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Circulating levels of C-type natriuretic peptide (CNP) are strongly linked to pregnancy but not to liveweight change in ruminants

B.A. MCNEILL[1]*, T.C.R. PRICKETT[2], M. WELLBY[1], M.J. RIDGWAY[1], E.A. ESPINER[2] and G.K. BARRELL[1]

[1]Faculty of Agriculture and Life Sciences, P.O. Box 84, Lincoln University, Lincoln 7647, New Zealand
[2]Department of Medicine, University of Otago, Christchurch 8015, New Zealand
*Corresponding author: bryonymcneill@gmail.com

ABSTRACT

Marked increases in plasma C-type natriuretic peptide (CNP) during ovine pregnancy, and decline during periods of imposed caloric restriction, raise the possibility that CNP concentrations in ruminants are associated with changes in live weight (LWT) and metabolic demands. Accordingly we measured plasma concentrations of CNP and a related amino-terminal fragment of proCNP (NTproCNP) at weekly intervals in pregnant (n = 8) and non-pregnant red deer hinds (n = 4) and non-pregnant ewes (n = 8) run concurrently. Plasma CNP forms increased markedly during cervine pregnancy to achieve peak concentrations (NTproCNP 132 ± 9.5 pmol/L, CNP 2.14 ± 0.2 pmol/L) at 38 and 10 days pre-partum respectively, falling in the final week of gestation to reach pre-pregnancy levels immediately post-partum (P >0.05). There was no association between the concentration of CNP forms and change in LWT in either group of non-pregnant ruminants. We conclude that maternal concentrations of CNP forms are markedly raised in pregnant deer exhibiting similar changes to those found previously in pregnant ewes, and that the observed changes in plasma levels are likely to be independent of change in LWT. Maternal plasma concentration of CNP forms may be a useful marker of fetal well-being and maturation in ruminant livestock.

Keywords: CNP; NTproCNP; red deer; ewe; pregnancy; placenta; live weight.

INTRODUCTION

C-type natriuretic peptide (CNP) is a paracrine/endocrine factor expressed in a variety of tissues. Circulating concentrations are low in adult healthy sheep but higher in lambs soon after birth, with the levels declining to adult levels in the course of skeletal maturation (Prickett *et al*., 2005). Much interest has focussed on CNP's potential role as a regulator of post-natal skeletal development (Chusho *et al*., 2001; Prickett *et al*., 2007a). However there is also evidence that CNP secretion is responsive to metabolic interventions in adult animals. For instance, the caloric restriction of ewes to 25% of maintenance diet for 16 days, reduced the circulating concentration of CNP (Prickett *et al.,* 2010). Although linear growth of the skeleton has ceased by adulthood, mature animals undergo annual and other temporal cycles of LWT change and skeletal remodelling. These events are likely to be accompanied by metabolic changes that would be expected to generate appropriate signalling responses. On this basis, changes in circulating levels of CNP in association with changes in LWT would provide evidence for CNP's role in metabolic/nutritional homeostasis.

In addition to roles in skeletal development, a novel function for CNP in pregnancy has recently emerged. Studies in sheep (McNeill *et al*., 2009; Prickett *et al*., 2007b) have revealed that the extremely high CNP concentration in maternal plasma directly reflects peptide production by the fetal zone of the placenta where the peptide may function as a signalling factor ensuring an optimal uterine environment for the fetus during pregnancy. The previous studies in sheep were the first to describe the changes in maternal CNP forms throughout the duration of pregnancy in any species. It remains to be determined whether CNP's role in pregnancy is unique to sheep or extends to ruminants in general.

CNP is regarded as the bioactive fragment of the pro-peptide. The amino-terminal, presumably a bio-inactive fragment (NTproCNP), is co-secreted with CNP and serves as a more stable plasma marker of CNP secretion (Prickett *et al*., 2001) and, because of its longer half life, circulates at substantially higher concentrations. Consequently, it has been informative to measure concentrations of both peptide forms in plasma simultaneously. In addition to examining the changes in CNP secretion during gestation in another ruminant species, this study examines a possible link between changes in LWT of non-pregnant ewes or hinds and the circulating levels of NTproCNP and CNP throughout the autumn, winter and spring months of the year.

