNP in non-pregnant animals were very similar in sheep and deer, suggesting that the apparent difference in peptide concentration between the two species during gestation can not be explained entirely by assay specificity. Structural differences between sheep and deer placentae may instead be a contributing factor. In particular, although the basic structure of the placenta is conserved in the two species, the oligocotyledonary placenta of deer consists on average of only ten placentomes whereas the polycotyledonary ovine placenta often has 100 or more placentomes (Klisch & Mess, 2007), indicating that there may be fundamental species differences in placental function at the level of the placentome. Possibly, the reason for these species differences will become apparent as our understanding of the role of CNP in pregnancy improves.

Regarding speciation, it is relevant to note that maternal values of plasma CNP concentration in human pregnancy (at term) do not appear to be different from those measured in non-pregnant adult women (Walther & Stepan, 2004). There are no reports of longitudinal studies of CNP concentration (nor of NTproCNP) during gestation in humans but Stepan *et al.* (1998) report that maternal plasma CNP concentration did not increase with gestational age in a cross sectional study . Many factors including placental structure and production of other key hormones during pregnancy may account for these differences. However more focused and serial measurements of both CNP forms in pregnant women would seem to be indicated in the light of the subtle but significant changes in maternal CNP observed here in deer hinds.

The current findings in pregnant ewes, together with evidence of reciprocal changes in fetus (in which CNP falls) and ewe (in which CNP rises) during nutritional stress (Prickett *et al.*, 2007), strongly support the view that CNP is carefully regulated in pregnancy and that change in maternal plasma CNP concentration, at least in sheep, may reflect local utero-placental secretion and fetal distress. On this hypothesis, higher circulating CNP concentration with increasing fetal number in ewes at day 120 gestation may be seen as an adaptive change to fetal nutrient restriction. This view is reinforced by the finding that fetal plasma CNP concentration is lower in a twin when compared with that in a single fetus (Prickett *et al.*, 2007). In late-gestation ewes carrying twin and triplet fetuses, nutritional requirements are 120 % and 140 % above maintenance, respectively (Bell *et al.*, 2005), which if not met, will result in caloric restriction. Although total blood flow and placenta size are greater in multiple pregnancies, when these factors are evaluated on a per fetus basis, they are actually lower than in single fetus pregnancies (Ferrell & Reynolds, 1992), with the same being true of lamb weight, placental weight, placental efficiency and cotyledon number (Dwyer *et al.*, 2005). Furthermore, as the total placental weight increases with increasing litter size, so too do its metabolic demands. Therefore, the larger but less efficient placenta in twin-bearing ewe may consume more energy for its own metabolic needs and transfer relatively less to the developing fetus (Dwyer *et al.*, 2005), further compromising fetal nutrient uptake.

Reduced energy uptake by twin and triplet fetuses can result in a state of hypoxia (Ferrell & Reynolds, 1992; Etchernkamp *et al.*, 2006). CNP production is stimulated by hypoxia (Scotland *et al.*, 2005), and elevated CNP in multiple pregnancies may be an adaptive response by the placenta to promote vasodilation and increase blood flow to ensure that fetal energy demands are being met in adverse conditions. Similar mechanisms may be the basis

for higher maternal concentrations of NTproCNP, and lower fetal values, in women presenting with preeclampsia when compared with normal pregnant women at term (Prickett *et al.*, 2004). Clearly these hypotheses now require experimental support, possibly using models of placental insufficiency.

In conclusion, this study reveals that concentrations of CNP forms are greatly elevated in maternal circulation during pregnancy in two species of ruminant; sheep and red deer. The pattern of hormone production, and positive relationship with fetal number suggest that CNP is produced by uteroplacental tissues in response to placental and fetal maturation. These findings suggest that CNP has important roles in ruminant pregnancy, elucidation of which may provide new insight into the role of the hormone in other species.

Chapter 5:
CNP production in ovine placenta

Declaration

A substantial amount of the material described in this thesis chapter has been published in *Placenta** and therefore includes significant contributions from co-authors. I was involved in all aspects of experimental design and sample collection, and all of the hormones assays, HPLC and frozen section immunohistochemistry were conducted by me. The CNP gene expression assays were performed by Dr Tim Prickett. Immunohistochemistry to locate trophoblast binucleate cells was conducted by Dr FBP Wooding at the Centre for Trophoblast Research, University of Cambridge, UK.

* McNeill, BA; Barrell, GK; Wooding, FBP; Prickett, TCR and Espiner, EA. (2011) The trophoblast binucleate cell is the source of maternal circulating C-type natriuretic peptide in ovine pregnancy. *Placenta* **In press**

5.1 Abstract

Maternal plasma concentrations of CNP and NTproCNP are elevated during ovine pregnancy. Although the uteroplacental unit has been implicated as a likely source of CNP, the relative contributions of specific uterine and placental tissues, and identity of the cellular site/s of production remain unknown. Therefore, CNP and NTproCNP concentration was measured in intercaruncular uterine tissue and maternal (caruncle) and fetal (cotyledon) placental tissues throughout gestation. Concentrations of CNP forms in placental tissues greatly exceeded those in intercaruncular uterine tissue throughout pregnancy ($P < 0.05$). Mean caruncular concentrations (CNP 32 ± 4, NTproCNP 56 ± 6 pmol g^{-1}) peaked at day 60 whereas in the cotyledon there was a progressive increase in CNP forms to peak values (CNP 66 ± 6, NTproCNP 134 ± 9 pmol g^{-1}) at day 100-135 followed by a sharp decline just prior to term (day 143). At term CNP gene expression was 6-fold greater in placental tissue compared with intercaruncular uterine tissue. Changes in maternal plasma concentration of CNP forms closely followed those in cotyledonary tissue whereas fetal plasma levels fell progressively throughout gestation. Immunohistochemistry revealed staining in trophoblast BNCs and around placental blood vessels.

CNP's localization to the BNC suggests a novel endocrine role during pregnancy, in addition to its paracrine actions within the placental vasculature. The function of CNP in maternal circulation remains to be determined, but as proposed for other BNC products, may involve manipulation of maternal physiology and placental function to favour fetal growth.

5.2 Keywords

C-type natriuretic peptide, NTproCNP, pregnancy, placenta, fetus, sheep, binucleate cell

5.3 Introduction

During ovine and cervine pregnancy, maternal circulating concentrations of CNP increase at the end of the first trimester to levels exceeding 20 pmol l^{-1} before rapidly declining during the final week of gestation (Chapter 4). The temporal pattern of CNP forms in maternal circulation (Chapter 4) and high venoarterial concentration gradient across the gravid uterus (Prickett *et al.*, 2007) strongly implicate the uteroplacental unit as an important source of maternal CNP during pregnancy. However the relative contributions from uterine and placental tissues are unknown, as is the identity of the cell type/s within the placenta responsible for CNP production.

A unique feature of the ruminant placenta is the presence of large, granulated binucleate cells of fetal placental origin which appear at the time of implantation and persist for the remainder of pregnancy. BNC are formed from uninuclear trophoblast cells which can undergo horizontal division resulting in one daughter cell without a tight junction attachment. This cell then undergoes nuclear division and is released from the trophoblast basement membrane, thus becoming a potentially motile cell which differentiates into the characteristic, heavily granulated binucleate phenotype (Wooding, 1982). In ovine pregnancy, the mature BNC migrate through the trophoblast tight junction and fuse with individual uterine epithelial cells or their derivatives. This gradually replaces the uterine epithelium with a fetomaternal hybrid syncytium formed by these migrating BNC and the uterine epithelial cells (Wooding, 1982). The cytoplasmic contents of the BNC fuse into the fetomaternal syncytium and the contents of the characteristic BNC granules are released by exocytosis at the basal plasmalemma of the original uterine epithelial cell, from where they diffuse into the maternal circulation. This provides a method of hormone delivery from fetal placental tissue to mother without apparent

activation of any maternal immune response (reviewed in Wooding, 1992; Wooding & Burton, 2008).

Preliminary experiments from our laboratory demonstrated that CNP and NTproCNP protein concentration in the fetal portion of the placenta (cotyledon) exceeded that in the maternal aspect (caruncle) in near-term ewes, despite a higher peptide concentration in maternal versus fetal plasma (McNeill *et al.*, 2009a). This finding, taken with the similarity between the pattern of CNP production during pregnancy and abundance of BNC in the ovine placenta (Wooding, 1982), suggests that CNP may be a product of these unique cells of pregnancy. Therefore, the aim of the current study was to further our understanding of CNP's role in ovine pregnancy by characterising the pattern of CNP expression in uterine and placental tissue at early, mid- and late-pregnancy and to address the hypothesis that maternal circulating CNP is produced by fetal BNC.

5.4 Methods

5.4.1 Collection of tissue and plasma samples

Mixed-age Coopworth ewes of known gestational age from the Lincoln University Research Farm carrying single (n = 9) or twin (n = 11) lambs were killed by captive bolt gun on day 30, 60, 100, 135 and 143 of pregnancy (n = 4 at each time-point, term = day 145). In the case of twins, samples of plasma and placental tissue were collected from each fetus and the hormone concentration averaged to give a single value for that animal. Prior to slaughter, a maternal blood sample was collected from each ewe. Fetal blood samples were collected immediately after slaughter at all time-points except day 30, when the fetal blood volume was too low to enable measurement of CNP forms. The uterus was excised and four randomly-selected placentomes were removed from each ewe as well as four samples of intercaruncular uterine tissue. In order to determine the individual contribution of maternal and fetal tissues to placental CNP concentration, the maternal (caruncle) and fetal (cotyledon) aspects of the placentome were separated at the time of tissue collection. This was achieved by gently pulling on the two sides of the placentome, yielding a clean separation of the two tissues. At day 135, samples of skeletal muscle (gracilis), intercaruncular uterine tissue and placental tissue were also collected for measurement of CNP gene expression. After dissection, all tissues were immediately frozen by immersion in liquid nitrogen and stored at -80 °C. Eight mixed-age non-pregnant Coopworth ewes were also killed at similar times (corresponding to

days 30 and 135 in the pregnant group, n = 4 at each time-point). Blood and uterine caruncular and intercaruncular tissue samples were collected from these animals and stored in an identical manner as for the pregnant ewes.

5.4.2 CNP gene expression

Total RNA was isolated using TRIzol (Invitrogen). Tissue samples (~300 mg) were homogenised by grinding in a Retsch MM301 tissue mill at 30 Hz for 10 minutes in 800 µl TRIzol. First-strand cDNA was synthesized from 1 µg total RNA with Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR analyses were performed with a Rotor-Gene RG-3000 real-time PCR machine (Corbett Research, Sydney, Australia). The CNP transcript was detected and quantified with the following set of primers: 5′-CAGAAGAAGGGCGACAAGAC-3′ (sense) and 5′-CCCTTGGACAAACCCTTCTT-3′ (antisense) – amplified product size 179 bp. PCRs were performed in a total volume of 20 µl containing 1 µl cDNA, 0.4 mM primers, 0.3 µl AccuPrime Taq DNA polymerase (Invitrogen), 1x PCR buffer I, 0.2 mM deoxynucleotide triphosphates, 4 µl 5 x Q solution (Qiagen) and 1 µl (1:5000 dilution) Sybr Green 1 (Roche). Following a hot start at 96 °C for 2 minutes, each sample underwent 45 cycles of annealing at 60 °C for 35 seconds, extension for 30 seconds at 72 °C, and denaturation at 90 °C for 30 seconds. Samples were assayed in duplicate and gene expression levels quantified against a standard curve and expressed as pg of message per microgram of total RNA.

5.4.3 Immunohistochemistry

In order to investigate site/s of CNP localisation within the placentome, whole placentomes were collected from twin-bearing Coopworth ewes and processed for immunohistochemistry. Samples were collected at approximately day 124 of pregnancy, frozen in liquid nitrogen and stored at -80 °C. Placentomes were embedded in optimal cutting temperature compound and sectioned at 6 µm. Endogenous peroxidise activity was blocked by incubation in 1 % peroxide solution for 20 minutes at room temperature. Slides were washed three times in phosphate buffered saline solution (PBS) containing 1 % normal goat serum and incubated with primary antibody (CNP 1:250; NTproCNP 1:500) or PBS (negative control) overnight at 4 °C. The slides were then subjected to successive overnight incubations at 4 °C - first with biotinylated secondary antibody (1:1000) followed by a second incubation with an extravidin-HRP complex (1:1000). A PBS washing step was performed between each incubation. The slides

were then washed in HRP reaction buffer prior to staining using HRP substrate solution (30 minute incubation at room temperature followed by dehydration steps using a series of alcohols and xylene) in preparation for viewing by microscopy.

To determine whether CNP is a product of the trophoblast BNC, additional studies were performed in Cambridge using araldite resin-embedded sections to locate CNP, NTproCNP and pregnancy associated glycoprotein (PAG). PAG is produced only by the fetal trophoblast BNC and therefore served as a positive control for identification of these cells.Two Welsh Mountain ewes were mated naturally and killed with an overdose of sodium pentothal at 80 days of pregnancy. After laparotomy the uterus was cut out, the fetus removed and the uterine and umbilical arteries cannulated in turn and perfused with 1 % glutaraldehyde plus 4 % formaldehyde in 0.1 M phosphate buffer and finally embedded in epoxy (araldite) resin by standard procedures (Wooding *et al.*, 2007). For immunocytochemistry, 1 μm resin sections were picked up on aminopropyltriethoxysilane coated coverslip pieces and dried at 60 °C for 30 minutes. Resin was removed from the epoxy resin sections by flotation on sodium ethoxide (15 g NaOH in 15 ml absolute ethanol) for 15 minutes. The sections were washed with 100 % alcohol, and then sequentially floated on 50 % alcohol, PBS, PBS plus 1 % bovine serum albumin (the blocking solution and diluent for all antibodies), and finally on antibody solution (polyclonal CNP 1:50; NTproCNP 1:50; PAG M4 1:1000 (Wooding *et al.*, 2007); monoclonal SBU3 1:1000 (Wooding *et al.*, 2007)) overnight at 4 °C.
After mouse monoclonal antibody incubation and a PBS wash, the sections were floated on rabbit anti-mouse serum (Dako Ltd., Ely, Cambridgeshire, UK) diluted 1:1000 for 30 minutes, washed with PBS and then joined by the polyclonal incubations on goat anti-rabbit 5 nm gold colloid solution (Stratech Scientific, Soham, Cambridgeshire, UK; diluted 1:40) for 40 minutes. All sections were washed with PBS and then water, left on water for 10 to 30 minutes, and finally transferred to the silver intensification reagent (Amersham Intense M) (15 to 25 minutes) to increase the size of the initial gold (monitored by eye) for light microscope visualization. The sections were then water washed and dried, counterstained with acidified 1 % Fast Green solution, and finally mounted section side down in Biomount (BBI, Cardiff, UK). Antibody controls replaced the antibody or lectin with equivalent non-specific solutions or omitted the gold colloid or intensification stages. Digital photomicrographs were taken on a Zeiss Axiomat microscope.

5.4.4 Statistical analyses

Differences between maternal and fetal plasma hormone concentrations were determined by two-way ANOVA and Bonferroni *post hoc* test. Differences in hormone concentration between uteroplacental tissues, and changes in hormone concentration over time within tissues, were determined using a restricted maximum likelihood (REML) test and a method of statistical differentials using day of gestation and number of fetuses as the dependent variables. Data were log-transformed where appropriate and a P value of ≤ 0.05 was considered statistically significant. Analyses were performed using Genstat Version 10 (VSN International Ltd., Hemel Hempstead, UK).

5.5 Results

5.5.1 CNP and NTproCNP in uteroplacental tissues

CNP and NTproCNP concentrations in uteroplacental tissues are expressed per gram of tissue (wet weight) extracted. The concentration of both CNP forms in placental tissues (cotyledon and caruncle) exceeded those in intercaruncular uterine tissue at all stages of gestation ($P < 0.05$, Figure 5-1and Table 5-1). Within the placenta, the concentration of CNP and NTproCNP was higher in the fetal cotyledon compared with the maternal caruncle at days 30, 100 and 135 of pregnancy ($P < 0.01$). Caruncular CNP and NTproCNP concentrations were lowest at day 30, rising to peak concentration at day 60 before declining (day 100) and remaining steady thereafter. In contrast, the concentration of CNP and NTproCNP in the cotyledon increased progressively from day 30 until day 135, before declining sharply just prior to term ($P < 0.05$).

In the uterus, the concentration of CNP and NTproCNP was low and unchanging throughout pregnancy except for values obtained on day 30 (0.72 ± 0.17 and 3.4 ± 1.2 pmol g^{-1} respectively) which were increased and significantly higher ($P < 0.05$) than concentrations of CNP (0.26 ± 0.1 pmol g^{-1}) and NTproCNP (0.42 ± 0.17 pmol g^{-1}) in non-pregnant intercaruncular uterine tissue. There was no difference in the concentration of CNP forms between pregnant and non-pregnant intercaruncular uterine tissue at any other stage of pregnancy (P = NS, data not shown). Similarly caruncular concentrations of CNP forms in non-pregnant ewes were not different from intercaruncular concentrations at either sampling period (P = NS). There was no effect of fetal number on the concentration of NTproCNP in uterine or placental tissues ($P = 0.2$). In contrast, there was evidence of a significant effect of

fetal number on CNP concentration in these tissues ($P < 0.05$), reflecting a trend for higher concentrations of CNP in cotyledon extracts from single- versus twin-bearing ewes at days 100 and 135.

