High dietary salt during pregnancy in ewes alters the responses of offspring to an oral salt challenge

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By

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TABLE OF CONTENTS

ABSTRACT

Most research to date has focused on non-pregnant sheep grazing saltbush to fill the summer/autumn feed gap in temperate regions of southern Australia. However, the summer/autumn period coincides with late pregnancy for autumn- or winter-lambing ewes, and feeding saltbush may reduce the amount and cost of supplementary feed that is required to meet the energy demands of late pregnancy. The challenge of dealing with a high-salt diet may be exacerbated during pregnancy since pregnancy is a salt-retaining physiological state, yet a high-salt intake requires an increase in mechanisms to excrete salt. The effect of high dietary salt on the developing foetus(es) has been studied in rodent models, but less so in sheep. Hence the aims of this thesis were to determine whether pregnant ewes can manage a high dietary salt content resembling that found in saltbush, and whether there are consequences to the offspring's physiological responses to ingested salt.

Merino ewes were synchronized for ovulation and artificially inseminated. To mimic the concentration of salt in animals grazing saltbush-based pastures in summer and autumn, a diet of 13% NaCl was fed from insemination through to parturition. It was found that pregnant ewes can be fed a 13% NaCl diet and manage the physiological conflict of high salt and pregnancy by decreasing their aldosterone concentrations and increasing their water consumption. There was no effect of high dietary salt on pregnancy rates, lamb birth weights, lamb survival or milk composition (fat and protein percentages).

A series of experiments were conducted to test if the high-salt intake of ewes during pregnancy was associated with a change in the dietary preference for salt and/or changes in physiological responses to ingested salt in the offspring ('S lambs' vs. control, 'C lambs'). C lambs and S lambs were exposed to short- and long-term preference testing to determine if there were differences in their voluntary selection for salt in their diet. There were no significant differences in dietary salt preference between C and S lambs.

The lambs were subjected to salt 'challenges' (oral dose of 40 g NaCl in 25% w/v solution) from 3-10 months of age and their water intake, urinary output, sodium excretion and hormone concentrations were measured over the ensuing 23 hours, and compared against counterparts dosed with an equal volume of water without salt. Following the initial salt challenge further experiments were conducted with slight alterations; water intake was manipulated immediately following the salt challenge; two consecutive salt challenges, 8 hours apart, were administered; and C and S lambs were offered salty water (1.5% NaCl) over a period of two days.

The results of these salt challenge experiments showed that C and S lambs excreted a salt load at a similar rate, but they differed in the magnitude of changes in water intake and hormone concentrations required to achieve sodium homeostasis. S lambs were able excrete sodium at the same rate as C lambs but without decreasing aldosterone concentrations to the same extent and whilst consuming 400 mL less water in the first two hours post challenge. The aldosterone results suggested a lowered responsiveness to aldosterone and the lower water consumption suggested an altered thirst threshold. The experiment in which water consumption was manipulated suggested that when the supply or access to fresh water is limited, the capacity to remove a salt load is likely to be less impaired in S lambs than C lambs; S lambs were able to excrete the salt load faster than the C lambs when the availability of drinking water was limited. From the experiment in which lambs were treated with two consecutive salt challenges, the rate of sodium excretion increased after the second dose, but there remained no difference in the rate of excretion between C and S lambs; all animals were able to excrete 95% of the administered dose of sodium within 23 hours. The final experiment in which animals were given salty water (1.5% NaCl) for a period of two days showed consistent results with the previous experiments for water consumption and aldosterone concentrations between C and S lambs. There was no difference in sodium excretion between

C and S lambs. A novel finding was a markedly lower voluntary feed intake in S lambs than C lambs. Although mechanisms for this are unknown, it may have profound effects on the productivity of the animals.

The experiments reported in this thesis provide new information of relevance to pregnant ewes grazing halophytic forages. It is apparent that they can withstand a high NaCl content typical, of a saltbush-based pasture. Further work is warranted to conclude whether high salt during pregnancy is (i) beneficial to the offspring in regards to a higher capacity to deal with excess salt under farming conditions and (ii) consistently associated with a lower voluntary feed intake of the offspring.

DECLARATION

I hereby declare that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis does not contain material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Serina Digby

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ABBREVIATIONS

µg:	Microgram
µL:	Microlitre
˚C:	Degrees Celcius
ACE:	Angiotensin-converting enzyme
ad libitum:	Without restraint
ADF:	Acid Detergent Fibre
Ang II:	Angiotensin II
ANP:	Atrial natriuretic peptide
AQP2:	Aquaporin 2
AT_1:	Angiotensin II receptor 1
AT_2:	Angiotensin II receptor 2
AVP:	Arginine Vasopressin
BSA:	Bovine serum albumin
C:	Control lambs
cm:	Centimetres
CP:	Crude protein
cpm:	Cycles per minute
DM:	Dry Matter
DOC:	Desoxycorticosterone
EDTA:	Ethylenediamine tetraacetic acid
et al:	and others
g:	Grams
GPB:	Gelatin phosphate buffer
H_2O:	Water
HCl:	Hydrochloric acid
hrs:	Hour
IGF1:	Insulin like growth factor-1
IU:	International Units
IUGR:	Intrauterine growth retardation
kg:	Kilogram
L:	Litres
m:	Metre
M:	Molar
mg:	milligrams

mins:	Minutes
mL:	Millilitre
mm:	Millimetre
mmHg:	Millimetres of Mercury
mmol:	Millimolar
mOsm:	Milliosmole
mRNA:	Messenger RNA
n:	Number
NA_2HPO_4:	Disodium hydrogen phosphate
NaCl:	Sodium chloride (salt)
$NAH_2PO_4.2H_2O$:	Sodium dihydrogen phosphate (aqueos)
ng:	Nanograms
NRS:	Normal rabbit serum
NSB:	Non specific binding
P:	Probability
PBS:	Phosphate buffered saline
PCO_2:	Partial pressure of carbon dioxide
PEG:	Polyethylene Glycol
pg:	Picogram
PGI_2:	Prostacyclin
PMSG:	Pregnant Mare Serum Gonadotropin
PRA:	Plasma renin activity
RAS:	Renin-angiotensin system
S:	Salt lambs
SE:	Standard error
TC:	Total counts
V_2R:	Vasopressin 2 receptor
w/v:	Weight per volume

CHAPTER ONE:

Introduction and Literature Review

1.1 INTRODUCTION

The land area affected by human-induced salinity is increasing at a rapid rate and presents both economic and environmental land use problems (Masters *et al*. 2001). The use of saltbush, which can contain 15-30% NaCl on a digestible matter basis (Wilson 1975), is one option available to some landholders to revegetate saline landscapes and convert it from close to zero productivity into an asset (Condon *et al*. 1994) as part of a grazing enterprise. There is a large body of research (Wilson 1966; Hanjra and Rasool 1993; Morcombe *et al*. 1996; Warren and Casson 1996; Masters *et al*. 2001; Franklin–McEvoy 2002) on sheep performance on saltbush (and other salt-tolerant forages). In Australia the majority of this research has considered only the performance of dry (non-pregnant and non-lactating) sheep, with particular focus on the use of saltbush to fill the summer/autumn feed gap. During this time, autumn-lambing ewes are in mid to late gestation, and hence saltbush could be used as a feed source for pregnant or lactating ewes, if they can tolerate the high salt content without detrimental effects on themselves or their offspring. There is little information on potential consequences of high dietary salt in pregnant or lactating sheep. Potential effects on ewe health, foetal development and persistent postnatal ramifications are yet to be defined.

The maternal physiological adaptations to pregnancy are profound and vital for successful foetal growth and development (Bocking 2001). The adaptations that occur during pregnancy involve physiological changes to the cardiovascular, renal, gastrointestinal and endocrine systems, as well as alterations to carbohydrate metabolism and the immune system (Kincaid-Smith and Fairley 1993). When these changes do not take place, foetal growth and development can be compromised (Bocking 2001).

Ewe health during this time may be affected by high dietary salt intake as a result of increased water reabsorption required to maintain a salt/water balance. When a large amount of salt is ingested, the absorption of water into the rumen increases to maintain fluid balance (Wilson

and Dudzinski 1973), and the rate of passage of feed through the gut is increased (Elam 1961). This influx of water can result in a higher osmotic pressure and a lower microbial population and activity in the rumen of sheep (Elam 1961). Following absorption of salt, reabsorption of water by the kidneys is necessary to maintain the salt/water balance in the body. As the pregnant ewe already has increased water retention as part of the normal physiological responses to pregnancy, any additional water reabsorption would further increase the volume of extracellular fluid in the body, and consequently blood pressure may rise. High blood pressure can damage the kidney glomeruli, affecting the filtration of sodium, water and waste products from the blood stream. Glomerular destruction due to hypertension is one of the most common causes of a reduction in kidney function, or in extreme cases, renal failure. Impaired kidney function may cause acute oedema due to salt and water retention and a raft of other metabolic disorders (Laing *et al*. 2005; Takahashi *et al*. 2005). Hypertension during pregnancy can lead to maternal kidney failure, breathing problems, stroke or seizure. Complications for the foetus may include intrauterine growth restriction, oxygen complications and premature labour (Roberts *et al*. 2003).

There are little data on the long-term consequences of high dietary salt during pregnancy on foetal or postnatal metabolism in sheep. It is possible that an elevated salt load in the pregnant dam affects renal development in the foetus, with longer-term consequences to the foetus in terms of regulating salt and water balance. There is growing evidence of prenatal events having long-term postnatal consequences (Stevens and Lumbers 1986; Hegarty *et al*. 2000; Fowden 2001; Marsh *et al*. 2001; Revell *et al*. 2002; Wintour 2002; Mohamed and Phillips 2003) and in particular, effects of an altered *in utero* environment on postnatal kidney function (Marsh *et al*. 2002; Wintour 2002; Dickinson *et al*. 2005; Mortiz *et al*. 2005; Rattanatray *et al*. 2005). Studies with rats in relation to high-salt diets prenatally and/or postnatally have shown an increase in salt preference (Kosten *et al*. 1983; Smriga *et al*. 2002;

Curtis *et al*. 2004) and changes in renin-angiotensin system (RAS) and blood pressure (Contreras 1993; Arguelles *et al*. 1996; Butler *et al*. 2002; Alves da Silva *et al*. 2003).

In the following sections salt and water balance in dry and pregnant sheep will be discussed, including the effects on the renin-angiotensin system and hypertension during pregnancy. Foetal growth and development, including placental function, and studies investigating foetal programming will also be discussed in relation to pre and/or postnatal manipulations. The evidence from the literature outlines the importance for the current study of physiological effects of high dietary salt intake in pregnant ewes and the effects on the offspring.

1.2 SALT AND WATER BALANCE IN DRY SHEEP

1.2.1 Kidney function

The kidneys play an important role in the regulation of water balance, electrolyte balance, acid/base balance, maintenance of osmotic pressures of body fluids and in the removal of metabolic waste products and other toxic substances (Sherwood 1997). The effectiveness of this regulation is directly related to the rate of renal blood flow, glomerular filtration and renal tubular excretion and reabsorption. Water and electrolyte homeostasis is maintained by osmoreceptors that detect changes in the concentration and osmolality of solutes in body fluids (Randall *et al*. 2002). Osmoreceptors stimulate the kidneys to maintain water and electrolyte balance. For example, when there is excess salt present, osmoreceptors stimulate the kidney to decrease sodium reabsorption, thus maintaining homeostasis. If this process is impaired, fluid retention increases and salt concentrations may also rise. This can be detrimental to the health of the animal through increasing plasma volume, resulting in hypertension and associated health disorders.

To ensure that changes in plasma volume and cardiac output have little effect on the glomerular filtration rate under normal circumstances, a number of regulatory processes exist that control blood flow through the nephrons of the mammalian kidney. Firstly, the afferent arteriole responds to the initial stretch that occurs with increased plasma volume by contracting, thus reducing the diameter of the arteriole and increasing the resistance to flow (Randall *et al*. 2002). This myogenic mechanism reduces variations in flow to the glomerulus in the face of oscillations in blood pressure. Secondly, granular and macula cells in the juxtaglomerular apparatus secrete substances that modulate renal blood flow. The granular cells release the enzyme renin, which indirectly affects blood pressure and therefore renal blood flow (August 2000). The macula densa releases various substances that act in a paracrine fashion causing vasoconstriction or vasodilation of the afferent arteriole in response

to variable flow through the tubule. In addition the glomerular filtration rate is subject to neuronal control originating from outside the digestive tract (Randall *et al*. 2002).

1.2.2 The renin – angiotensin system (RAS)

Renin is an enzyme that triggers a cascade of responses involved in regulating blood volume and pressure (**Figure 1**). Its release by the kidneys is controlled by several factors (Davis and Freeman 1976). The juxtaglomerular cells act as miniature pressor transducers that sense renal perfusion pressure, which are perceived via distortions in the stretch on the arterial wall. For example, a reduction in renal perfusion pressure and afferent arteriolar pressure occurs with a reduction in circulating blood volume. The release of renin is increased when low pressure is sensed by the juxtaglomerular cells. This leads to normalised blood volume and blood pressure via the effect of angiotensin II on aldosterone (these hormones promote sodium reabsorption in the kidney; see later) and vascular tone (Seely and Moore 1994). Thus the RAS controls blood volume by appropriate modification of renal tubular sodium transport (Rhoades and Pflanzer 1996).

A second control mechanism for renin release is centred in the macula densa cells. It has been suggested that these cells may function as chemoreceptors, monitoring the sodium load present in distal tubules, and relaying that information back to the juxtaglomerular cells, where appropriate modifications of renin release take place (Seely and Moore 1994). A third mechanism involves the sympathetic nervous system via direct effects on the juxtaglomerular cells leading to renin release (Rhoades and Pflanzer 1996). Finally, a number of circulating factors may alter renin release, such as potassium (increased dietary potassium directly decreases renin release) and angiotensin II (increased angiotensin II concentration decreases renin release via a direct short feedback loop) (Dluhy *et al*. 1970; Williams *et* al. 1978).

An increase in renin specifically causes an increase in concentration of angiotensin II in the blood. Angiotensin II has several actions, one of which is to cause general constriction of arterioles throughout the body, raising blood pressure and thereby increasing both renal blood flow and the rate of glomerular filtration (August 2000). Because the efferent arterioles in the kidney are especially sensitive to angiotensin II, small increases in the hormone cause constriction of the efferent arterioles only, raising glomerular blood pressure and increasing filtration. High levels of angiotensin II constrict both afferent and efferent arterioles and reduce glomerular filtration (Randall *et al*. 2002). An increase in blood levels of angiotensin II also leads to an increase in aldosterone from the adrenal gland. Aldosterone has two important mineralocorticoid activities: (1) it is a major regulator of extracellular fluid balance, and (2) it is a major regulator of potassium metabolism (Williams *et al*. 1972). Extracellular fluid volume is regulated through a direct effect on the renal distal tubular transport of sodium. Aldosterone enhances the active reabsorption of sodium ions from the filtrate, and increases the synthesis of arginine vasopressin (AVP). AVP is responsible for maintaining water balance (Randell *et al*. 2002).

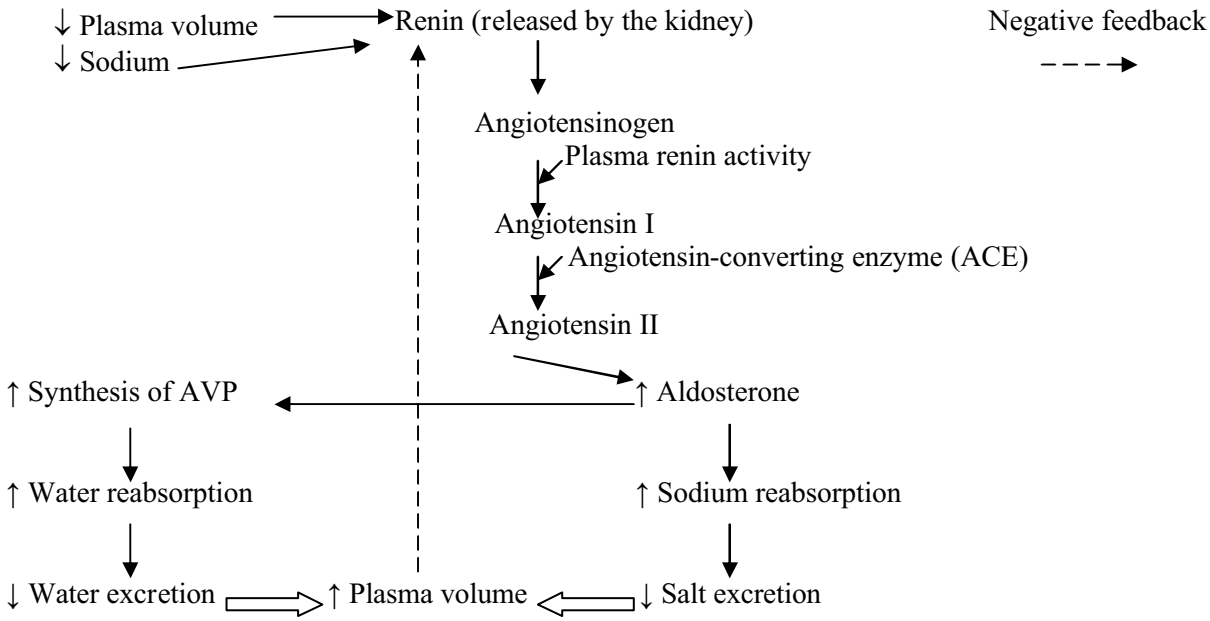

Figure 1: The renin-angiotensin system (RAS).

When plasma volume is increased, due to aldosterone-induced expansion, it is usually accompanied by high blood pressure. This is also due to angiotensin-mediated vasoconstriction independently affecting kidney function (August 2000). Also when plasma volume is high there is an independent decrease in AVP from the posterior pituitary (Rhoades and Pflanzer 1996).

AVP synthesis increases permeability of the distal tubule and collecting duct to water, promoting its reabsorption. Aldosterone then acts with AVP to enhance both sodium and water reabsorption by the kidney (Randall *et al*. 2002).

Atrial natriuretic peptide (ANP) released by the cells in the heart in response to an increase in venous pressure causes an increase in urine production and sodium excretion (Rhoades and Pflanzer 1996). ANP inhibits the release of AVP and renin and the production of aldosterone by the adrenal gland. ANP acts directly on the kidney to reduce sodium and therefore, water reabsorption (Randall *et al*. 2002).

1.2.3 Salt in feed or water

Research has shown that the means of ingestion of salt (feed or water) alters the effects that salt may have on food intake and the health of the animal and, that the acceptability or tastes of food or water containing high levels of salt is a factor in determining the salt tolerance of sheep (Wilson 1966). There are differences in the tolerable concentrations of salt when ingested with water or with feed. The upper concentration of salt in water (1.3% NaCl; Peirce 1968) is lower compared to the upper concentration of salt in food (13.1%; Meyer and Weir 1954); however the total daily load is similar given that feed intake is often about 1kg/day and water intake can be up to 10L/day (Wilson 1966). The tolerance of higher concentrations of salt in food is due to the availability of fresh water, which helps avoid high osmolality (Wilson 1966).

1.2.4 Salt tolerance in dry sheep

Research to date of animal responses to high-salt intake has focused on dry sheep grazing saltbush (and other halophytic plants), dissolved salts in drinking water and added sodium chloride in the diet.

Studies by Wilson in the 1960s showed that the intake of salty feed is influenced by both salt content and the quality of the feed. As salt concentrations increased the intake of feed decreased but this also depended on the type of feed (halophytic plants or constituents of diets with salt added); the higher the quality and digestibility of feed, or the more moisture in the leaves (halophytic plants), the more tolerant the animals were of the salt concentration in relation to depressed feed intake. The supply of fresh water also has a significant bearing on intake of halophytic plants such as *Atriplex* (saltbush) and *Kochia* (bluebush) (Wilson 1966; Wilson and Hindley 1968). When access to water is restricted to once daily, there is a reduction in food intake, which is exacerbated as the salt content increases. Wilson (1966) showed that sodium intake is related to voluntary water consumption where the ratio of sodium chloride intake to total water intake was between 1.8-2.2%. Studies through the 1990s (Hanjra and Rasool 1993; Morcombe *et al*. 1996) further showed that saltbush could be used as maintenance feed during the summer/autumn feed gap and that animals are capable of maintaining weight.

A number of researchers (Wilson 1966; Wilson and Hindley 1968; Hemsley 1975; Hopkins and Nicholson 1999; Masters *et al*. 2005) have also investigated the effects of high sodium chloride when added to the diet on sheep. There are differences between studies in the responses of feed intake and wool production, which may reflect differences in breed, diet constituents and quality and availability of water between experiments. However the overall conclusions are that high-salt diets decrease feed intake and wool growth, although wool growth efficiency (wool gown per kg of organic matter) is increased. The most recent study

(Masters *et al*. 2005) found that increasing sodium in the diet for 45 days significantly decreased feed intake, digestibility, live-weight gain and wool growth. This study suggested a physiological limitation to the intake of sodium chloride and that there was no long-term adaptation or recovery in daily intake over the 45 days. Although total wool growth decreased at high sodium intakes, the amount of wool grown per kg of organic matter intake increased indicating that improvement in the efficiency of wool growth could be achieved, as similarly reported by Hemsley (1975).

The tolerance of sheep to dissolved salts in their drinking water has also been studied extensively (Peirce 1957; Potter 1963; 1968; Wilson 1966; Wilson and Dudzinski 1973; Hamilton and Webster 1987). Sheep are tolerant of NaCl concentrations <1.0%, but are very sensitive as the concentration increases to 2.0%. Drinking water containing 1.3 – 1.5% NaCl results in a small decrease in food intake, but a concentration of 2.0% is associated with a severe reduction in food intake and possibly death (Peirce 1957).

Potter (1963, 1968) also studied sheep consuming salty water (1.3% NaCl) and found that the ability of sheep to tolerate salty water was associated with an adaptation, which increases the rate of renal excretion of ingested sodium and chloride ions. The adaptation involves adjustments leading to increases in glomerular filtration rate and filtration fraction without any pronounced change in renal plasma flow. The elimination of additional salts is accomplished by a reduction of reabsorption of sodium chloride in individual nephrons of the sheep kidney. This suggests the renal response in these animals becomes more efficient when sheep are exposed for months to 1.3% salty water. Potter *et al*. (1972) further investigated the effect of 1.3% NaCl in drinking water on intraruminal function and found significant increases in osmotic pressure. The rate of passage of rumen fluid was increased due to the greater influx of water when fresh water was replaced with saline water. An increase in fluid

flow was observed in the chaffed diet compared to an increased rate of passage in total digesta when fed the pelleted diet and this consequently reduced microbial population. These changes may indirectly attribute to depressed feed intake that has been observed when animals are consuming >1.5% sodium chloride.

Further studies (Wilson and Dudzinski 1973; Hamilton and Webster 1987) also showed that an increase of sodium chloride in drinking water (Wilson and Dudzinski 1973) or in a daily oral dose (Hamilton and Webster 1987) causes a reduction in feed intake and wool production, and increases water consumption, presumably in an attempt to increase the excretion of sodium through increased urinary output.

Therefore dry sheep tolerate salt concentrations of up to 1.5% in drinking water by adapting the RAS. Angiotensin II concentration increases causing increases in the rate of glomerular filtration and aldosterone release. However, the tolerance of pregnant ewes to salts in drinking water has been shown to be dramatically reduced when compared to their dry sheep counterparts. Concentrations as low as 1.3% can cause neonatal mortalities in lambs (Peirce 1968; McIntosh and Potter 1972; Potter and McIntosh 1974).

Pregnancy is characterised by important changes in fluid and electrolyte balance, which are largely dictated by pregnancy-induced changes in the RAS and in AVP (Seely and Moore 1994). Therefore the ability of the pregnant animal to maintain homeostasis is altered due to the considerable alterations in cardiovascular and renal haemodynamics. These changes in physiological status during pregnancy will now be discussed in detail.