MATERIALS AND METHODS

Ethics

All procedures involving animals were approved by the Lincoln University Animal Ethics Committee.

Animals

Twelve red deer hinds of mixed age and eight Coopworth, or South Hampshire x Coopworth cross ewes were randomly selected from the Lincoln University Research Farm for this study. Animals were grazed outdoors at the Lincoln University Research Farm, with supplementary feed supplied during winter. Synchronisation of oestrus was achieved using CIDRs (Eazi-breed; Pharmacia, Auckland, New Zealand) in accordance with published protocols for sheep (Wheaton *et al*., 1993) and deer (Rhodes *et al*., 2003). Hinds were randomly allocated to two groups and run with (n = 8) or without (n = 4) a red deer stag. 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 deer calf. As conception dates were estimates, results are expressed relative to actual parturition date. Ewes were run with a vasectomised ram for the first two weeks of the study, and were then run with a group of pregnant ewes. Non-fasting LWT was recorded fortnightly.

Blood samples

Blood samples were collected weekly beginning one week pre-conception until three weeks post-partum in pregnant hinds and, for the same time period, in non-pregnant hinds. Blood samples were collected weekly from non-pregnant ewes during the months of April to September. Blood collection was by jugular venipuncture into 10 mL evacuated blood tubes containing EDTA as anticoagulant (Vacutainer; Franklin Lakes, New Jersey, USA).

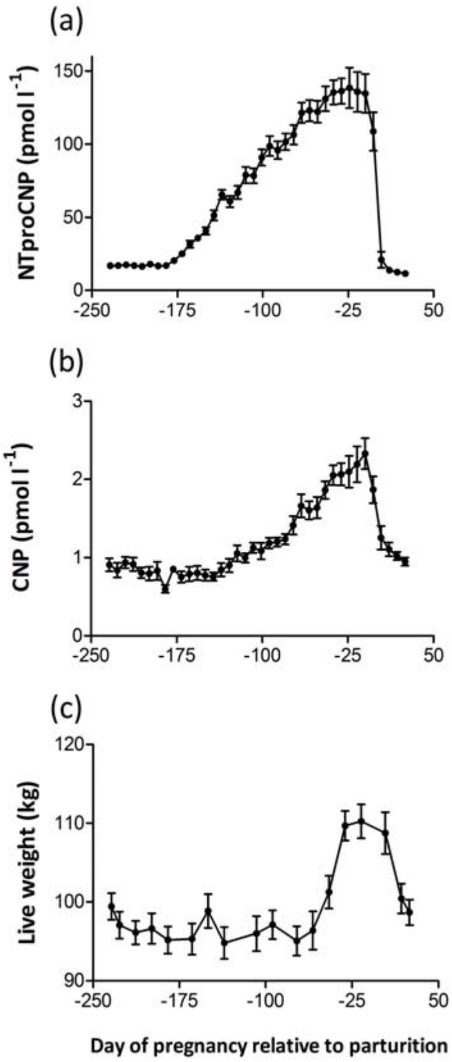
Assays

NTproCNP and CNP were measured by radioimmunoassay as previously described (McNeill *et al*., 2009). The entire set of samples from an individual animal was processed in duplicate in a single assay.

Statistical analyses

Statistical analyses were performed using Systat 7.0 (SPSS Inc., Chicago, Illinois, USA) or Genstat 10 (VSN International Ltd., Hemel Hempstead, UK). Repeated measures analysis of variance with Bonferroni *post hoc* analyses were used to assess changes in maternal NTproCNP, CNP, and LWT with time as the independent variable. Correlations between LWT and peptide concentration were investigated using regression analysis. Data were $\log_e$ transformed where appropriate. A P value of <0.05 was considered statistically significant.

FIGURE 1: Mean (± standard error of the mean) plasma concentration of (a) an amino-terminal fragment of proCNP (NTproCNP), (b) C-type natriuretic peptide (CNP) and (c) live weight in eight pregnant red deer hinds throughout the course of gestation. Hinds were grazed on pasture with supplementary feed supplied during winter. Conception occurred at approximately Day -233, and parturition occurred at Day 0.