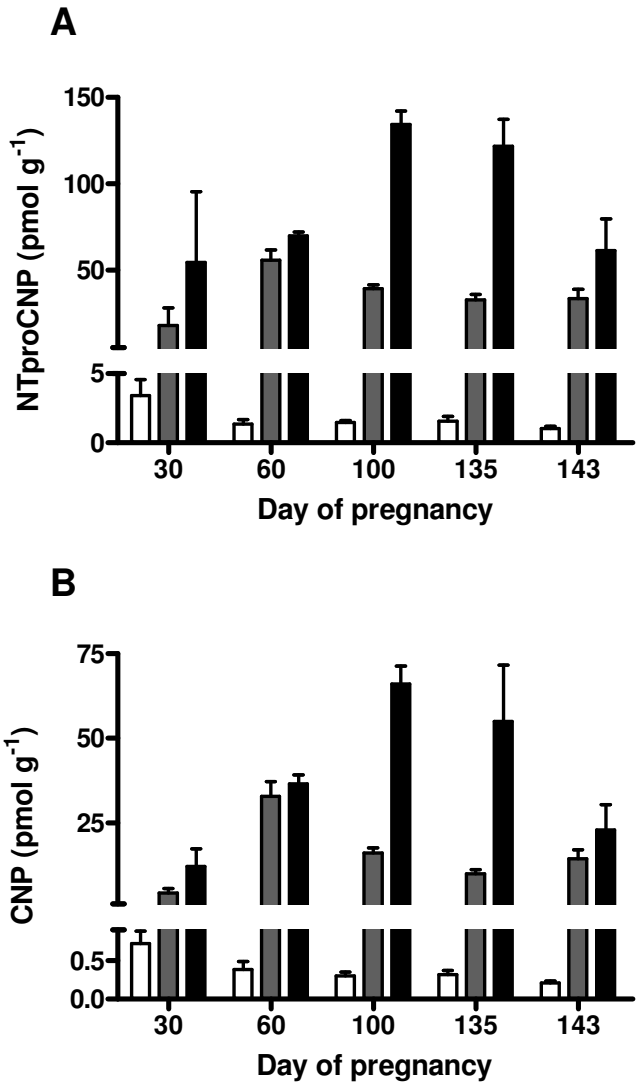

Figure 5-1.

Mean (+ SEM) protein concentration of (A) NTproCNP and (B) CNP in intercaruncular uterine tissue (white bars), maternal caruncle (grey bars) and fetal cotyledon (black bars), n = 4 for each. Differences between tissues at each time-point are summarised in Table 1.

Table 5-1.

Summary of differences in CNP and NTproCNP concentration in uterine and placental tissues during ovine pregnancy.

Day of pregnancy	Comparison	P-value	
		CNP	NTproCNP
30	uterus vs caruncle	< 0.01	< 0.001
30	uterus vs cotyledon	< 0.001	< 0.001
30	caruncle vs cotyledon	0.001	< 0.001
60	uterus vs caruncle	< 0.001	< 0.001
60	uterus vs cotyledon	< 0.001	< 0.001
60	caruncle vs cotyledon	NS	0.02
100	uterus vs caruncle	< 0.001	< 0.001
100	uterus vs cotyledon	< 0.001	< 0.001
100	caruncle vs cotyledon	< 0.001	< 0.001
135	uterus vs caruncle	< 0.001	< 0.001
135	uterus vs cotyledon	< 0.001	< 0.001
135	caruncle vs cotyledon	< 0.001	< 0.001
143	uterus vs caruncle	< 0.001	< 0.001
143	uterus vs cotyledon	< 0.001	< 0.001
143	caruncle vs cotyledon	NS	< 0.001

5.5.2 CNP gene expression in placental and uterine tissues

CNP mRNA expression at day 135 was similar in placental cotyledon and caruncular tissue, and greatly exceeded that observed in intercaruncular uterine tissue ($P < 0.001$) and skeletal muscle (Figure 5-2).

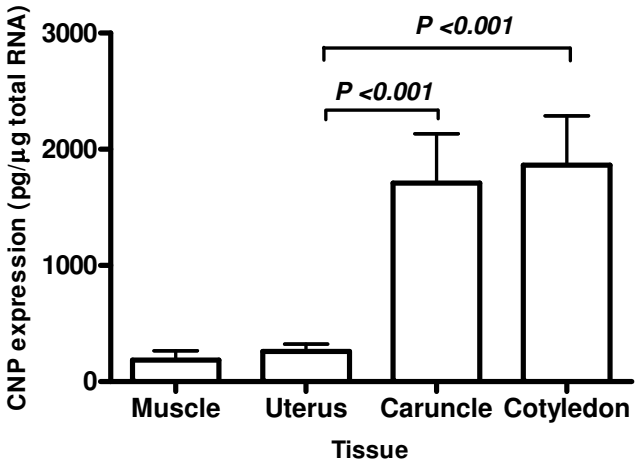

Figure 5-2.

Mean (+ SEM) CNP mRNA levels in tissues obtained from pregnant ewes at day 135 of gestation, (n = 3, 4 replicate samples per tissue source from each animal). Quantitated gene levels are expressed as picograms of message per microgram of total RNA (pg/µg total RNA).

5.5.3 Plasma concentration of CNP forms in mother and fetus

As shown in Figure 5-3, in contrast to the increasing concentrations of CNP forms in the maternal circulation, there was a progressive decline in the concentration of both peptides in fetal circulation during gestation. In the fetus, the concentration of CNP forms was highest at day 60 – i.e. at first sampling. Circulating CNP concentration was higher in maternal compared with fetal plasma at all time-points ($P < 0.05$, Figure 5-3), and maternal circulating NTproCNP was higher than fetal from day 100 onwards ($P < 0.05$). Maternal circulating CNP concentration increased between day 30 and day 100. Coinciding with the decline in cotyledonary CNP concentration (Figure 5-1), plasma CNP concentration fell abruptly just before term ($P < 0.001$). Maternal circulating NTproCNP concentration increased between day 30 and day 60 ($P < 0.001$) and remained elevated for the duration of pregnancy.

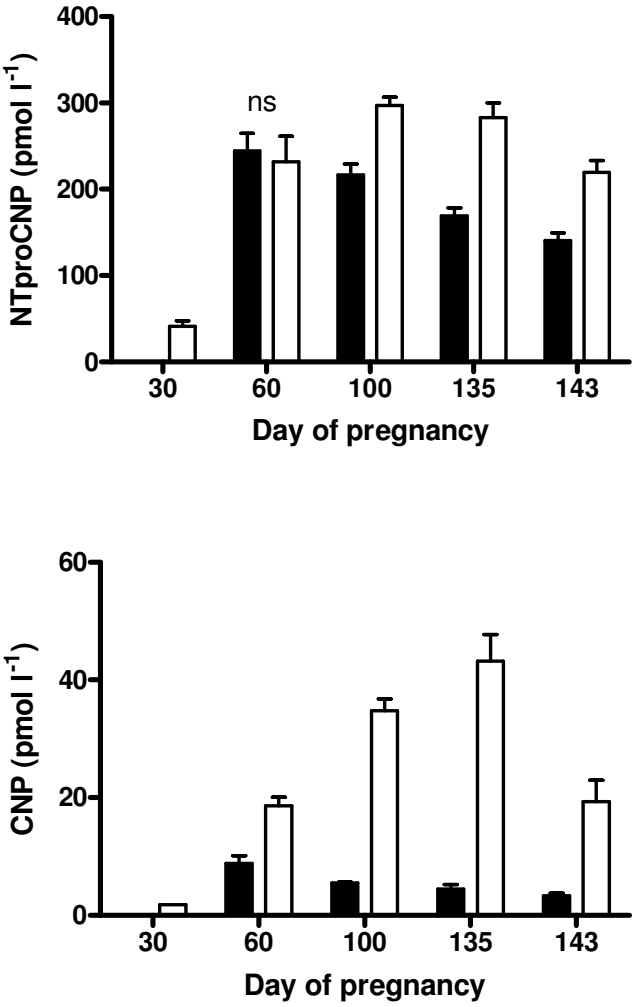

Figure 5-3.

Mean (+ SEM, n = 4) plasma concentration of (A) NTproCNP and (B) CNP in the maternal (white bars) and fetal (black bars) circulations. Except where indicated by ns, the concentration of both CNP forms was significantly higher in the maternal compared with fetal circulation.

5.5.4 Cellular localisations of CNP forms in placental tissue

As shown in Figure 5-4, in sections processed from frozen tissue, strong staining for both CNP and NTproCNP was evident in the placentome, particularly in cells surrounding placental blood vessels. Staining was absent in control sections. Figure 5-5 shows sections prepared after resin embedding, and demonstrates that CNP (Figure 5-5 c) and NTproCNP (Figure 5-5 b) are strongly labelled in a minority of BNC, and when seen, are colocalised with PAGs (Figure 5-5 a and d) as shown on the semi-serial sections. No labelling was seen on control sections (Figure 5-5 e).

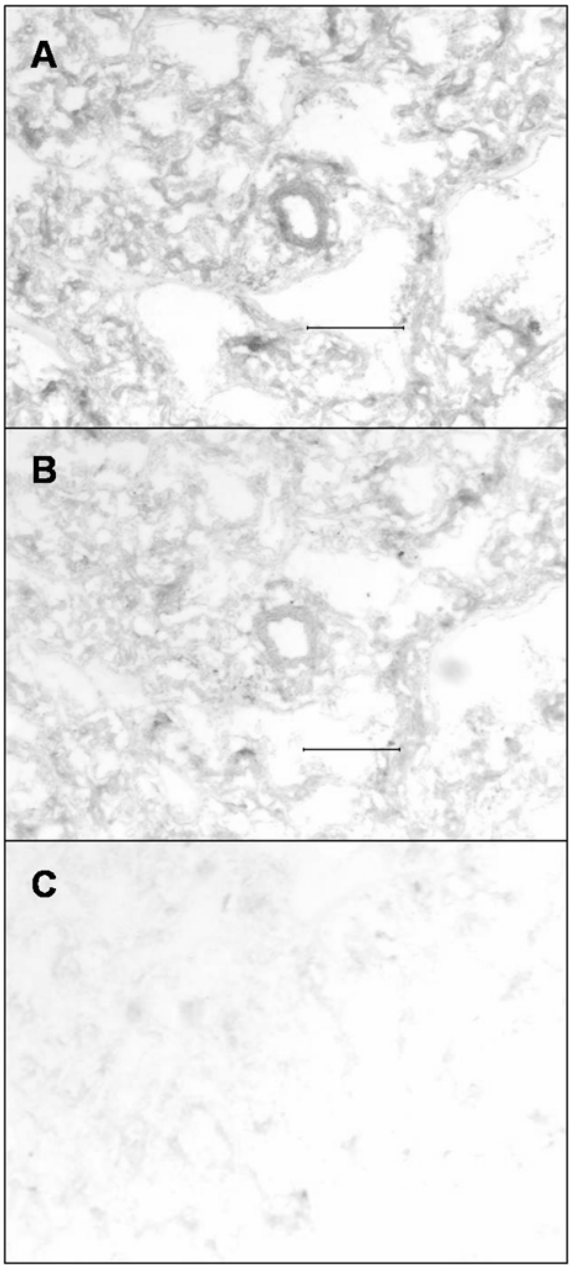

Figure 5-4.

Immunohistochemical localisation of (A) NTproCNP and (B) CNP in cotyledon trophoblasts and vascular tissue and (C) negative control on frozen sections at day 124 of pregnancy. Bar equals 200 µm. Note deposition of stain surrounding blood vessel near centre of the figure in A and B.

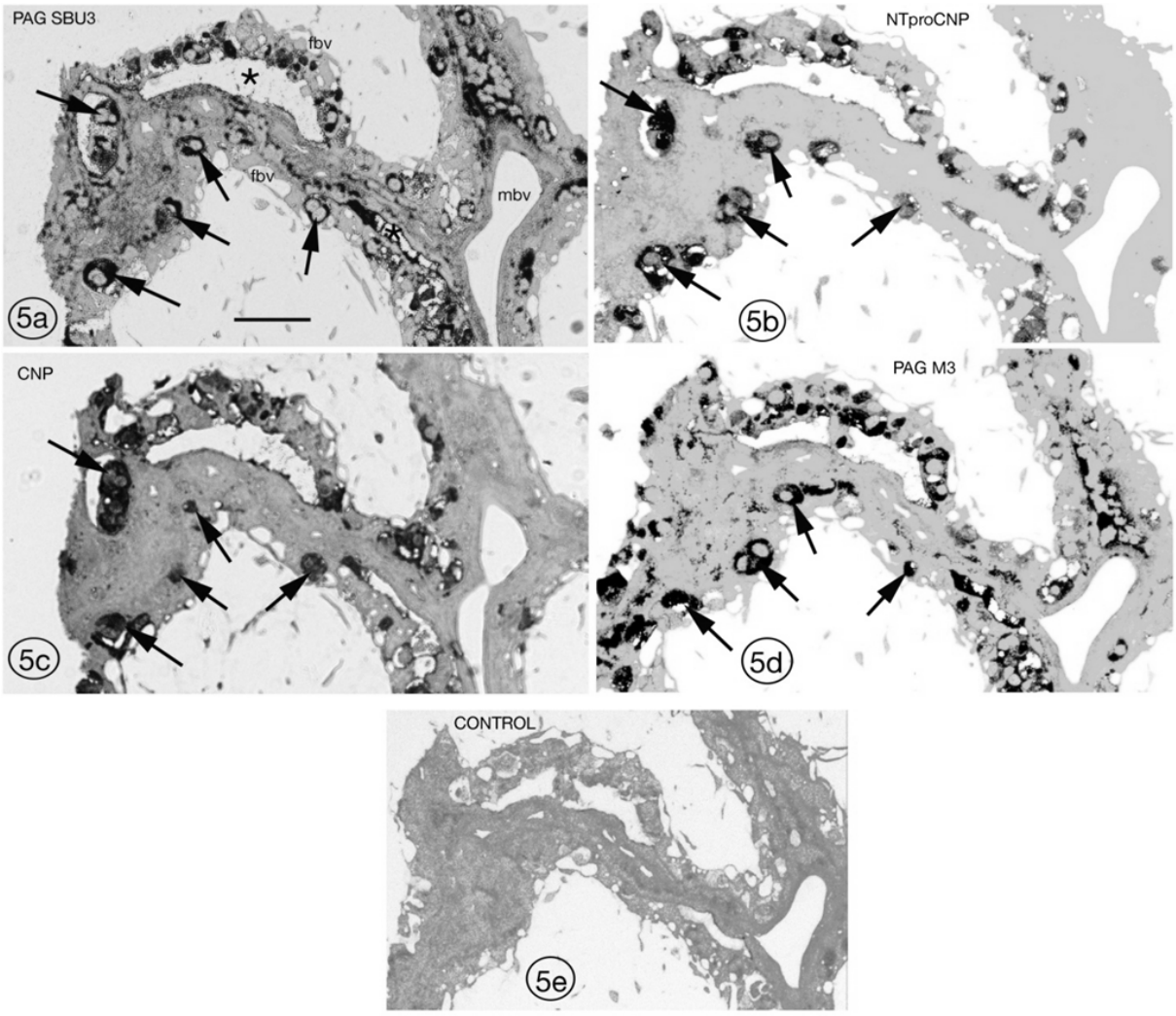

Figure 5-5.

Immunocytochemistry of deresinated sections prepared after aldehyde perfusion, fixation and araldite embedding. The arrows demonstrate the co-localisation of CNP (5c), and NTproCNP (5b) with the two PAGs, SBU3 (5a) and M3 (5d), in the same BNCs on semi-serial sections. Control section (5e) prepared with non-specific antibody. fbv: fetal blood vessel; mbv: maternal blood vessel; asterisks: artefactual separation of the microvillar junction between trophoblast and fetomaternal syncytium. Bar (5a) equals 60 µm.

5.5.5 Relationship between CNP forms in maternal circulation and fetal cotyledon

When subjected to size exclusion HPLC/RIA, a major peak of immunoreactive CNP eluted in a position consistent with CNP-53 (Figure 5-6 A). A smaller peak (fractions 31–33) consistent with CNP-22 was also identified. As shown in Figure 5-6 B, a major peak of immunoreactive

proCNP 1-15 eluted in a position consistent with the 5-kDa (or similar) aminoterminal fragment (proCNP 1-50). A smaller (unidentified) and later eluting peak was also present. The early eluting shoulder (fractions 22–25) of immunoreactive material identified in both Figure 5-6 A and B is consistent with the presence of proCNP (1–103).

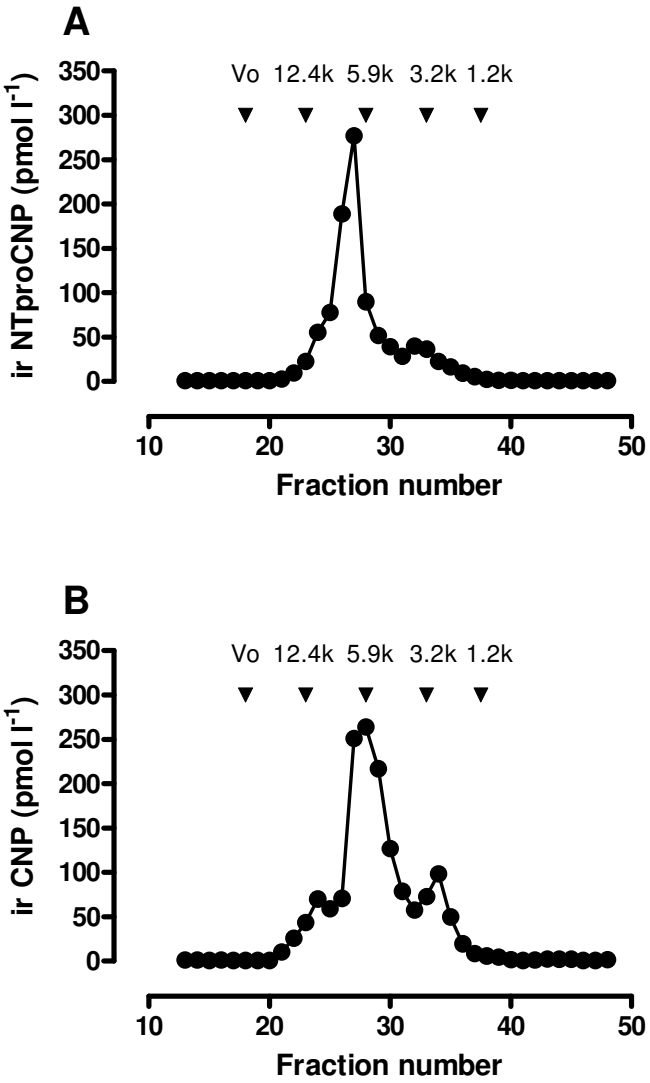

Figure 5-6.

Immunoreactive (ir) (A) NTproCNP and (B) CNP size exclusion HPLC profiles of ovine placental extract from day 143 of gestation. Column void volume (V_0) and elution positions of molecular markers are shown by arrows.