1.3 SALT AND WATER BALANCE IN THE PREGNANT EWE

1.3.1 Cardiovascular, renal and haemodynamic alterations

Marked alterations in cardiac, renal and hemodynamic parameters are apparent at the beginning of gestation. Cardiac output rises 30-40% relative to the non-pregnant state in humans, and is a result of increased stroke volume and heart rate (August 2000). The increased stroke volume is due to the increase in plasma volume. Despite these increases, blood pressure falls during normal pregnancy and is associated with a reduced peripheral vascular resistance due to the vascular effects of ovarian steroid hormones (Wilson *et al*. 1980) and nitric oxide (August 1999). In the latter half of pregnancy blood pressure gradually rises until the mean blood pressure in most near-term pregnant subjects is approximately equal to that in the non-pregnant state.

Accompanying the increase in renal blood flow observed during pregnancy is a substantial increase in glomerular filtration rate. Filtration fraction, which is the fraction of plasma flowing through the glomeruli that is filtered into the tubule, falls in early pregnancy due to a smaller increase in glomerular filtration rate than in effective renal plasma flow (the amount of plasma perfusing the kidney tubules per unit time) (Kincaid-Smith and Fairley 1993). Filtration fraction rises again in late pregnancy. An increased filtered load inevitably accompanies the substantial increase in glomerular filtration rate during pregnancy (Kincaid-Smith and Fairley 1993); hence changes in tubular function are necessary to avoid a considerable loss in urine of various substances such as water, nutrients and electrolytes present in the glomerular filtrate. Tubular reabsorption increases in order to prevent rapid depletion from the body of sodium, chloride, glucose, potassium and water (Blackburn 2003). Conversely, tubular reabsorption rates cannot always accommodate the increased filtered load and leads to excretion of substances such as glucose and amino acids.

In pregnant women, the filtered load of sodium increases during pregnancy from 20,000 to 30,000 mmol/day (Kincaid-Smith and Fairley 1993). The increased filtered load of sodium requires a considerable increase in sodium reabsorption by the renal tubule to avoid massive sodium loss (Kincaid-Smith and Fairley 1993). The capacity to avoid excessive sodium retention during pregnancy may be enhanced by high circulating levels of progesterone, which has an antagonistic effect on aldosterone (Landau and Lugibihl 1958). Although progesterone is a competitive inhibitor of aldosterone, it also induces a natriuretic effect that activates the RAS by the same mechanisms as dietary sodium restriction (Braley *et al.* 1996). Thus, sodium balance is achieved during periods of increased progesterone concentrations via control of opposing effects: increased plasma concentrations of aldosterone through a direct effect on the RAS balanced by an antagonistic effect on aldosterone through competitive inhibition.

Plasma osmolality decreases from conception and persists throughout pregnancy. In humans the decrease is approximately 8 to 10 mOsm/kg below non-pregnant values, 280 vs. 270 mOsm/kg (Duvekot and Peeters 1994). This change is associated with changes in the plasma concentration of sodium, urea and other ions and may arise from the decrease in PCO_2 and subsequent compensatory adjustments in renal ion excretion (Duvekot and Peeters 1994). Massive water diuresis due to the inhibition of AVP secretion would normally occur with a decrease in osmolality of this degree; however, this does not occur in pregnancy. The threshold osmolality that stimulates AVP secretion and thirst is decreased (Kincaid-Smith and Fairley 1993), and the mean AVP concentration in plasma increases. An increase in total body water during pregnancy is associated with and contributes to this decrease in osmolality (6-8 litres in humans, 60-70% of which is extracellular and distributed approximately 50/50 between the maternal and foetal-placental compartments; Seely and Moore 1994). The mechanisms controlling intracellular and extracellular volume during pregnancy are poorly

understood, but renal sodium handling is a major determinant of the changes that occur. Several factors including high oestrogen and plasma desoxycorticosterone (DOC) levels may enhance sodium retention during pregnancy (Hutchinson-Williams and Decherney 1992). The increase in aldosterone secretion during pregnancy (Seely and Moore 1994) provides another salt-retaining mechanism.

1.3.2 Renin – angiotensin system (RAS) during pregnancy

Plasma renin activity (PRA) increases substantially during normal pregnancy. Angiotensin II concentrations also increase to nearly twice normal, due to the increase of the renin substrate, angiotensinogen, produced by the liver in response to increased levels of oestrogen (Hutchinson-Williams and Decherney 1992). As a result of the increased angiotensin II, aldosterone concentrations increase, and cause increases in blood volume (**Figure 2**). Oestrogen infusions into the body also increase blood flow; however this effect is secondary to a rise in angiotensin II. Angiotensin II increases uterine blood flow and local production of prostaglandin E or prostacyclin within the vessel wall leads to vasodilation (Resnick 1981).

Figure 2: The effect of oestrogen during pregnancy on angiotensin and blood volume (Hutchinson-Williams and Decherney 1992).

Even though angiotensin II concentration is significantly elevated during the course of pregnancy, the elevated levels in a normal pregnancy do not produce an increase in blood pressure because of a reduced responsiveness to angiotensin II (Abdual-Karim *et al*. 1961). Reduced responsiveness to angiotensin II occurs via an increased production of prostaglandins by the uterus or placenta. A critically important adaptive response is the decrease by mid pregnancy of systolic and diastolic pressures. These fall by 10 to 20mmHg. If blood pressure does not decrease there is an increased risk of developing pregnancy-induced hypertension (Hutchinson-Williams and Decherney 1992). Circumstances in which a defective response may occur include genetics, where individuals are predisposed to high blood pressure, or gestational diabetes.

Associated with high concentrations of angiotensin II are elevated concentrations of aldosterone during pregnancy (Kincaid-Smith and Fairley 1993). Even though plasma aldosterone concentrations are markedly increased during pregnancy, the regulation of aldosterone secretion appears to be normal. Some studies suggest that pregnancy would be a chronic sodium-losing state without the elevated levels of aldosterone necessary to maintain normal sodium balance (Seely and Moore 1994). Thus aldosterone secretion is increased to compensate for this salt-losing tendency and when its secretion is inhibited, salt wasting occurs (Ehrlich 1971).

An interesting feature of the increased levels of mineralocorticoids in pregnancy is that potassium wasting does not occur. Thus even though there is an 8- to 10-fold increase in aldosterone secretion rates, serum potassium levels are normal (Kincaid-Smith and Fairley 1993). The elevated levels of progesterone observed in pregnancy can probably best explain this apparent paradox (Tulchinsky and Okada 1975). Progesterone acts as an antagonist to aldosterone at the renal tubule and inhibits both its sodium-retaining and potassium-losing

tendencies (Landau and Lugibihl 1958). Therefore the RAS responds to the hormonally-mediated changes of pregnancy and maintains and supports blood pressure and blood flow as well as sodium and potassium balance.

1.3.3 Salt tolerance in pregnant sheep

Studies have shown that ewes have adverse reproductive effects when drinking water with high salt concentration. McIntosh and Potter (1972) showed that concentrations of sodium chloride up to 1.3% in drinking water produced distress in some ewes and neonatal mortalities in their lambs. These results were associated with adaptive changes similar to those found in dry sheep that interfered with normal sodium chloride status of the pregnant animal (McIntosh and Potter 1972). Peirce (1968) also found that the reproductive performance of ewes was adversely affected by excess (1.3% or above) salt in drinking water, with ewes failing to conceive and mortality of neonatal lambs prevalent. The susceptibility of pregnant ewes to salt water ingestion increases with age and/or multiple births (Potter and McIntosh 1974). Elevated progesterone levels were observed in ewes that consumed salty water (Potter and McIntosh 1974). Progesterone has been shown to be a natriuretic agent in some species and exerts an antagonistic effect on the salt-retaining influence of aldosterone (Landau and Lugibihl 1958). This indicates the elevated progesterone results from a need to maintain sodium homeostasis by increasing sodium clearance from the body. However, under salt loading, an increase in progesterone concentration may contribute to an extended gestation. If progesterone stays elevated during pregnancy in sheep, the onset of parturition may be delayed as normal pregnancy in sheep involves a rapid decline in progesterone before the onset of parturition (Rurak 2001).

Potassium and chloride concentrations in plasma, both significantly increase in response to increased salt intake, while calcium and magnesium are reduced in sheep (Potter *et al*. 1972). Plasma potassium increases were associated with reduced secretion of corticosteroids and the

increase of plasma chloride might be a reflection of greater rumen chloride concentrations as observed previously in non-pregnant sheep drinking 1.3% saline water (Potter *et al*. 1972). Therefore the effect of drinking water containing 1.3% sodium chloride on pregnant ewes, particularly those carrying twins, is associated with changes in plasma progesterone and electrolyte concentration, but the significance of these changes is not clear. It is possible that the biological mechanisms available to the animal for removing excess sodium chloride may conflict with salt retention associated with pregnancy.

The reproductive success of ewes fed a high-salt diet could be compromised because the availability of energy for reproduction, which is an energy-dependent physiological state, might become limited if it reduces voluntary feed intake and the efficiency of energy use for production to the same extent as it does in dry sheep (Wilson, 1966; Arieli *et al.,* 1989; Masters *et al.,* 2005; Blache *et al.,* 2007).. The metabolic status of the animal is the most powerful internal regulator of reproductive function. When the metabolic status of an animal decreases either due to a decrease in voluntary feed intake or a decrease in fat reserves it is usually associated with a decrease in the concentration of metabolic hormones such as insulin and leptin (Chilliard *et al.,* 2005). Indeed, high salt ingestion has recently been shown by Blache *et al.* (2007) to affect energy metabolism through changes in insulin concentrations in wethers fed high-salt (20% NaCl) diets. Thus, the ingestion of large amount of salt may impact on energy availability and impair reproductive performance.

1.3.4 Consequences of hypertension during pregnancy

Hypertension causes glomerular destruction and is one of the most common causes in reducing kidney function, or in extreme cases, renal failure. Impaired kidney function may cause acute oedema due to salt and water retention. Acidosis can also be caused by impaired kidney function due to the inability of the kidneys to excrete acidic substances (Laing *et al*. 2005). Other effects include increased blood concentrations of urea due to impaired renal

excretion of metabolic waste products, elevated potassium levels than can lead to cardiac arrest and anaemia because the kidneys no longer produce enough erythropoietin for adequate red blood cell production (Takahashi *et al*. 2005). Chronic kidney failure decreases glomerular filtration rate and increases blood levels of nitrogen containing wastes and creatinine.

If hypertension is induced in pregnancy due to excess salt loads a number of health situations, such as stroke, heart attack, loss of kidney function or renal failure and impaired vision and even blindness can arise (Roberts *et al*. 2003). These in turn can affect the growth and development of the foetus. The maternal RAS may also play a significant role in the regulation of foeto-placental blood pressure (Hutchinson-Williams and Decherney 1992). If the RAS is impaired and blood pressure increases, the foetus may experience an increase in blood pressure resulting in hypertension and other health problems after birth. Hypertension may also damage maternal blood vessels resulting in a deficient maternal blood supply, foetal retardation of growth, or even foetal death as adequate maternal blood supply is essential for foetal growth and development (Liggins 1982).

Therefore hypertension during pregnancy may have direct effects on maternal health and have both direct and indirect effects on foetal development. Feeding high-salt diets to ewes could result in hypertension due to the increase in extracellular water from pregnancy in addition to the increased salt loads. This may have important consequences to both the ewe and her offspring.

Factors other than hypertension in the mother during pregnancy have been shown to either directly or indirectly effect foetal growth and development through placental function and cardiovascular and renal changes. These factors in turn can influence foetal programming and

thus life-long consequences. The major factors in relation to maternal influences and foetal growth and development will now be discussed.

1.4 MATERNAL INFLUENCES ON FOETAL GROWTH AND DEVELOPMENT

Maternal nutritional status is one of the major extrinsic factors programming nutrient partitioning and ultimately growth, development and function of the major foetal organ systems. Prenatal growth is sensitive to the direct and indirect effects of maternal dietary intake and body composition from the earliest stages of embryonic life (Wallace 2000) and manipulation of maternal dietary intake can lead to behavioural and physiological changes of the offspring later in life. For example, restricted maternal nutrition in early to mid-gestation increases body fatness and the incidence of coronary heart disease (Symonds *et al*. 2001) and maternal undernutrition during late gestation increases arterial blood pressure in the sheep foetus (Edwards and McMillen 2001). Therefore, nutritional manipulation either throughout and/or at specific stages of pregnancy can have profound effects on the embryo, placenta or foetus.

1.4.1 Placental function

The placenta combines to perform many functions, all of which assist the growth and development of the foetus (Renfree 1982). It is a partial barrier to the transfer of cells from the mother to the foetus or vice versa and hence provides an immunological 'fence'. It facilitates the delivery of oxygen to the foetus; assists in the preferential transfer of amino acids and limits the transfer of large molecules; provides the substrates for foetal metabolism and disposes of waste products; is a site of hormone production and adapts to the increasing metabolic demands of the foetus (Rurak 2001). Diffusion is the most common, and quantitatively the most significant, transfer mechanism in the placenta (Liggins 1982). It accounts for the transfer of oxygen, carbon dioxide, fatty acids, glucose, steroids, nucleosides, some electrolytes, fat-soluble vitamins and most therapeutic agents.

Placental effects can influence foetal growth and development. The weight of the placenta is an important predictor of birth weight (Sanin *et al*. 2001). Experimental reduction in placental

size also reduces foetal growth in a number of species. In the ewe, removal of the placental implantation sites before pregnancy reduces placental mass and lowers foetal body weight at term by as much as 70% (Fowden 2001). Reductions in the functional mass of the placenta by the umbilical artery ligation or partial immobilisation of the foetal placental vasculature with microspheres also result in intrauterine growth retardation (IUGR) (Anthony *et al*. 2003). Wallace *et al.* (2004) also showed that overnourishing adolescent dams resulted in a major restriction in placental growth, due to competition for nutrients between the maternal body and the gravid uterus, which leads to decreased lamb birth weight. Foetal growth is therefore highly dependent on the functional and structural integrity of the placenta. The nutrients taken up by the placenta provide energy for the transport and biosynthetic activities of the placenta. They also provide precursors for the synthesis of hormones and other molecules that are released into both the umbilical and uterine circulations. For growth and oxidative metabolism the foetus uses some of the substances synthesised by the placenta, such as lactate and certain amino acids. The hormones produced by the placenta, such as progesterone may also have effects on foetal growth and development either directly or indirectly via changes in placental or maternal metabolism (Fowden 2001).

The RAS has regulatory functions in the placenta (Schauser *et al*. 1998) but these regulatory effects differ between species. Wilkes *et al*. (1985) have identified and characterised human placental angiotensin II receptors and Schauser *et al*. (1998) have identified bovine placental angiotensin II receptors, however the proportion of AT_1 and AT_2 receptors at specific locations are different between the two species and thus the regulatory functions also differ. Angiotensin II is one of the factors that regulate the blood flow in the uteroplacental unit, and it thereby indirectly influences the foetal volume homeostasis and oxygenation (Wilkes *et al*. 1985). Angiotensin II also stimulates placental lactogen in humans, pregnancy-specific $ß_1$-

glycoprotein, and estradiol secretion in human trophoblast cells via AT_1 receptors (Schauser *et al*. 1998).

1.4.2 Foetal development of the cardiovascular and renal system

The cardiovascular system is composed of the heart and blood vessels and is the first system in the embryo to begin to function (Blackburn 2003). Blood formation in the embryo begins in the liver after a few weeks and can be seen circulating through the embryonic body at the end of those few weeks. As tissues and organs differentiate, the regional networks elaborate to meet metabolic demands. Development of the heart is controlled by a group of cardiac genes and transcription factors (Blackburn 2003). Alterations in these genes or factors or sequencing may to lead to failure in development, incomplete or defective development (hypoplasia), abnormal development (dysplasia), malposition, failure of adjoining parts, abnormal fusion, incomplete resorption, persistence of a vessel or early obliteration of a vessel and thus cardiac defects (Carlson 1999). Environmental factors may also play a role in the etiology of congenital cardiac malformations. Foetal exposure to teratogens through maternal ingestion of drugs such as antiepileptic drugs or warfarin, or alcohol, as well as viral infections, can result in alterations in cardiac development. Forty to fifty percent of the foetal cardiac output is directed toward the placenta, with 14% to the brain and 10-12% to the lungs. The remainder is divided among the gastro-intestinal tract, kidneys and the rest of the body.

Kidney development begins in early gestation with the formation of the adult number of nephrons by mid to late gestation. Urine formation is occurring by 5 weeks and by mid gestation urine production by the foetus is a major component of amniotic fluid. The kidney develops through three successive overlapping stages. The initial steps involve formation of transient non-functional structures on either side of midline, from which the permanent kidney develops. Formation of the kidney involves two separate, interrelated processes. These processes are under the control of genes that are differentially expressed to form proteins that

encode for extracellular matrix, cell adhesion, growth factors and cell receptor proteins (Blackburn 2003). Factors influencing nephrogenesis include platelet-derived growth factor, protein proteases and the RAS (Guillery 1997). Maternal hyperglycemia, alterations in the RAS, a reduction in the supply of vitamin A to the foetus and pharmacologic agents and maternal undernutrition alter renal development. Lumbers *et al*. (1996) have proposed that one pathway by which maternal undernutrition may do so is through its effects on the developing RAS. Lumbers *et al*. (1996) showed that the RAS is essential for maintenance of the foetal glomerular filtration rate. Both angiotensin converting enzyme inhibitors and angiotensin receptor antagonists administered to the dam cause acute renal failure in the foetus due to the direct effects on the foetal RAS. Zhang *et al*. (2000) have shown that in placental insufficiency, which is associated with IUGR, renal renin levels are low. On the other hand infusion of insulin like growth factor-1 (IGF1), which stimulates foetal renal growth, is associated with up-regulation of the activity of the foetal RAS in the absence of significant changes in foetal arterial pressure (Marsh *et al*. 2001). Since kidney disease and dysfunction are intimately involved in the aetiology of hypertension, abnormal renal development may leave the foetus predisposed to hypertension in adult life (Lumbers *et al*. 1996).

Foetal ability to concentrate urine and conserve sodium is limited. Urine is hypotonic due to greater tubular reabsorption of more solute than water. The major solute in foetal urine is sodium. The foetus is not dependent on the kidney for sodium conservation since sodium is readily transported across the placenta. The RAS is active in the foetus and is necessary for normal development. However the foetus is very susceptible to changes in maternal fluid balance. For example, foetal urine flow rate falls and urine osmolarity rises when the ewe is dehydrated or infused with hypertonic mannitol (Lumbers and Stevens 1983). By contrast, the foetus appears to be protected from both a high maternal salt intake (0.17M NaCl) and

moderate salt depletion (Stevens and Lumbers 1986). This protection is probably largely because of the efficiency of maternal homeostatic mechanisms. However, if the mother has renal disease these homeostatic mechanisms may be impaired. Therefore if the pregnant ewe has a salt load, it may not be the salt that directly affects foetal growth and development, but rather the effect that the salt has on the mother, i.e. if the dam can't maintain homeostasis due to a loss in kidney function or a malfunction in the RAS, this is likely to affect the foetus predisposing it to high blood pressure, or impairing its ability to excrete or reabsorb salt and water.

The adaptations that the foetus makes to its pattern of growth to survive adverse nutritional conditions *in utero* may therefore program tissues for subsequent pathophysiology. Hence, the availability of nutrients *in utero* and their metabolic fate in the foetus have important implications for adult morbidity and mortality.

1.4.3 Foetal programming

There is growing evidence that prenatal events can have long-term postnatal consequences. Studies with rats have found that adults exposed to high salt pre and/or postnatally have an increased preference for salt and a change in RAS and blood pressure. Alves da Silva *et al*. (2003) found that offspring exposed to 8% NaCl through gestation until weaning had increased angiotensin II, high blood pressure and blood pressure that was less responsive to salt intake. Arguelles *et al*. (1996) also found rats exposed *in utero* and perinatally to a high salt environment increased angiotensin II sensitivity. This may have been the result of a feedback mechanism in which angiotensin II receptors are up regulated in the foetus in response to lower activity of the RAS in the mother (Arguelles *et al*. 1996; Butler *et al*. 2002). Evidence by Mohamed and Phillips (2003) has shown that maternal high-salt intake does effect foetal development with postnatal consequences. Their studies showed that calves from cows that had received supplementary sodium during pregnancy had a higher appetite for

sodium, indicating that appetite regulation of the offspring could be entrained by the sodium intake of the dam during late pregnancy. Similarly, Curtis *et al*. (2004) found results where adult rats exposed to dietary NaCl and born to dams exposed to high NaCl during gestation had a profound effect on NaCl intake, particularly on stimulated NaCl intake. Results showed striking differences in the temporal patterns of water and salt solution intake in offspring from dams that received 3% NaCl in their diet and that these rats drank less water for each unit of NaCl consumed. It was concluded that early dietary NaCl manipulations had selected consequences that occur despite unimpaired sodium regulation, and that taste driven alterations in NaCl intake and the behavioural response to preabsorptive taste signals is associated with NaCl (Curtis *et al*. 2004).

Smriga *et al*. (2002) also found that adult rats whose tongues were exposed to a NaCl-enriched milk formula during 7-8 days of postnatal development had an increased acceptance of salty and sweet solutions. However when exposed 14 -15 days of age the same results was not observed, indicating that the time of taste exposure is important. Contreras and Kosten (1983) hypothesise that the mechanisms behind the changes in offspring may be due to changes in sensitivity of taste receptors and/or changes in salt and water balance. However, as salt appetite can be increased by inducing extracellular dehydration (Nicolaidis *et al*. 1990) during pregnancy, thus not exposing the dam or offspring to high-salt intakes, the RAS may be the primary mechanism involved in the changes of the offspring.

There is evidence to suggest that similar postnatal effects would be found in sheep as in rats exposed to high-salt diets during gestation. Wintour (2002) found that when sheep were stressed in early pregnancy, through infusing the pregnant ewes with cortisol, their lambs had stunted kidneys that predisposed them to high blood pressure as adults. Stress forces the cells destined to form the kidney to mature too fast, giving the organ less time to grow, resulting in

animals that have only two thirds of the normal number of fluid-filtering units in their kidneys. Over time, the inability of the kidney to expel water and salts efficiently may cause blood pressure to rise. Hegarty *et al*. (2000) also found that cortisol infusions into foetuses caused a physiological increase in plasma cortisol concentration in the foetus and this was associated with an increase in systolic blood pressure as foetuses.

1.5 LONGER TERM POSTNATAL EFFECTS OF HIGH SALT DIETS

It is possible, as stated previously, that high-salt intake when the mother is pregnant could lead to maternal hypertension, which can result in kidney damage and impairment of the dam's RAS. This, in turn, could possibly result in abnormal renal development and profound changes in foetal development. This may then predispose the foetus to hypertension and further complications after birth. A study by Bogdarina *et al.* (2007) showed a direct link between maternal environmental factors that cause hypertension and epigenetic modification of a gene promoter in the offspring. The exact mechanisms that underlie the alteration have not yet been determined, however maternal undernutrition resulted in offspring with an increased expression of the AT_{1b} receptor mRNA and protein in the adrenal and an undermethylated AT_{1b} gene promoter, which control blood pressure. However, if the high salt load does not induce hypertension during pregnancy, the increase in salt may predispose the lamb to an increased preference to salt, thus influence their response to high-salt feeds (such as saltbush). It may also increase the capacity to deal with high salt ingestion through the renal adaptations originating during foetal development. Thus not only might the animal prefer high-salt feeds, it may also be able to manage the high salt through more efficient sodium excretion.

The general hypotheses of this thesis are:

1). High-salt diets during pregnancy will interfere with the ability to conceive and continue pregnancy to term.

2.) The offspring will differ in responses to high salt ingestion and will have an increased preference for salt.