RESULTS

Circulating CNP forms in pregnant red deer hinds

Plasma concentration of both CNP forms increased during pregnancy in red deer hinds (Figure 1). Plasma NTproCNP concentration became significantly elevated (P <0.001) above pre-pregnancy levels by 178 days before parturition. Gestation in deer is approximately 233 days. In contrast the rise in CNP concentration was not evident until around 100 days before parturition

FIGURE 2: Mean (± standard error of the mean) plasma concentration of (a) an amino-terminal fragment of proCNP (NTproCNP), (b) C-type natriuretic peptide (CNP) and (c) live weight in four non-pregnant red deer hinds between the months of April and December. Hinds were grazed on pasture with supplementary feed supplied during winter.

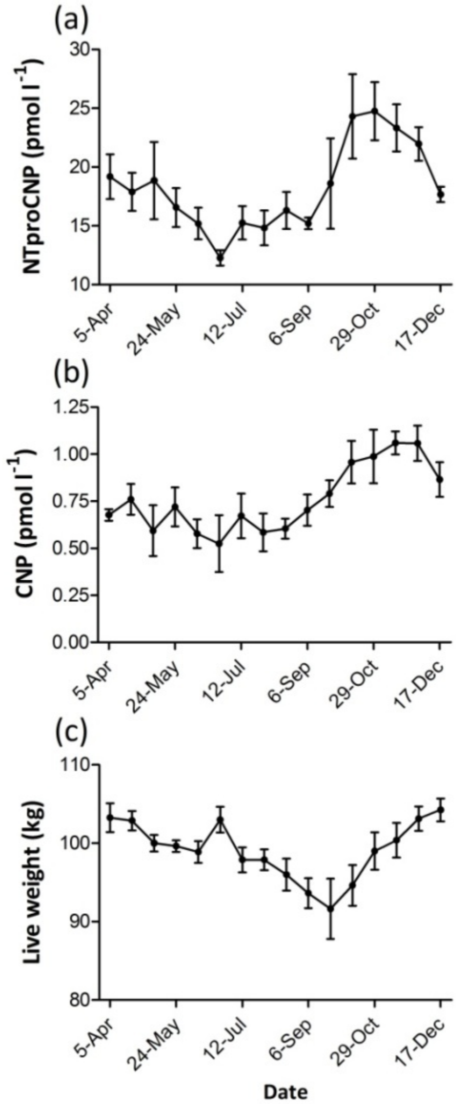
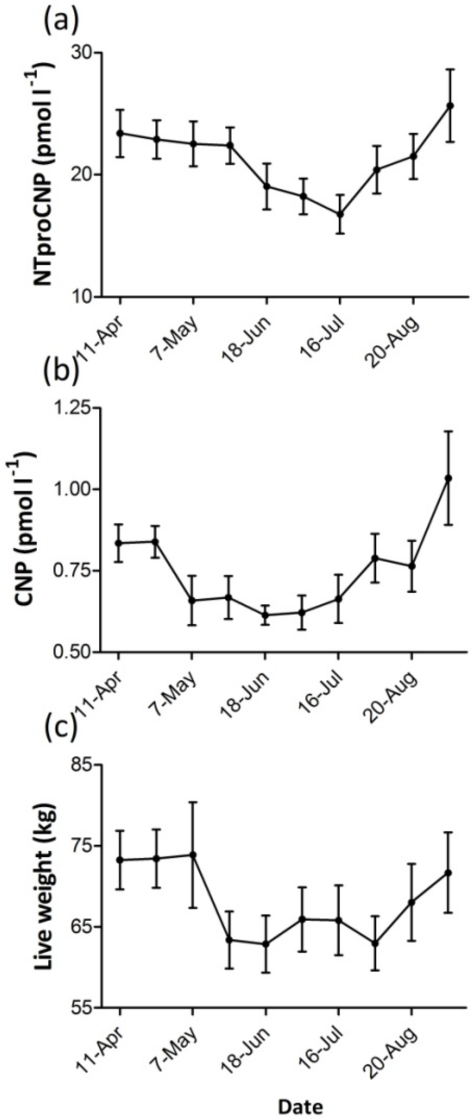


FIGURE 3: Mean (± standard error of the mean) plasma concentration of (a) an amino-terminal fragment of proCNP (NTproCNP), (b) C-type natriuretic peptide (CNP) and (c) live weight in eight non-pregnant ewes between the months of April and September. Ewes were grazed on pasture with supplementary feed supplied during winter.