Comparison of the concentration of CNP forms in the fetal cotyledon (current study) with maternal circulating CNP forms throughout pregnancy (Chapter 4) revealed a strong similarity in the temporal pattern of CNP in these two situations (Figure 5-7).

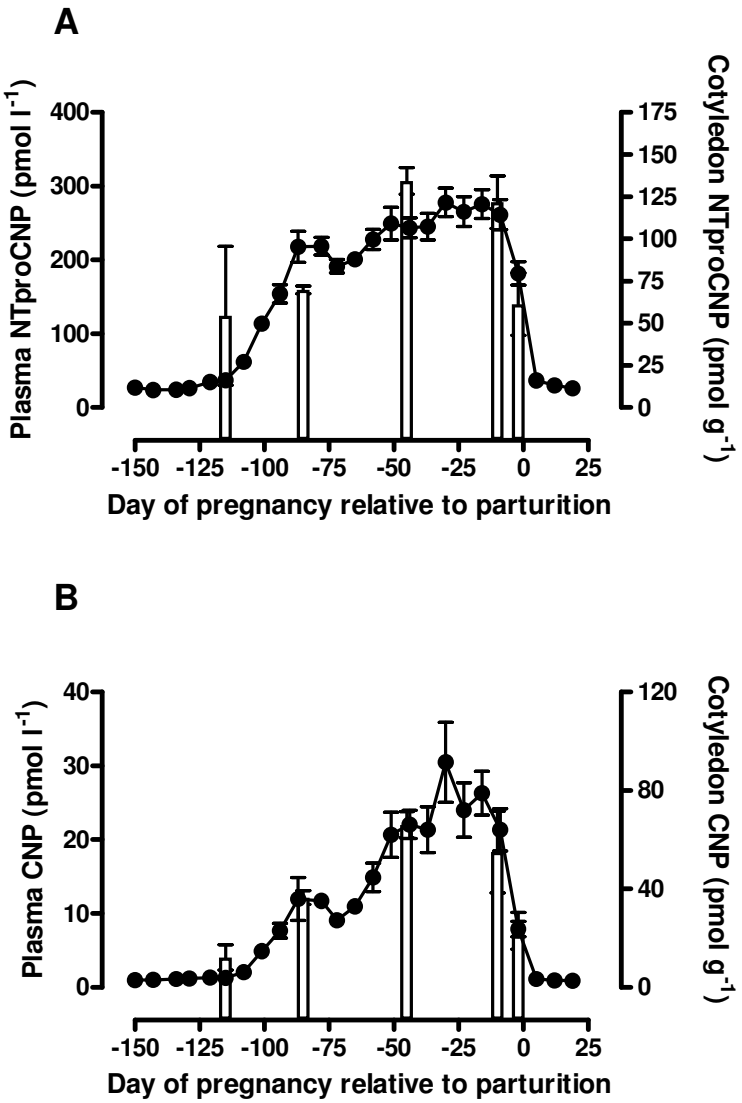

Figure 5-7.

Mean (± SEM) concentration of (A) NTproCNP and (B) CNP in the maternal circulation (•) and fetal cotyledon (white bars).

5.6 Discussion

This study reports a number of novel observations relating the concentration of CNP peptides in placental and uterine tissues to blood concentrations throughout gestation in ovine pregnancy - findings that contrast with the progressive decline in their concentration in fetal plasma. Importantly, for the first time the trophoblast BNC has been identified as an important source of CNP during pregnancy. Collectively, the evidence of sustained and increasing participation of (placental) paracrine and (maternal) endocrine CNP activity, in the

face of decreasing CNP concentration in the fetal circulation, point to separate and distinct actions in the dam and the fetus.

Using well-recognised immunohistochemical techniques, both CNP and NTproCNP were localised to trophoblasts and vascular tissue in the ovine placentome. The identity of CNP-positive BNCs is confirmed by the co-localised staining of CNP forms and the BNC marker PAG. Ruminant BNC are capable of producing a variety of steroid and other hormones (Wooding, 1992), but this is the first study to report localisation of CNP to these cells, strongly implicating them as a site of production of this peptide. Of note, obvious CNP peptide-specific staining was observed in a minority of BNCs in contrast to the more prevalent staining of PAG. Presumably the much higher concentrations of PAGs (nanomolar versus picomolar for CNP (Kappes *et al.*, 1992)) contribute to this apparent discrepancy. Other factors such as the lability of CNP (half-life less than 2 minutes (Charles *et al.*, 1996)) and loss of antibody binding epitopes in the course of immunocytochemical processing are additional factors reducing positive labelling on the resin sections. The demonstration of staining of both CNP and NTproCNP around the placental blood vessels on the frozen sections, a site of prominent CNP gene expression in the mouse embryo (Cameron *et al.*, 1996), suggests that CNP has two distinct roles during pregnancy; exerting its effect in an endocrine fashion in the maternal circulation and also acting as a paracrine/autocrine factor within the placental vasculature.

A striking result of this study is the close temporal relationship between changes in the concentration of CNP forms in the fetal cotyledon and in maternal plasma throughout the course of gestation. An exception applies to samples drawn on day 30 where, for both CNP forms, the ratio of maternal plasma concentration to placental content is comparatively low, suggesting a possible impairment of secretory ability at this early stage of placental development. Although the relative weights of the caruncle and cotyledon were not recorded in the current study, previous reports indicate that cotyledonary mass is generally 2-3 times greater than caruncular mass (Reynolds *et al.*, 2005a). Taken together with the immunolocalisation of CNP and PAG to the BNC, these data strongly implicate the fetal placenta as the primary source of maternal circulating CNP during ovine pregnancy. A similar temporal relationship between maternal and placental ovine placental lactogen (oPL) has been reported, where oPL mRNA expression in the cotyledon was related to maternal, but not to fetal serum oPL concentration (Kappes *et al.*, 1992). Although the precise function of BNC-derived oPL and PAG has not been confirmed, it appears that oPL may promote fetal growth

by manipulating maternal metabolism to favour preferential delivery of glucose from mother to fetus (Byatt *et al.*, 1992). A function for PAG in cell adhesion and immunomodulation at the feto-maternal interface has been proposed (Wooding *et al.*, 2005). Of note, there was some evidence of higher CNP concentrations in uteroplacental tissues in single- versus twin-bearing ewes. This result is in contrast to the positive relationship between maternal CNP concentration and fetal number reported in Chapter 4, indicating that further work with larger numbers in the two groups is required to investigate a possible effect of fetal number on placental CNP concentration.

The similarities between the site of production and temporal secretion of oPL (Kappes *et al.*, 1992) and CNP as demonstrated in the current study, provide new evidence to suggest that CNP may also represent a communication from the fetus to its dam, presumably for the benefit of the fetus. This view is further supported by the previous finding of increased maternal concentrations as fetal levels fall in response to acute nutrient deficiency in late ovine gestation (Prickett *et al.*, 2007). Infusion studies will be an important next stage to identify the physiological effects elicited by CNP in the pregnant female, which may include maintenance of myometrial quiescence (Carvajal *et al.*, 2009) and mediating a reduction in peripheral vascular resistance as blood volume expands. On the other hand, CNP's role as a predominantly paracrine/autocrine factor likely contributes importantly to the greatly increased blood flow to uteroplacental tissues during pregnancy (Cameron *et al.*, 1996; Itoh *et al.*, 1998). In addition to its vasodilatory function, CNP is also a potent inhibitor of vascular smooth muscle cell growth (Furuya *et al.*, 1991) and may therefore be involved in regulation of uteroplacental vascular development, a process which must be carefully controlled during pregnancy to prevent the development of pathological conditions associated with excessive capillary growth (Reynolds & Redmer, 2001).

Until the current report, the relative changes in CNP production within uterine and placental tissues during ovine pregnancy were unknown. In the current study, CNP protein concentrations in ovine uterine tissue were low and stable as gestation proceeded to term, apart from a transient increase early in pregnancy coinciding with the phase of rapid uterine growth (Spencer *et al.*, 2004). These results are in sharp contrast to the progressive increase in uterine CNP mRNA reported in murine pregnancy (Stepan *et al.*, 2001). Furthermore, the finding of low CNP gene expression in uterine tissue – similar to skeletal muscle at term – provides further evidence of different patterns of uterine CNP production in the two species. It has been suggested that augmented uterine CNP expression in murine pregnancy reflects

increasing oestrogen production which is known to stimulate CNP gene transcription in rodent uterine tissue (Acuff *et al.*, 1997). In previous studies in non-pregnant sheep, oestrogen has been identified as a potent stimulus of CNP secretion (Prickett *et al.*, 2008b). However the lack of an association of uterine CNP content to fluctuations in plasma oestradiol concentration during ovine pregnancy (Chapter 4) suggests that alternative mechanisms regulate CNP production within uterine tissues in ovine pregnancy.

The current study is the first to document the time course of CNP forms in fetal plasma in any species, from early in the 2nd trimester through to term. In marked contrast to the increase in placental and maternal concentrations during the same period, both CNP and NTproCNP declined progressively in the fetus to attain concentrations at term approximating 40 % (CNP) and 60 % (NTproCNP) of initial values observed on day 60.This is a very similar decline to that shown for oPL in the fetal circulation (Chan *et al.*, 1978). These findings, and the absence of a concentration gradient of CNP across the fetal umbilical artery and vein (McNeill *et al.*, 2009b), constitute strong evidence that the sheep placenta is not a source of CNP in the fetal circulation. Conceivably, changes in distributional volume (plasma volume per body weight increases with fetal maturation), and/or increased degradation could contribute to the observed decline in fetal CNP forms. However, evidence that fetal concentrations fall during acute caloric restriction (Prickett *et al.*, 2007), and during physiological increments in fetal cortisol levels (Prickett *et al.*, 2010a), indicates that CNP production itself is likely to be carefully regulated. Since the peptide is widely expressed in fetal tissues (Stepan *et al.*, 2001), future studies using trans-organ regional sampling should help to clarify tissue contributions to circulating levels. It is interesting to note that whereas maternal concentrations of CNP forms in humans (Prickett *et al.*, 2004) appear to differ markedly from those in pregnant sheep, fetal concentrations at term are very similar (3-4 pmol l^{-1}). Furthermore, the umbilical vein concentration of NTproCNP is inversely related to gestational age in both humans and sheep and the concentration ratio of NTproCNP to CNP in fetal plasma is some 5-6 fold that in the maternal circulation (Prickett *et al.*, 2008a). These observations suggest that circulating CNP concentration in the fetus is tightly regulated.

Equally intriguing is the role of CNP in the fetal circulation. A possible role in longitudinal growth has been proposed due to the relationship between fetal CNP concentration and linear growth rate in humans (Prickett *et al.*, 2008a) and the crucial role for CNP in postnatal skeletal development (Chusho *et al.*, 2001). However, the pattern of CNP in fetal circulation does not reflect ovine patterns of linear growth, which are characterised by accelerated growth

in the final stages of pregnancy (Barbera *et al.*, 1995) - a period of decreasing circulating CNP in the fetus. Furthermore, as discussed by Prickett *et al.* (2007), in CNP-knockout mice the skeletal effects of disrupted CNP signalling are generally not evident at birth, and manifest only during the postnatal growth period. In keeping with this view, recent studies in the fetal lamb (Prickett *et al.*, 2010b) suggest that changes in fetal growth and plasma NTproCNP concentration during glucocorticoid administration are less tightly coupled than in the postnatal period but further study of these relationships are clearly needed in humans and other mammals.

In conclusion, positive localisation of CNP to the trophoblast BNC is reported during ovine pregnancy, suggesting an important new endocrine role for CNP in the pregnant female. The direct relationship between maternal circulating CNP concentration and placental peptide production indicates that measurement of maternal concentrations may provide a novel tool for monitoring feto-placental well-being and identifying at-risk pregnancies. This study has revealed a new mediator of fetal-maternal communication in ruminant pregnancy with possible relevance to understanding the regulation of placental function in other species as well.

Chapter 6:
Regulation of CNP during ovine pregnancy – the role of nutrition

Declaration

I was involved in all aspects of the design, execution and analysis of the experiment described in this chapter. Furthermore, all CNP and NTproCNP assays were performed by me. Other analytes were assayed by Gribbles Veterinary Pathology Laboratories (β-OHB), Lincoln University (glucose, urea, progesterone) or Canterbury District Health Board (IGF-1). I received significant technical assistance with sample collection and animal procedures as well as comments on drafts of this chapter from my PhD supervisors.

6.1 Abstract

Extremely high concentrations of CNP and NTproCNP occur in the maternal circulation during ruminant pregnancy; probably arising from production by the BNC of the placental trophoblast. As further increases in maternal circulating CNP forms have been reported during fasting in late pregnancy, the aim of this study was to test the hypothesis that CNP production by the uteroplacental unit is homeostatically regulated by its nutritional status. At day 85 of pregnancy, twin-bearing ewes were allocated to one of three treatment groups: caloric restriction (CR, n = 10, fasted between days 121-124 of pregnancy), caloric loading (CL, n = 6, fed to 200 % of ME requirements between days 110-124 of pregnancy) or control (n = 10, fed to 100 % of ME requirements). Maternal blood samples were collected twice weekly from day 80 until day 100 and then daily until day 124. At day 124 of pregnancy, ewes were killed and samples of fetal plasma, uterine and placental tissues collected. Maternal circulating NTproCNP concentration, expressed as a percentage of pre-fasting concentration (day 121), was significantly elevated 24 hr later in CR ewes compared with controls (117 ± 5.1 % vs 107 ± 3.5 %, $P < 0.05$). Fetal plasma CNP and NTproCNP concentrations were lower in CR compared with control fetuses (CNP: 4.6 ± 0.28 vs 5.2 ± 0.23 pmol l^{-1}; NTproCNP: 191.5 ± 5.9 vs 208.8 ± 5.1 pmol l^{-1}, $P < 0.05$). Fetal weight was also reduced in CR fetuses ($P = 0.02$). There was no effect of dietary manipulation on the concentration of CNP forms in uterine or placental tissues. There was no effect of CL on the concentration of either CNP form in plasma or uteroplacental tissues (P = NS), despite a significant increase in maternal body condition score and fetal plasma IGF-1 concentration ($P < 0.05$).

The results of this study do not support the hypothesis that CNP production during pregnancy is homeostatically regulated by nutrition, instead they suggest that production of the peptide is selectively upregulated by caloric restriction.

6.2 Keywords

Sheep, placenta, CNP, NTproCNP, caloric loading, caloric restriction

6.3 Introduction

Extremely high concentrations of CNP and NTproCNP have been identified in plasma and placental extracts from pregnant ruminants, suggesting that CNP is likely to be involved in fetal and/or placental development. However, the role of CNP during pregnancy and the factor/s involved in regulating its production have not been identified. In ewes, Prickett *et al.* (2007) reported a significant increase in maternal circulating CNP and NTproCNP concentration following a three-day fast in late gestation, which was reversed on refeeding. This finding was used to formulate the hypothesis that the increase in CNP production may be an adaptive response by the uteroplacental unit, as part of a homeostatic mechanism regulating nutrient supply to the conceptus.

Therefore, the aim of the current study was to test this hypothesis; by monitoring the effects of nutrient restriction (maternal fast) and, conversely, abundance (caloric load) on plasma and placental concentrations of CNP forms in late pregnancy. In keeping with the hypothesis, it is proposed that caloric loading during ovine pregnancy will result in a decrease in the concentration of CNP forms in maternal plasma. In contrast, maternal circulating concentrations of CNP forms are expected to increase following nutrient restriction. Having identified the trophoblast BNC as a likely source of CNP in maternal circulation (Chapter 5), it is hypothesised that changes in maternal plasma concentration of CNP forms will be reflected by similar changes in CNP protein concentration within the cotyledon.

6.4 Methods

6.4.1 Animals

Mixed-age Coopworth ewes of known gestational age from the Lincoln University Research Farm were used in this study. Pregnancy was confirmed and fetal number determined by transabdominal ultrasonography at day 70 of pregnancy, at which time the first 30 ewes from a large mob scanned as carrying twin fetuses were selected for inclusion in the study.

6.4.2 Feeding details

Prior to day 70 of pregnancy, all ewes were fed *ad libitum* on pasture. Between days 70 and 80 of pregnancy the ewes remained on pasture but were provided with supplementary pellets (concentrate diet consisting of 13.5 MJ/kg, 85 % dry matter; providing the complete dietary requirements for pregnant ewes. Weston Animal Nutrition, Rangiora, New Zealand) to allow them to become accustomed to the concentrated feed. On day 80 of pregnancy all ewes were transferred to an indoor animal housing facility with individual pens and fed only pellets for the remainder of the experiment. Feed requirements in terms of ME were determined according to live weight, stage of pregnancy and number of fetuses using the method described by Nicol and Brookes (2007). Feed allowances were calculated on an individual basis for each ewe and were adjusted weekly as pregnancy progressed. Fresh water was available at all times throughout the study period.

On day 80 of pregnancy ewes were allocated to one of three treatment groups (n = 10 per group): control, caloric restriction (CR) or caloric loading (CL). Allocation to groups was random but balanced for live weight. Control ewes were fed to requirement until slaughter at day 124 of pregnancy. CR ewes were also fed to requirement until day 121, after which they were fasted for three days until slaughter at day 124. CL ewes received their requirement feed until day 94 of pregnancy, thereafter gradually increasing to 200 % of requirement by day 110 of pregnancy and fed at that level until slaughter at day 124 of pregnancy.

Ewes were fed once daily in the morning after collection of a blood sample. As voluntary intake of CL ewes did not reach the desired level of 200 % of ME requirements, these ewes were provided their daily feed allowance in two equal allocations, in the morning and in the afternoon, in an attempt to encourage increased food intake. Fresh feed was provided daily to all animals and refusals were weighed to determine individual feed intakes.

6.4.3 Blood samples and live weight measurements

Maternal blood samples were collected twice weekly between days 84 and 100 of pregnancy and then daily from day 101 until slaughter for measurement of circulating CNP, NTproCNP, glucose, β-OHB and urea concentrations. On all occasions, blood samples were collected in the morning, between 8 and 10 am. A fetal blood sample was collected immediately after

slaughter on day 124 of pregnancy for measurement of plasma CNP, NTproCNP and IGF-1 concentration.