CHAPTER TWO:

Physiological effects of high dietary salt on pregnant ewes

2.1 INTRODUCTION

Pregnancy is characterised by sodium retention and increased extracellular volume both in humans (Hytten and Klopper 1963) and in sheep (Davison 1974). These changes are mediated by alterations in renal function and the renin-angiotensin system (RAS) (Blackburn 2003). The specific mechanisms for water and sodium retention during pregnancy are unclear but are important for the maintenance of the mother and growth of the foetus (Davison and Lindheimer 1989). In humans, the maintenance of sodium balance during pregnancy is related to a balance between sodium-conserving factors such as increased renin, aldosterone and oestrogen and sodium excretion factors such as increased glomerular filtration rate, vasodilating prostaglandins and progesterone (Blackburn 2003). The sequence of events involves an increase in sodium retention and plasma osmolality due to an increase in oestrogen. This change is detected by osmoreceptors that signal to the pituitary gland to increase AVP (Blackburn 2003). Water excretion is consequently decreased and water retention is increased. The osmolality threshold for the release of AVP is reset during pregnancy causing an increase in extracellular volume at a lower plasma osmolality (Lindheimer *et al*. 1987). However, other studies (Durr *et al*. 1981; Olsson *et al*. 1982; Davison *et al*. 1984) suggest that there is considerable species variation in the effect of pregnancy on plasma osmolality and the concentration of AVP. Bell *et al*. (1986) found that pregnant ewes do not have an altered AVP concentration or plasma osmolality.

High salt consumption leads to an increase in plasma osmolality that induces an AVP response similar to that which has been reported (Blackburn 2003) to occur in pregnant humans. However, an increase in plasma osmolality in this case also has a negative feed back on the RAS, decreasing aldosterone release and therefore decreasing sodium retention and increasing sodium excretion. High salt consumption is also associated with an increase in water intake (Wilson and Hindley 1968; Meintjes and Olivier 1992) due to changes in osmolality detected in the thirst centre located in the hypothalamus.

Therefore, the challenge of dealing with a high-salt diet may be exacerbated during pregnancy since pregnancy is a salt-retaining physiological state, yet a high salt intake requires an increase in mechanisms to excrete salt. Thus pregnancy and high salt consumption require opposing physiological responses to regulate sodium retention. It is not clear how pregnant ewes resolve this apparent conflict. The aim of this experiment was to determine if ewes fed a high-salt diet could conceive, continue pregnancy to term and avoid hypertension. It also investigated how pregnant ewes fed a high-salt diet manage the potential physiological conflict of salt retention for pregnancy and salt excretion for the overload of salt, through the hormones involved in the RAS.

2.2 MATERIALS AND METHODS

2.2.1 Experimental Design

A total of 76 Merino maiden ewes were divided randomly into four small paddocks (approx. 6 m x 12 m), with 19 in each paddock (**Figure 2.2.1**). Ewes in paddocks 1 and 3 received a high-salt diet and ewes in paddocks 2 and 4 received a control diet (see section 2.2.2 for dietary compositions).

Figure 2.2.1: A small holding paddock housing 19 Merino ewes.

Upon arrival, ewes were fed oaten and lucerne hay *ad libitum* and weighed. Intravaginal progesterone sponges (Lyppards, S.A.) were inserted to synchronise ovulation. After 12 days, sponges were removed and each ewe was injected with 2 mL of Pregnant Mare Serum Gonadotropin (PMSG) (Lyppards, S.A.). Two 'teaser' wethers were introduced to each paddock to identify those ewes in oestrus. The wethers were injected with 2 mL of duratestone (Lyppards, S.A.) 12 days prior to being introduced to the paddocks. Forty-eight hours after progesterone sponges were removed, laparoscopic artificial insemination was performed on each ewe identified as being in oestrus (day 0) and dietary treatments commenced. Animals were weighed on days 0, 21, 51, 79, 115 and 140 of gestation to monitor liveweight changes. Blood samples were also taken on these days for haematocrit, glucose and hormone analysis (see section 2.4).

2.2.2 Diets

Two diets were used, with half of the ewes receiving a high-salt diet and the other half a control diet. The concentration of 'high-salt' during pregnancy was selected to be 13% in order to mimic the likely salt load of ewes grazing a saltbush annual pasture mixture during the summer/autumn period (D. Thomas, personal communication). Both diets were based on barley and lupin grain, and their ingredient composition and estimated nutrient specifications are shown in **Table 2.2.2**. The diets were prepared by a commercial manufacturer (Lauckes, Murray Bridge, SA) and analysed for nutritive value by Feedtest®, Hamilton, Victoria.

Table 2.2.2: Ingredient composition and nutrient specifications of the high-salt and control diet

	Control	*High-salt*
***Ingredients** (% air-dry basis)*		
Lupins	38.9	33.8
Barley	17	14.8
Mill mix	25	21.7
Oat offal	5	4.3
Rice hulls	10	8.8
Canola oil	3	2.61
Limestone	1	0.9
Mineral mix	0.1	0.09
Added NaCl	0	13
***Nutrient composition** (% of dry matter)*		
In vitro digestibility of dry matter	70.9	74.9
CP	18.8	18.1
ADF	18.8	14.1
Na content	0.19	4.7

Ewes receiving the high-salt diet were fed *ad libitum*, whilst the ewes on the control diet were fed the same amount of organic matter as their paired high-salt ewes. Paired animals were selected to be of similar liveweight to each other. This paired feeding was calculated daily

with the organic matter intake of high-salt ewes on day 'n' being fed to paired control ewes on day 'n+1'. From day 0 to day 45 (ultrasound scanning) ewes were paired fed on a group basis of similar average liveweight. From day 45 through to parturition, each ewe fed the control diet was paired with a ewe of similar liveweight fed the high-salt diet.

2.2.3 Ultrasound

Pregnancy diagnosis was performed by ultrasound on day 44 of gestation (**Figure 2.2.3.1**). Ewes were identified as non-pregnant, single or twin bearing.

Figure 2.2.3.1: Pregnancy scanning at day 44 of gestation performed by ultrasound.

Forty ewes (20 from the high-salt diet and 20 from the control group) identified by ultrasound scanning as single-bearing were placed in individual pens in the Livestock Research Centre, The University of Adelaide, Roseworthy, to permit individual measurements of feed intake (**Figure 2.2.3.2**).

Figure 2.2.3.2: Individually penned ewes.

On day 78 of gestation, pregnancy diagnosis by ultra-sound was performed again to clarify pregnancy status. Of the 40 diagnosed, one from the high-salt diet was not pregnant and was removed from the experiment along with the control partner.

2.2.4 Blood sampling

A 9 mL blood sample was collected by venipuncture from the jugular vein on days 0, 21, 51, 79, 115 and 140 of gestation and 1 week into lactation. Capillary tubes were filled with whole blood, centrifuged at 276 g for 5 mins and a haematocrit reader was used to determine packed cell volume (%) for each individual ewe. Plasma glucose concentrations were also measured on whole blood using a glucometer (HemoCue® Glucose 201+) measuring from 0 - 22.2 mmol/L. The remaining blood sample was then centrifuged at 1106 g for 15mins and plasma was extracted, divided into three eppendorf tubes, and frozen for later analysis of the hormones AVP, aldosterone and progesterone at The School of Animal Biology, The University of Western Australia.

2.2.4.1 Progesterone assay

The concentration of plasma progesterone was measured using a double antibody radioimmunoassay after extraction with hexane as described by Gales *et al*. (1997).

Buffers:

0.1M phosphate

1.226% NA_2HPO_4, 0.212% $NAH_2PO_4.2H_2O$, 0.10% sodium azide, pH 7.5.

Phosphate buffered saline (PBS)

0.01 M phosphate, 0.15 M sodium chloride, 0.1% sodium azide, pH 7.5

Buffer 2

PBS, 0.1% bovine serum albumin (BSA, Fraction V, Sigma), pH 7.5

Buffer 3

PBS, 0.1% BSA, 0.05M EDTA and normal rabbit serum (NRS), pH 7.5

Buffer 4

PBS, 0.001 M EDTA, 1% BSA, pH 7.5

Extraction:

Two mL of distilled hexane were added to 100 µL duplicate aliquots of plasma and pools in disposable glass tubes of 12 x 75 mm. The tubes were then vortexed for 5 minutes and placed in a dry ice/acetone bath for a few minutes. The hexane was poured off into 10 x 75 mm disposable glass assay tubes and dried under a stream of compressed air at 37°C. The recovery of progesterone from plasma was determined by adding labelled steroid to pooled samples, incubating them for 60 min at 37°C, and then extracting.

Standards:

Powdered progesterone (Sigma Australia) dissolved in ethanol was used to prepare the standards by serial dilution to the following concentrations: 32, 16, 8, 4, 2, 1, 0.5, 0.25 0.125,

0.0625, 0.03125 ng/mL which were dried under compressed air after adding 2 mL of distilled hexane.

Antiserum:

The antibody (GT1) was raised in a rabbit against progesterone -11α-carboxymethyloxime-human serum albumin and the major cross-reactions with deoxycorticosterone (2% w/v) and 20α-OH progesterone, 17β-OH progesterone, 17α-OH progesterone and allopregnenolone were all <1%.

Tracer:

[1,2,6,7-^{3}H](N)-progesterone (Amersham) was diluted in Buffer 3 to give approximately 20,000 cpm/100 μL

Assay procedure:

The assay included 6 replicates of three quality controls pools. On the first day, 200 μL of antiserum at 1:12.000 in buffer 2 were added to the extraction samples and standard tubes but not to those for total counts (TC) and non specific binding (NSB). They were vortexed and left overnight at 4°C. On the second day, 100 μL labelled progesterone was added to all tubes, which were incubated for a further 24h at 4°C prior to the addition of 100 μL of second antibody (Donkey 5, Batch1) 1:7 in PBS. The tubes were vortexed and incubated overnight at 4°C. On the fourth day, 1mL of 2% polyethylene glycol 6000 in PBS was added to all tubes, except TC, and centrifuged at 3000 g for 25 min at 4°C, and the supernatant was aspirated. The precipitate was dissolved in 0.5 mL of 0.05M HCl. The dissolved pellet was aspirated from assay tubes and dispensed into scintillation vials with 2 mL scintillation fluid (Starcint, Packard). Vials were shaken and left to stand for one hour before counting for 3 minutes in a

beta counter (Packard Tri Carb 1500). The Intra-assay variation was 6.7% at 1.03 ng/mL, 5.3% at 2.18 ng/mL and 5.2% at 4.10 ng/mL. The limit of detection was 0.10 ng/mL.

2.2.4.2 AVP assay

The concentration of plasma AVP was measured using a double antibody radioimmunoassay from The University of W.A.

Iodination:

Arginine vasopressin (Sigma Australia) was dissolved in 0.2 M Acetic acid to a concentration of 0.1 mg/mL. Aliquots of 2 µg/20 µL was stored at -80^{o}C. Arginine vasopressin is not stable in buffer.

To a 2 µg aliquot 20 µL 0.5M phosphate was added followed by 5 µL ^{125}I and then 5 µL chloramine T (5 mg/10 mLs in 0.5M phosphate) and incubated for 20 seconds with mixing. Finally 100 µL 25% BSA was added and the solution was then added to 3 mL plastic tubes containing 150 mg Dowex 2-2x 8–5 and 1 mL H_2O and inverted constantly for 10 minutes. During this time 500 µL NRS was added to G25 column and allowed to run in to the top of the column. The liquid from the tube containing Dowex was added to the column and 1 mL fractions were collected. The peak fractions were pooled and run through a second G25 column also with NRS. Peak fractions were again pooled and stored at 4^{o}C.

Extraction:

C18 sep-Pak cartridges were pre wet with 5 mL methanol and then washed with 10 mLs DDW. One mL of plasma was acidified by adding 0.1 mL 1M HCL and the sample was loaded into 1 mL syringe and run into the cartridge over the course of a minute. The cartridge was rinsed with 2 x 10 mL of 4% acetic acid. Elute with 4.5 mLs 75% aqueous acetonitrile

with 25% of 4% acetic acid into glass extraction tubes. The elute was evaporated to dryness at 37°C under compressed air and reconstituted in 500 µL of buffer #1. 200 µL/tube in assay (400 µL of plasma) was used. To reuse sep-Pak flush between extractions with 5 mL 8M Urea followed by 10 mL distilled water prior to methanol wash.

Assay:

200 µL of extract was added in duplicate to plastic assay tubes and then 100 µL buffer #1 and 100 µL buffer #2 was added. Standards ranging from 0.63 pg/mL – 320 pg/mL (stable for 2 weeks only) were assayed in triplicate. The standards were made in buffer #2 100 µL/tube and 300 µL buffer #1 was also added. To the extract in plastic assay tubes 50 µL of the 1st antibody diluted 1 in 30K in buffer #1 was added and incubated overnight at 4°C. Then 50 µL of tracer diluted to 5000 cpm in buffer #1 was added and incubated overnight at 4°C. 100 µL of the 2nd Antibody diluted 1 in 15 in buffer #1 containing 1 in 800 normal rabbit serum was added followed by 500 µL of 10% PEG and incubated overnight at 4°C. The solution was then spun at 2000 g for 25 minutes and the supernatant was aspirated and counted.

The first antibody was purchased from Fitzgerald Industries International (USA); it had been raised in a rabbit using arginine vasopressin-thyroglobulin as the immunogen. The antibody reacts with rat, mouse, human, sheep and rabbit and has less than 1% reactivity to oxytocin.

The intra-assay coefficient of variation for AVP concentration was 4.90% and the inter-assay coefficient was 5.02%. The limit of detection was 1.7pg/mL.

2.2.4.3 Aldosterone assay

The concentration of plasma aldosterone was measured using a radioimmunoassay described previously by James and Wilson (1976) and modified at The University of Western Australia.

Assay buffer:

Phosphate buffered saline (PBS): 1 litre 0.1 M stock phosphate buffer; 0.14 M sodium chloride (89 g); 0.1% sodium azide (10 g); double distilled water to 10 litres (pH 7.5). 0.1 M stock phosphate buffer: 61.3 g Na_2HPO_4 + 10.6 g $NaH_2PO_4.2H_2O$ + 5 g sodium azide, dissolved in 5 litres of double distilled water (pH7.5). Gelatin phosphate buffer (GPB*):* PBS with 0.1% gelatin (pH 7.5).

Tracer:

The tracer (1,2,6,7-)^{3}H-aldosterone (Amersham) was diluted to give 10,000 dpm per 100 µL.

Charcoal:

Charcoal solution was made 24 hours before use with 3 g of activated charcoal, 0.3 g of dextran and 300 mL of GPB. The solution was mixed thoroughly for 1hr prior to use.

Aldosterone Standards:

A stock solution of aldosterone (Sigma Aust) was prepared in ethanol 100 ng/mL. Standards were made by serial dilution to concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.0195, 0.0095 and 0.0047 ng/mL in ethanol.

Antiserum:

The antiserum (rabbit) was raised against aldosterone-3-BSA and was purchased from Fitzgerald Industries International (USA). The antisera cross reactivity was 1.1% with11-deoxycorticosterone, 0.01% with testosterone and less than 0.001% with estradiol, 11-deoxycortisol, androsterone, 21-deoxycortisol, estriol, cortisone, estrone and dihydrotesterone.

Assay method:

0.2 mL of sample was added to glass test tubes (12 x 75 mm) and the aldosterone was extracted using 2 mL dichloromethane (AR) by vortexing for 5 minutes and the tubes were placed in a dry ice/acetone bath to freeze the plasma layer. The solvent containing the aldosterone was poured off into 10 x 75 mm disposable glass assay tubes and dried under a stream of compressed air at 37°C. The recovery of aldosterone from plasma was determined by adding labelled steroid to pooled samples incubating them for 60 min at 37°C and then extracting. The efficiency was greater than 95% and solvent blank read less than the minimum standard. 100 µL of standards in triplicate were also extracted.

The dried extracts were reconstituted in 200 µL PBS + 0.5% BSA and 100 µL 1st antibody was added at 1:30,000 dilution followed by 100 µL of tracer (approx 10,000 dpm/tube) which was also diluted in PBS + 0.5% BSA. The tubes were incubated at 4^{o}C for 48 hours and then 200 µL cold Dextran coated charcoal (1%) made in GPB was added. The tubes were incubated at 4^{o}C for 20 minutes and then spun for 10 minutes at 4^{o}C. The supernatant was removed and dispensed into counting vials with 2 mL of scintillant (Starcint, Packard Chemical Operations). Vials were capped, shaken and allowed to stand for 1 hour before counting in a liquid scintillation counter (Packard Tri Carb 1500) for 3 minutes.

The intra-assay coefficient of variation for aldosterone concentration 6.60% and the inter-assay coefficient was 4.91%. The limit of detection was 1.7 pg/mL .

2.2.5 Blood pressure

On day 112, 113, and 114 of gestation four sheep from the high salt and four from the control group were randomly selected to have catheters surgically implanted into the carotid artery to permit measurement of blood pressure. The surgical procedure is described in the Appendix .

Blood pressure was measured on days 115 and 116 of gestation using two approaches. The first involved removing the cap on the external end of the catheter so the blood began to rise up the catheter tubing. The catheter was held vertically and the height of the blood column in the catheter was measured (**Figure 2.2.5**).

Figure 2.2.5: Measuring the height of blood in the catheter.

The second method involved using a pressure transducer (PowerLab MLT1050/D using the PowerLab system hardware, analysed by Chart 4.0 software; ADInstruments, Sydney). The catheter was joined to the transducer and a systolic and diastolic reading was displayed on a computer.

2.2.6 Water intake and urine analysis

It was not possible to measure water consumption of individual animals housed in the Livestock Research Centre, but water intake was measured when the ewes were in the paddocks from day 0 to day 26 of gestation. During this time, water consumption was determined on a group basis by fitting a flow meter to the water line supplying each small holding paddock.

Spot samples of urine were collected in the last month of gestation and tested for renal dysfunction using Multistix 10 SG (reagent strips for urinalysis: Bayer, Australia) testing for leucocytes, nitrite, protein, glucose, blood and pH. The reagent strips were dipped into urine samples and changes in the colour along the sticks after a specific time indicated the level of each parameter outlined above.

2.2.7 Lambing

From day 146 through to day 155 the ewes gave birth to single lambs (**Figure 2.2.6**). Three hours after birth each lamb was weighed and crown to rump length measured. Once the ewe had lambed the high-salt diet was removed and ewes were fed 1kg of control pellets and 500g of lucerne for one week. Ewes and lambs were then placed into grazing paddocks for 8 weeks until weaning where they had *ad libitum* access to pasture.

Figure 2.2.6: The birthing process:

Sac of amniotic fluid; expulsion of the lamb; suckling of the lamb.

2.2.8 Udder volume and milk analysis

Approximately one week after lambing the udder was measured longitudinally over the left and right teats. These measurements were then used to calculate udder volume through the following equation: (R.Bencini, personal communication).

Average circumference = average of length and width measurements

Average radius = average circumference / 3.14

Udder volume = $(4/3*3.14*\text{average radius}^3)/2$

A milk sample was also taken at this time by injecting 1 IU of synthetic oxytocin intramuscularly and milking 5 mL from each teat. The samples were then frozen for later fat

and protein analysis. Samples of milk were analysed with a Milko Scan 133 (Foss Electric, Denmark) calibrated for sheep as described by Bencini (1999).

2.2.9 Statistical Analysis

The data were analysed using Genstat 8th Edition. The analysis performed on pregnancy rates was an analysis of variance. The analysis performed on hormone data, liveweight, feed intake, water intake and lambing was a repeated measures analysis of variance, using conservative F-tests, with the data transformed to better approximate the assumption of equal variance:

AVP concentration - log transformed

Aldosterone concentration - log transformed

Progesterone concentration – transformed to the fourth root. One outlier and replaced with a missing value.

2.2.10 Ethics

The University of Adelaide's Animal Ethics Committee approved the experiment. Approval Number: W-28-2003

2.3 RESULTS

2.3.1 Pregnancy rates

There was no significant difference in pregnancy rates across both treatments (**Table 2.3.1**). Twenty-two out of 38 on the high-salt diet had singletons and twenty-four of the 38 in the control group. Four of the 38 were twins in the high-salt diet compared to 6 out of the 38 in the control group.

Table 2.3.1: Average pregnancy rate (%) for each dietary treatment at day 44 of gestation

Diet	n	Pregnant	Singles	Twins	Non-pregnant
Control	38	81.5	63.0	18.5	18.0
High-salt	38	73.0	57.0	15.5	26.0

2.3.2 General animal responses

2.3.2.1 Death rates

Four ewes fed the high-salt diet and one in the control group died during the experiment. Post-mortems were carried out on three of the high-salt ewes and kidney size was recorded (**Table 2.3.2.1**). The ewe from the control group was euthanased after complications during surgery that resulted in rumen fluid passing into the lungs causing pneumonia; this ewe had aborted 2 days prior to euthanasia. The ewe from the high-salt diet that died but for which no post-mortem was conducted, may have died as a result of a blood clot being dislodged when the carotid catheter was being flushed. Two of the three deaths, for which post mortems were performed, were considered unrelated to the dietary treatment of high salt. One ewe experienced difficulties during lambing and was euthanased, and the other had multiple dysfunctions that could not be attributed to any particular cause (**Table 2.3.2.1**). The third ewe appeared to die from dropsy (oedema), which may conceivably have been related to the consumption of a high-salt diet.

One lamb from the control group and one lamb from the high-salt group died 2 days after birth and another high-salt lamb died after being abandoned when moved from the Livestock Research Centre to the paddock.

Table 2.3.2.1: Post-mortem results from animals that died throughout the experiment

Sheep No.	Dietary treatment	Gestation day	Likely cause of death	Kidney weight (g)		Cortex	Medullar
				Left	Right		
Ewe 4	High salt	140	Dropsy	70g	70g	15mm	10mm
Ewe 134	High-Salt	151;	Lambing paralysis/euthanased (ewe & lamb	105g	105g	12mm	20mm
Ewe 306	High-salt	150	Bronchi pneumonia, pleurisy, retained placenta and pericarditis. Lamb died from being squashed by its mother.	105g	95g	15mm	20mm
Ewe 326	High-salt	140	Unknown, possible dislodgement of clot when catheter flushed	65g	55g	15mm	12mm
Ewe 226	Control	120	Complications during surgery				
Lamb 3	High-salt	2 days old	Suffocation	15g	15g	4mm	10mm
Lamb 17	High-salt	6 days old	Mother abandoned once moved to paddock	20g	20g	5mm	10mm
Lamb 27	Control	2 days old	Pneumonia	15g	15g	5mm	12mm

2.3.2.2 Live weight

There was a significant difference ($P<0.05$) in the live weight of the ewes between the two dietary treatments from the second month of pregnancy onwards (**Table 2.3.2.2**). The ewes fed the high-salt diet weighed 3 kg more than their counter parts from the second month of pregnancy to parturition.

Table 2.3.2.2: Live-weight of the ewes for each month during pregnancy (Means ± SE)

Month	**Control**		**High salt**	
	Mean	**SE**	**Mean**	**SE**
0	44.8	0.90	45.0	0.87
1	43.7	1.04	42.9	0.67
2	46.9[a]	1.05	49.0[b]	0.87
3	50.8[a]	0.85	53.0[b]	0.82
4	53.4[a]	1.08	56.7[b]	1.13
5	58.9[a]	1.22	62.5[b]	1.40

[a,b] Values within a row with different superscript are significantly different (P<0.05)

2.3.2.3 Feed Intake

Feed intake did not differ between the two dietary treatments (**Table 2.3.2.3**). This was expected as those fed the control diet were pair-fed to partners fed the high-salt diet.

Table 2.3.2.3: Monthly feed intake for each dietary treatment (Mean ± SE)

Days of gestation	**Control (g OM/day)**		**High salt (g OM/day)**	
	Mean	**SE**	**Mean**	**SE**
0-31	0.85	1.02	0.98	1.10
31-62	0.95	1.07	1.07	1.13
63 - 94	0.98	0.05	1.04	0.05
95 – 126	0.91	0.07	0.99	0.07
127 - 155	0.83	0.05	0.84	0.07

2.3.2.4 *Water intake*

Ewes in the high-salt group drank nearly twice as much water than those in the control group by day 25 of pregnancy. ($P<0.05$). Water consumption of the ewes was still increasing at this point, and more so for the ewes fed high salt than those in the control group (**Figure 2.3.2.4**).