(P =0.02). Peak mean plasma NTproCNP concentration (132 pmol/L) was attained 38 days before parturition, whereas the peak CNP concentration (2.14 pmol/L) did not occur until 10 days before parturition. Concentrations of both CNP forms appeared to decline in the final week of pregnancy, although this did not reach statistical significance. Circulating NTproCNP and CNP concentrations returned to pre-pregnancy values immediately after parturition (P >0.05).

CNP forms in non-pregnant ewes and red deer hinds

In non-pregnant red deer hinds, there appeared to be seasonal changes in plasma concentration of CNP forms with low concentrations occurring in winter and rising to peak values in late spring. However, these changes did not achieve significance for either peptide form (Figure 2). In non-pregnant ewes there was a significant change in plasma concentration of NTproCNP (P <0.001) and CNP (P = 0.014) during the study period (Figure 3), with a similar trend of lower values in winter followed by an increase in spring.

Liveweight changes

In pregnant hinds, LWT, as would be expected, was dominated by the growth of the fetus. Peak LWT was attained in the final week of gestation followed by a sharp decline after parturition

(Figure 1). In non-pregnant red deer hinds, LWT declined significantly between the months of July and October ($P < 0.05$) before steadily increasing until the conclusion of the study in mid-December (Figure 2). Non-pregnant ewes also displayed seasonal fluctuation in LWT, with a significantly lower LWT recorded during the winter months of May to August compared with those recorded in April and September ($P < 0.05$; Figure 3).

Relationship between CNP forms and Live weight

In non-pregnant red deer hinds there was no correlation between LWT and circulating concentration of either NTproCNP or CNP ($P > 0.05$, Table 1). In non-pregnant ewes, although some individual animals showed a significant relationship between CNP and LWT, this did not hold for NTproCNP and the overall trend was for no relationship between LWT and CNP forms in non-pregnant ewes (Table 1).

TABLE 1: Table of correlations between plasma concentration of an amino-terminal fragment of proCNP (NTproCNP), C-type natriuretic peptide (CNP) and live weight between the months of April and November in four red deer hinds and eight non-pregnant ewes grazed on pasture with supplementary feed supplied during winter in order of decreasing correlation for NTproCNP within the ruminant type.

Type of ruminant	Tag number	NTproCNP		CNP	
		r^2	Significance	r^2	Significance
Hind	96	7.1	NS	0.8	NS
	823	2.9	NS	0.0	NS
	128	0.4	NS	0.6	NS
	207	0.0	NS	11.27	NS
Ewe	126	51.8	*	51.3	*
	143	32.9	NS	17.9	NS
	464	32.5	NS	1.5	NS
	1071	29.9	NS	59.1	**
	330	26.2	NS	40.8	*
	108	10.0	NS	35.6	NS
	141	8.6	NS	1.5	NS
	132	4.8	NS	60.9	**

DISCUSSION

CNP has been described as a paracrine/autocrine factor with actions within the vasculature and growth plates of the skeleton (Hama et al., 1994; Chusho et al., 2001). The recent findings of very high concentrations of the hormone in maternal plasma during ovine pregnancy (McNeill et al., 2009) broadens the field and suggests novel roles for CNP as a hormone of pregnancy. Prior to the current study, ovine pregnancy was the only species in which a profile of CNP forms in maternal circulation was available for the duration of pregnancy. The relevance of these findings to ruminants in general had not been addressed. In this study, we demonstrate elevated plasma concentrations of CNP forms during cervine pregnancy with a similar temporal pattern to that previously reported in sheep. This provides further evidence in support of an important role for CNP in the endocrinology of pregnancy in ruminants. Whereas the current study also reveals novel seasonal variations in plasma CNP forms in non pregnant adult ruminants, our observations do not support any association with LWT.