Non-fasting live weights were recorded weekly from day 70 until day 110 of pregnancy, and then twice weekly from day 110 until slaughter. A final live weight and body condition score were recorded on day 124 immediately prior to slaughter.

6.4.4 Tissue collection and slaughter procedure

All ewes were killed by captive bolt followed by exsanguination on day 124 of pregnancy. The pregnant uterus was excised immediately after slaughter and samples of intercaruncular uterine tissue, caruncle and cotyledon collected and processed for measurement of CNP and NTproCNP protein concentration as described in Chapter 5. Placentomes were separated into A-, B-, C- and D-type according to the classification of Vatnick *et al.* (1991). As the majority of placentomes were A-type and everted placentome types were infrequent or absent in many ewes, CNP protein concentration was measured only in A-type placentomes. Two randomly-selected A-type placentomes were collected from regions of placenta associated with each fetus (a total of four placentomes per ewe). CNP and NTproCNP concentration was measured in each of these four samples and the results averaged to give a single value for each ewe.

Fetal weight, sex and crown-rump length were recorded immediately following collection of a fetal blood sample. Total placental weight - taken as the combined weight of all placentomes (cotyledon plus caruncle) - was recorded as well as the total number and weight of placentomes in each category.

6.4.5 Statistical analyses

Statistical analyses were performed using Genstat Version 10 (VSN International; Hemel Hemstead, UK). A P value of ≤ 0.05 was considered statistically significant. Data were log-transformed where appropriate to meet ANOVA assumptions. Differences in maternal feed intake, live weight and maternal CNP concentration were assessed by 2-way repeated measures ANOVA followed by Bonferroni *post hoc* tests with treatment group and day of gestation as fixed effects. Differences in the concentration of CNP forms and IGF-1 in fetal plasma between control and treatment groups were determined by one-tailed unpaired *t*-test. Effect of diet on maternal glucose, urea and β-OHB concentration were analysed by one-way

ANOVA followed by Bonferroni *post hoc* test. Relationships between plasma and tissue CNP forms and fetal and placental characteristics were examined by fitting the data with linear regression best-fit models and testing the deviation from zero slope using an F-test.

6.5 Results

6.5.1 Maternal feed intake, live weight and condition score

Maternal feed intake and live weight throughout the study period are shown in Figure 6-1. There was no difference in feed intake between the three groups at the beginning of the study (P = NS). By day 97 feed intake in the CL group was significantly elevated above that of controls ($P < 0.001$) and remained elevated for the remainder of the study ($P < 0.05$), with the exception of days 106, 108, 110, 112, 115 and 117. Although CL ewes were offered 200 % of ME requirements, voluntary intake was lower than this; with a mean daily intake of 126.6 ± 2.7 % of ME requirements between days 95 and 124 of pregnancy. Four ewes in the CL group consistently had feed intakes of less than 100 % of requirements and were therefore excluded from the study. There was no significant difference in live weight between the three groups of ewes at any time point except at day 124, at which time CL ewes were significantly heavier than CR ewes ($P < 0.05$). Maternal body condition score at slaughter was significantly higher in CL ewes compared with controls ($P < 0.001$, Figure 6-2). There was no difference in body condition score between CR and control ewes (P = NS).

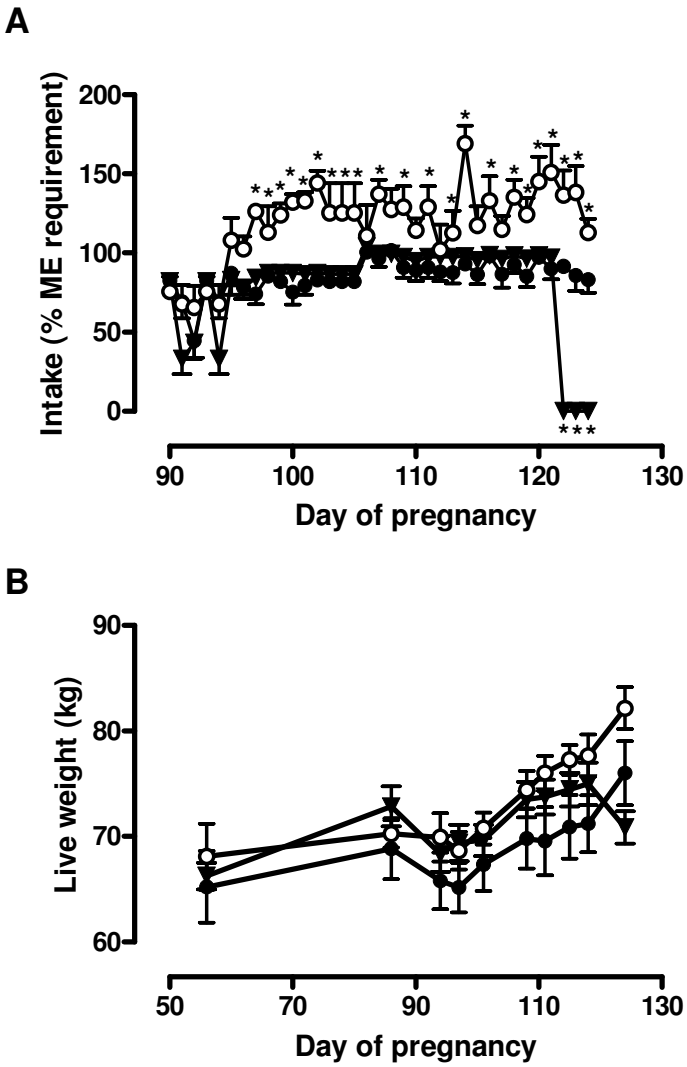

Figure 6-1.

Mean (± SEM) (A) maternal feed intake (% ME requirement) and (B) live weight in pregnant ewes from control (closed circles, n = 10), caloric loading (open circles, n = 6) and caloric restriction (▼, n = 10) treatment groups. Feed allocation to ewes in the caloric loading group was gradually increased from 100 % to 200 % of ME requirements between days 95 and 110 of pregnancy. Significant difference (P < 0.05) from control values is indicated by an *.

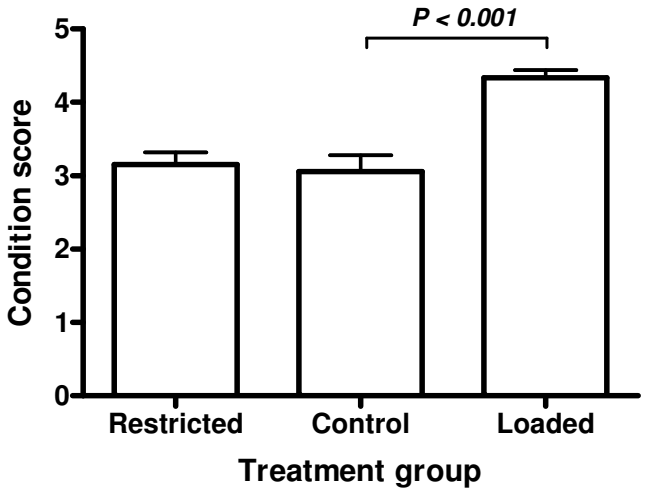

Figure 6-2.

Mean (+ SEM) maternal body condition score of pregnant ewes at slaughter in caloric restriction (n = 10), control (n = 10) and caloric loading (n = 6) treatment groups.

6.5.2 Fetal and placental characteristics

Fetal weight was significantly lower in CR fetuses compared with controls (P = 0.02, Figure 6-3), and there was a trend for reduced crown-rump length in the CR fetuses (P = 0.07). There was no difference in fetal weight or crown-rump length between control and CL fetuses (P = NS, Figure 6-3).

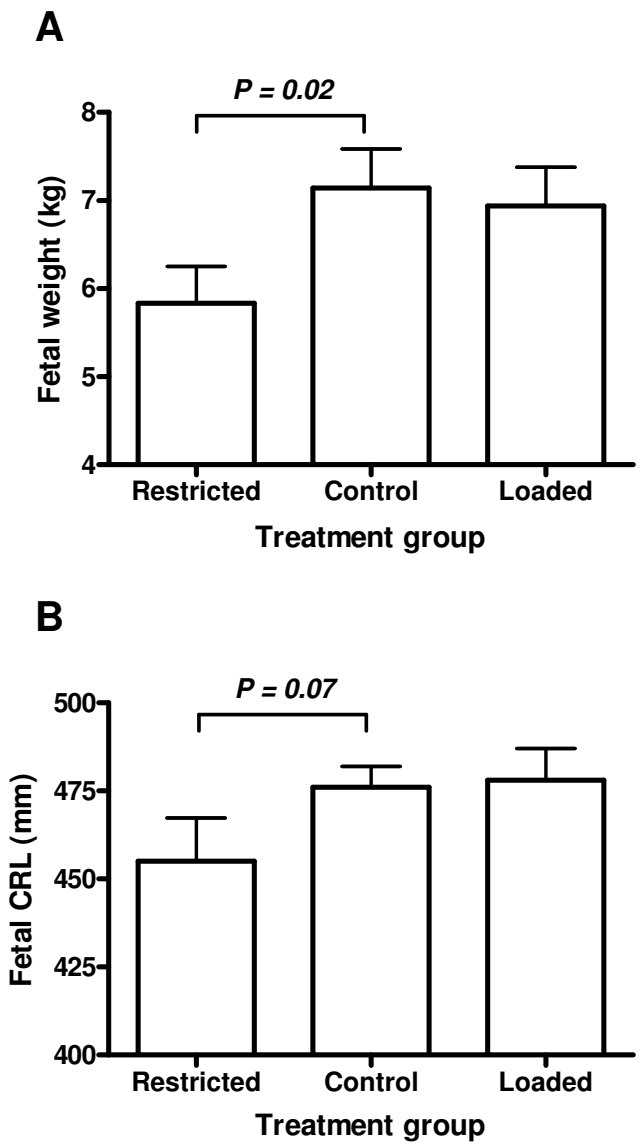

Figure 6-3.

Mean (+ SEM) (A) weight and (B) crown-rump length (CRL) of sheep fetuses from caloric restriction (n = 10), control (n = 10) and caloric loading (n = 6) treatment groups. Fetal weight represents the combined weight of both twins whereas crown-rump length is an average of each pair of twins.

There was no difference in total placental weight or placental efficiency, calculated as the ratio of fetal:placental weight (Fowden *et al.*, 2009), between the three treatment groups (P = NS, Figure 6-4). There were significantly fewer placentomes in CL compared with control ewes (P = 0.03), although there was no difference in average placentome weight between the two groups (P = NS).

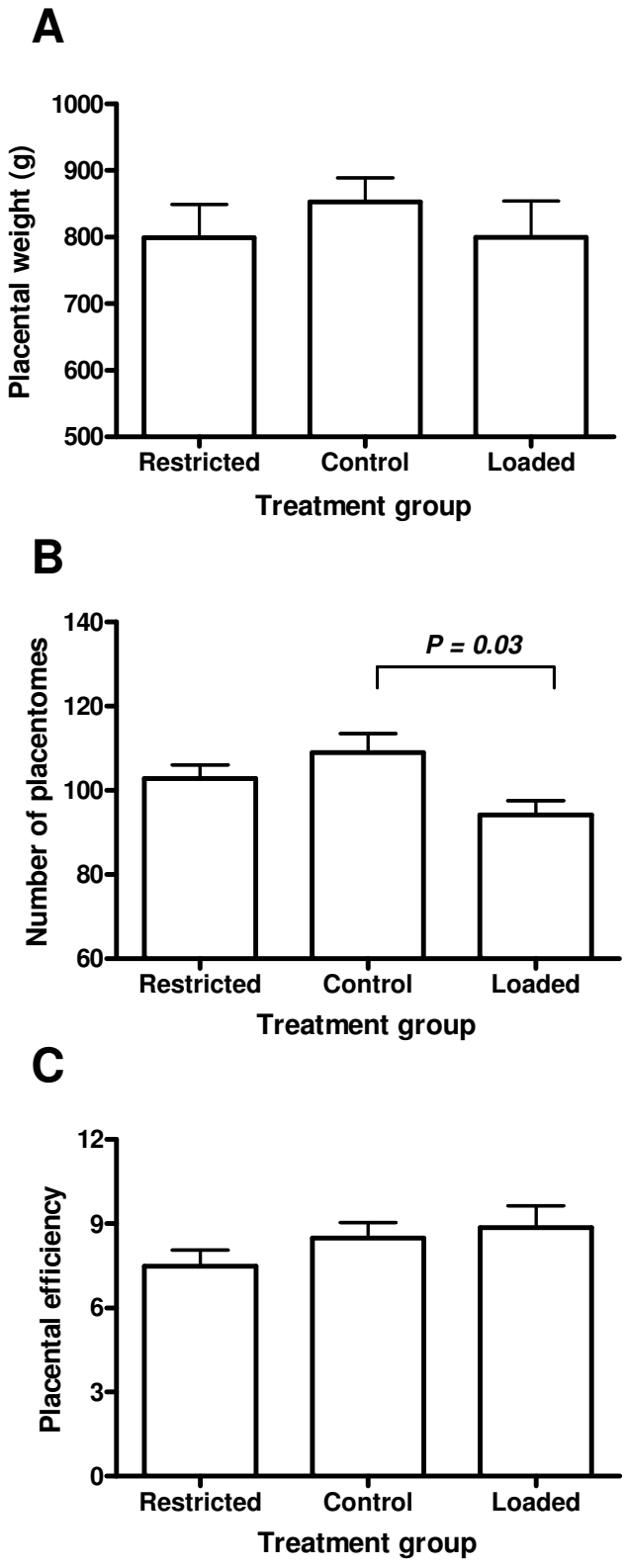

Figure 6-4.

Mean (+ SEM) (A) total placental weight (B) number of placentomes and (C) placental efficiency at day 124 of pregnancy in pregnant ewes from caloric restriction (n = 10), control (n = 10) and caloric loading (n = 6) treatment groups.

The placentae of all three groups consisted predominantly of A-type placentomes, accounting for 81, 69 and 76 % of all placentomes in the control, CR and CL groups, respectively. The frequency of each placentome type did not differ between the three treatment groups (P = NS, Figure 6-5).

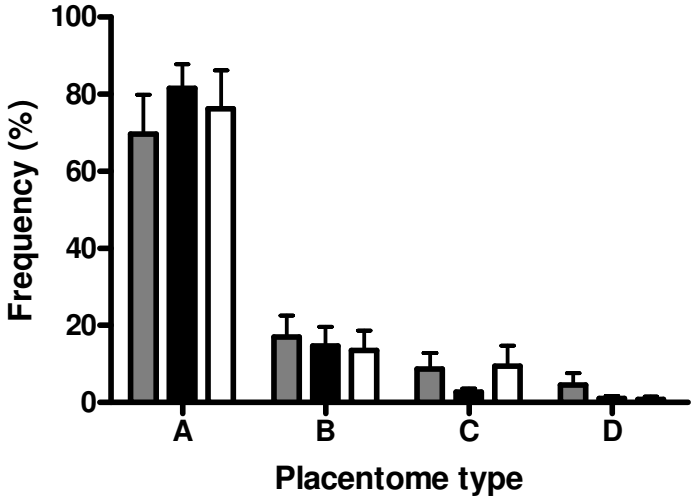

Figure 6-5.

Mean (+ SEM) placentome distribution (%) at day 124 of pregnancy in pregnant ewes from caloric restriction (grey bars, n = 10), control (black bars, n = 10) and caloric loading (white bars, n = 6) treatment groups.

6.5.3 CNP and NTproCNP concentration in maternal plasma

Effect of CR

There was no difference in maternal plasma concentration of either CNP form between control and CR ewes at day 85 of pregnancy (P = NS). However, immediately prior to fasting, circulating concentration of CNP forms were higher in control compared with CR ewes. Therefore, to allow comparison to be made between these two groups, the data were normalised to express fasting hormone concentration as a percentage of day 121 values (immediately prior to the commencement of the fasting period). This transformation revealed a significant elevation in maternal circulating NTproCNP concentration in CR ewes compared with control ewes on day 122 ($P < 0.05$, Figure 6-6). There was no change in maternal circulating CNP concentration between control and CR ewes during the fasting period (Figure 6-6). Fasting was not associated with a change in the ratio of NTproCNP:CNP in maternal plasma (P = NS, Figure 6-6).

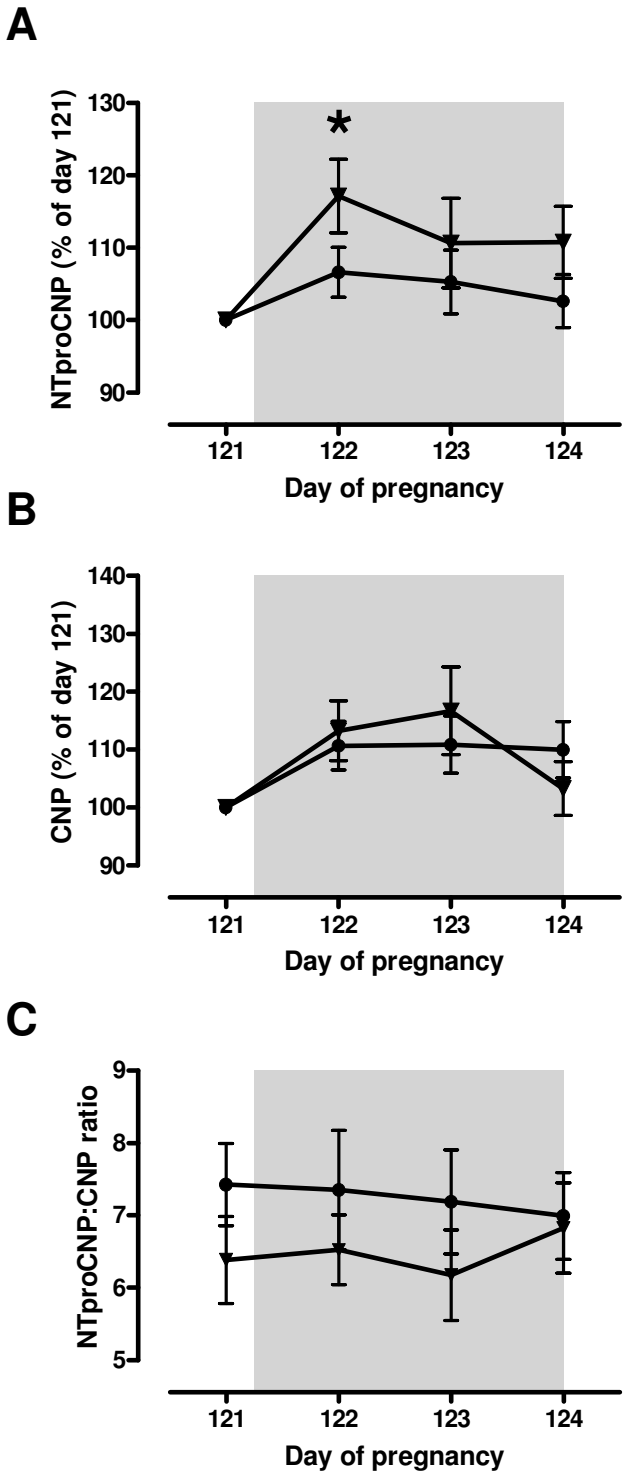

Figure 6-6.