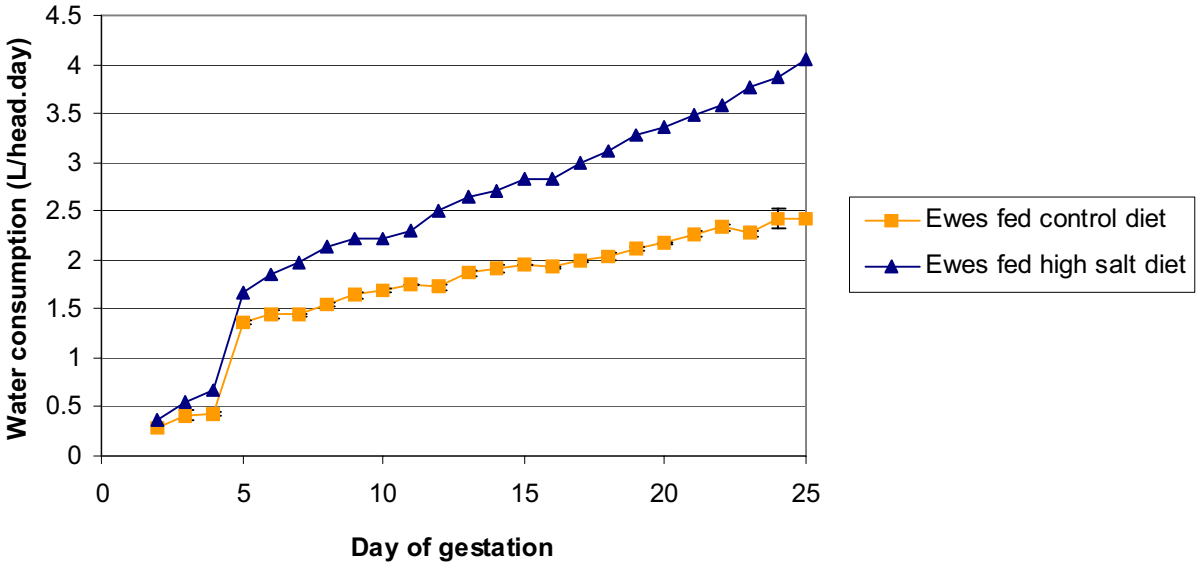

Figure 2.3.2.4: Water consumption over the first 25 days of pregnancy for ewes fed the control or the high-salt diet (Mean ± SE).

2.3.2.5 Haematocrit, glucose and urinary analysis

There was no significant difference in haematocrit or glucose concentration (**Table 2.3.2.5**) and urinary analysis indicated no renal dysfunction in any ewes in either group. There was a significant difference between the two treatments in urinary pH at day 136 of pregnancy, with the ewes fed the high-salt diet having a higher pH than the control group (6.6, ± 0.18 vs. 5.3, ± 0.18).

Table 2.3.2.5: Haematocrit (%) and plasma glucose levels (mM) for each dietary treatment (Mean ± SE)

Gestation day	Dietary Treatment	Haematocrit		Glucose mmol/L	
		Mean	SE	Mean	SE
21	Control	28.4	1.26	4.45	0.204
21	High-salt	29.6	0.63	3.84	0.083
106	Control	33.5	0.58	3.2	0.09
106	High-salt	33.7	0.47	3.3	0.10
140	Control	32.6	0.75	n.a.	
140	High-salt	34.1	0.80	n.a.	

n.a samples not analysed

2.3.3 Hormone analysis

There was no significant difference in plasma progesterone concentration between the two treatments (**Figure 2.3.3.1).** Prior to pregnancy progesterone was quite low (0.69 ng/mL and 0.47 ng/mL for control and high-salt ewes respectively). When the animals were pregnant there was a gradually rise in progesterone, with a substantial increase at 4 months (approximately 5 ng/mL) and again near parturition (approximately 6 ng/mL).

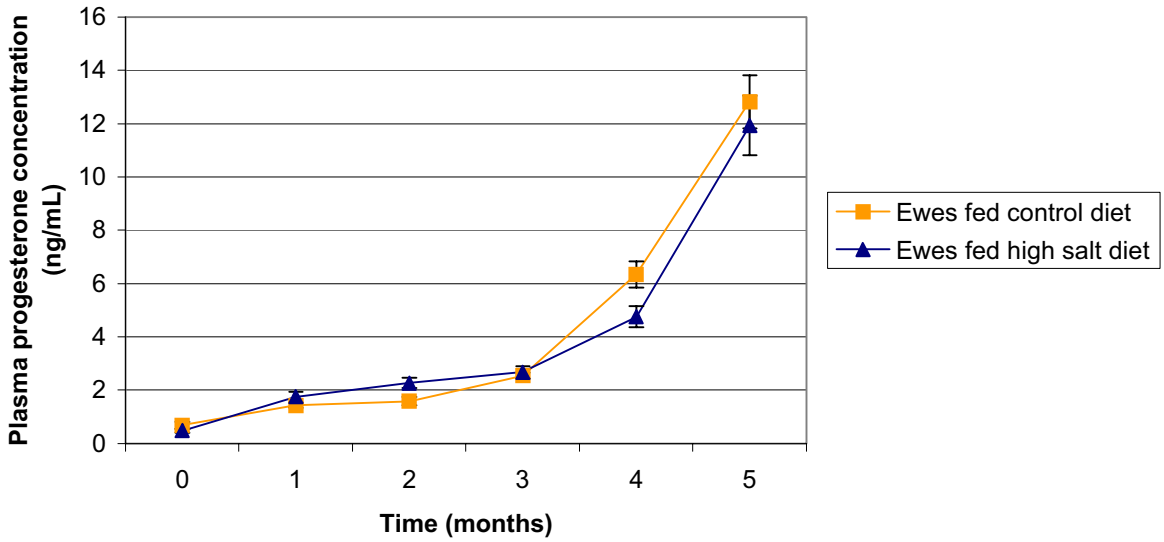

Figure 2.3.3.1: Plasma progesterone concentration (ng/mL) for each month of pregnancy for the high-salt and control ewes (Means ± SE).

Prior to insemination, aldosterone concentrations were not significantly different between control ewes and high-salt ewes (38 pg/mL and 42 pg/mL, respectively). There was a significant difference ($P<0.05$) in aldosterone concentration from the first month of pregnancy onwards. Those fed the high-salt diet had a lower aldosterone concentration than ewes fed the control diet during pregnancy **(Figure 2.3.3.2)**. In the last month of pregnancy there was an increase in aldosterone concentrations of approximately 20 pg/mL in ewes fed both diets.

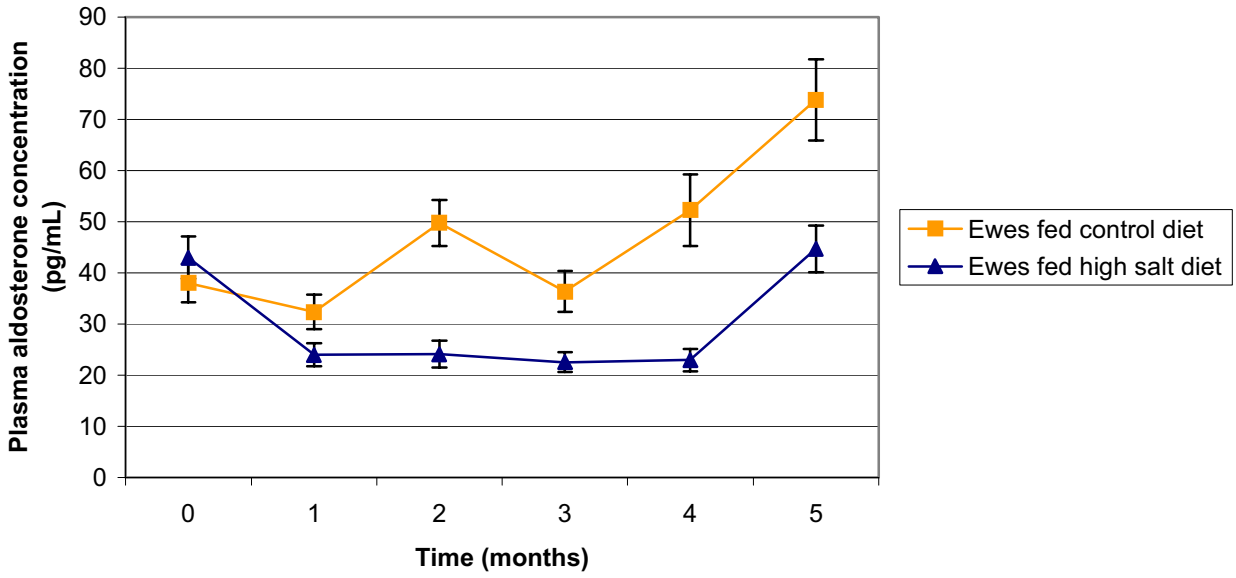

Figure 2.3.3.2: Plasma aldosterone concentration (pg/mL) for each month of pregnancy for the high-salt and control ewes (Means ± SE).

There was no significant difference between the ewes treatment for plasma AVP concentration during pregnancy **(Figure 2.3.3.3)**. Prior to pregnancy AVP concentrations were quite high (27 pg/mL and 15 pg/mL for the high salt and control ewes respectively). When the ewes became pregnant AVP concentration decreased (approximately 18 pg/mL and 10 pg/mL) although in the first month of pregnancy high-salt ewes were 8 pg/mL higher in AVP concentration than the control ewes. AVP concentration remained fairly constant until the last month of pregnancy where there was a slight increase of approximately 5 pg/mL.

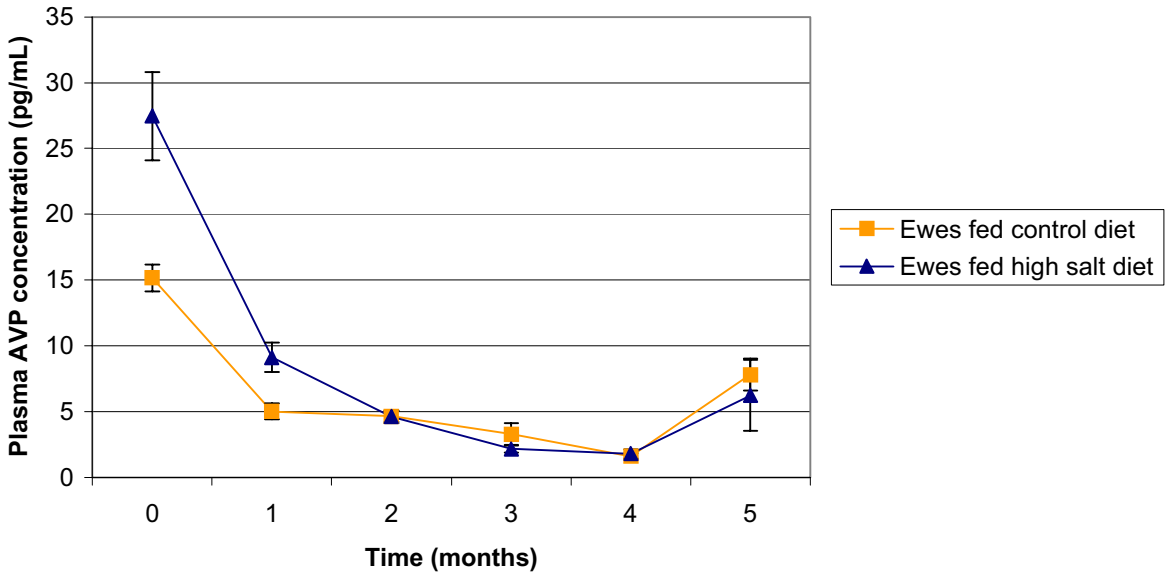

Figure 2.3.3.3: Plasma AVP concentrations (pg/mL) for each month of pregnancy for the high-salt and control ewes (Means ± SE).

2.3.4 Blood pressure

There was no significant difference between blood pressure measured by the height of blood in the column (116 cm and 117 cm for high-salt and control respectively), and the diastolic pressures (60 mmHg and 45 mmHg for high-salt and control respectively) recorded with the pressure transducer (**Table 2.3.4**). Due to the low numbers of animals measured using the pressure transducer statistical analysis wasn't performed. However results show a trend of high salt ewes having an increased systolic and diastolic pressure but further experimental work is needed to make a conclusion.

Table 2.3.4: Height of blood in column and systolic and diastolic pressures recorded from pressure transducer for each treatment on day 116 of gestation (Means ± SE)

				Pressure transducer (mmHg)				
	n	**Height (cm)**	**SE**	**n**	**Systolic**	**SE**	**Diastolic**	**SE**
Control	4	117.0	3.3	2	80.0	0.0	45.0	5.0
High-salt	4	115.8	4.8	3	103.3	3.3	60.0	10.0

2.3.5 Lambing

There was no significant difference in gestation length (150 days), lamb weight (4.85 kg) and crown-to-rump measurements (45 cm) between treatment groups (**Table 2.3.5).**

Table 2.3.5: Gestation length, lamb weight and crown to rump measurements for the lambs born to the ewes fed the salt and control diets (Means ± SE)

Dietary treatment	**Gestation length (days)**		**Weight of lamb (kg)**		**Crown to Rump (cm)**	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	150.2	0.45	4.81	0.15	44.9	0.96
High-salt	150.0	0.57	4.90	0.17	46.1	1.06

2.3.6 Udder volume and milk analysis

There was no significant difference in udder volume between the two treatments (high-salt 1487 mL vs. control 1670 mL). There was also no significant difference between fat and protein content in the milk samples between the two treatments (high-salt 8.1% fat and 4.7% protein vs. control 8.8% fat and 4.8 % protein).

2.4 DISCUSSION

This study has shown that feeding ewes on a high-salt (13% NaCl) diet from the time of insemination until lambing did not compromise insemination rates, pregnancy rates or lamb birth weights. Conception rates based on ultrasound scanning at day 44 after insemination were 73% and pregnancy rates were 98% when ewes were identified as pregnant on day 78 after ultrasound scanning. These figures are typical for laparoscopic artificial insemination in sheep (Hill *et al*. 1998). There is therefore no evidence that the ingestion of a large amount of salt from insemination onwards, or the consequential high water consumption, altered the environment of the oviduct or uterus to affect conception or implantation. Gestation length and lamb birth weights were unaffected by feeding the high-salt diet. Some degree of caution is required in making this conclusion because one ewe fed high salt died of a condition caused by excessive fluid accumulation, and this could conceivable have been related to a high-salt diet. Oedema is generally a symptom of cardiac insufficiency, which may be secondary to kidney disease, such as nephritis, leptospirosis or other kidney functional abnormality (Hungerford 1975). In this case, it is possible that cardiac insufficiency was associated with ascites (excess fluid in the peritoneal cavity). No other ewes from the high-salt treatment showed any signs of excessive fluid accumulation, and haematocrit values (packed cell volume) were not elevated in any ewes.

The apparent tolerance of the ewes to the high-salt diet may have been enhanced by their young age (two years old) and the fact they were bearing single lambs. Potter and McIntosh (1974) found that drinking water containing 1.3% NaCl only had a negative affect on twin-bearing, older ewes (7 years old) or older single-bearing ewes indicating that age and/or multiple births are important in the apparent susceptibility of pregnant ewes to salt water. Whether this susceptibility occurs with high-salt feed is yet to be determined.

The adaptive mechanisms by which the ewes tolerated the high-salt diet will now be discussed.

AVP concentration and water retention

Plasma AVP concentrations in this current study did not differ between the two dietary treatments from the second month of pregnancy to parturition. The reason that the high-salt diet did not induce in increase in AVP was probably because the ewes were able to considerably increase their intake of non-salty water, a response frequently observed with sheep fed salty feeds (eg, Wilson and Hindley 1968; Meintjes and Olivier 1992). Exposure to high salt was likely to have been perceived physiologically by the animal as dehydration due to an increase in osmolality of the extracellular fluid. This would have stimulated the thirst centre (Bie 1980) and triggered an increase in the amount of water consumed. As water intake increased, plasma osmolality would have begun to return to normal levels. In non-pregnant animals, a declining osmolality would normally decrease AVP release but during pregnancy, AVP is not suppressed at the usual levels of body tonicity (at least in humans; Lindheimer and Davison 1995), as part of the normal water-retaining mechanisms of pregnancy. Therefore, the physiological 'objective' of sheep in the present experiment to increase water retention to sustain a normal pregnancy was achieved without a change in AVP concentration because they adapted by drinking more water.

Body water content was not determined in the present experiment due to a technical error, so the increase in water retention could not be quantified. However, a comparison of live weight during pregnancy between ewes fed the high-salt diet and control diet can provide some indication of extra water retention. Since the ewes were 'pair fed' on an equal organic matter basis, and because lamb birth weight was unchanged (and therefore foetal weights during pregnancy were unlikely to have differed), any difference in live weight between the groups is likely to be due to different water retention. By the end of pregnancy, ewes fed the high-salt

diet were 3 kg heavier than their control counterparts. Warren *et al*. (1995) found an increase of 10 to 15% in total body water, which equates 3 to 5 kg more in weight when animals were fed saltbush containing 15-20% salt. This is consistent with the current data. Masters *et al*. (2005) suggested that the increase in live weight associated with high salt consumption is due to a change in body composition. A high-salt diet increases protein flow to the small intestine (Hemsley *et al*. 1975), which may increase protein accretion. Protein retained in muscle is accompanied by water (Searle and Graham 1975). Thus the increase of 3 kg in live weight of the high-salt ewes may be a combination of increased water retention to maintain osmolality of body fluids and water retained in muscle.

As gestation progresses, body water is continually monitored (via changes in osmolality and blood pressure) by the animal, and there are continual adjustments in the volume-sensing AVP release mechanisms (Lindheimer *et al*. 1989). This mechanism could possibly explain the increase in AVP in the last month of pregnancy in both groups of sheep. Although plasma osmolality may have decreased due to high water retention in late pregnancy, the osmotic threshold that triggers a decline in AVP release may have been adjusted to a higher set point, resulting in an elevated AVP concentration.

Aldosterone concentration

The ewes fed a high-salt diet managed the physiological conflict of salt retention for pregnancy and salt excretion for an overload of salt by reducing their plasma aldosterone concentration by approximately 50% of control values. When pregnant ewes are fed a normal or low-salt diet, aldosterone is required to increase water reabsorption and thus increase extracellular fluid. When pregnant ewes are fed a high-salt diet, aldosterone is not required as the high salt from the diet results in increased water intake and thus extracellular volume is increased. Therefore pregnant ewes fed high salt were able to avoid complications such as hypertension (Rafestin-Oblin *et al*. 1991) or neonatal mortalities (Potter and McIntosh 1974)

by reducing their plasma aldosterone concentrations and increasing water intake to increase in extracellular fluid without accumulating excessive fluid.

Blood pressure

The techniques used to assess blood pressure in this experiment were not as successful as intended due to difficulties in maintaining patency of carotid artery catheters. Consequently blood pressure was measured only in a small number of ewes over two days. Results seem to be within a normal blood pressure range reported from other studies although pressures appear to be quite variable (**Table 2.4**). It is not possible to make conclusions from these limited data and further work is required to provide a conclusive case. There are two main forms of gestational hypertension; preeclampsia which is characterised by high blood pressure and protein in the urine and gestational hypertension, which has no typical symptoms other than, elevated blood pressure. As neither of these symptoms was found in the ewes it can be suggested that blood pressure was probably not elevated. The consequences of gestational hypertension include decreased birth weight (August 2000), pre-term delivery, and placental abruption causing massive bleeding from the vagina. Again, these consequences were not found in the pregnant ewes and lamb birth weight was not affected by feeding high-salt diets, and this suggests that blood pressure was not above normal levels in the ewes of this experiment.

Table 2.4: Systolic and diastolic pressures cited from other studies

Reference	**Systolic (mmHg)**	**Diastolic (mmHg)**	**Status of animal**
Dukes (2004)	140	90	Adult sheep (Mean)
Grunberger and Szalay (1983)	110	90	Pregnant (Individual)
	90	70	Pregnant (Individual)
	115	95	Pregnant (Individual)
	105	75	Pregnant (Individual)
	115	90	Pregnant (Individual)
	110	85	Pregnant (Individual)
Xu *et al*. (2004)	108	68	Pregnant (Mean)
	109	65	Pregnant (Mean)
	105	66	Pregnant (Mean)

Pregnancy outcomes and early lactation

Lamb birth weights were unaffected by feeding the high-salt diet during pregnancy, suggesting that foetal growth was unaffected. However, foetal development may have been affected even though any changes were not manifested in an altered birth weight. This aspect is investigated in the following Chapters of this thesis.

Lambing rates, milk volume and composition were not significantly different between the two treatments. The milk fat percentage was higher in this study (approximately 8%) than the average reported fat content of sheep milk (Sakul and Boylan 1992), which suggests removal of residual milk. This could be due to excessively high doses of oxytocin being injected or the milk samples being collected after the lambs had removed some 'fore' milk. Protein percentage was slightly lower (approximately 4.7%) than average protein content 5.8% reported by Sakul and Boylan (1992). Other studies (Meyer and Weir 1954; Seynaeve *et al*.

1996) found that there was no difference in protein percentage when sheep (Meyer and Weir, 1954) or sows (Seynaeve *et al*. 1996) were fed high-salt diets during lactation.

In summary pregnant ewes can consume 13% NaCl diet without experiencing pregnancy difficulties and neonatal mortalities. However, future studies should be undertaken before it can be categorically concluded that the ewe fatalities during pregnancy in this experiment were unrelated to the high-salt treatment. The adaptive mechanisms involved in maintaining a successful pregnancy outcome are a decrease in the RAS, where aldosterone concentration decreased in pregnant ewes fed a high-salt diet and AVP remained the same as control ewes. It is important to note however, that further research is required before it can be concluded that pregnant ewes can graze on saltbush as other factors present in saltbush may influence outcomes. There are also further investigations required on the effect of the offspring because if the increased salt intake of the pregnant ewes lowered the activity of the RAS, there may be affects on the kidney function of the offspring (Alves da Silva *et al*. 2003). The exact mechanisms by which these changes in the offspring occur are largely unknown, however there is mounting evidence that high-salt intake pre and/or postnatally effects the offspring (Arguelles *et al*. 1996; Smriga *et al*. 2002; Alves da Silva *et al*. 2003; Curtis *et al*. 2004). Consequences on kidney function and salt tolerance in the offspring are investigated in the following Chapters.

CHAPTER THREE:

Responses of the offspring to an oral salt challenge

3.1 INTRODUCTION

Evidence from experiment one (Chapter 2) has shown that pregnant ewes fed a high-salt diet (13% NaCl) do not experience adverse effects in pregnancy rates, blood pressure or lamb birth weights. The ewes managed the potential physiological conflict between salt retention for pregnancy and salt excretion for high-salt intake by reducing their plasma aldosterone concentration by approximately 50% of control values and increasing water consumption at least two-fold. Reducing aldosterone concentrations during pregnancy may have consequences beyond the successful completion of pregnancy as it has been implicated in altering kidney function and development of the offspring (Arguelles *et al*. 1996; Alves da Silva *et al*. 2003).

Studies with rats have indicated that high maternal salt intake (3-8% NaCl) during gestation, or exposure to high salt during early postnatal development, affects the offspring. They display increased preference for dietary salt and changes in the RAS and blood pressure. Smriga *et al*. (2002) found that rats whose tongues had been exposed to a sodium chloride-enriched milk formula (85 mmol/L NaCl) during 7-8 days of postnatal development had an increased acceptance of salty and sweet solutions as adults. However when exposed to the high-salt milk at 14 -15 days of age, the same results were not observed, indicating that the time of salt or taste exposure is important. Curtis *et al*. (2004) similarly found that; rats born to dams exposed to high NaCl (3%) during gestation and lactation and then exposed 9 days post weaning to a high-salt (3%) chow consumed, as adults, more of a 0.5M NaCl diet after 10 days of dietary Na deprivation than offspring from dams fed either 1% or 0.1% NaCl during pregnancy. The temporal patterns of intake of water and salt solution in offspring from dams that received 3% NaCl in their diet during pregnancy and lactation also differed from controls. These rats drank less water for each unit of NaCl consumed. It was concluded that manipulating dietary NaCl early in life did not impair sodium regulation, but did affect

the dietary preference for NaCl (i.e., feeding behaviour) and the intake of NaCl (Curtis *et al*. 2004). Contreras and Kosten (1983) hypothesised that mechanisms behind these changes may relate to the sensitivity of taste receptors and/or changes in salt and water balance. However, as salt appetite of offspring can also be increased by inducing extracellular dehydration of their dam during pregnancy (Nicolaidis *et al*. 1990), the RAS, rather than the salt *per se*, may be the primary mechanism involved in the changes in the offspring. Alves da Silva *et al*. (2003) found, with rats, that offspring whose mothers were exposed to 8% NaCl through gestation until weaning increased angiotensin II, had higher blood pressure and lower responsiveness of blood pressure to salt intake. Arguelles *et al*. (1996), using a similar experimental design, reported that offspring of dams fed high-salt had increased sensitivity to angiotensin II. This may have been the result of a feedback mechanism in which angiotensin II receptors were up-regulated in the foetus in response to lower activity of the RAS in the mother (Arguelles *et al*. 1996; Butler *et al*. 2002).