The profile of CNP and NTproCNP in the maternal circulation during cervine pregnancy presented in this study is almost identical to that reported previously in sheep (McNeill *et al*., 2009), with peaks recorded at comparable stages of pregnancy and the same drop in concentration of both peptide forms at the end of pregnancy. The close alignment of the data from pregnant ewes and red deer hinds suggests that, as in sheep, the cervine placenta is an important source of CNP and although CNP's function during pregnancy has not yet been determined, this too is likely to be conserved within ruminant species.

Despite these similarities, a marked difference was evident in the absolute hormone concentrations achieved during pregnancy; CNP levels were some 15-fold lower and NTproCNP 2-fold lower in deer than previously reported in sheep (McNeill *et al*., 2009). This difference cannot be attributed to fetal number alone as peak plasma NTproCNP concentration in cervine pregnancy is still approximately 100 pmol/L lower than that previously reported for single-bearing ewes in late gestation (McNeill *et al*., 2009). Lack of information on the amino acid sequence of deer proCNP leaves open the possibility that cervine NTproCNP binds with lower affinity to the antiserum used in the current study. Nevertheless, previous studies using size-exclusion high performance liquid chromatography analysis of deer and sheep pituitary extracts, enriched with CNP forms, demonstrate identical immunoreactive NTproCNP peaks of the 5 Kd protein in these two species (Prickett *et al*., 2003). Furthermore, since the amino acid sequence of CNP 22 (proCNP $_{82\text{-}103}$) is conserved in all mammals studied to date, it is unlikely that the much lower CNP concentrations we observe in maternal deer plasma result from differences in immunoreactivity.

Also 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 cannot 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 similar in the two species, the oligocotyledonary placenta of deer consists of only ten placentomes in the average pregnancy 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.

A relationship between CNP concentration and LWT in non-pregnant ruminants was not evident in this study, despite previous data indicating that the concentration of circulating CNP forms may reflect changes in nutritional/metabolic status in sheep (Prickett *et al.*, 2007a; Prickett *et al.*, 2010). The absence of a relationship may be explained by the duration and severity of the metabolic changes likely to have been experienced by the ewes and hinds in our study. Prickett *et al.*, 2010 restricted feed to 25% of maintenance causing a significant and abrupt metabolic disturbance with a weight loss of approximately 10 kg in 16 days. A weight loss of this magnitude is unlikely to occur in healthy animals in response to normal fluctuations in feed quality and abundance. It also needs to be noted that in the previous study (Prickett *et al.*, 2010) increases in caloric intake resulting in an increase in LWT of 5 to 6 kg over 16 days, failed to affect the plasma concentration of either CNP or NTproCNP in adult sheep. Although comparable reductions in LWT occurred in both studies, the decrease observed in ewes and hinds fed *ad libitum* on pasture are unlikely to be the result of severe feed restriction, as supplementary feed was provided during winter. Thus the observed LWT changes may reflect normal changes in voluntary food intake which occur in seasonal animals in response to changes in photoperiod (Archer *et al*., 2004).

Nonetheless there were significant changes in circulating CNP concentration in the non-pregnant ruminants in this study, with a trend for reduced concentrations in the winter months. This was particularly evident in non-pregnant ewes. The lowest concentrations of circulating CNP occurs during the expected period of reproductive anoestrus, and therefore, may relate to the animals' reproductive state and concentration of other hormones, particularly oestradiol (Jankowski *et al*., 1997) which strongly stimulates plasma CNP forms in adult ewes (Prickett *et al*., 2008).

Other factors which undergo seasonal fluctuations during the study period include temperature, daylength and stress, which could all result in a change in CNP production, either directly or indirectly. Significant changes in bone turnover also occur during the year in adult sheep, characterised by low bone mineral density and bone alkaline phosphatase activity, which is a marker of bone formation, during winter (Arens *et al*., 2007). Although CNP is believed to act primarily in the growing skeleton, a role in the adult animal should not be excluded (Prickett *et al*., 2010).

In conclusion, elevated CNP concentration in maternal plasma appears to be a common feature of ruminant pregnancy, and may serve as a useful index of feto-placental growth and development. Serial observations in non-pregnant female deer and sheep, while showing no association with LWT, reveal novel seasonal fluctuations of plasma CNP forms which deserve further study.

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