Mean (± SEM) change in maternal circulating (A) NTproCNP and (B) CNP concentration expressed relative to day 121 values and (C) mean molar plasma NTproCNP:CNP ratio in pregnant ewes from control (•) and caloric restriction (▼) treatment groups (n = 10 per group). The shaded area represents the fasting period. * P < 0.05 compared with control group.

Effect of CL

There was no significant difference in maternal plasma concentration of either CNP form in control and CL ewes at the beginning of the study, before the period of caloric loading (P = NS), although there was a trend for lower maternal circulating concentration of CNP forms in CL ewes. Therefore, the data are expressed as a proportion of day 86 values to remove a possible confounding effect of this difference in baseline values (Figure 6-7). Analysis of both the raw data and normalised values revealed no significant difference in maternal circulating concentration of either CNP form between the control and CL groups at any time point (CNP: P = 0.6; NTproCNP: P = 0.7)

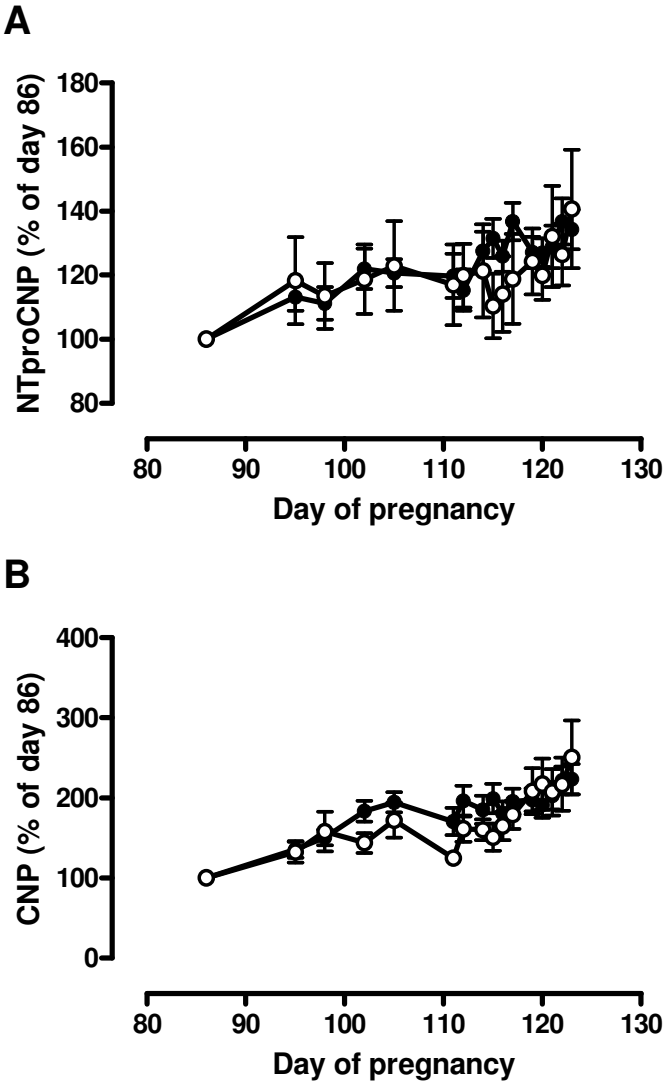

Figure 6-7.

Mean (± SEM) change in maternal circulating (A) NTproCNP and (B) CNP concentration expressed relative to day 86 values in pregnant ewes from control (closed circles, n = 10) and caloric loading (open circles, n = 6) treatment groups. Feed allocation to CL ewes was gradually increased from 100 % to 200 % of requirements between days 95-110.

6.5.4 CNP and NTproCNP concentration in fetal plasma

Maternal fasting was associated with significantly lower fetal plasma concentration of CNP ($P = 0.05$) and NTproCNP ($P = 0.02$) in CR fetuses compared with controls (Figure 6-8). There was no effect of CL on fetal plasma concentration of either CNP form (P = NS).

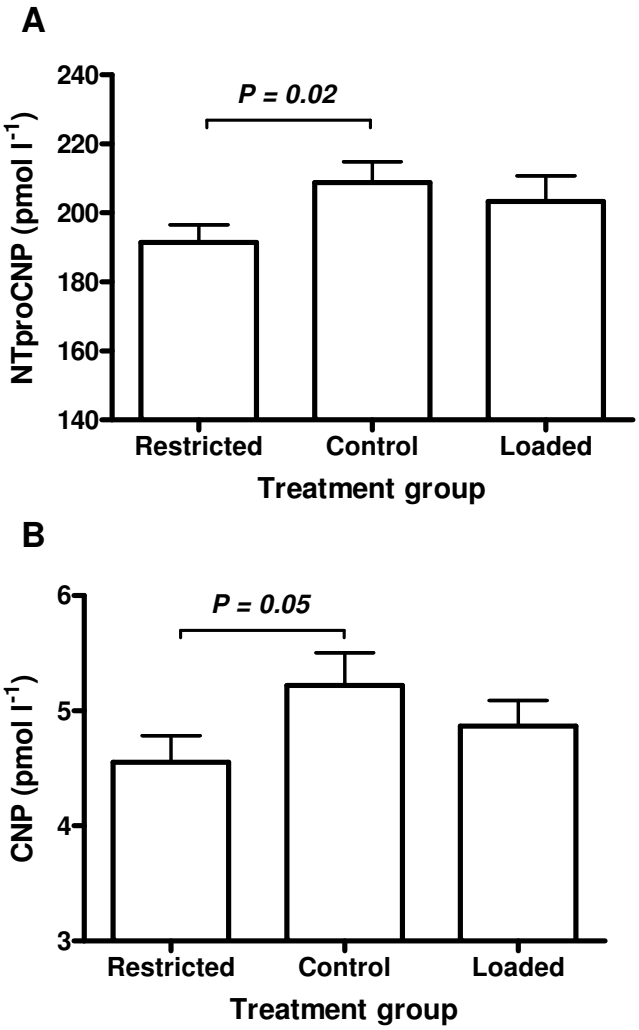

Figure 6-8.

Mean (+ SEM) plasma concentration of (A) NTproCNP and (B) CNP in ovine fetuses from caloric restriction (n = 10), control (n = 10) and caloric loading (n = 6) treatment groups. Hormone concentrations are averages for each pair of twins.

6.5.5 CNP and NTproCNP concentration in uterine and placental tissue

In all groups, the concentration of CNP forms in the placenta exceeded that in intercaruncular uterine tissue ($P < 0.05$). Within the placenta, the concentration of both CNP forms was higher in the cotyledon compared with the caruncle ($P < 0.05$). There was no effect of diet on the concentration of either CNP form in uterine or placental tissue at slaughter (P = NS, Figure 6-9).

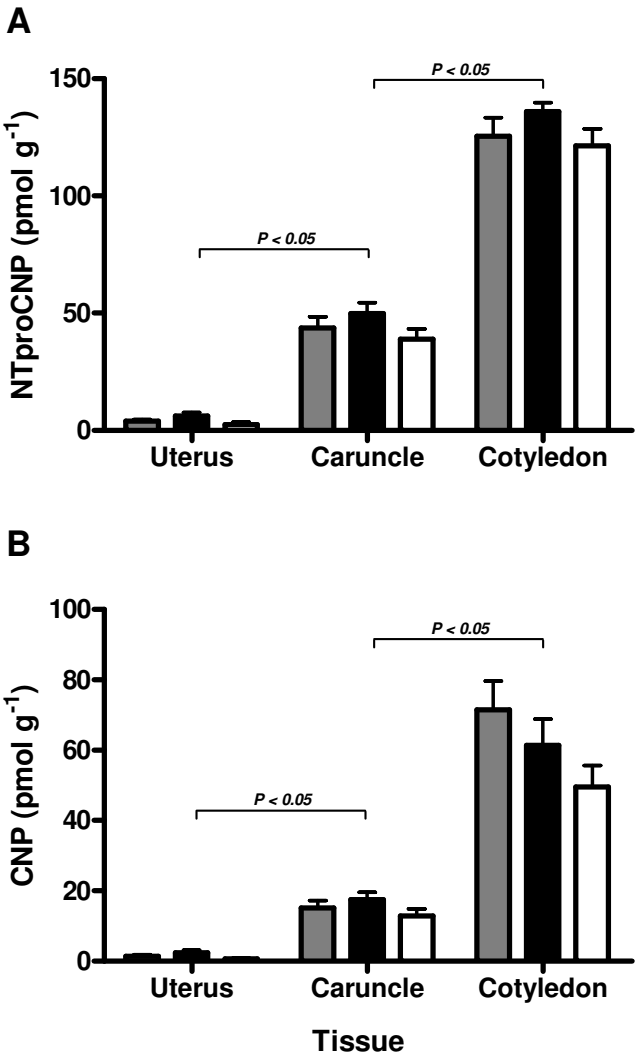

Figure 6-9.

Mean (+ SEM) concentration of (A) NTproCNP and (B) CNP in uterine and maternal (caruncle) and fetal (cotyledon) placental tissues in pregnant ewes from caloric restriction (grey bars, n = 10), control (black bars, n = 10) and caloric loading (white bars, n = 6) treatment groups at day 124 of pregnancy. Within each tissue type, diet had no effect on the concentration of either CNP form (P = NS).

6.5.6 Relationship between CNP forms and fetal and placental weight

There was a significant positive relationship between maternal plasma NTproCNP, but not CNP, concentration at slaughter and total placental weight (NTproCNP: $r^2 = 0.24$, P = 0.01; CNP: $r^2 = 0.10$, P = 0.13). There was also a significant positive relationship between maternal plasma NTproCNP concentration at slaughter and NTproCNP concentration in the cotyledon ($r^2 = 0.24$, P = 0.01), but again no relationship between CNP concentration in maternal plasma and in the cotyledon (P = 0.99).

There was no relationship between fetal weight and maternal circulating concentration of either CNP form at slaughter (NTproCNP: P = 0.28; CNP: P = 0.07). Likewise, there was no relationship between fetal plasma concentration of either CNP form at slaughter and fetal weight (NTproCNP: P = 0.88; CNP: P = 0.96) or crown-rump length (NTproCNP: P = 0.85; CNP: P = 0.60).

6.5.7 Maternal progesterone and maternal and fetal nutritional biochemistry

Maternal progesterone:

There was no difference in maternal circulating progesterone concentration between control and caloric loading groups at any time-point during the study period (P > 0.05, Figure 6-10).

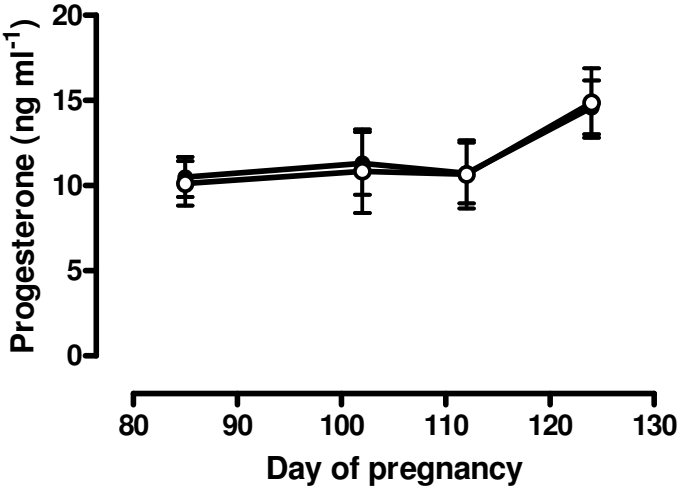

Figure 6-10.

Mean (± SEM) maternal circulating progesterone concentration in pregnant ewes from control (closed circles, n = 10) and caloric loading (open circles, n = 6) groups.

Maternal β-OHB, glucose and urea:

There was no difference in maternal plasma concentration of β-OHB, glucose or urea between the three groups at the beginning of the study (day 85 of pregnancy), or immediately prior to the fasting period (day 121) (P = NS; Figure 6-11). Caloric restriction was associated with a significant increase in the circulating concentration of β-OHB and urea, and a significant decrease in maternal plasma glucose concentration compared with controls (P < 0.05). Caloric loading was associated with a significant elevation in maternal plasma glucose concentration at day 122 of pregnancy (P < 0.05).

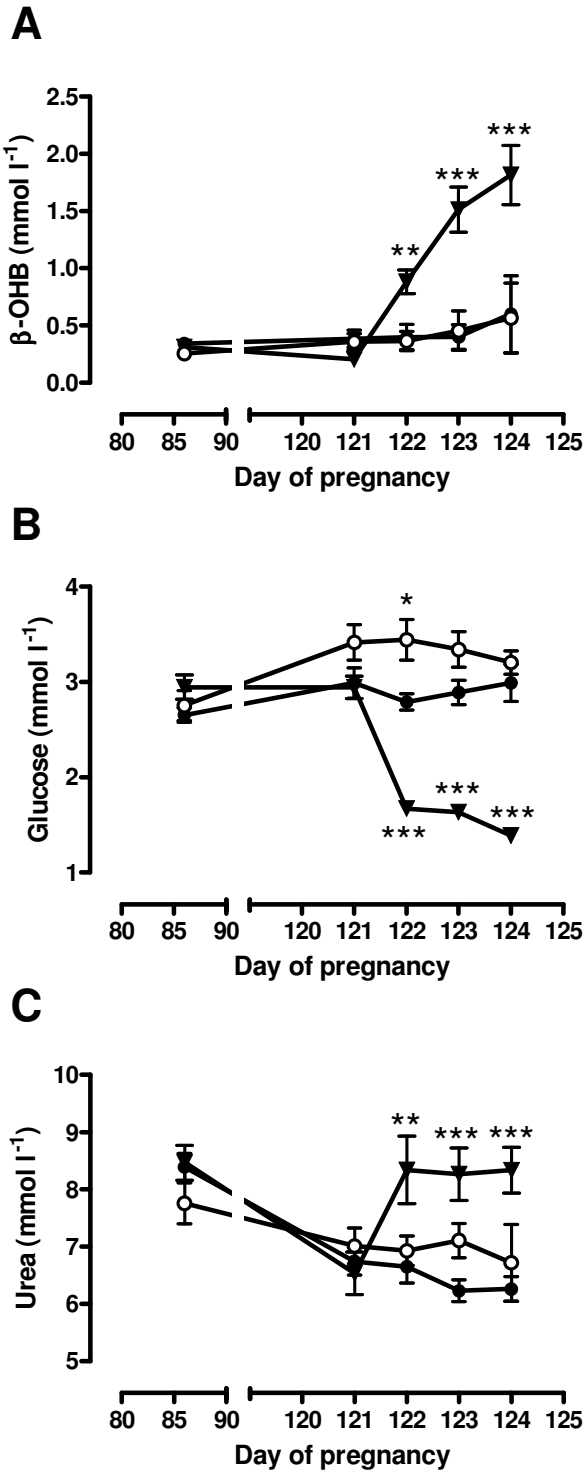

Figure 6-11.

Mean (± SEM) maternal circulating (A) β-hydroxybutyrate, (B) glucose and (C) urea concentration in pregnant ewes from control (closed circles, n = 10), caloric loading (CL) (open circles, n = 6) and caloric restriction (CR) (▼, n = 10) groups. Fasting in CR ewes commenced after collection of day 121 blood sample. CL commenced on day 95. (* P < 0.05, P < 0.01, *** P < 0.001 compared with control group.)**

Fetal IGF-1:

IGF-1 concentration in fetal plasma was significantly reduced ($P < 0.001$) in CR fetuses, and significantly elevated ($P < 0.05$) in CL fetuses, when compared with controls (Figure 6-12).

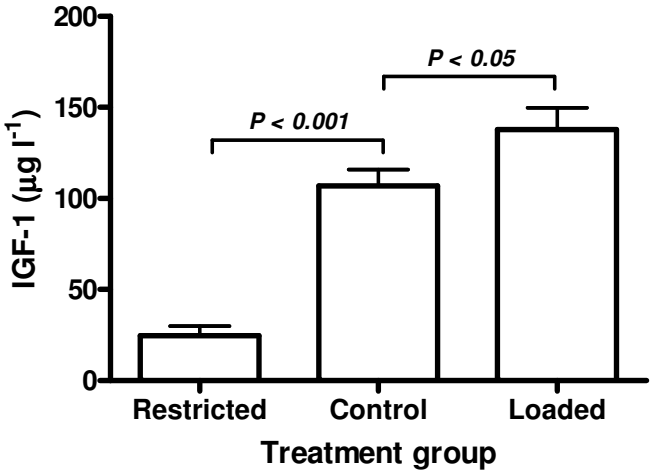

Figure 6-12.

Mean (+ SEM) insulin-like growth factor-1 concentration in ovine fetal plasma in caloric restriction (n = 10), control (n = 10) and caloric loading (n = 6) groups. Hormone concentrations are an average for each pair of twins.

6.6 Discussion

The results of this study show no effect of caloric loading on maternal circulating or placental concentration of CNP forms and therefore do not support the hypothesis that CNP production by the uteroplacental unit is homeostatically regulated by nutritional status. However, the significant increase in maternal circulating NTproCNP concentration during fasting in late pregnancy reported here substantiates a previous finding (Prickett *et al.*, 2007) and suggests that CNP release into maternal circulation is selectively upregulated by nutrient restriction but unaffected by nutrient abundance during pregnancy.