There is evidence that similar pre- and postnatal effects as described above with rats may also be found in sheep. Dodic *et al*. (2002) found that when pregnant sheep are infused with cortisol for 2 days at 27 days of gestation, their lambs had stunted kidneys that predisposed them to high blood pressure at 5 months of age. Hegarty *et al*. (2000) also found that cortisol infusions into foetuses caused an increase in plasma cortisol concentration in the foetus and this was associated with an increase in systolic blood pressure. However, Stevens and Lumbers (1986), found that the ovine foetus appears to be protected from both a high dietary salt intake by the pregnant dam and moderate salt depletion (achieved using furosemide) presumably because of the efficiency of maternal homeostatic mechanisms. Data with cattle (Mohamed and Phillips 2003) have shown that maternal high-salt intake affected foetal calf development with postnatal consequences. Their studies indicated that calves from cows that had received supplementary sodium (70 g NaCl per day) during the last two months of

pregnancy had a higher appetite for sodium, indicating that sodium appetite regulation of offspring could be entrained by the sodium intake of the dam during late pregnancy.

The hypotheses underlying this experiment are that lambs born to ewes fed a high-salt diet ('S lambs') will have a higher preference for a salty diet than their control counterparts ('C lambs'), and S lambs will exhibit differences in urinary output, water intake, sodium excretion and hormone (AVP and aldosterone) concentration when administered a salt tolerance test. The latter would be consistent with a developmental change in the sensitivity and function of the RAS for regulating sodium and water balance.

3.2 MATERIALS AND METHODS

3.2.1 Short term preference testing

At eight weeks of age a total of 24 lambs (12 C and 12 S) born in Experiment 1 (Chapter 2) were weaned and placed into individual pens in the Livestock Research Centre, Roseworthy Campus, University of Adelaide. In this and following chapters, the two groups of lambs are referred to as 'C' and 'S' lambs to indicate that they were born to ewes fed the control or high-salt diet during pregnancy. After weaning, the lambs were fed 1 kg of lucerne chaff and 500 g of the same control pellets as used in the preceding chapter (Refer to Chapter 2 section 2.2.2). Two weeks after an adaptation period the lambs were offered high-salt pellets and control pellets simultaneously in adjacent feeders for 5 minutes. After each animal was offered the two feeds for 5 minutes the uneaten feed was weighed and the intake of each was calculated. The preference for the high-salt diet was determined as a percentage of the total amount of feed consumed. The high-salt pellets and the control pellets were of the same formulation as fed to pregnant ewes in the preceding chapter (see Section 2.2.2, Table 2.2.2 for dietary composition). After the preference test the feed was taken away for one hour. This was repeated 4 times per day and over 3 days. At the end of each trial all lambs received 500 g lucerne chaff and 500 g control pellets overnight.

3.2.2 Long term preference testing

One week after completing the short-term preference testing the animals were subjected to longer term preference testing. They were offered the high-salt and control pellets (as above) in adjacent feeders for 24 hours and, after each day, the feed was weighed and the amount of each diet consumed was calculated. This was repeated for four consecutive days and preference for the high-salt diet was determined as a percentage of the total amount of feed consumed expressed on a daily basis.

3.2.3 Salt tolerance test on lambs

One week after the preference testing was completed, 12 lambs (six C lambs and six S lambs) were placed into metabolism crates. Between and during experiments all animals were fed 1kg of lucerne and 500 g of maintenance sheep pellets each day (8.5% crude protein; 8.5 MJ ME/kg; J.T Johnson and Sons, Kapunda, S.A.). On the first day, half the lambs from each treatment (three C lambs and three S lambs) were given an oral dose of 40 g NaCl in solution whilst the other six lambs received a dose of deionised water of equal volume. A dose of 40 g NaCl was selected to represent approximately 20% of maximum daily intake of salt (Masters *et al*. 2005). Thus, as a single dose, it represents a significant physiological dose but within the range encountered by animals on halophytic forages (Masters *et al*. 2005). The salt solution was made up as a 25% w/v solution in 160 mL of distilled water. Before the oral dose was given a 9 mL blood sample was collected by venipuncture of the jugular vein for basal concentrations of AVP and aldosterone. Sequential blood samples were taken at 4, 8 and 23 hours after the oral salt dose to monitor hormone concentrations in response to the salt tolerance test. Every two hours for 10 hours, and at 23 hours, urine output was determined. A 10 mL sample of urine at each time point was taken and frozen for later analysis of sodium concentration. Water intake was also measured over the same time intervals as urine output.

On the second day the treatments were switched, so those that had received the oral salt dose received the water (control) dose, and vice versa, and the same sampling protocol was followed. These two days of tolerance testing were repeated the following week with another group of 12 lambs, such that a total of 24 lambs (12 C lambs and 12 S lambs) were tested.

3.2.3.1 Sodium Concentration

Urine samples were analysed for Na^+ concentration by inductively coupled plasma atomic emission spectrometry (Dahlquist *et al*. 1978) using a Spectro CIROS ICPAES machine (Waite Analytical Services, The University of Adelaide). Samples were digested with nitric

acid and finished with hydrochloric acid as described by McQuaker *et al*. (1979). The intra-assay coefficient of variation for sodium concentration was 2.88%.

3.2.3.2 Hormone Analysis

Hormone analysis was carried out at the University of Western Australia, School of Animal Biology. Refer to section 2.2.4 for methodology and intra- and inter-assay coefficients of variation and limit of detection.

3.2.4 Statistical Analysis

The data were analysed using Genstat 8th Edition and used a balanced set of data. The model used was a mixed model as it contains both fixed and random effects (**Table 3.2.4**).

For a given transformed response variable γ the full model equation is

$$\gamma = \mu + u + e$$

where μ represents a vector of fixed effects, **u** represents a vector of random effects and **e** a random error term.

Each of the fixed effects were tested using a variance ratio statistic obtained from the Mean Square of the fixed effect of interest divided by the Residual Mean Square for that strata of the ANOVA table. Interactions that were not significant were removed from the model. Most of the data had to be transformed to meet the assumptions of the model; outliers were replaced with missing values. These transformations are listed below:

Aldosterone concentration in plasma: Log transformation minus two outliers;

AVP concentration in plasma: Square root transformation minus three outliers;

Water intake: Square root transformation minus one outlier;

Urinary output: Square root transformation minus three outliers;

Sodium excretion: Square root transformation minus five outliers

Table 3.2.4: Fixed and Random effects

Variable	Description
Lambs	Random factor (blocking factor)
Time	Random factor (blocking factor) nested in lambs
Days	Random factor (blocking factor) nested in days
Lamb group	Treatment fixed factor (salt or control)
Challenge	Treatment fixed factor (salty or water)
Days	Treatment fixed factor (1 or 2)
Order	Treatment fixed factor (which drench was received first
Time	Treatment fixed factor (2, 4, 6, 8, 10 and 23 hours)

Data presented in tables and figures are untransformed values, even though the statistical analysis may have required transformation as described above.

3.2.5 Ethics

The University of Adelaide's Animal Ethics Committee approved the experiments. Approval Number: W-28-2003

3.3 RESULTS

3.3.1 Short and Long term preference testing

There was no significant difference in short-or long-term preference for the salt diet between the C or S lambs (**Table 3.3.1 and Table 3.3.2**). There was however approximately a four-fold increase for C lambs and approximately a two-fold increase for S lambs in preference for the salt diet from the short-term to long-term testing.

Table 3.3.1: Short term preference testing for sodium intake in C and S lambs

	Day 1			Day 2			Day 3		
	Na intake (g/5min)	Na intake (g/kg DM intake)	Preference for high salt (% of total intake)	Na intake (g/5min)	Na intake (g/kg DM intake)	Preference for high salt (% of total intake)	Na intake (g/5min)	Na intake (g/kg DM intake)	Preference for high salt (% of total intake)
C lambs	0.87	4.9	6	0.31	2.7	4	0.54	4.6	5
S lambs	0.62	4.5	7	0.59	4.3	7	0.76	5.7	9
SE	0.22	0.95	1.9	0.17	1.01	3.6	0.24	1.41	2.2
P-value	0.46	0.80	0.90	0.29	0.33	0.58	0.54	0.59	0.18

Table 3.3.2: Longer term preference testing for sodium intake in C and salt S lambs

	Na intake (g/day)	Na intake (g Na/kg intake)	Preference for the high salt (% of total intake)
C lambs	23.0	10.7	20
S lambs	29.2	13.4	26
SE	6.64	2.08	4.7
P- value	0.53	0.38	0.45

3.3.2 Salt tolerance test

3.3.2.1 Urinary output

C lambs excreted more urine per hour (mL/hr) between 4 and 10 hours after the water or salt dose compared to the S lambs (P=0.037). When lambs received the salt challenge, their rate of urinary output was higher than after the water treatment, from 2-23 hours after receiving their dose. From 2 to 10 hours this difference in urinary output was about two-fold. As the

experiment was conducted over two days, each day had to be considered as a factor and analysis showed that on day one all lambs excreted more urine (P=0.034) than on day two irrespective of diet fed during pregnancy or dose type. The data shown in **Figure 3.3.2.1** are the averages across both days 1 and 2.

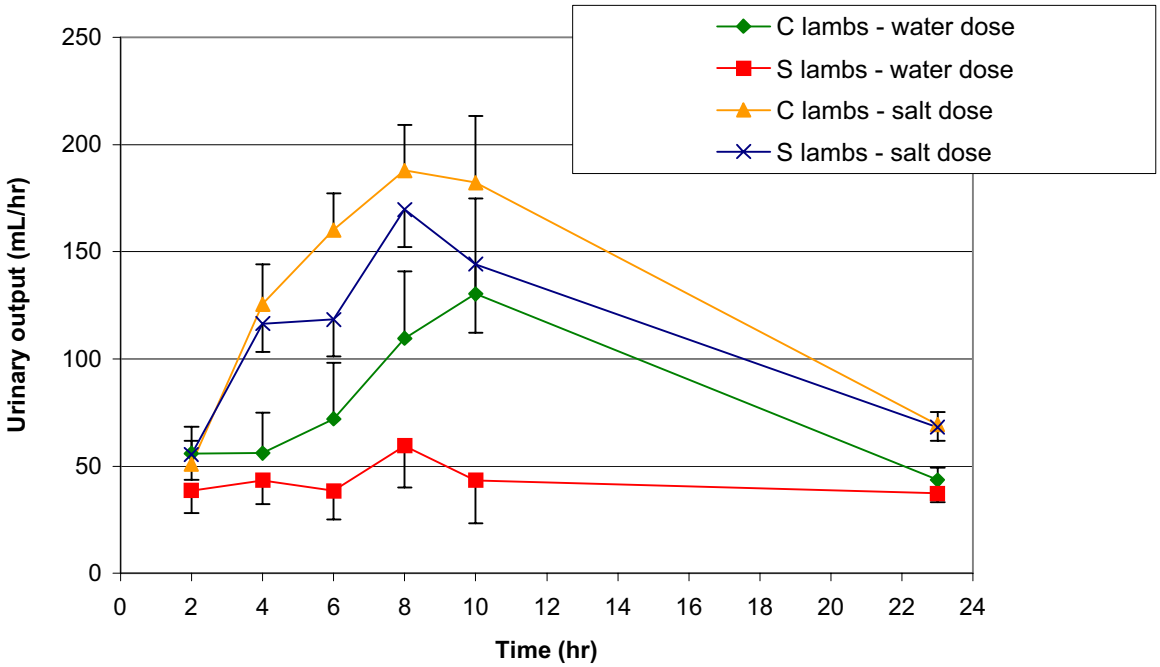

Figure 3.3.2.1: Rate of urinary output over time for lambs born to ewes fed a high-salt diet (S lambs) or a control diet during pregnancy (C lambs), receiving either an oral dose of salt or water (Mean ± SE).

3.3.2.2 Water intake

There was a significant interaction between the diet of the lamb group (i.e. C and S lambs), dose and time (P=0.037) for water intake where C and S lambs had a different profile when administered the salt or water doses. During the first two hours after the salt dose, C lambs had a higher water intake by approximately 200 mL/hour (P<0.05) compared to the S lambs (**Figure 3.3.2.2**). Over the following two hours the C lambs dropped their water intake from about 800 to 400 mL/hour, but S lambs remained elevated and consequently consumed about 100 mL/hour more than their control counter parts. At 6 hours, water consumption of both

treatment groups was the same, and remained so for the remaining 17 hours. There was no significant difference in water consumption between C and S lambs when administered a water (control) dose.

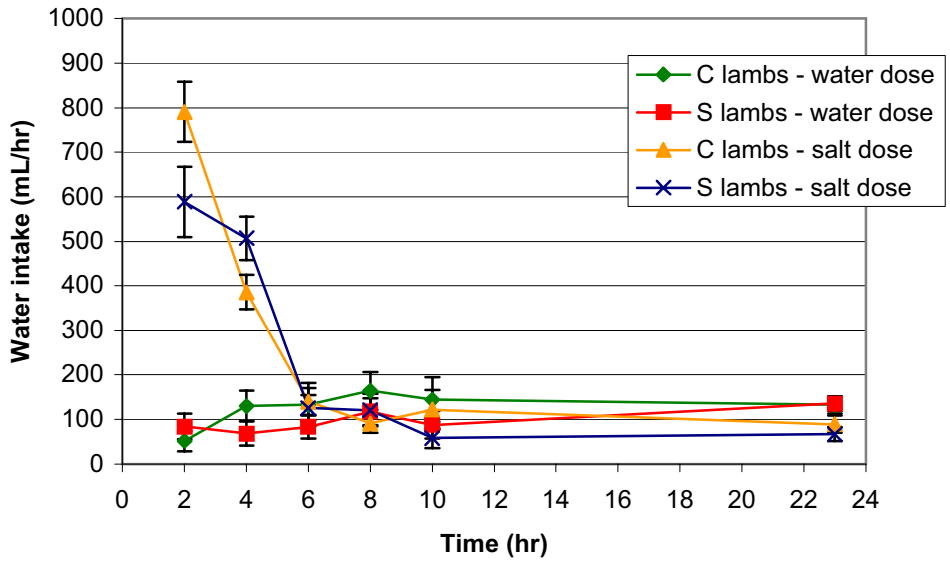

Figure 3.3.2.2: The average water intake (mL/hr) over 23 hours for C and S lambs administered either a salt or water dose (Mean ± SE).

3.3.2.3 Sodium excretion

There was no significant difference between S and C lambs in sodium excretion; over the 23 hour period C lambs excreted 10.5 g of sodium and S lambs excreted 10.4 g. There was a significant interaction between dose (water vs. salt) and time ($P<0.001$) in that lambs that received the salt dose, regardless of mother, order or day, had a higher rate of sodium excretion than lambs that received the water drench (**Figure 3.3.2.3**).

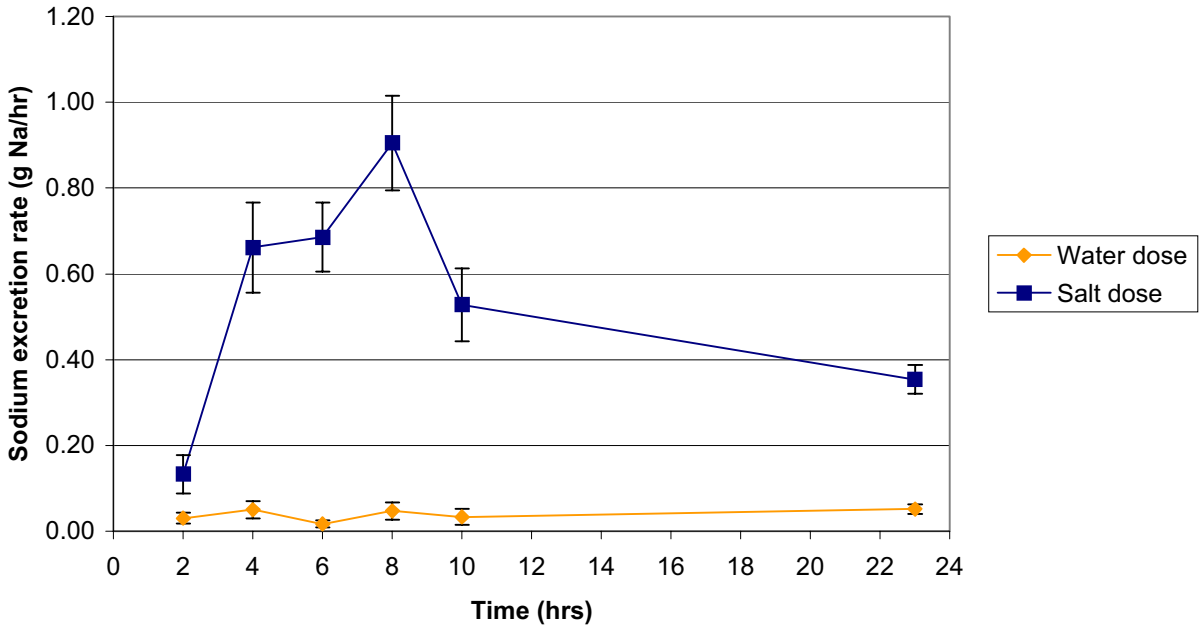

Figure 3.3.2.3: Rate of sodium excretion over time for lambs receiving either an oral dose of salt or water (Mean ± SE). Values pooled across C and S lambs within each 'dose' treatment.

3.3.2.4 AVP concentration

There was a significant interaction between lamb group (i.e. C and S lambs), time and day (P=0.03) where AVP concentration was lower in all offspring on day 2 than day 1 at 0, 4 and 23 hours after the dose (consistent with the lower urinary output). At 8 hours only, S lambs had a low AVP concentration on day 2 than day 1.There was also a significant difference between challenge (water vs. salt) and time (P=0.046) where lambs given the salt dose had increased AVP concentrations at 4 and 8 hours after the dose but by 23 hours the values were the same. The data shown in **Figure 3.3.2.4** are the averages across both days 1 and 2 for the salt and water dose pooled for C and S lambs.

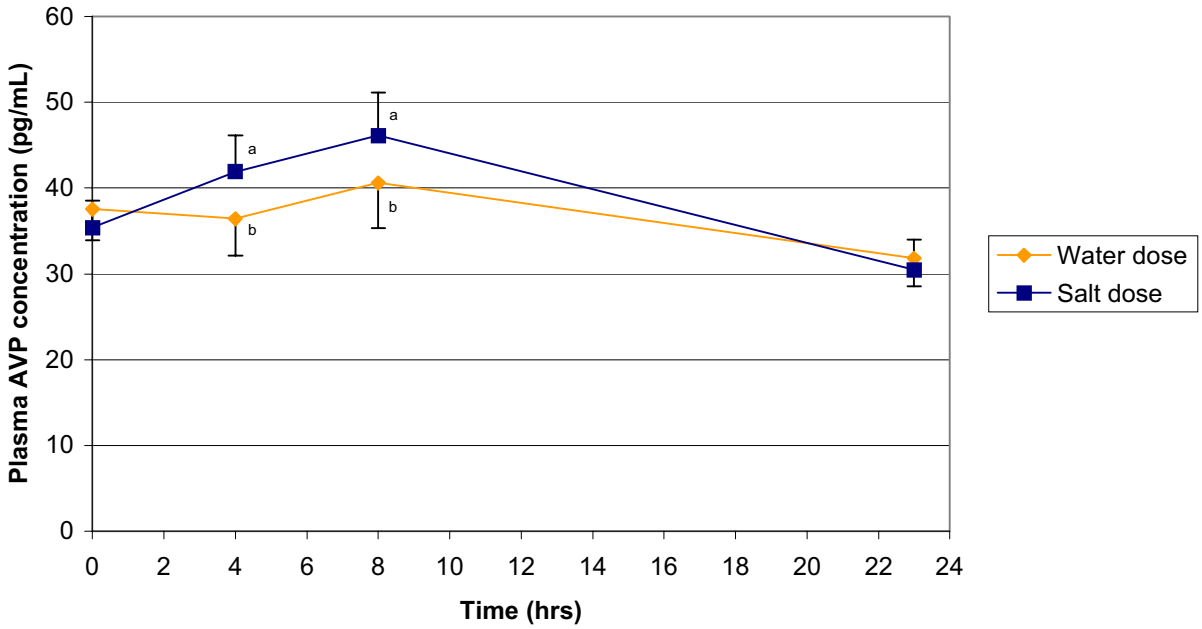

Figure 3.3.2.4: Plasma AVP concentration over time pooled for C and S lambs receiving either an oral dose of salt or water. [a, b] values with different superscripts are different (P<0.05) (Mean ± SE).

3.3.2.5 Aldosterone concentration

Overall, S lambs had a higher concentration of aldosterone (P=0.013) over the 23 hours irrespective of the dose (water or salt) (**Figure 3.3.2.5**). This means that following the water dose the average aldosterone concentration for S lambs was 29 pg/mL compared to 19 pg/mL for the C lambs. Following the salt dose, S lambs decreased aldosterone concentration by 50% (from 20 to 10 pg/mL) whilst C lambs decreased by 68% (from 22 to 7 pg/mL). At 4 and 8 hours after the dose, all lambs that had received the salt dose had a lower (P=0.042) aldosterone concentration of (7-10 pg/mL) compared with those receiving the control (water) dose (22-32 pg/mL).

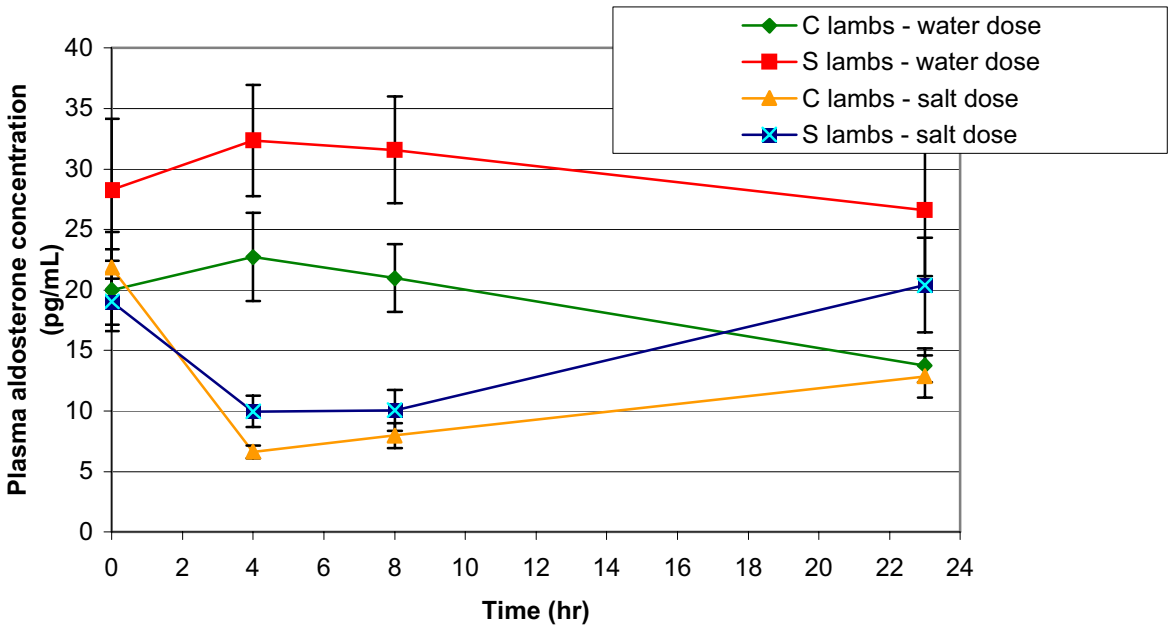

Figure 3.3.2.5: Plasma aldosterone concentration over time for C and S lambs receiving either an oral dose of salt or water (Mean ± SE).