Maternal fasting for three days in late gestation was associated with a 20 % increase in maternal circulating NTproCNP concentration; a magnitude of increase similar to that reported previously by Prickett *et al.* (2007). This increase is in direct contrast to the effects of caloric restriction in non-pregnant ewes, in which feeding to 25 % of ME requirements is associated with a marked decrease in plasma CNP concentration (Prickett *et al.*, 2010c). In the

current study, the changes in NTproCNP were accompanied by a marked increase in maternal circulating β-OHB and urea concentration and a decrease in plasma glucose concentration, indicating that a three-day fast represents a significant physiological challenge to the ewe in late pregnancy. Surprisingly, and contrary to the original hypothesis, the changes in NTproCNP concentration in maternal plasma were not reflected in placental tissues, with no difference in protein concentration of either CNP form in the cotyledon evident between CR and control ewes. In this study, the number of ewes with everted placentome types (e.g. D-type) was too low to allow for comparison of CNP concentration between categories. There is evidence to suggest that these more everted placentome types may improve nutrient supply to the fetus during periods of undernutrition, and therefore it is possible that differences in CNP concentration within this placentome type may exist. However, the functional differences between placentome types are likely to be present only if the nutrient restriction is present from early pregnancy (Vonnahme *et al.*, 2006). Furthermore, the different placentome categories do not differ with respect to vascularity, vasoreactivity or gene expression levels of various angiogenic factors (Vonnahme *et al.*, 2008b), suggesting that a difference in CNP synthesis/secretion between placentome types is unlikely to explain the increase in maternal plasma NTproCNP concentration in CR ewes in the current study.

The absence of an increase in cotyledonary CNP protein concentration in CR ewes does not necessarily exclude the BNC as the source of elevated NTproCNP in maternal circulation during fasting. The concentration of BNC-derived hormones in placental tissues and maternal circulation can be affected by BNC abundance, as well as the rate of migration, maturation, fusion and granule release (Wooding *et al.*, 1986; Lea *et al.*, 2007). An increase in the rate of granule release, for example, would elevate CNP concentration in maternal circulation with no effect on protein concentration within the cotyledon, providing a possible explanation of the current results. Elevated maternal circulating oPL concentration has been reported following maternal fasting (Brinsmead *et al.*, 1981) and in ewes on low-quality feed during pregnancy (Oddy & Jenkin, 1981) suggesting that the BNC is sensitive to nutrient restriction and remains a likely source of the elevated NTproCNP in maternal plasma reported here during CR. However, as the current study investigated CNP protein concentrations only in uterine and placental tissues, extra-placental contributions to maternal circulating CNP forms during fasting can not be excluded. Ultrastructural studies of BNC migration and granule release, as well as regional sampling of other organs will be required to confirm the BNC as the source of the additional CNP in maternal plasma during fasting.

Nutrient restriction during pregnancy can have a multitude of effects on maternal, fetal and placental tissues, and targeted experiments are required to determine the factor/s responsible for stimulating the observed increase in CNP synthesis and/or secretion. A reduction in uterine blood flow (UBF) during fasting emerges as a likely candidate, as UBF is extremely sensitive to nutrient restriction (Prada *et al.*, 1992) and provides a linkage to the likely role for CNP in regulating blood flow during pregnancy (Itoh *et al.*, 1998). UBF may be reduced by as much as 43 % during a three-day maternal fast in late pregnancy (Prada *et al.*, 1992), and although UBF was not measured in the present study, the significant reduction in fetal weight and crown-rump length suggests that reduced UBF was a likely consequence of the caloric restriction treatment.

A reduction in UBF during nutrient restriction may be attributable, at least in part, to a decrease in fetal and placental NO concentration (Lassala *et al.*, 2010). A relationship between CNP and NO in maintaining homeostasis of placental blood flow during fasting is plausible, as there is significant cross-talk between the NO and natriuretic peptide systems. For example, cGMP, the second messenger produced by binding of CNP to the NPR-B receptor, stimulates NO synthesis in VSMCs (Inoue *et al.*, 1995). Furthermore, temporal changes in the concentration of NO in maternal circulation during ovine gestation follow a similar pattern to that of CNP forms, and likewise NO concentration in maternal plasma has a similar positive relationship with fetal number (Vonnahme *et al.*, 2005). A coordinated relationship between NO and CNP has been proposed, with a compensatory increase in one system occurring if the other is dysfunctional (reviewed by Ahluwalia *et al.*, 2004); providing a possible explanation for the increase in CNP, in the face of decreasing NO concentration, during fasting.

Clearly, further studies are required to investigate the relationship between UBF, CNP and NO both in normal pregnancy and during experimental manipulations to determine whether CNP production is affected by changes in UBF. In such experiments, the model used to manipulate UBF will be important. For example, the widely-utilised embolism model is associated with placental necropsy in addition to reduced UBF (reviewed by Lang *et al.*, 2000), which could independently affect the production of CNP and NO. Likewise, a reduction in UBF is associated with various fetal pathologies, such as hypoxia (McLellan *et al.*, 1992) which must also be considered.

Although significantly higher than control intake, the daily feed intake of CL ewes during the treatment period did not reach the desired level of 200 % of requirements. However, CL ewes had significantly higher body condition score at slaughter compared with controls. Body condition score in ewes is a reliable marker of energy reserves (Sanson *et al.*, 1993), and together with their elevated plasma glucose concentration, indicates that CL ewes were on a significantly higher plane of nutrition than control ewes. Furthermore, although there was no difference in fetal or placental weight, fetal plasma IGF-1 concentration was elevated in the CL group, indicating that the increased nutrient supply in CL ewes was being conferred to the fetus. In this study, fetal fat mass and individual organ weights were not recorded and it is possible that these measurements may also have revealed differences between the CL and control treatments. Of note, there was a trend for a reduction in the number of placentomes in CL pregnancies. Placentome number is generally determined in the first trimester of ovine pregnancy, and nutritional insult later in gestation is more likely to be associated with changes in placentome size and/or classification (reviewed by Wathes *et al.*, 1998), suggesting that the apparent difference in placentome number reported here was not a consequence of the nutritional disturbance.

The normal fetal and placental weights reported here following CL treatment are in contrast to those reported in the obese pregnant adolescent ewe, a popular model of overfeeding during pregnancy. In that model, maternal obesity is associated with hypoxia as well as a significant reduction in fetal and placental weight and UBF (reviewed by Reynolds *et al.*, 2005b). These adverse pregnancy outcomes have been attributed to enhanced nutrient uptake by the growing adolescent mother at the expense of fetal tissues (Redmer *et al.*, 2009). Some of the detrimental effects of overfeeding on fetal and placental development in the adolescent sheep model have been attributed to a reduction in maternal circulating progesterone concentration (Lea *et al.*, 2007; Redmer *et al.*, 2009). An inverse relationship between maternal circulating progesterone concentration and feed intake has been reported, probably due to increased metabolism as a result of elevated blood flow in the gut and liver (Parr, 1992). In the current study, there was no difference in maternal plasma progesterone concentration between CL and control ewes at any time-point and this may explain the differences in fetal and placental attributes between the current results and those reported previously in the obese adolescent model.

In contrast to the popular adolescent model, there are few studies investigating the effect of overfeeding in adult ewes. These studies generally report no effect of overfeeding on fetal

weight (or lamb birth weight, depending on study design) (Wallace *et al.*, 2005; Muhlhausler *et al.*, 2006; current study), despite significant increases in fetal plasma IGF-1, glucose and insulin concentration (Wallace *et al.*, 2005; Zhu *et al.*, 2009) and increased fat mass (Muhlhausler *et al.*, 2006) in fetuses from overfed dams. Excessive fetal growth can be detrimental to maternal and fetal survival at parturition due to dystocia, and thus fetal nutrient delivery and growth potential is constrained (reviewed by Gluckman & Pinal, 2003), which may explain the absence of a birth weight response in studies of overfeeding during pregnancy. Interestingly, in both adolescent and adult models, fetal weight is generally greater in overfed animals in mid-pregnancy, but by late-pregnancy is significantly reduced (Redmer *et al.*, 2009) or in keeping with that of controls (Ma *et al.*, 2010). This is unlikely to reflect an inability of the placenta to meet the demands of these fetuses, or lack of uterine capacity, as the combined weight of larger litter sizes illustrates the ability of the uteroplacental unit to support a large fetal mass and suggests that some factor/s are in place to constrain the growth of individual fetuses.

A reduction in cotyledonary vascularisation and gene expression of a number of angiogenic factors has been reported in adult ewes overfed from 60 days pre- until 75 days post-conception (Zhu *et al.*, 2009; Ma *et al.*, 2010); changes which might be expected to affect local production of CNP within the placenta. The absence of a difference in CNP concentration in placental tissues from CL ewes compared with controls in the current study may be related to the late timing of this nutritional challenge; as it is the periconceptual period, targeted in previous studies, that is particularly sensitive to nutritional challenges (Bloomfield *et al.*, 2004). Nutritional manipulation in early pregnancy may have a significant effect on establishment and development of the placenta. In contrast, the period of caloric loading in the current study did not commence until day 95, by which time placental growth is complete.

The decrease in fetal plasma concentration of CNP forms reported here during nutrient restriction is supported by previous studies in fetal (Prickett *et al.*, 2007) and postnatal (Prickett *et al.*, 2005) lambs, and may reflect the apparent cessation of growth in nutrient-restricted fetuses during the fasting period (Prickett *et al.*, 2005). Fetal circulating cortisol concentration increases during maternal undernutrition (reviewed by Gluckman & Pinal, 2003; Fowden & Forhead, 2004), and may be involved in mediating the reduction in fetal plasma concentration of CNP forms during CR (Prickett *et al.*, 2010b). Identification of the

sites of production and role of CNP in the fetus will be important for understanding how fetal CNP production is regulated, and the possible implications this has for fetal well-being.

In summary, the results of this study firmly establish that there is an increase in maternal circulating NTproCNP concentration during fasting in late gestation ewes. Unexpectedly, the change in peptide concentration in maternal circulation was not associated with similar changes in protein concentration within uterine and placental tissues, possibly reflecting an altered rate of granule release from the BNC. There was no effect of caloric loading on the concentration of CNP forms in maternal circulation or placental tissues. Collectively these findings do not support the hypothesis that CNP production by the uteroplacental unit is homeostatically regulated by nutrition, and instead suggest that CNP production is selectively upregulated in situations of fetal growth restriction. The stimulus for increased CNP release during fasting and the physiological implications of elevated CNP in maternal circulation of pregnant ewes remain to be elucidated.

Chapter 7:
Effect of mid-pregnancy shearing on maternal circulating CNP forms

Declaration

This study was conducted at Massey University, Palmerston North, New Zealand as part of a study led by Dr PR Kenyon investigating the effects of mid-pregnancy shearing on lamb birth weight. I travelled to Massey University to assist with blood sampling and weighing ewes, and I performed and analysed all of the CNP and NTproCNP assays, but the remainder of the experimental work was conducted by Dr Kenyon's group. Consequently, discussion of the results is limited to those which directly relate to CNP.

7.1 Abstract

Shearing ewes in mid-pregnancy is associated with an increase in lamb birth weight and therefore may provide a means of improving productivity and lamb survival. Although the mechanism whereby shearing increases lamb birth weight has not been determined, changes in placental nutrient transfer capabilities and production of placental hormones have been implicated. To investigate a possible involvement of CNP, maternal plasma concentration of CNP and NTproCNP was measured at day 69, 75, 95, 110, 125 and 140 in single- and twin-bearing ewes of Cheviot or Suffolk breed, which were either shorn at day 70 or remained unshorn for the duration of pregnancy (n = 7-12 of each birth rank and breed per treatment group). Mean individual lamb birth weight was higher in single versus twin lambs (5.9 ± 0.28 kg vs 5.1 ± 0.15 kg, $P < 0.05$), and in Suffolk versus Cheviot lambs (6.0 ± 0.23 vs 4.9 ± 0.18 kg, $P < 0.05$). Shearing had a positive effect on lamb birth weight in Cheviot ($P < 0.05$), but not Suffolk, lambs (P = NS). There was no effect of shearing on maternal circulating CNP or NTproCNP concentration at any stage of pregnancy, regardless of breed or birth rank (P = NS). In contrast, maternal circulating concentration of both CNP forms was higher in Cheviot compared with Suffolk, and twin- compared with single-bearing ewes at all stages of pregnancy ($P < 0.05$). These data reveal that the relationship between fetal number and maternal plasma CNP concentration is already established by the second trimester of ovine

pregnancy. The absence of a relationship between CNP and shearing suggests that this peptide is unlikely to mediate the increase in fetal growth following shearing. Together with the lack of effect of caloric loading (Chapter 6), these findings demonstrate that CNP synthesis and secretion during pregnancy are not affected by nutrient abundance.

7.2 Keywords

Sheep, mid-pregnancy shearing, CNP, NTproCNP, fetal growth

7.3 Introduction

Low birth weight, particularly in litters of two or more lambs, is a common occurrence in sheep production. A birth weight outside the optimal range is associated with reduced lamb survival and poor performance and is therefore a significant issue for the sheep production industry (Gootwine *et al.*, 2007). There is ample evidence from experimental studies that shearing ewes in mid-pregnancy is associated with a significant increase in lamb birth weight (reviewed by Kenyon *et al.*, 2003), potentially providing a simple and cost-effective means of increasing productivity. The beneficial effects of shearing on lamb birth weight are more pronounced in twin compared with singleton pregnancies (Corner *et al.*, 2006; Jenkinson *et al.*, 2009), suggesting that a response to shearing is possible only if potential lamb growth is restricted under normal conditions, as is the case for twin pregnancy (Wu *et al.*, 2006).

Despite a consistent effect of shearing, particularly in twin-bearing ewes, the mechanism/s responsible for the increase in lamb birth weight remain unknown, and cannot be attributed to an increase in voluntary food intake in response to cold stress in shorn animals (Symonds *et al.*, 1986; Revell *et al.*, 2002). Instead, enhanced fetal growth may be the result of changes in the production of and/or sensitivity to key nutrients or growth factors including glucose, insulin (Symonds *et al.*, 1986), IGF-1 (Jenkinson *et al.*, 2009), fat oxidation pathways (Symonds *et al.*, 1989) or thyroid hormones (Symonds *et al.*, 1989). Although placental weight does not appear to be affected by shearing (Revell *et al.*, 2002), increased caruncle occupancy and higher liver respiration rates in lambs of shorn ewes (Revell *et al.*, 2002) suggest that shearing may indirectly promote fetal growth by enhancing utero-placental nutrient transfer and placental function.

According to the findings of this thesis, CNP is produced by the fetal trophoblast BNC and exported into the maternal system to circulate at very high concentrations during ovine

pregnancy (Chapter 5). In addition to likely (but unidentified) endocrine actions in the maternal circulation, CNP is likely to have a local vasodilatory role within the placental vasculature (Chapter 5, Cameron *et al.*, 1996). Elevated maternal circulating CNP concentrations have been reported in situations of fetal growth restriction such as maternal fasting (Prickett *et al.*, 2007) and multiple pregnancy (Chapter 4). Furthermore, in humans, placental NTproCNP expression is elevated in pre-eclampsia (Prickett *et al.*, 2004), and myometrial CNP expression is increased in IUGR (Stepan *et al.*, 2002a). Both of these conditions are classified as diseases of the placenta, suggesting that CNP may have a key role in regulating placental function and nutrient partitioning to feto-placental tissues.

The aim of the current study was to investigate whether mid-pregnancy shearing is associated with changes in CNP production, by measuring maternal circulating CNP forms in mid- to late-pregnancy in shorn and unshorn pregnant ewes. As maternal CNP concentration appears to be elevated in situations of natural or imposed growth restiction and reduced nutrient availability, it is hypothesised, conversely, that maternal plasma CNP concentration will be lower in ewes shorn in mid-pregnancy than in their unshorn counterparts.

7.4 Methods

7.4.1 Animal procedures

Mixed-age ewes of Cheviot (n = 118) or Suffolk (n = 99) breed at the Massey University Research Farm were used in this study. Animals were run on pasture at Massey University's Tuapaka Farm, 15 km southwest of Palmerston North, New Zealand. Oestrus was synchronised in the entire flock by the insertion of CIDRs ('Eazi-breed' type G CIDRs, Pfizer New Zealand Ltd., Auckland) for 12 days. On the day of CIDR removal (= day 1 of pregnancy) the two breeds of ewe were separated and mated with either 6 Cheviot rams (Cheviot ewes) or 6 Suffolk rams (Suffolk ewes). Ewes were reunited after removal of rams. At day 72 of pregnancy approximately half of the ewes from each breed were shorn using a cover comb (Sunbeam New Zealand Ltd, maximum depth of teeth 7-9 mm). All other ewes remained unshorn, with approximately 8 months wool growth.

Non-fasting live weight was recorded at day 1, 39, 71, 97, 127 and 142 of pregnancy and then at 96 days after the mid-point of the lambing period. Lambs were identified to their dam and their sex, weight and birth rank recorded within 12 hours of birth.

Blood samples were collected at day 69, 75, 95, 110, 125 and 140 of pregnancy from a randomly-selected sub-sample of the flock consisting of approximately equal numbers of shorn and unshorn twin- and single-bearing ewes of both breeds for measurement of plasma CNP and NTproCNP concentration. Final analyses were performed on data from animals in this sub-sample only. The number of animals in each group is summarised in Table 7-1.

Table 7-1.

Number of single- and twin-bearing Cheviot and Suffolk ewes in the two treatment groups (shorn or unshorn) included for analysis in this study.

		Unshorn	Shorn
Cheviot	Single-bearing	10	9
	Twin-bearing	7	7
Suffolk	Single-bearing	**7**	**7**
	Twin-bearing	12	9

7.4.2 Statistical analyses

Statistical analyses were performed using Genstat version 10 (VSN International Ltd., Hemel Hempstead, UK). A P value of ≤ 0.05 was considered statistically significant. Effect of breed, birth rank and shearing treatment on hormone concentration and lamb birth weight, and changes in hormone concentration over time within treatments were analysed using a REML test and a method of statistical differentials using day of gestation, breed, birth rank and shearing treatment as the dependent variables. Relationships between plasma and tissue CNP forms and fetal and placental characteristics were examined by fitting the data with linear regression best-fit models and testing the deviation from zero slope using an F-test. Data were log-transformed where appropriate to meet REML assumptions. Data are mean and SEM.