3.4 DISCUSSION

Animal responses to the oral salt dose (40 g NaCl) in this study are consistent with other research (Wilson and Dudzinski 1973; Hamilton and Webster 1987) where high-salt consumption has increased water intake, urinary output and sodium excretion. The single dose of salt used in this experiment (and following experiments) is a novel approach in testing the response to oral salt load. This has shown for the first time that there are differences in the responses to a salt or water challenge between lambs born to dams fed high salt (S lambs) and lambs born to dams fed a control diet (C lambs) were different. In response to the high salt challenge, S lambs drank 400 mL less in the first two hours compared to C lambs. S lambs also had a higher concentration of aldosterone following the control (water) dose and exhibited a smaller decline in aldosterone concentration following the salt challenge. Furthermore, C lambs showed an increased urinary output compared to S lambs when receiving a control (water) dose. This is consistent with aldosterone results under 'normal' conditions where decreased aldosterone results in increased water excretion. These results partially support the second part of the hypothesis in that lambs born to ewes fed a high-salt diet during pregnancy would differ in their responses to manage a salt load and also provides evidence that C and S lambs differ under 'normal' conditions. The first part of the hypothesis is rejected as there was no difference between C and S lambs for the preference of high-salt diets.

Water consumption

Total water intake over the 23-hour period was similar for both lamb groups (i.e. C and S lambs) after they received the salt drench, but S lambs drank approximately 400 mL less than the C lambs over the first 2 hours after the salt dose. This suggests that the thirst set points for the S lambs were higher and the drive to increase water consumption immediately after the salt load was comparatively less than for C lambs. In the following two hours (2-4 hours), the water intake of S lambs remained elevated, more so than the C lambs, possibly suggesting

that the initially lower water intake (at 2 hours) of S lambs meant that their plasma osmolality was not reduced as quickly as the C lambs and hence their water intake did not decline much over the second 2-hour period (2-4 hours) compared with C lambs. The different pattern of water intake between the two groups of lambs, and the importance of water consumption immediately after a salt load on the sequalae of physiological events (i.e., urinary output and sodium excretion) is investigated in more detail in the following chapters.

Aldosterone

The data suggest a lowered responsiveness to aldosterone for S lambs. The S lambs had a higher mean aldosterone concentration following the water (control) dose than the C lambs and also the required drop in aldosterone concentration, following a salt dose, was less than the C lambs. A lowered responsiveness to aldosterone would result in Na-retaining effects being less potent that normal. A similar study in rats by Alves da Silva *et al*. (2003) showed that adult rats exposed to perinatal salt overload had increased kidney angiotensin II. High angiotensin II leads to increased aldosterone, so although not measured by Alves da Silva *et al*. (2003) aldosterone concentrations may also have been elevated. The implications of increased aldosterone concentrations are higher blood pressure. This was not measured in the current experiment, however Alves da Silva *et al*. (2003) did find elevated blood pressure and hypothesized it to be due to increased aldosterone concentration. Alves da Silva *et al*. (2003) suggested that higher renal angiotensin II was a possible mechanism responsible for the shift of the pressure natriuresis curve and consequently higher blood pressure. Their results also showed that blood pressure was less responsive to salt intake in animals with high perinatal exposure the salt.

Both C and S lambs increased sodium excretion after the salt challenge and no differences could be attributed to the diet during pregnancy. Under these experimental conditions S lambs excreted sodium at the same rate as C lambs however they did not consume as much water

during the first 4 hours after the salt dose or decrease their aldosterone concentration to the same degree as C lambs. This suggests that although sodium excretion rates were the same, the magnitude of difference within the mechanisms by which the animals dealt with the excess salt were different. For example both lamb groups drank more water and decreased aldosterone concentrations after a salt dose, but S lambs were less perturbed by the salt dose than the C lambs.

Figure 3.4.1 shows from 2 hours to 23 hours there was a 2 phase linear increase in sodium excretion; i.e.; increased rapidly up to 10 hours, then the rate declined. Over 23 hours, both C and S lambs only excreted approximately 64% of Na from the 16 g of Na administered. Soon after the dose, the increase in osmolality of body fluids would trigger an increase in water intake and sodium renal excretion. As osmolality began to return to normal, so too would the rate of sodium excretion. With a repeated dose, or with continual exposure to high salt (as found in field studies with animals grazing halophytic plants), the rate of excretion would need to increase to avoid an accumulation of sodium in the body. This is dealt with in the following chapters in more detail.

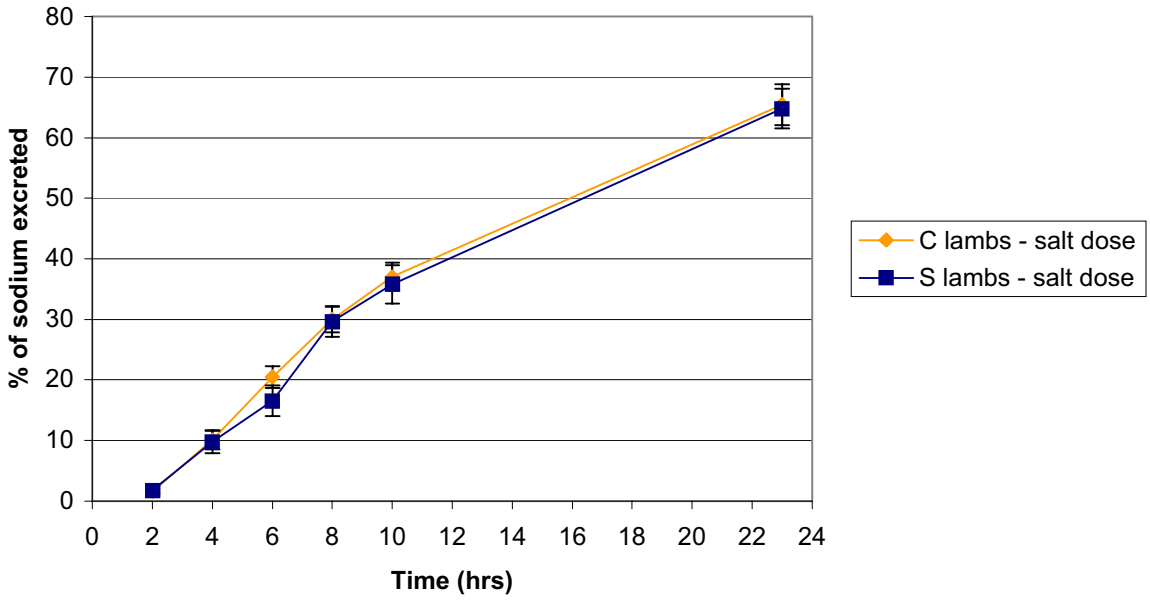

Figure 3.4.1: Cumulative sodium excretion expressed as a percent of administered sodium for both C and S lambs over 23 hours (Mean ± SE).

Dietary salt preference

Preference for salt has been investigated in offspring who were exposed during gestation and/or during early postnatal development (Pittman and Contreras 2002; Smriga *et al*. 2002; Mohamed and Phillips 2003; Curtis *et al*. 2004). Results from these studies found that when exposed to high salt during prenatal and/or postnatal development there was an increased preference for salt. This was not found in the current experiment (P=0.45). The non-significant responses in this experiment may be because the ewes were not fed a high-salt diet after parturition, as the findings with rats have been obtained when animals were exposed to high salt early in their postnatal life (Smriga *et al*. 2002). Another explanation is that the level of 'high salt' in the current experiment was higher compared to previous work in the literature (13% compared with 3%). Therefore, the preference for salt may have been increased in the 'S lambs' of this experiment, but because the dietary salt concentration was so high, their salt appetite was reached with a modest increase in consumption of the 13% salt diet.

Results in the longer-test for preference showed an increase of approximately 6 g Na/day for some S lambs, although not statistically significant. Sodium requirements for sheep are typically reported to be in the range of 0.7-0.9 g/kg DM intake (McDonald *et al*. 1995). Based on these requirements, the animals in this experiment required approximately 1.6 g Na per day (2 kg intake of DM per day). An increase of 6 g Na/day represents a considerable load above basal requirements. Sodium intake was considerably higher than this requirement in all animals in the current study; 10.7 and 13.4 g /day intake for C and S lambs, respectively. It is interesting to note that only two of the eight C lambs tested consumed >20 g Na/day, whereas four of the eight S lambs consumed >20 g Na/day (**Figure 3.4.2**).

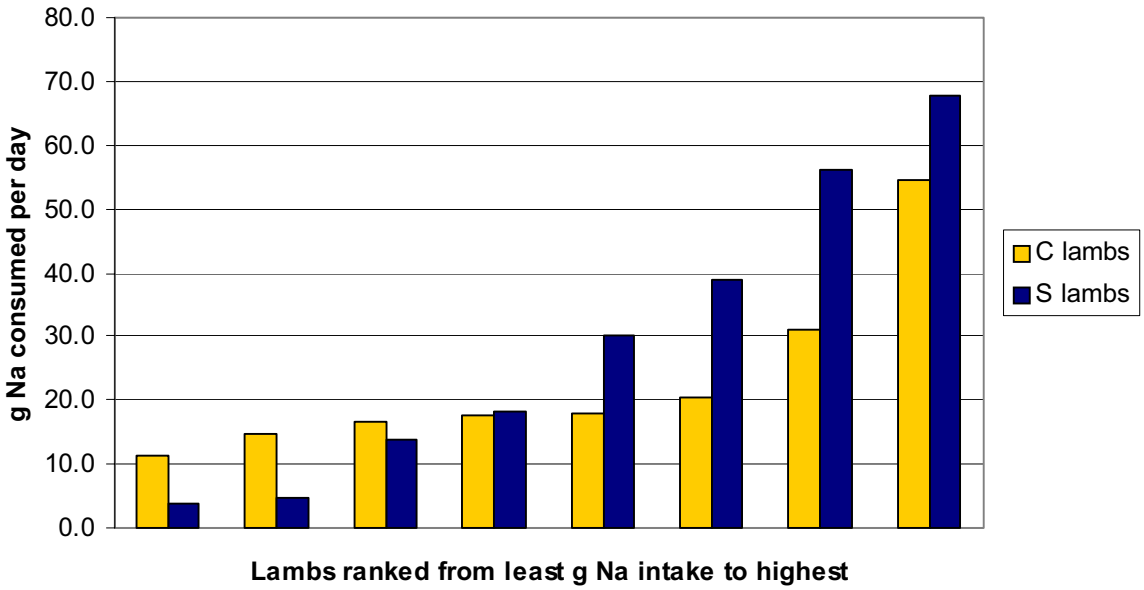

Figure 3.4.2: Sodium intake for C and S lambs, ranked from lowest to highest sodium intake during the long-term preference test (each bar represents an individual animal).

In summary, feeding a high-salt diet through pregnancy affected the aldosterone concentrations of the offspring and their water intake following an oral salt challenge. This is possibly due to the changes in the mother's RAS (Alves da Silva *et al*. 2003). The differences observed between the C and S lambs were: 1). Water intake in the first two hours after an oral salt challenge was 400 mL less for the S lambs suggesting an altered thirst threshold. 2).

Aldosterone concentrations were higher in S lambs when administered a water (control) dose and, when administered a salt dose, the decrease in aldosterone was not as great compared to the C lambs, possibly reflecting a lowered responsiveness to aldosterone. S lambs excreted sodium at the same rate as C lambs however, they did not have to consume the initial high levels of water or decrease their aldosterone concentrations to the same degree as C lambs indicating that physiological set points and/or hormone responsiveness had been altered in offspring by the consumption of a high-salt diet by their mothers (dams) during pregnancy.

CHAPTER FOUR:

Response of the offspring to an oral salt challenge with restricted water intake or to consecutive salt challenges

4.1 INTRODUCTION

Evidence from experiment two (Chapter 3) showed that lambs born to dams fed high-salt during pregnancy (S lambs) drank approximately 400 mL less than the control (C) lambs during the first two hours after a salt dose. This suggests that the thirst set points for the S lambs were higher and the drive to increase water consumption immediately after the salt load was comparatively less than for C lambs. This difference in initial water consumption may influence urinary output and sodium excretion between C and S lambs.

Experiments with humans (Shepard *et al*. 1987; Lindheimer *et al*. 1989; Penney and Hampton 1990; Gordon *et al*. 1997; Evbuomwan *et al*. 2001; Stachenfeld and Keefe 2002; Smith *et al*. 2004) have shown that the thirst threshold is not fixed for an individual, but can change in response to physiological state or dehydration. For example, during pregnancy a decreased threshold stimulates an increase in water intake and dilution of body fluids (Lindheimer *et al*. 1989) that are necessary to maintain pregnancy (refer to Chapter 1, Section 1.3.1). The control of thirst in humans is affected by sodium balance, possibly mediated via endogenous angiotensin II (Gordon *et al*. 1997). Therefore, any change in the angiotensin II or other components of RAS in S lambs in the current work may be manifested in an altered thirst threshold and a greater capacity to deal with excess salt if access to fresh water is limited. Previous results (Chapter 3) showed that C lambs excreted sodium at the same rate as S lambs but their water consumption was initially higher, indicating a greater reliance on increased water intake to deal with excess salt. Limited access to fresh water may compromise the capacity of C lambs to excrete sodium. Thus the hypothesis tested in this chapter is that when C and S lambs have an equal intake of fresh water, restricted to that below the voluntary intake of C lambs over the first 6 hours post-salt challenge, S lambs can excrete sodium faster.

The differences between C and S lambs in response to a single (bolus) salt challenge (Chapter 3) were expressed over the first 4-6 hours and began to diminish over time. Indeed, by the 23-hour point, cumulative sodium excretion was the same across both groups (at approximately 64% of the sodium dose being excreted). This raises two issues. First, it was unexpected to find only two-thirds of a 40 g dose of NaCl excreted in urine over the course of 23 hours. Second, it may be that the differences between C and S lambs evident in the short term do not confer any longer-term advantages to S lambs unless the challenge of salt ingestion is maintained. Therefore in this chapter, the consequences of administering a second dose of salt within one day were investigated. The hypothesis tested is that S lambs will be better able to respond to a secondary dose of salt because of a greater capacity to increase sodium excretion immediately after each oral dose.

4.2 MATERIALS AND METHODS

The same lambs that were used in the salt tolerance test in Chapter 3 were used in the following experiments. Before each experiment animals, were placed into the metabolism crates and given two days to allow them to adapt to the conditions. Between and during experiments all animals were fed to 1.5 maintenance (1kg of lucerne chaff and 500 g of sheep pellets) (8.5% crude protein; 8.5 MJ ME/kg; J.T Johnson and Sons, Kapunda, S.A.). The animals were 8 months of age at the commencement of these experiments.

4.2.1 Salt challenge with restricted water intake

The same protocol was followed as **3.2.3 Salt tolerance test**, however water intake was restricted to a maximum of 500 mL/hr for each animal. Water intake and urine output was measured every hour for 10 hours and then again at 23 hours. Urine samples were analysed for sodium concentration as described in ***3.2.3.1 Sodium concentration analysis.***

4.2.2 Salt challenges at 0 and 8 hours

The same protocol was followed as **3.2.3 Salt tolerance test**, however a second salt challenge was administered at 8 hours. Water intake and urine output was measured at 2, 4, 6, 8, 10, 12, 14, 16 and 23 hours. Eight hours was chosen as the time for the second salt challenge because this is when water intake and urine output has returned to baseline in the previous chapter. Urine samples were analysed for sodium concentration as described in ***3.2.3.1 Sodium concentration analysis.***

4.2.4 Statistical Analysis

Salt challenge with restricted water intake

The data were analysed using Genstat 8th Edition. The model used was a mixed model as it contained both fixed and random effects (**Table 4.2.4**).

For a given transformed response variable $\boldsymbol{y}$ the full model equation was

$$\boldsymbol{y} = \boldsymbol{\mu} + \boldsymbol{u} + \boldsymbol{e}$$

where $\boldsymbol{\mu}$ represents a vector of fixed effects, $\boldsymbol{u}$ represents a vector of random effects and $\boldsymbol{e}$ a random error term.

Each of the fixed effects was tested using a variance ratio statistic obtained from the Mean Square of the fixed effect of interest divided by the Residual Mean Square for that strata of the ANOVA table. Interactions that were not significant were removed from the model. Most of the data had to be transformed to meet the assumptions of the model; outliers were replaced with missing values. These transformations are listed below:

Water intake: Arcsin transformation;

Urinary output: Square root transformation minus 3 outliers;

Sodium excretion: Square root transformation minus 13 outliers

Table 4.2.4: Fixed and Random effects

Variable	Description
Lambs	Random factor (blocking factor)
Time	Random factor (blocking factor) nested in lambs
Days	Random factor (blocking factor) nested in days
Lamb group	Treatment fixed factor (salt or control)
Challenge	Treatment fixed factor (salty or water)
Days	Treatment fixed factor (1 or 2)
Order	Treatment fixed factor (which drench was received first
Time	Treatment fixed factor (2, 4, 6, 8, 10 and 23 hours)

Salt challenge at 0 and 8 hours

In the double dose experiment the data were analysed using the model above. However, due to the abundance of zero values at specific time points for water and/or urine output following the water (control) dose, data were only analysed for the salt challenge. Most of the data had to be transformed to meet the assumptions of the model; outliers were replaced with missing values. These transformations are listed below:

Water intake: Square root transformation minus one outlier;

Urinary output: No transformation required minus four outliers;

Sodium excretion: No transformation but minus six outliers.

As data were analysed for the salt challenge only, the fixed effects sub-model was

$\mu_{ijl} = M_i * O_k * T_l$

where $i = 1,2$ (No. of Lamb group); $k = 1,2$ (No. of Orders); $l = 1,2,...t$ (No. of Times).

The random effects for the sub-model can be expressed as

$u_{al} = \alpha_a + \gamma_{al}$

where $a = 1,2,...,24$ (No. of lambs); $l = 1,2,...,t$ (No. of times). These random effects have assumed distributions:

$\alpha_a \sim N(0, \sigma_\alpha)$: Random effect associated with lambs

$\gamma_{al} \sim N(0, \sigma_\gamma)$: Random effect associated with times nested in lambs.

All data in tables and figures are presented as untransformed values, even though the statistical analysis may have required transformation as described above.

4.2.5 Ethics

The University of Adelaide's Animal Ethics Committee approved the experiments. Approval Number: S-99-2004

4.3 RESULTS

4.3.1 Salt challenge with restricted water intake

The aim to equalise water intake across both groups after the salt challenge was achieved as both C and S lambs consumed the same amount of water throughout the 23-hour period of measurement (**Figure 4.3.1**).

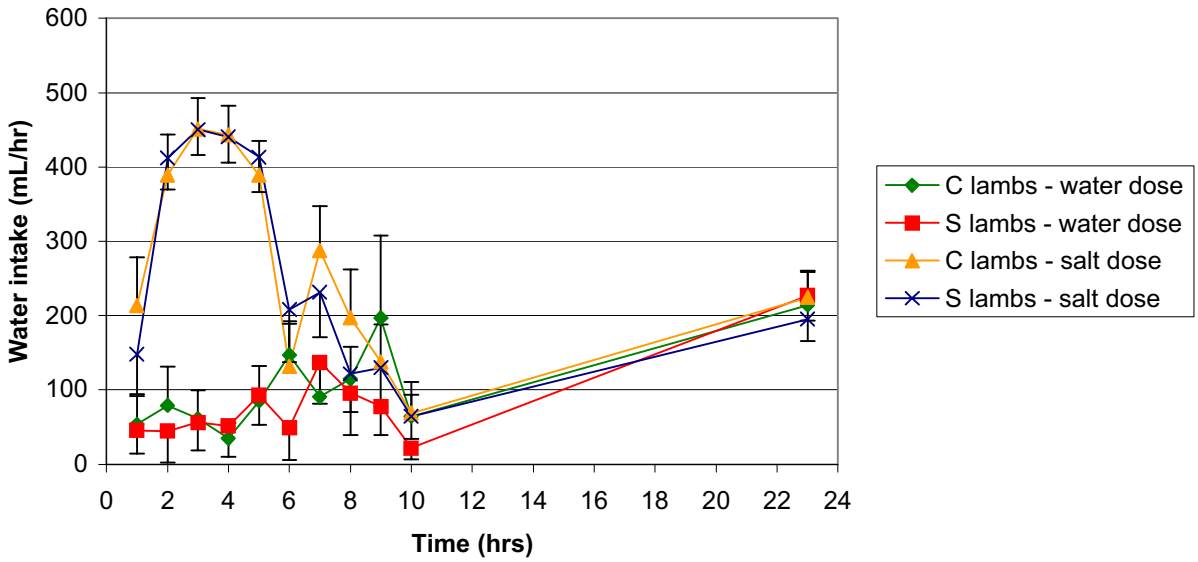

Figure 4.3.1: The average water intake (mL/hr) over 23 hours for C and S lambs administered either a salt or water dose (Mean ± SE).

There was a significant interaction ($P<0.001$) between time, lamb group (ie C and S lambs) and dose for sodium excretion following the salt or water challenges (**Figure 4.3.2**). S lambs excreted less sodium during the first two hours after the salt dose, but rapidly increased sodium excretion to c. 1 g Na/hr within 4 hours, which was approximately double the rate for C lambs at that time. C lambs peaked in sodium excretion at c. 1.3 g Na/hr at 8 hours after the salt dose, by which time the S lambs had begun to decrease their rate of sodium excretion. Thus, S lambs excreted more sodium over the first 4-6 hours, but by 23 hours, both the sodium excretion rate and the total amount of sodium excreted did not differ between C and S lambs.

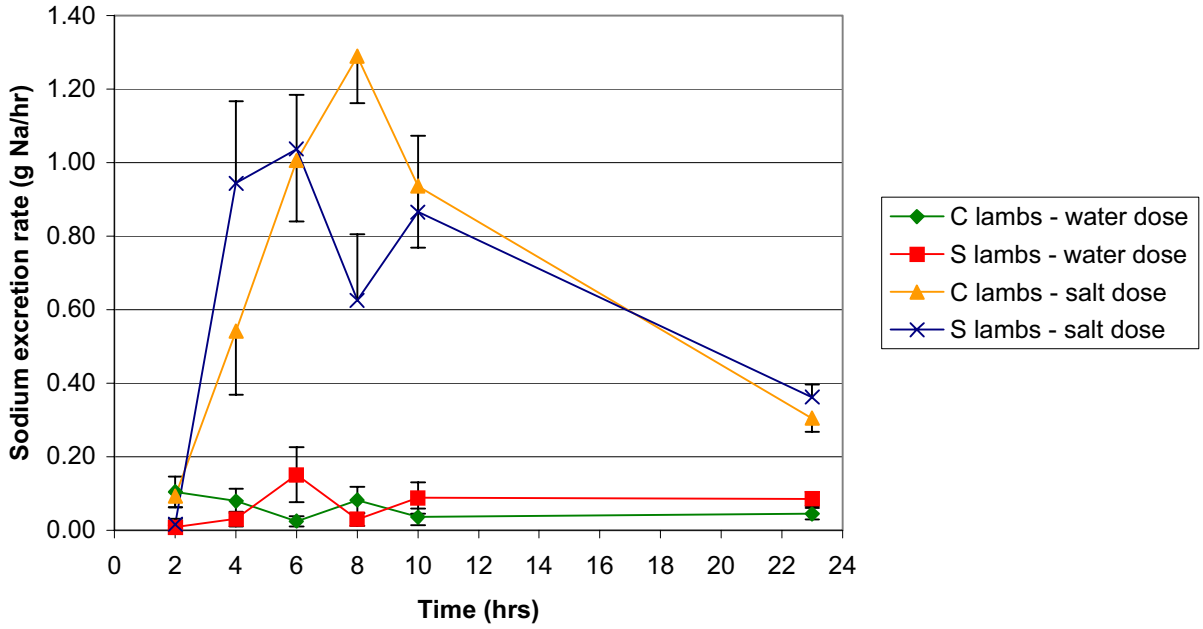

Figure 4.3.2: The rate of sodium excretion (g Na/hr) over 23 hours for C and S lambs administered a salt or control dose (Mean ± SE).