7.5 Results

7.5.1 Lamb birth weights

Mean lamb birth weight was significantly greater in Suffolk (6.0 ± 0.2 kg) compared with Cheviot (4.9 ± 0.2 kg) lambs ($P < 0.05$, Figure 7-1). Total litter weight was greater in twins versus singletons ($P < 0.001$), although mean individual lamb birth weight was higher in single lambs (5.9 ± 0.3 kg) compared with twins (5.1 ± 0.2 kg) ($P < 0.05$, Figure 7-1). Mid-pregnancy shearing had a significant positive effect on lamb birth weight in both twin and singleton Cheviot lambs ($P < 0.05$, Figure 7-1). There was no effect of shearing on lamb birth weight in Suffolk lambs of either birth rank (P = NS, Figure 7-1).

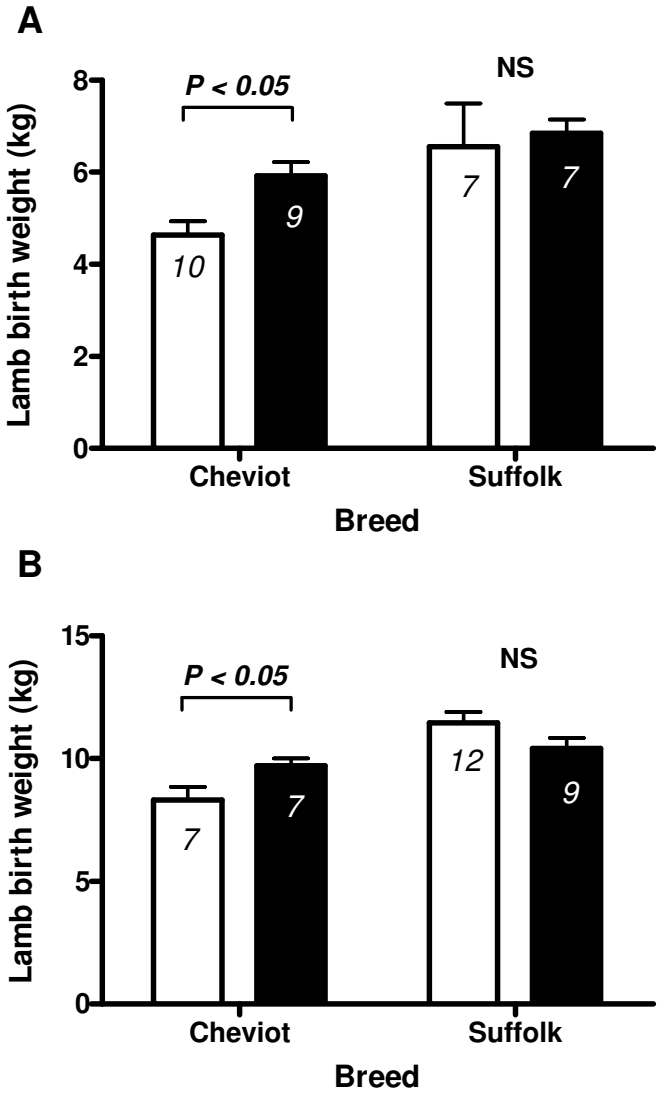

Figure 7-1.

Mean (+ SEM) lamb birth weight in (A) singleton and (B) twin lambs of Cheviot or Suffolk breed born to ewes which were unshorn for the duration of pregnancy (white bars), or shorn in mid-pregnancy (black bars). In the case of twins, lamb birth weight is the combined weight of both lambs. The number of animals in each group is indicated on the bars.

7.5.2 Maternal circulating CNP and NTproCNP concentration

Maternal plasma concentration of CNP forms increased progressively between day 69 and day 140 in all groups of ewes, regardless of breed, shearing treatment or birth rank ($P < 0.001$). Mid-pregnancy shearing had no effect on maternal circulating CNP or NTproCNP concentration at any stage of pregnancy (P = NS, Figure 7-2). There was no interaction

between breed and shearing treatment (CNP: P = 0.17; NTproCNP: P = 0.63), indicating that the absence of a shearing effect was consistent across the two breeds.

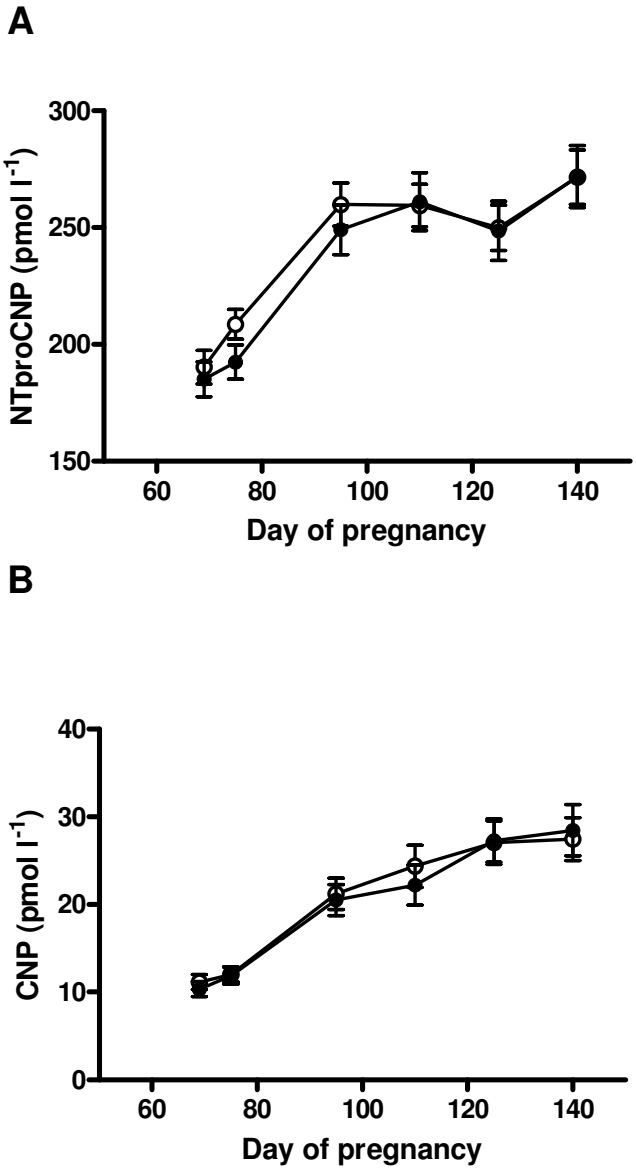

Figure 7-2.

Mean (+ SEM) concentration of (A) NTproCNP and (B) CNP in maternal circulation during pregnancy in unshorn controls (open circles, n = 36) and ewes shorn at day 70 of gestation (closed circles, n = 32). Single- and twin-bearing ewes of both Cheviot and Suffolk breed are shown here.

The concentration of both CNP and NTproCNP was elevated in twin- compared with single-bearing ewes at all time points examined in both breeds (P < 0.001, Figure 7-3). Furthermore, there was a significant effect of breed on maternal circulating CNP forms, with higher plasma

concentration of both CNP forms in Cheviot versus Suffolk ewes at all time points (P = 0.001, Figure 7-3).

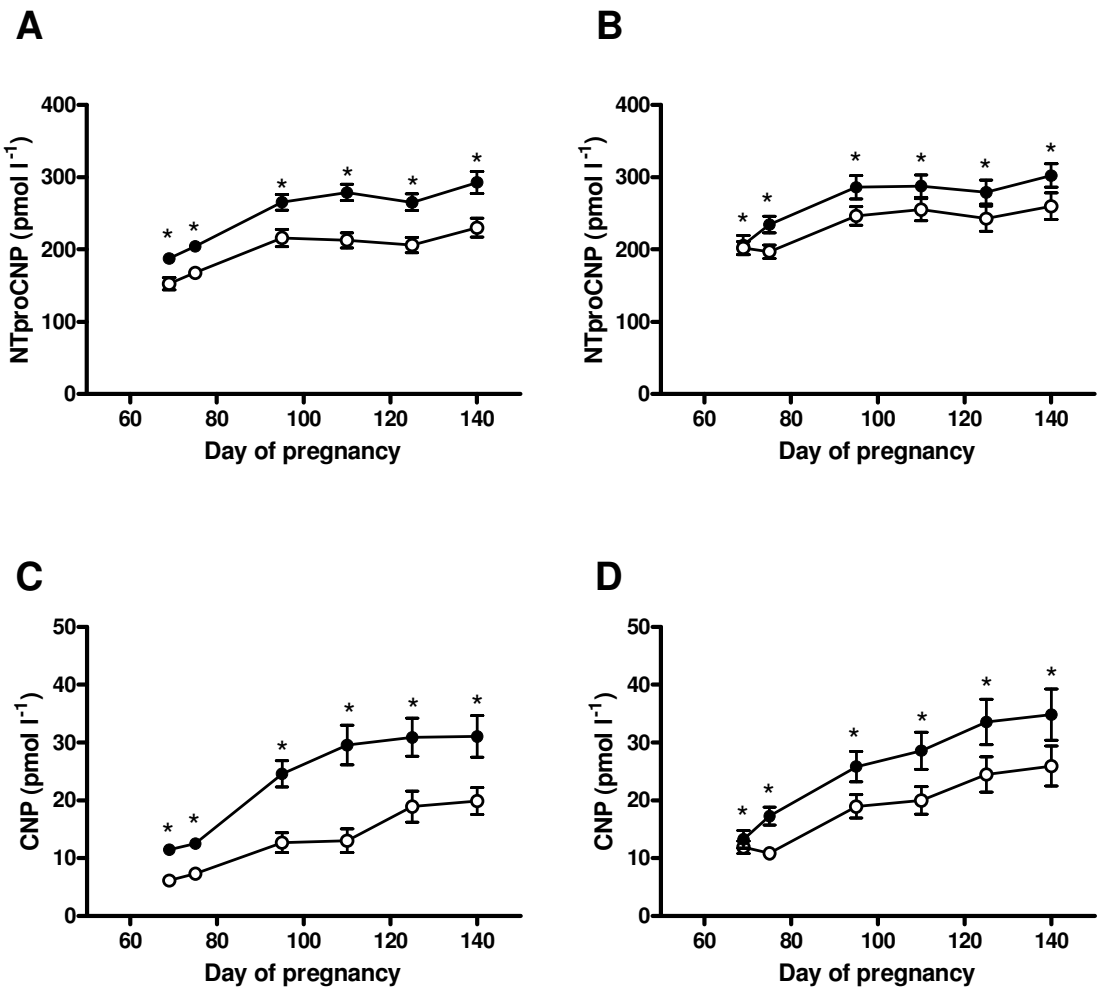

Figure 7-3.

Mean (+ SEM) concentration of NTproCNP (A and B) and CNP (C and D) in maternal circulation during pregnancy in two breeds of ewe: Suffolk (A and C) and Cheviot (B and D) carrying singleton (open circles, Cheviot: n = 19, Suffolk n = 14) or twin (closed circles, Cheviot: n = 14, Suffolk n = 21) fetuses. (A) NTproCNP in Suffolk breed; (B) NTproCNP in Cheviot breed; (C) CNP in Suffolk breed; (D) CNP in Cheviot breed. A significant difference (P < 0.05) in the circulating concentration of CNP forms between singleton and twins is indicated by an *.

7.5.3 Relationship between maternal circulating CNP forms and lamb birth weight

There was some evidence of a modest positive relationship between maternal circulating concentration of CNP and lamb birth weight seen only in the twin-lamb cohort. There was no

relationship between NTproCNP and lamb birth weight in twins, and no relationship between either CNP form and lamb birth weight in singletons (Table 7-2).

Table 7-2.

Relationship between maternal circulating CNP forms during pregnancy and lamb birth weight in singleton (n = 33) and twin (n = 35) pregnancies.

	Results of linear regression (r^2 and *P* value)			
	CNP		NTproCNP	
Day of pregnancy	Singletons	Twins	Singletons	Twins
69	ns	ns	ns	ns
75	ns	0.19, *P= 0.01*	ns	0.14, *P= 0.053*
95	ns	0.13, *P = 0.037*	0.17, *P = 0.02*	ns
110	ns	0.13, *P = 0.03*	ns	ns
125	ns	0.13, *P = 0.037*	ns	ns
140	ns	0.19, *P = 0.01*	ns	ns

7.6 Discussion

In contrast to the original hypothesis, mid-pregnancy shearing was not associated with a reduction in the concentration of maternal circulating CNP forms in the current study. This result strongly suggests that the effects of shearing on fetal growth are unlikely to involve changes in CNP synthesis and/or secretion. The absence of a relationship between maternal plasma CNP concentration and shearing treatment, taken with the results of Chapter 6 – in which caloric loading did not affect maternal circulating CNP concentration – support the hypothesis that CNP secretion into the maternal circulation during ovine pregnancy is not homeostatically regulated by fetal nutrient supply.

The results of the current study, that demonstrate unchanged maternal plasma CNP concentration despite a significant increase in lamb birth weight, confirm the finding presented in Chapter 6 showing that an increase in fetal nutrient supply had no effect on the concentration of CNP forms in the maternal circulation. An increase in maternal plasma glucose, and decrease in plasma insulin concentration has been reported in shorn ewes; possible reflecting an increase in energy demands by maternal tissues in response to cold-

stress (Symonds *et al.*, 1986). Consequently, the increase in fetal nutrient supply following shearing may simply reflect an increase in glucose transfer across the placenta, as fetal growth is directly related to maternal glucose concentration (Schlumbohm & Harmeyer, 2008). This increase in nutrient supply may occur without significant changes in placental structure, UBF or fetal oxygen supply, providing a possible explanation for the absence of any change in maternal plasma CNP concentrations.

It is noteworthy that higher concentrations of CNP forms were measured in the plasma of Cheviot compared with Suffolk ewes in this study. Breed differences in placental characteristics have been reported previously in sheep, including placental weight, cotyledon number, placentome distribution (proportion of A-, B-, C- and D-type placentomes) and placental efficiency (Dwyer *et al.*, 2005). Placental characteristics were not measured in the current study, but a previous embryo-transfer study of Cheviot and Suffolk ewes, conducted on the same research farm at Massey University, revealed significantly fewer cotyledons in placentae from Cheviot ewes (Sharma *et al.*, 2009). Cheviot ewes are smaller and lighter than Suffolk ewes, and maternal constraint is therefore inherently greater in this breed (Gluckman & Hanson, 2004). This, taken with the lower cotyledon number relative to Suffolk ewes, could result in a greater degree of fetal restriction in this breed and may therefore contribute to higher maternal circulating CNP concentrations.

A similar embryo transfer study involving Cheviot and Suffolk ewes is currently being conducted at Massey University, with plasma samples being collected for analysis of CNP forms. It is hypothesised that these samples will show differences in maternal plasma CNP concentration between the different treatment groups. Interestingly, Sharma *et al.* (2009) found that birth weight and placentome number of Cheviot lambs in the previous embryo transfer study were the same regardless of the breed of the recipient dam. This suggests that the Cheviot fetuses are unable to exploit the larger uterine capacity and increased potential for nutrient availability provided by the Suffolk host (Sharma *et al.*, 2009). Nonetheless, measurement of maternal circulating concentration of CNP forms following embryo transfer may provide some insight into the mechanism/s contributing to the breed differences in CNP concentrations which occurred in the current study.

In both breeds of sheep a trend for higher circulating CNP forms in twin- versus single-bearing ewes was already pronounced in the first blood sample collected on day 69 of pregnancy. This finding extends the findings reported in Chapter 4 showing a positive relationship between maternal circulating CNP forms and fetal number at day 120 of

pregnancy to reveal that this difference is already established by mid-pregnancy. The positive relationship between maternal plasma CNP concentration and fetal number may reflect an increase in total UBF, shear stress or uterine stretch in multiple pregnancies. Maternal circulating NO concentration is also positively related to fetal number during ovine pregnancy (Vonnahme *et al.*, 2005). It has been proposed that the increase in NO production in multiple pregnancy may be mediated by VEGF (Vonnahme *et al.*, 2008a). However, as discussed in Chapter 6, it is possible that CNP and NO may constitute a local system of blood-flow regulation within the placenta. As there is no relationship between maternal circulating VEGF concentration and fetal number (Vonnahme *et al.*, 2005), CNP may be a more likely candidate for this communication.

The difference between maternal plasma CNP concentration in single- and twin-bearing ewes at all time-points studied suggests that maternal plasma CNP concentration may provide a potential index of placental health and development from mid-pregnancy. An understanding of the factors regulating placental development has important consequences for postnatal development and productivity, as placental function can influence a host of postnatal traits including standing and suckling vigour in early life (Dwyer *et al.*, 2005) and future meat quality (Jenkinson *et al.*, 1995). The developmental differences between singleton and multiple pregnancies also has important consequences for human medicine, due to the increased prevalence of multiple pregnancies, which are associated with a high incidence of premature birth and other complications (Alexander *et al.*, 1998).

In conclusion, the absence of a reduction in maternal circulating CNP concentration following mid-pregnancy shearing suggests that the factor/s mediating increased fetal growth following shearing are not key regulators of CNP production during pregnancy. However, the effects of breed and fetal number on CNP support the hypothesis that some feature of the growth restricted fetus/placenta stimulates CNP production, elucidation of which may provide important insight into the peptide's role in pregnancy.

Chapter 8:
Final discussion and future direction

8.1 Major contributions of this thesis

The findings presented in this thesis contribute significantly to our understanding of CNP's involvement in ruminant pregnancy. Prior to these studies, the temporal changes and source of CNP forms in plasma and placental tissues had not been described for any species. Consequently, elucidating these parameters in two representative mammalian species were important aims of this thesis. The demonstration of a sustained elevation of CNP and NTproCNP in maternal plasma and placental tissues during ovine pregnancy provides strong evidence to suggest that CNP plays an important role in the hormonal regulation of pregnancy. Furthermore, the similarity of the pattern of CNP forms in maternal plasma during ovine and cervine pregnancy (Chapter 4) suggests that the peptide's actions in the pregnant female are likely to be conserved among ruminants, and possibly other mammals.