4.3.2 Salt challenge at 0 hours and 8 hours

There was no significant difference in urinary output after both salt challenges between C and S lambs. After the first dose, urinary output increased from about *c.* 60 to 200 mL/hr over the first 4 hours. During the 4 hours immediately after the second dose, it increased further to *c.* 350 mL/hr before declining to *c.* 180 mL/hr at the 23-hour time point.

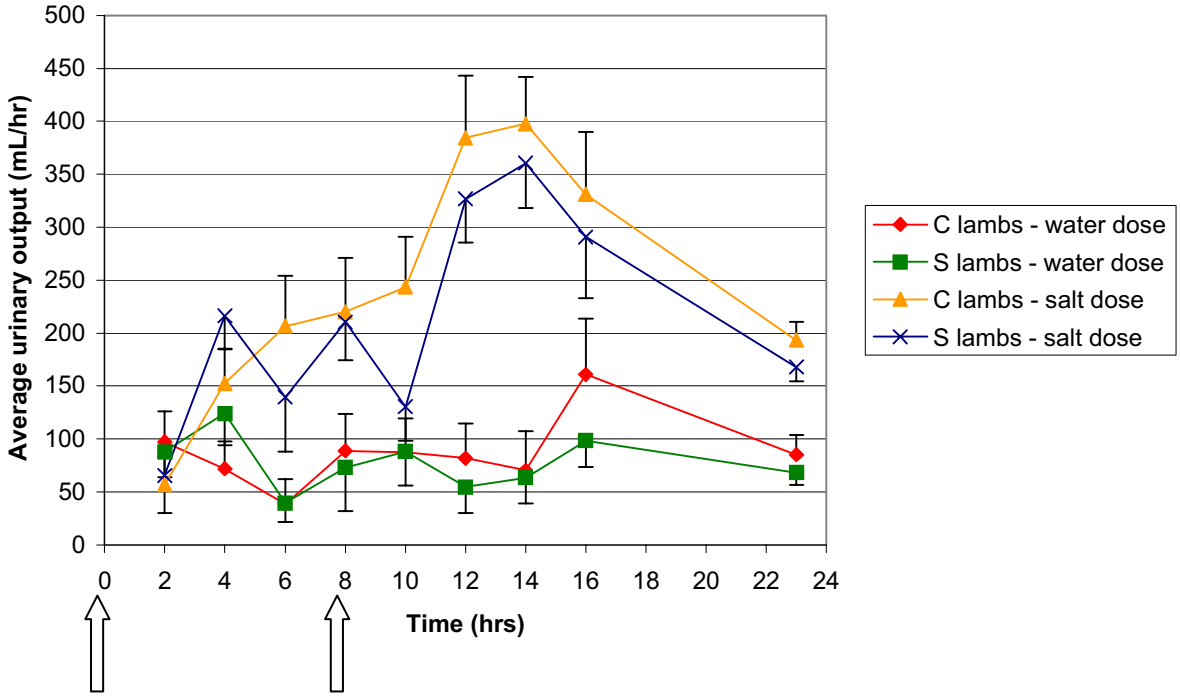

Figure 4.3.2.1: The average urinary output (mL/hr) over 23 hours for C and S lambs administered either a salt or water dose. Arrows indicate the timing of the two salt doses (Mean ± SE).

Sodium excretion (g/hr) reflected the pattern of urinary output. There was an interaction between lamb group (ie C and S lambs) and time (P=0.08) on sodium excretion caused by the lower sodium excretion at 8 hours for C lambs in comparison to S lambs. After the second salt challenge there was a marked increase in the rate of sodium excretion for both C and S lambs, a pattern that was the same for C and S lambs (**Figure 4.3.2.2**). This equal sodium excretion rate was achieved even though S lambs had a tendency to produce less urine between 10 and 23 hours (**Figure 4.3.2.1**), which indicates that S lambs had a more concentrated urinary output (**Figure**. **4.3.2.3**).

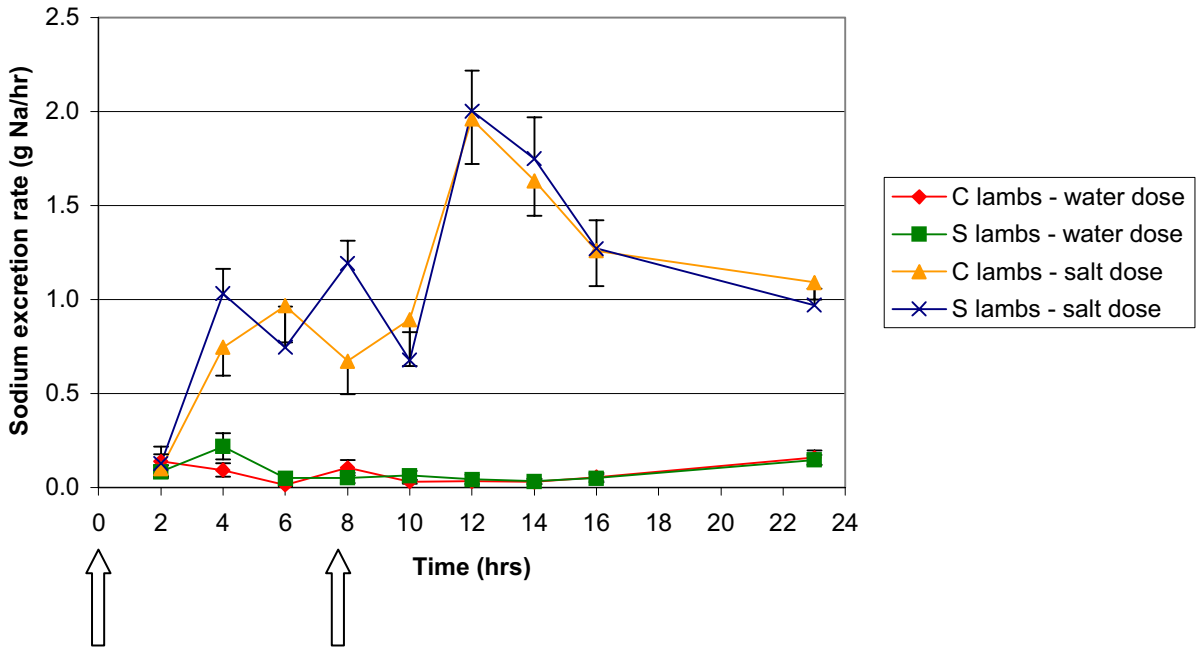

Figure 4.3.2.2: The average sodium excretion (g Na/hr) over 23 hours for C and S lambs administered either a salt or water dose (Mean ± SE).

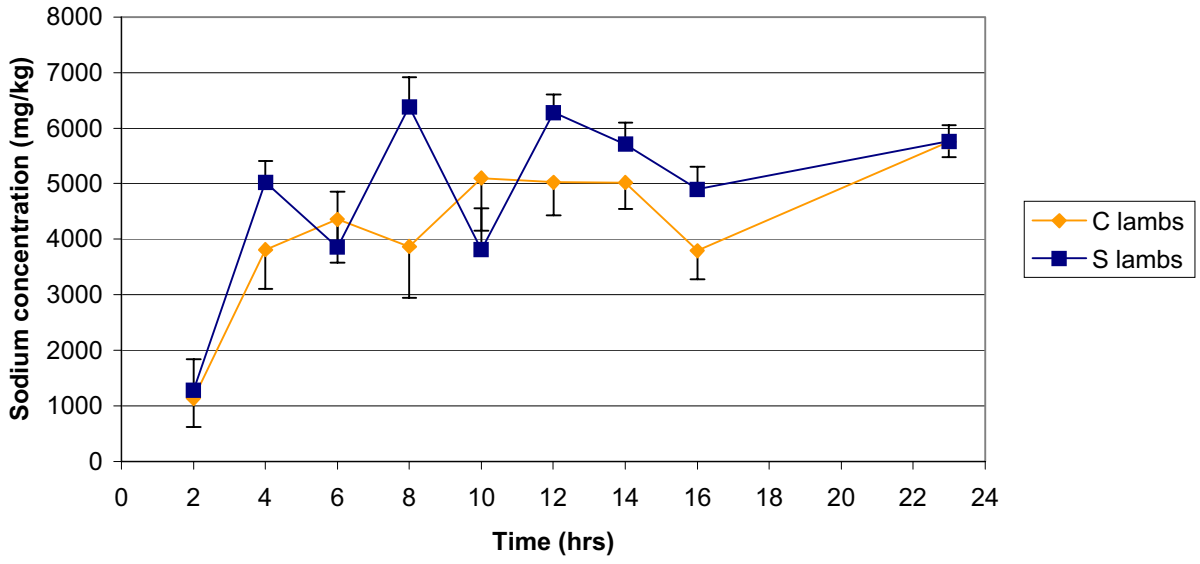

Figure 4.3.2.3: The average sodium concentration in urine for C and S lambs when administered a salt dose (Mean ± SE).

Figure **4.3.2.4** shows that only about 40% of the first dose of sodium was excreted by the time of the second dose, but by 23 hours c. 95% of the two doses had been excreted. This indicates that under the conditions of the 'double dose' experiment, all lambs were able to return to sodium balance within one day.

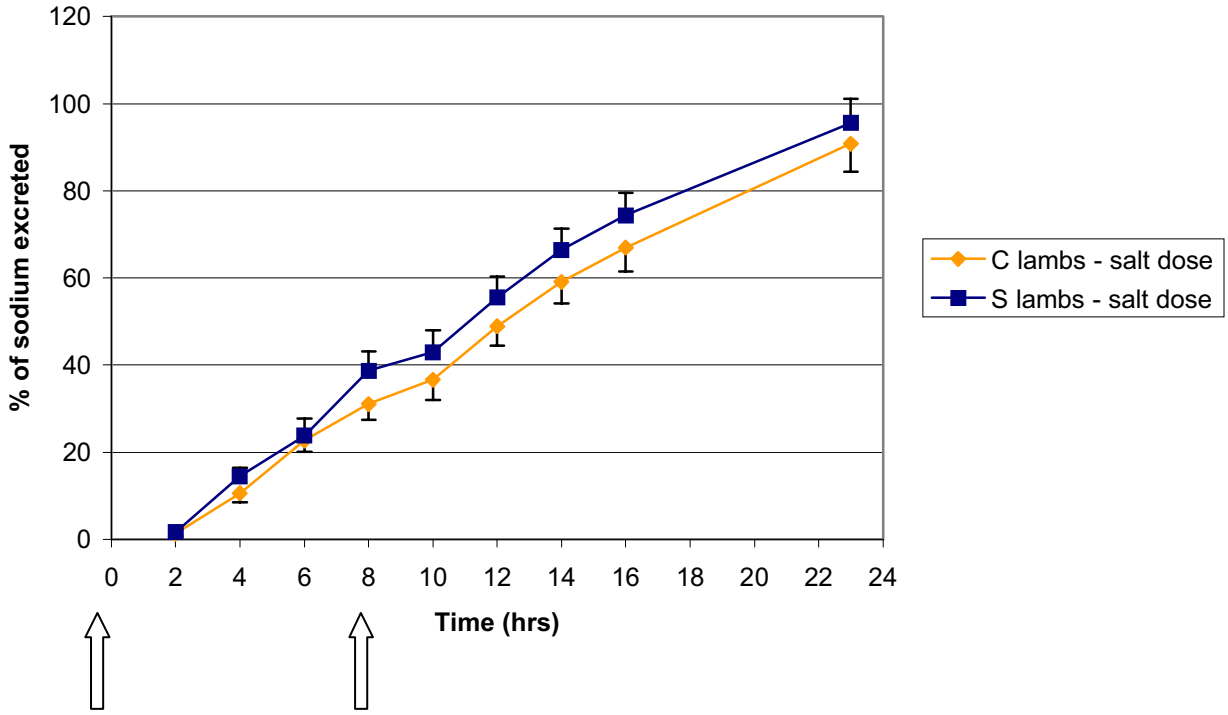

Figure 4.3.2.4: Cumulative sodium excretion expressed as percent of sodium administered for both C and S lambs over 23 hours (Mean ± SE).

4.4 DISCUSSION

The first hypothesis of this Chapter can be accepted because restricting the water intake of the C lambs below their *ad libitum* intake of fresh water over the first few hours reduced their short-term (2-4 hours) capacity to excrete sodium. Therefore, the S lambs appeared to have a physiological advantage during the first 4-6 hours after the oral salt dose. When C and S sheep both had free access to fresh water in Chapter 3, this advantage was not apparent. These data suggest that (i) when sheep are consuming a high-salt diet and not able to regularly consume fresh water immediately after ingesting the salt, S lambs may be better able to cope; and (ii) if fresh water is freely available, over time and in close proximity, then the differences between C and S sheep may not be apparent.

The observed short-term increase in excreting the salt load for S lambs indicates a greater capacity to rapidly increase the sodium concentration in urine (5500 vs 4000 mg Na/kg urine). This could possibly be due to decreased reabsorption of sodium, fewer renal receptors for AVP, or an altered distribution of aquaporins in the tubules. These possibilities will now be discussed briefly.

The amplitude of the cortico-medullary gradient governs the final urine concentration and is generated by the counter current multipliers of the descending and ascending limbs of the loop of Henle (de Rouffignac 2001). An increase in the length of the loop of Henle is likely to increase the hypertonicity of the solution in the tubules. An example of this change in architecture is found in desert animals which produce extremely concentrated urine, and evidence shows that a correlation exists between the length of the thin limb and the ability to concentrate urine (Mbassa 1988). Therefore the longer the loop of Henle, the more efficient retention of water and the more concentrated urine is produced by building an osmotic gradient along the extended loop of Henle (Shanas and Haim 2004). There is also evidence

that dietary salt concentration can influence the area of short and long loops of the medullary thick ascending limb in rat kidneys (Stillman *et al*. 1994). In addition to morphological changes, dietary salt is known to cause physiological changes in the kidney, such as medullary osmolality, endothelin production (Herrara and Garvin 2005) and expression of endothelial nitric oxide synthase (Mattson and Higgins 1996). These changes may be involved in the adaptive responses to a dietary salt load. It remains to be tested if such changes noted in other species also occur in sheep and if any of these characteristics may be altered by the foetal environment associated with high-salt feeding during pregnancy.

Higher aldosterone concentrations observed in S lambs (Chapter 3) could reflect a lowered responsiveness to aldosterone. Aldosterone promotes sodium reabsorption so the lowered responsiveness to aldosterone results in a decreased reabsorption of sodium in the kidney, and higher concentrations of sodium in the urine immediately after a single dose of NaCl.

Another important factor in determining the sodium concentration of urine is AVP. When AVP concentrations are increased, the final urinary output is hypertonic as the kidneys retain as much water as possible to compensate for a deficit in body water. No differences were observed between AVP concentrations for the C and S lambs in Chapter 3, but the number of receptors for AVP may have differed. If receptors for AVP were lower in the S lambs there would be a decreased reabsorption of water from the loop of Henle, resulting in a more concentrated urinary output. Park *et al*. (1998) found that vasopressin V_2 receptor (V_2R) mRNA decreased in a time dependent manner in the cortex and medulla through 48 hours of water restriction in rats. Their data suggested that water restriction leads to a regional time-dependent down-regulation of V_2R mRNA and protein within the rat kidney allowing for maximisation of concentrating urine and minimising water loss. The current experiment did not measure AVP receptors, so it is not possible to confirm if receptor numbers or their

binding affinity were down regulated when water was restricted, or whether differences existed between C and S lambs.

Another possible explanation for a higher urinary sodium concentration is a decreased expression of aquaporin 2 in S lambs. Aquaporin 2 plays a pivotal role in renal water regulation. An example of changes in aquaporin 2 expression without differences in AVP concentration is shown in a study by Ohara *et al*. (1998). They found that AVP concentration in pregnant rats did not differ significantly from non-pregnant rats but the mRNA of aquaporin 2 was increased in pregnancy, as was aquaporin 2 protein. This indicated that up-regulation of AQP2 contributes to water retention during pregnancy even though AVP remains unchanged. Therefore hormonal changes associated with pregnancy appear to influence AQP2 expression without influencing AVP concentration. It remains to be tested if the changes induced by feeding a high-salt diet during pregnancy, such as an alteration in the ewes' RAS system, may cause similar changes in renal aquaporin 2 expression in the offspring.

A practical implication of an adaptation that allows a rapid increase in the renal capacity to concentrate sodium in urine is that if fresh water is limited when animals are grazing high-salt forages (eg brackish water, or if fresh water is distant from foraging sites), 'adapted' animals may (i) be able to consume more feed before needing to stop and drink or (ii) take longer to reach their salt limit and thus maintain intake and liveweight above 'control' animals. Further experiments are required and warranted to quantify any practical benefits.

A marked difference between field conditions with sheep grazing high-salt forages and both the first experiment in this chapter and the preceding experiment in Chapter 3 is that in the former case, sheep receive a near-continuous salt 'challenge' rather than a moderately-sized,

one-off dose. The second experiment in this chapter was designed to test the effects of two salt challenges within 8 hours of each other (with free access to fresh water). This showed, consistent with the first experiment of this chapter, that S lambs excrete more sodium over the first 4 hours after one dose but, after the second dose, sodium excretion was identical. Therefore, the second hypothesis for this experiment is rejected. This suggests that any benefit conferred to S lambs in terms of adaptability to salt ingestion will only be evident in the immediate short-term, and that under conditions of repeated (or continual) oral salt 'challenge', all sheep (C and S lambs) have virtually the same capacity to excrete excess sodium. The consequences of a continual salt challenge (rather than two separate doses as used here), combined with a limited capacity to consume more water to maintain salt balance, will be investigated further in the next chapter.

CHAPTER FIVE:

Response of the offspring to salty drinking water

5.1 INTRODUCTION

In Chapter 3 it was found that C lambs and S lambs excreted sodium after an oral salt dose at the same rate, but the immediate increase in water consumption was less in S lambs than C lambs. S lambs also exhibited a smaller decrease in aldosterone concentration after the salt dose, indicative of a lowered responsiveness to aldosterone. In Chapter 4 it was found that limiting access to fresh water resulted in S lambs excreting sodium faster over the first 8 hours, suggesting that S lambs have a greater renal capacity to concentrate sodium in the urine. To further investigate whether S lambs do indeed have a greater capacity to concentrate sodium, a salt load was administered in the following experiment by replacing fresh drinking water with salty water (1.5% NaCl). This methodology was used in an attempt to restrict the opportunity for the animals to consume more water to reduce the osmolality of body fluids and promote the mechanisms that concentrate sodium in the urine. Thus the focus was on comparing renal capacities of the C and S lambs.

Peirce (1957) indicated that a NaCl concentration of 1.0% in drinking water had no adverse effects on sheep in terms of body weight or feed intake. A small proportion of sheep in the study where affected by 1.5% NaCl and all sheep in the study were affected by 2.0% NaCl, as evidenced by a decline in food consumption and body weight with those receiving the 2.0% NaCl becoming emaciated and weak. As the NaCl concentration in drinking water increases to 1.5%, the intake of water also increases (Peirce 1957; Potter 1961; Wilson 1966), as does the excretion of urine (Peirce 1957; Potter 1961). Potter (1961) found that the glomerular filtration rate and filtration fraction increased, together with a slight reduction in effective renal plasma flow for sheep exposed to 1.3% NaCl in the drinking water. This suggested that the sheep had efficient renal mechanisms for the rapid elimination of excess sodium chloride. Potter (1961) hypothesized that the increased glomerular filtration rate was probably the result of either an increase in the number of functioning glomeruli or an increased hydrostatic

glomerular pressure resulting from an increase in arterial blood pressure. Potter (1963) investigated this hypothesis and found that the prolonged ingestion of 1.3% NaCl did not induce an increase in the number of active tubules in the kidneys. However it confirmed that the elimination of additional salt is accomplished by a reduction of reabsorption in individual nephrons.

An increased renal capacity in S lambs would likely be beneficial when sheep are grazing halophytic plants. Animals may have a greater feed intake if salt could be excreted at faster rates. Further, if access to fresh water was limited, salt excretion would not be jeopardized as much compared to the animals that are more reliant on increased water consumption to deal with an excess salt load. In the following experiment the hypothesis tested was that lambs born to mothers fed high salt during pregnancy (S lambs) are able to excrete sodium faster after a 48-hour oral salt load in the drinking water. In addition to urinary and sodium excretion, water intake and feed intake were also measured.

5.2 MATERIALS AND METHODS

5.2.1 Salt tolerance test using salt water (1.5% NaCl)

The 24 lambs used in this experiment were the same lambs used in Chapters 3 and 4. The age of the animals at the commencement of these experiments was 10 months. Prior to the treatment being applied, all lambs were placed in metabolism crates for two days for adaptation and on the second day catheters were inserted into the jugular vein for blood sampling. The catheters were flushed with heparin saline solution (1000 IU heparin/mL) after each sample. Twelve lambs (six C lambs and six S lambs) were offered *ad libitum* amounts of drinking water containing 1.5% NaCl and a conventional feed (sheep pellets; 8.5% crude protein 8.5 MJ ME/kg; J.T Johnson and Sons, Kapunda, S.A.) for two consecutive days. Blood and urine samples were collected at 0, 2, 4, 8, 20, 24, 28, 32, 44 and 48 hours. Water and feed were measured at 0.5, 1, 1.5, 2, 4, 8, 20, 24, 28, 32, 44 and 48 hours. The experiment was then repeated on the remaining 12 lambs.

5.2.2 Statistical analysis

The data were analysed in S-PLUS 6.2 (Insightful Inc.) using a package called Spatial Analysis of Mixed Models. For a given transformed response variable $\boldsymbol{y}$ the full model equation is

$$\boldsymbol{y} = \boldsymbol{\mu} + \boldsymbol{u} + \boldsymbol{e} \qquad \textbf{(1)}$$

where $\boldsymbol{\mu}$ represents a vector of fixed effects, $\boldsymbol{u}$ represents a vector of random effects and $\boldsymbol{e}$ a random error term. This is a mixed model as it contains both fixed and random effects.

The fixed effects component of **(1)**, $\boldsymbol{\mu}$, can be expressed as

$$\mu_{il} = M_i * T_l \qquad \textbf{(2)}$$

where $i = 1,2$ (No. of Lamb group); $l = 1,2,...t$ (No. of Times). The random effects component of **(1)**, $\boldsymbol{u}$, can be expressed as

$$u_a = \alpha_a \qquad \textbf{(3)}$$

where $a = 1,2,...,24$ (No. of lambs). As the data were obtained at irregular intervals over the two-day period it was necessary to allow for correlations that might be present across the sampled time periods. To do this, the random error term, $\boldsymbol{e}$, in **(1)** was allowed to have a complex correlation structure of the form

$$\text{var}(e) = \sigma^2 (I_r \phi C) \qquad \textbf{(4)}$$

where r is the number of lambs in the experiment and the matrix $\boldsymbol{C}$ is a $l \times l$ matrix with ij th entry $\phi^{|t_i - t_j|}$, $i \neq j$ with t_i representing the i th irregularly sampled time period and ϕ, σ^2 are unknown parameters estimated from the data. For simplicity, in this particular case, the matrix $\boldsymbol{C}$ is a common correlation matrix for all the lambs. This form of correlation is known as an exponential correlation structure which is commonly used to model correlated data where samples are taken at irregular intervals.

Most of the data had to be transformed to meet the assumptions of the model; outliers were replaced with missing values. These transformations are listed below:

Aldosterone concentration in plasma: Log transformation;

AVP concentration in plasma: Square root transformation;

Water intake: Square root transformation;

Urinary output: Square root transformation;

Sodium excretion: Power 0.25 transformation;

Feed intake: Square root transformation

Data presented in tables and figures are untransformed values, even though the statistical analysis may have required transformation as described above.