Another key outcome of the current studies was the immunolocalisation of CNP and NTproCNP to the trophoblast BNC (Chapter 5), which strongly implicates these cells as a major source of maternal circulating CNP forms during pregnancy. Consequently, the previous suggestion that CNP acts primarily as a paracrine/autocrine factor in mammals (Walther & Stepan, 2004) must be challenged, based on the established role of the BNC as a transporter of fetal products into the maternal circulation. Furthermore, the identification of the BNC as a site of CNP production raises the intriguing possibility that CNP may have an important role as a feto-maternal communication signal. Although the stimulus for CNP production and its effects on maternal function remain to be determined, the results of the experiments presented in this thesis support the hypothesis that this communication pathway is activated during situations of fetal/placental restriction (possibly of key nutrients and/or oxygen), but is unaffected by an increase in fetal nutrient availability.

In addition to providing novel information regarding aspects of maternal and placental CNP biology during pregnancy, these studies extend previous reports of CNP signalling in the fetus. Although the role of CNP in fetal development has not been identified, the findings reported here – decreasing fetal plasma concentration of CNP forms as gestation progressed – do not support a role in fetal bone growth, suggesting distinct roles for the peptide in pre- and postnatal life. The novel contributions of this thesis are outlined in Table 8-1.

Table 8-1.

Summary of novel findings presented in this thesis.

Documentation of longitudinal profile of maternal circulating concentration of CNP and NTproCNP throughout pregnancy in sheep and red deer.
Identification of a positive relationship between maternal circulating concentration of CNP forms and fetal number in ovine pregnancy.
Localisation of CNP to trophoblast BNCs, suggesting these cells are a probable source of CNP forms in the maternal circulation during pregnancy.
Identification of CNP peaks in fetal cotyledon extracts by size-exclusion HPLC in identical positions to those previous identified in maternal plasma, suggesting the CNP forms are identical.
The finding that the concentration of CNP forms in placental tissue exceeds that in uterine tissue throughout ovine gestation, with the highest concentrations occurring in the fetal cotyledon.
Documentation of the steady decline in the concentration of CNP forms in fetal plasma throughout ovine gestation.
The demonstration that caloric loading for a two week period during late pregnancy had no effect on the concentration of CNP forms in maternal or fetal plasma, or in uteroplacental tissues.
Evidence that the fetal nutrient abundance model of mid-pregnancy shearing has no effect on maternal circulating concentration of CNP forms.
The finding that there are breed-specific differences in maternal circulating concentrations of CNP forms in Cheviot and Suffolk ewes during pregnancy.

8.2 CNP's role in fetal-maternal physiology

Collectively, the studies described in this thesis provide evidence that CNP has important and distinct actions within the caruncle, cotyledon and maternal circulation during pregnancy. These findings provide a strong basis for the generation of testable hypotheses to identify and distinguish the actions of CNP within these tissues. These are discussed below.

8.2.1.1 Local effects in uterine and placental tissues

Soon after the discovery of CNP in brain tissue (Sudoh *et al.*, 1990), significant concentrations of CNP and NPR-B were identified in the vasculature (reviewed by Woodard

et al., 2002) and CNP's actions as a potent vasodilator were confirmed (Itoh *et al.*, 1998). Consequently, a role for CNP in regulating vascular tone within the uteroplacental unit and mediating the necessary increase in blood flow during pregnancy was proposed (Cameron *et al.*, 1996; Itoh *et al.*, 1998). The results of the studies presented here support this hypothesis, with the demonstration that changes in the concentration of CNP forms in fetal and maternal placental extracts during ovine pregnancy (Chapter 5) are related to the discrete patterns of vascular growth and function in the cotyledon and caruncle (reviewed by Reynolds *et al.*, 2005b). Furthermore, the demonstration of immunolocalisation of CNP forms around placental blood vessels suggests involvement in the control of blood flow distribution. However, empirical evidence will be required to explore the ideas raised by these observational studies and to confirm the role of CNP in maintaining UBF. Possible experiments may involve *in vitro* myographical studies on the direct effects of CNP on isolated vessels, which may provide valuable insight into CNP's effects on specific uteroplacental tissues *in vivo*. Furthermore, measuring UBF and vascular changes (e.g. capillary density, vessel diameter, angiogenic factors) following CNP infusion to ewes in early gestation, before the commencement of the normal rise in CNP may be revealing.

During pregnancy, growth and development of uterine and placental tissues is highly dependent on the growth of new vessels (reviewed by Reynolds *et al.*, 2005b). In addition to promoting vasodilation of existing vessels, CNP may be involved in the development of new vessels through its actions as an angiogenic factor (Doi *et al.*, 2001). Capillary area density, a marker of angiogenesis (Reynolds *et al.*, 2005b), was not measured in the current studies, but would be an informative addition to future studies to determine the involvement of CNP in placental angiogenesis. Furthermore, known angiogenic factors could be assayed in specific tissues to determine if there is a relationship with CNP concentration.

In addition to a likely vasoactive role during pregnancy, maintenance of myometrial quiescence (discussed in Chapter 5) and immunomodulation at the maternal-fetal interface are also possible functions for CNP within the uteroplacental unit. An immunomodulatory role for CNP is consistent with its described interactions with various cytokines (Suga *et al.*, 1993). Furthermore, as the appearance of BNCs in the fetal trophoblast coincides with implantation, BNC-derived factors have been implicated in mediating local immunological adaptations which prevent destruction of the conceptus by the maternal immune system (Wooding *et al.*, 1992),

The placenta is the site of production of numerous hormones and growth factors including CNP, and it stands to reason that some of the peptide's effects may be achieved through coordinated interactions with these other factors. Therefore, the identification of these interactions may provide important insight into CNP's function. In this regard, future studies investigating a functional relationship between CNP and NO during pregnancy are warranted (discussed in Chapter 6). For example, in the pulmonary vasculature, it has been proposed that CNP and NO may function as a positive feedback loop (Simon *et al.*, 2009) and a similar relationship within the placenta is possible. The proposed compensatory relationship between NPs and NO (reviewed by Ahluwalia *et al.*, 2004) has implications for the design and interpretation of experimental studies, particularly those which involve blocking the actions of CNP, NO or their receptors. VEGF, which has been implicated as a likely mediator of NO-induced vasodilation during pregnancy (reviewed by Reynolds *et al.*, 2005b) has been shown to inhibit CNP secretion *in vitro* (Doi *et al.*, 1996), and its involvement in a possible feedback loop involving CNP and NO may also be worthy of consideration.

8.2.1.2 Endocrine role of CNP in the pregnant female

Extremely high concentrations of CNP in maternal circulation during ovine pregnancy and localisation to the BNC strongly suggest an endocrine mode of action during pregnancy. The physiological consequences of the extremely high maternal circulating concentration of CNP forms during ruminant pregnancy were not explored in the current studies, and there are very few reports in the literature of CNP infusion at concentrations similar to those reported here. Therefore, infusion studies using a high titre of CNP forms, to achieve the concentrations attained in maternal circulation during ovine pregnancy, should form a key component of future studies. In conscious sheep, CNP administration to achieve plasma concentrations of 10 - 115 pmol l^{-1} resulted in natriuresis, increased circulating cGMP concentration and a dramatic reduction in cardiac output (18 %), suggesting that a hemodynamic role in the pregnant female is a possibility (Charles *et al.*, 1995). Administration of CNP to achieve high circulating concentrations of the peptide into steroid-primed non-pregnant ewes may be one approach to determine CNP's effects in maternal peripheral tissues.

Charles *et al.* (1995) reported no effect of CNP administration on mean arterial blood pressure in conscious sheep, a finding that does not support a role for CNP in regulating blood pressure during normal pregnancy. However, CNP has a hypotensive effect when administered intravenously to rats (Sudoh *et al.*, 1990), and CNP-mediated inhibition of VSMC proliferation may prevent increases in peripheral vascular resistance and consequently reduce

hypertension (Woodard *et al.*, 2002). Maternal plasma NTproCNP concentration is elevated in preeclampsia (Prickett *et al.*, 2004), a condition associated with inappropriately elevated blood pressure. These experimental studies suggest a hypotensive role for CNP, with potential clinical applications for the peptide to be utilised as an agent to lower maternal blood pressure in pathological pregnancies.

Control of BNC production is believed to be primarily under fetal control, and the effects of BNC-derived products in the maternal circulation therefore may be expected to promote nutrient transfer for the benefit of the fetus. For example, oPL is believed to enhance fetal nutrient supply directly by facilitating glucose transfer from mother to fetus (reviewed by Gootwine, 2004). Other BNC products may enhance fetal nutrient supply indirectly, for example by increasing blood flow (reviewed by Fowden *et al.*, 2006). A comparable indirect role for CNP in promoting fetal growth via increased UBF is likely, but direct roles, such as promoting lipolysis from maternal fat cells are also possible, as suggested by recent studies in humans (Lafontan & Langin, 2009).

The amino acid sequence of CNP-22 is highly conserved amongst vertebrate species, which suggests that its function also is highly conserved. Therefore, examination of CNP's role in phylogenetically ancient species may provide important insight for understanding its function in mammalian pregnancy. For example, an endocrine role for CNP has been proposed in the Japanese eel (*Anguilla japonica*) due to the high circulating concentrations of the peptide following transition from a saltwater to freshwater environment (Takei *et al.*, 2001). This phenomenon suggests a possible role for CNP in osmoregulation which also may be operational in other vertebrate groups.

8.3 Conceptual model

A conceptual model to describe the control of CNP production during ruminant pregnancy is proposed in Figure 8-1 using the novel findings of this thesis together with data from the literature.

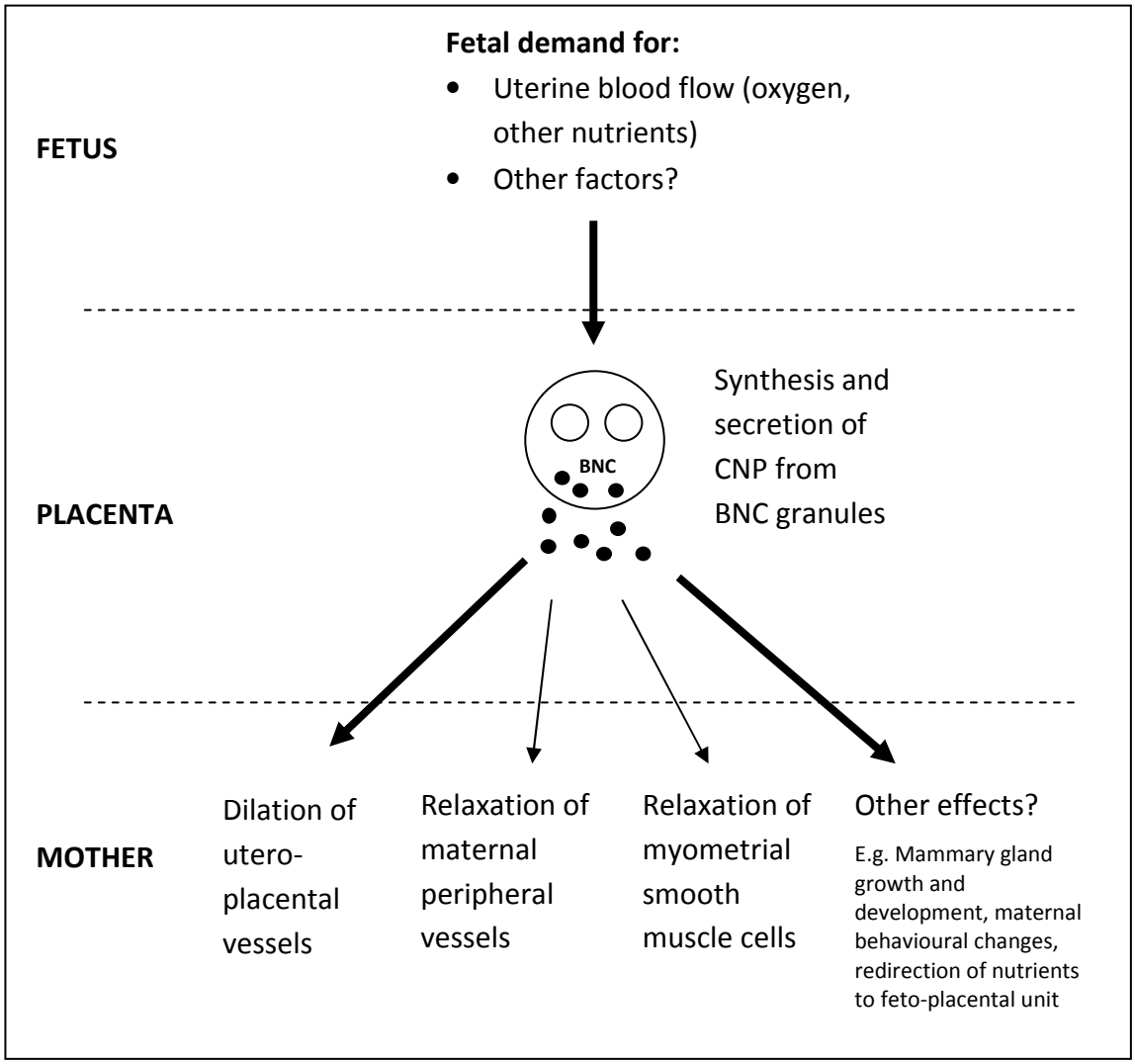

Figure 8-1.

Proposed conceptual model showing CNP's actions during ruminant pregnancy. Pathways highlighted with bold arrows are predicted to be upregulated during nutrient restriction (e.g. maternal fasting, multiple fetuses).

8.4 Implications

The current studies, in showing high concentrations of CNP in pregnant ruminants, identify CNP as an important new hormone of pregnancy. Furthermore, the novel finding that CNP

production is upregulated in situations of fetal restriction raises the possibility that CNP may be a marker of fetal and/or placental development and wellbeing. This potentially has applications for human medicine and animal production.

Clearly, elucidation of CNP's function in the pregnant female will be necessary to determine its potential suitability as a marker of wellbeing. Although the current RIA protocol used to determine CNP concentration provides results with very high sensitivity, the long duration of the assay (at least 4 days from sample collection to assay completion), requirement for highly specialised equipment and skilled personnel, high cost per sample and reliance on radioactive isotopes makes the assay unsuitable for use outside of a laboratory and consequently is of limited relevance in a field situation. Furthermore, the CNP assay currently requires collection of relatively large sample volumes, which must be continuously chilled and promptly centrifuged due to the labile nature of the peptide. Therefore, these issues will need to be addressed before CNP can be utilised as a routine diagnostic test in large-scale animal production systems. Despite these technical limitations, measurement of CNP concentrations during pregnancy may contribute significantly to effective animal production systems. In particular, the ability accurately to determine fetal number early in pregnancy would be particularly advantageous; suggesting that development of new, simple and cost-effective assays for CNP forms may be worthwhile. In this context, as circulating levels are very high during pregnancy the development of a rapid 15 minute analyser similar to the NTproBNP bedside test unit is feasible.

In light of the current studies on ruminants, and the potential clinical applications arising from these studies, the role of CNP in human pregnancy must now be readdressed. There have been relatively few studies investigating CNP's involvement in human pregnancy, and typically these have included low numbers of study participants, limited sampling frequency, cross-sectional design and analysis of CNP only, with no measurement of NTproCNP. The subtle changes reported in circulating maternal CNP concentration during cervine pregnancy despite dramatic changes in NTproCNP (Chapter 4) clearly illustrates the value of utilising NTproCNP concentration as a marker of CNP activity, in addition to CNP itself, and demonstrates the advantages of a longitudinal study over a cross-sectional design. With robust assay techniques for measuring NTproCNP now available, it will be important in future work to conduct similar studies in pregnant women to understand whether the changes that occur in ruminants are also a feature of human pregnancy. Further, although it is generally assumed that NTproCNP is biologically-inactive, the extremely high concentrations of NTproCNP

reported here during pregnancy require that this assumption must be confirmed equivocally experimentally to exclude a possible independent role for NTproCNP.

The finding of a marked decline in maternal plasma concentration of both CNP forms in the final week of ruminant pregnancy implicates the peptide as a possible marker of impending parturition. Consequently, future studies involving a more intense sampling regime to provide a detailed description of the changes in CNP production which occur in the final stages of pregnancy will be important. In humans, premature birth is a significant cause of neonatal morbidity and mortality and is associated with numerous developmental and physiological consequences which may persist into adulthood. Presently, the difficulty in predicting and therefore preventing premature birth is a significant challenge in clinical medicine (reviewed by Moss, 2005), further highlighting the need for new studies to characterise comprehensively the role of CNP in human pregnancy.

The role, if any, of maternal and fetal cortisol concentration in regulating CNP production during pregnancy remains unresolved. Despite the temporal association between the decline in maternal circulating CNP forms and the prepartum fetal cortisol surge, both low (Prickett *et al.*, 2010a) and high (our unpublished pilot study) doses of cortisol administered to ovine fetuses appeared to have no effect on maternal circulating CNP concentration. Experimental studies in sheep have shown that the normal decline in BNC in the final week of pregnancy can be prevented by fetal hypophysectomy (Wooding *et al.*, 1986) or adrenalectomy and induced prematurely by fetal (Ward *et al.*, 2002) or maternal (Braun *et al.*, 2007) glucocorticoid administration, suggesting that a possible role for cortisol in regulating maternal CNP production may warrant further investigation.

Since the pivotal reports of Barker and the development of the early origins of health and disease hypothesis (De Boo & Harding, 2006; Barker, 2007), there has been increasing evidence to support the idea that an individual's experiences *before* birth have a significant impact on future health and well-being, both in their own lifetime and also during that of their offspring. Consequently, the relationship between CNP and feto-placental development has relevance both for adult health in humans and future productivity in production animals. Recently, there has been a considerable focus on identifying factors involved in promoting UBF and angiogenesis for use as therapeutic agents in preventing and treating IUGR in compromised pregnancies (Reynolds *et al.*, 2010). The studies presented in this thesis provide important new evidence to implicate CNP as a novel candidate for such an agent. There is now an urgent requirement to identify the function/s of CNP within the placenta and maternal circulation, and to characterise fully the factor/s regulating its synthesis and secretion in order

to exploit the potential therapeutic benefits of this peptide for both human health and animal production.

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