5.2.3 Ethics

The University of Adelaide's Animal Ethics Committee approved the experiments. Approval Number: S-027-2005

5.3 RESULTS

5.3.1 Water intake

There was a significant interaction between lamb group (C and S lambs) and time for water intake (P=0.0003). During the first 20 hours, C lambs had a higher water intake compared to the S lambs. At 24 hours, the reverse occurred, with S lambs consuming more. From 28 hours onwards water intake did not differ between C and S lambs (**Figure 5.3.1**).

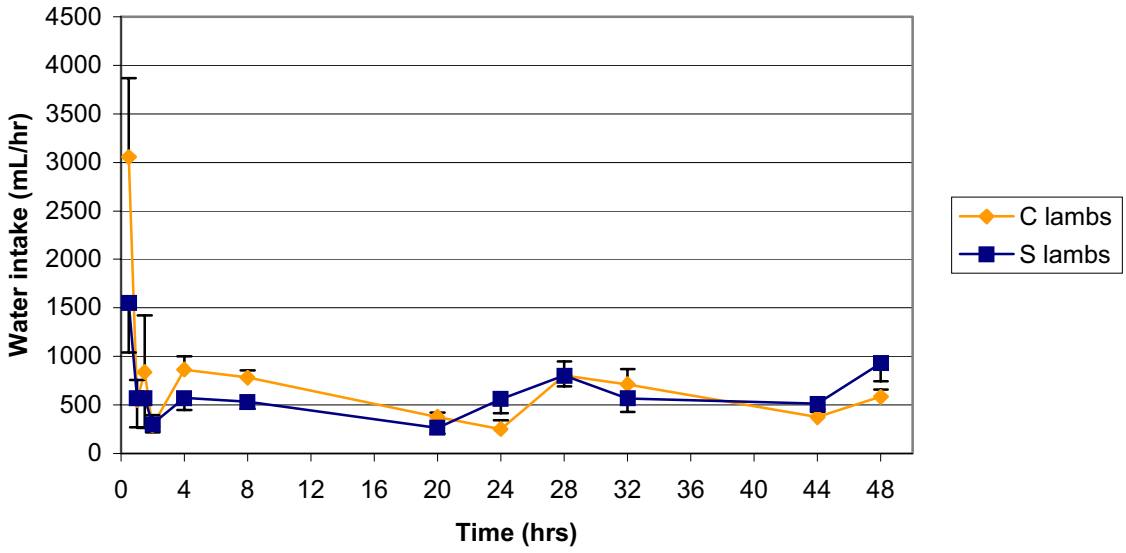

Figure 5.3.1: The rate of water intake (mL/hr) for C and S lambs over 48 hours when receiving *ad libitum* salty drinking water (Mean ± SE).

5.3.2 Urinary output

There was a significant interaction between lamb group (C and S lambs) and time for urinary output (P=0.038). In the first 24-hour period C and S lambs increased their urinary output, with C lambs urinating approximately 450-500 mL/hr and S lambs urinating 400 mL/hr. In the second 24-hour period the S lambs increased their urinary output again to approximately 550 mL/hr, where C lambs remained around 400mL/hr (**Figure 5.3.2**).

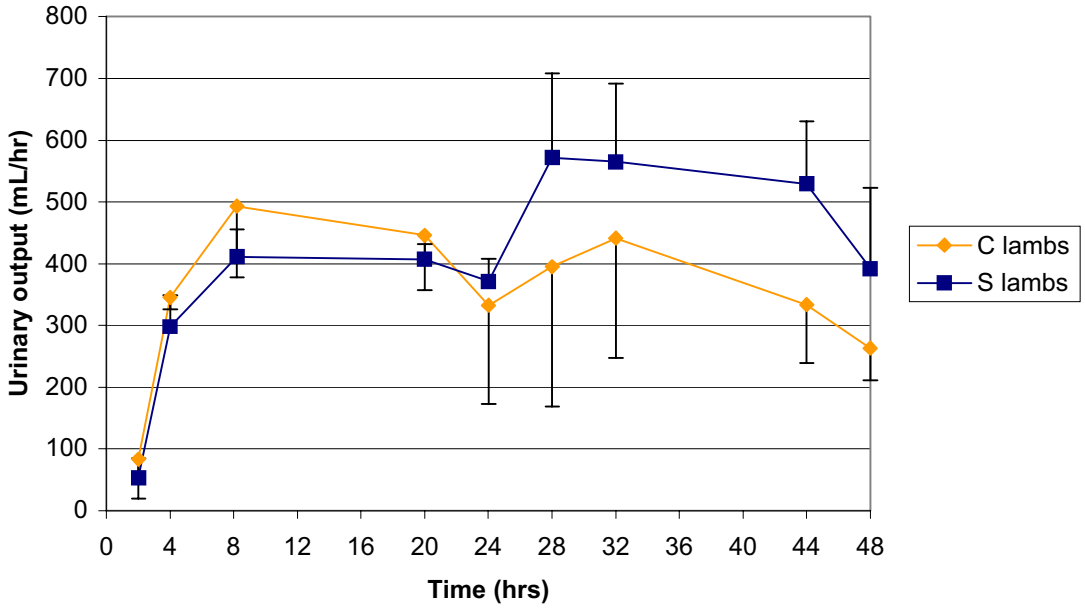

Figure 5.3.2: The rate of urinary output (mL/hr) for C and S lambs over 48 hours when receiving *ad libitum* salty drinking water (Mean ± SE).

5.3.3 Sodium excretion

There was no significant difference between C and S lambs for sodium excretion. All lambs rapidly increased the rate of sodium excretion from 0.2 g/hr to approximately 2.5 g/hr and maintained this rate over the 48-hour period (**Figure 5.3.3**). There was a tendency, though, for sodium excretion to decline in C lambs during the last 16 hours of the experimental period, which was similar to the output of urine described above.

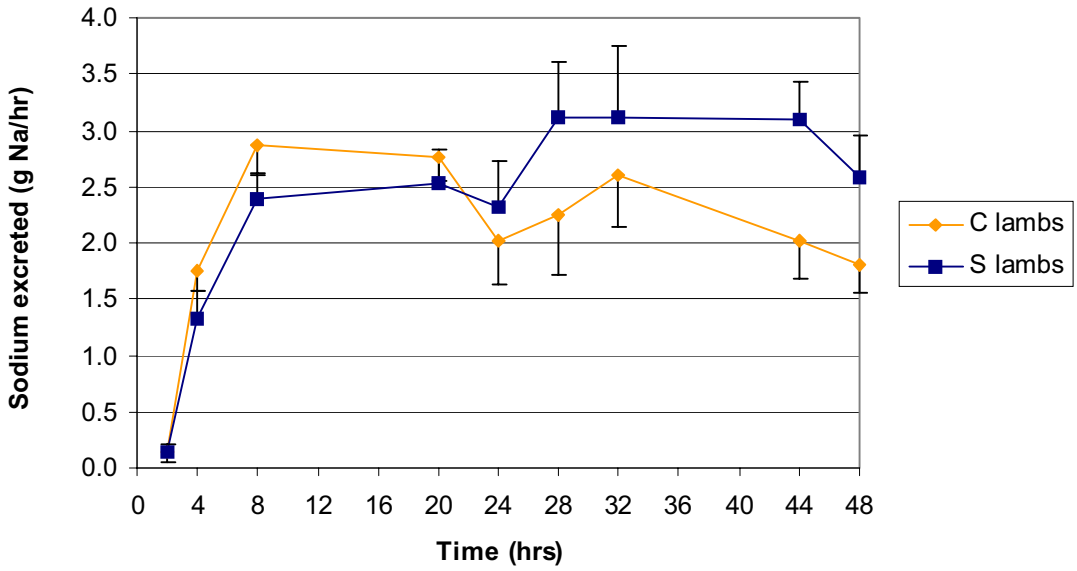

Figure 5.3.3: The rate of sodium excretion (g Na/hr) for C and S lambs over 48 hours when receiving *ad libitum* salty drinking water (Mean ± SE).

5.3.4 Feed Intake

There is a significant difference between C and S lambs for feed intake (P=0.009). After one hour of offering salty water, feed intake dropped severely from c. 800 g/hr to approximately 100 g/hr for both C and S lambs. On average over the two days, S lambs had a voluntary feed intake 35% lower than C lambs (0.89 kg/day vs. 1.36 kg/day). Data presented in **Figure 5.3.4** has excluded the first hour of feed intake and shows the average daily intake for C and S lambs for each of the two days.

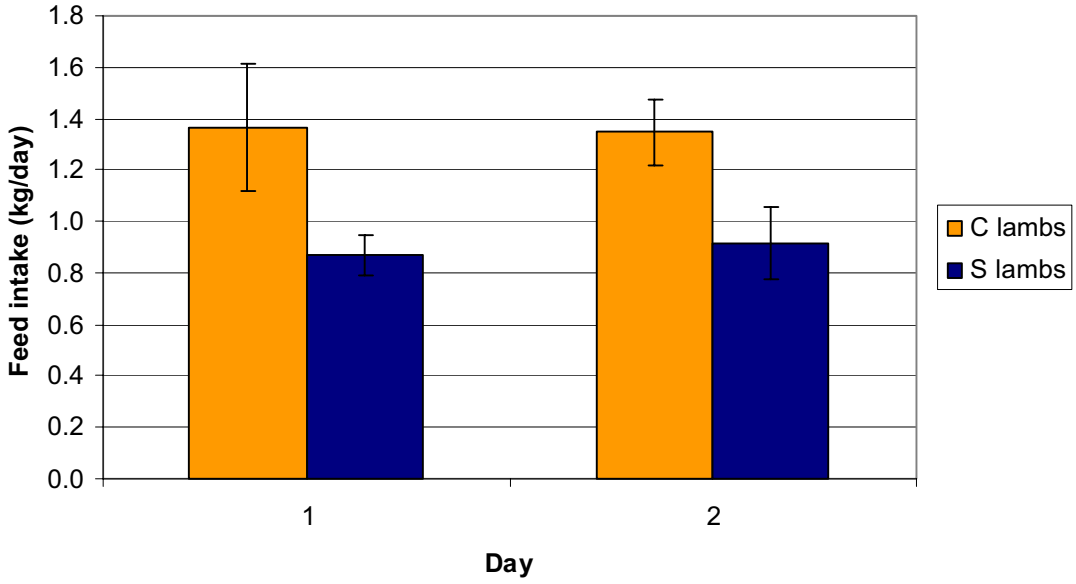

Figure 5.3.4: The average feed intake (kg/day) for C and S lambs over 2 days when receiving *ad libitum* salty drinking water (Mean ± SE).

5.3.5 Aldosterone and AVP concentration

For both aldosterone and AVP concentrations there was a significant interaction between lamb group (C and S lambs) and time (P=0.0216 and P=0.019 for aldosterone and AVP respectively).

Aldosterone concentration decreased in all sheep from 20 pg/mL to 10 pg/mL within the first 2 hours (**Figure 5.3.5**). C lambs continued to decline to 5 pg/mL at 20 hours before increasing to approximately 8 pg/mL during the second 24-hour period. S lambs had a slightly different pattern after 2 hours: the decline in aldosterone concentrations was smaller than C lambs and not until 28 hours did they reach 5 pg/mL. There was an increase between 28 and 32 hours before S lambs decreased again. At 48 hours, both C and S lambs had a concentration of 5 pg/mL (**Figure 5.3.5**).

At 8 hours C lambs had a higher AVP concentration than S lambs (**Figure 5.3.6**) and this was maintained until 20 hours. At 28 hours the S lambs increased their AVP concentration from 30 to 40 pg/mL before declining in the following 4 hours to 30 pg/mL, and both S and C lambs maintained this concentration over the last 16 hours of the 48-hour period.

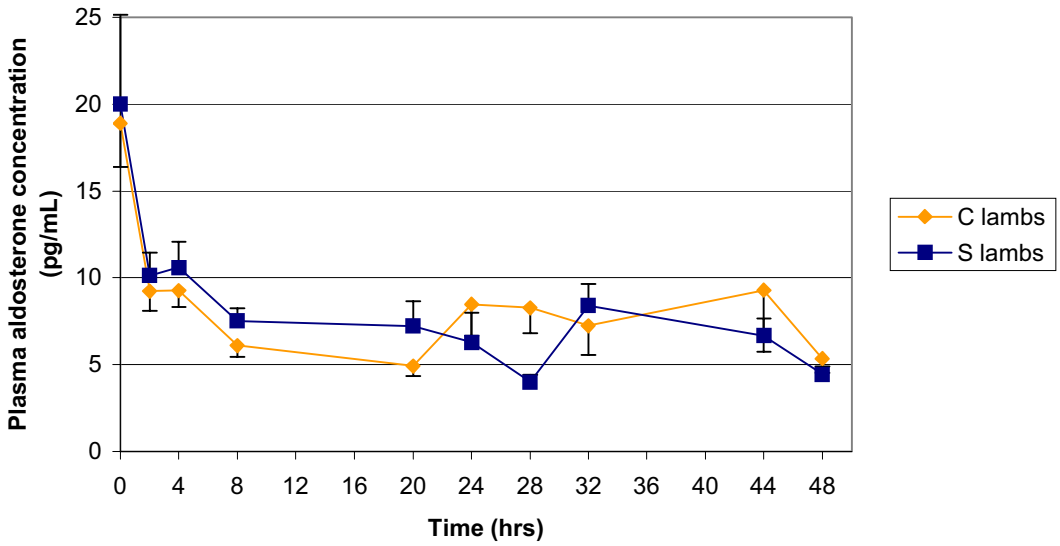

Figure 5.3.5: Plasma aldosterone concentration for C and S lambs over 48 hours when receiving *ad libitum* salty drinking water (Mean ± SE).

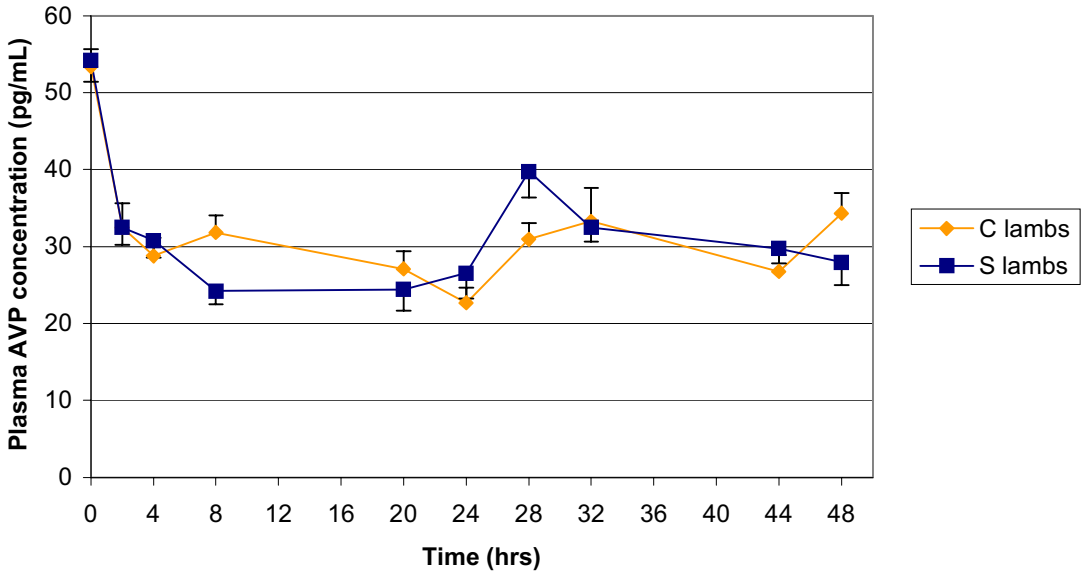

Figure 5.3.6: Plasma AVP concentration for salt and control lambs over 48 hours when receiving *ad libitum* salty drinking water (Mean ± SE).

5.4 DISCUSSION

Animal responses to *ad libitum* drinking water containing 1.5% NaCl showed that there was no significant difference between C and S lambs in sodium excretion rates. Therefore the hypothesis is rejected. There were, however, consistent results with Chapter 3 for water consumption and aldosterone concentrations. Again, results showed that C lambs had higher water consumption immediately after an increase in salt ingestion, indicating that the initial drive to drink in C lambs is greater than for S lambs. The drop in aldosterone concentration was, again, less in S lambs than C lambs. Collectively these results provide more evidence to support the hypotheses that S lambs have a higher thirst threshold (the initial drive to drink is reduced) and S lambs may have a lowered responsiveness to aldosterone.

AVP concentrations also differed between C and S lambs, a response that was not evident in the other salt challenge experiments (Chapters 3 and 4). AVP concentrations from 8 to 20 hours, after the start of the experiment, were higher in the C lambs than the S lambs. The difference in AVP concentrations could be linked to the patterns of change in plasma aldosterone concentration and water consumption observed in this and previous experiments. Both AVP, aldosterone and water consumption are involved in controlling osmolality, and AVP and aldosterone interact through effects of the RAS. Aldosterone enhances the active reabsorption of sodium ions from the filtrate, and increases the synthesis of AVP. AVP synthesis increases permeability of the distal tubule and collecting duct to water, promoting its reabsorption. Aldosterone then acts with AVP to enhance both sodium and water reabsorption by the kidney (Randall *et al*. 2002). As previously stated in earlier chapters, studies (Arguelles *et al*. 1996; Butler *et al*. 2002; Alves da Silva *et al*. 2003) have shown that high maternal salt (3-8% NaCl) during gestation can change the RAS in the offspring. Such changes include increased angiotensin II, higher blood pressure and lower responsiveness of blood pressure to salt intake (Alves da Silva *et al*. 2003) and increased sensitivity to angiotensin II (Arguelles *et al*. 1996). These changes may have been the result of a feedback

mechanism in which angiotensin II receptors were up-regulated in the foetus in response to lower activity of the RAS in the mother (Arguelles *et al*. 1996; Butler *et al*. 2002). This evidence provides support to the notion that changes in the mothers RAS may change sensitivity and/or secretion of hormones in the offspring's RAS resulting in differed responses to high salt.

The differences observed in AVP concentrations between this experiment and previous experiments are likely to have arisen because the salt challenge was administered via salty drinking water rather than a single oral dose. In the previous experiments, AVP concentrations did not need to increase because fresh water was readily available and animals were able to consume more water to control osmolality. Without fresh water being available in the present study, increased AVP concentrations were presumably required to increase water reabsorption in the kidneys to help cope with the excess salt load. A study by Cowley *et al*. (1986) provides evidence towards this hypothesis. Dogs fed high-salt diets with access to readily available fresh water did not increase AVP concentrations, as the primary adaptive mechanism was the consumption of water. However, if access to fresh water was restricted dogs fed high-salt diets increased AVP concentrations to control osmolality of body fluids.

In the present experiment, it was also hypothesised that S lambs, if able to excrete sodium at a faster rate, would have a higher feed intake than C lambs. The opposite response in feed intake was found even though no difference was observed in sodium excretion. S lambs had a voluntary feed intake 35% lower than C lambs (0.89 kg/day vs. 1.36 kg/day), which is approximately 0.5 kg/day less than C lambs. This is the first finding that shows offspring of dams fed high salt during pregnancy have a lower voluntary feed intake. Presumably, if this had continued, S lambs would have decreased in liveweight and thus it is important to determine the cause of a depressed feed intake. The immediate effect of salt on feed intake is

consistent with Thomas *et al.* (2007) where feed intake was high during the first half hour after a high-salt (18.5% NaCl) diet was introduced, followed by a dramatic decrease (approximately 200g DM/sheep h) and then a consistent rate of intake thereafter.

It is possible that the differences observed in feed intake may be due to changes in leptin or insulin secretion or plasma concentrations (Baskin *et al*. 1999). Increased leptin concentrations have been shown to suppress feed intake (Barb *et al*. 1998; Morrison *et al*. 2001) and does so by inhibiting glucose absorption in the small intestine. Leptin concentration is influenced by nutritional status (Morrison *et al*. 2001; Morrison *et al*. 2002) and further studies have shown that high-salt intake could contribute to elevated plasma leptin levels (Dobrian *et al*. 2003). The mechanism for possible increased leptin concentrations in response to a high-salt intake is unclear. However it is possible that the S lambs may have been influenced by their dam's diet of high-salt intake during pregnancy, which may have programmed S lambs to have higher concentrations of leptin postnatally. Leptin levels were not measured in this experiment thus further work should be carried out to determine whether leptin is a factor influencing the decreased feed intake in S lambs.

The regulation of feed intake and insulin are also related, however the evidence from various studies is conflicting. Some studies have found increased insulin results in increased feed intake (Houpt 1974; Rodin 1985; Roberts *et al*. 1994), where others have found that increased insulin decreases feed intake (Porte and Woods 1981; Woods *et al*. 1985; Woods *et al*. 1990; Isganaitis and Lustig 2005).

It has been reported that high-salt diets may be a factor in promoting insulin resistance (Ogihara *et al*. 2001) due to an increase in circulating free fatty acids (Donovan *et al*. 1993). Randle *et al*. (1963) proposed that via substrate competition, increased circulating free fatty

acids caused insulin resistance, from an inhibition of key rate limiting enzymes involved in glycolysis and glucose oxidation. The mechanism by which sodium intake influences circulating free fatty acids is unclear, however the observed insulin resistance to a high-salt diet may represent a normal physiological response to dietary salt loading (Donovan *et al*. 1993). Insulin resistance is coupled with increased insulin secretion, due to homeostatic mechanisms compensating the insensitivity (Isganaitis and Lustig 2005), thus resulting in higher concentrations of insulin. One report (Vidonho *et al*. 2004) has shown that low-salt diets during pregnancy and lactation in rats causes insulin resistance in the offspring, thus providing evidence that maternal salt during pregnancy and lactation have long term influences on insulin sensitivity of the adult offspring. It remains to be tested if high-salt feeding during pregnancy has an effect on insulin resistance in the offspring. The secretion of insulin and leptin is influenced by the amount of adipocytes and short-term changes in energy and consequently, leptin secretion under 'normal' conditions is under the control of insulin and glucocorticoids (Isganaitis and Lustig 2005). It is possible that both leptin and insulin act together to decrease feed intake in response to high salt ingestion.

Further work is essential to establish why offspring born to dams fed high salt diet during pregnancy have a lower feed intake as it could result in a decreased liveweight when animals are exposed to long periods of high salt.

In summary, when C and S lambs are exposed to 1.5% NaCl in their drinking water there was a difference in adaptive mechanisms between C and S lambs. As seen in previous chapters, C lambs had lower aldosterone concentrations and an initial higher drive to increase water consumption. AVP concentrations were increased to contribute to maintaining osmotic balance, which could not be achieved by increased water consumption and sodium excretion alone, and were higher in C lambs than S lambs. C lambs were found to consume more feed

than S lambs, although the mechanisms behind this remains unknown. Collectively, the differences observed between C and S lambs provides evidence to support the general hypothesis that high salt during pregnancy influences adaptive mechanisms in response to salt ingestion in the offspring.

CHAPTER SIX:
GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

Saline lands are increasing rapidly within Australia particularly in Southern and Western Australia. McFarlane *et al*. (2004) estimates that 4.4 million hectares in Western Australia are at risk with a predicted increase of approximately 14,000 hectares per annum. In South Australia 4.1 million hectares are at risk with some predictions suggesting up to 60% may be affected in 50 years (Australian Dryland Salinity Assessment, 2000). Landholders are using salt tolerant forages (halophytic plants that can tolerate saline soil) to re-vegetate these landscapes (Condon *et al*. 1994) and are incorporating them into grazing enterprises. Research to date of animal responses to high-salt intake has focused on dry sheep grazing saltbush (and other halophytic plants) to fill the summer/autumn feed gap and concluded that dry sheep can tolerate the high salt loads and maintain weight (Wilson 1966; Hanjra and Rasool 1991; Morcombe *et al*. 1996; Masters *et al*. 2001; Franklin – McEvoy 2002). During the summer/autumn period lambing ewes could be in mid to late gestation and hence the initial focus of this thesis was whether pregnant ewes could manage the high salt content found in saltbush.

Effects of high dietary salt during pregnancy

There are physiological 'conflicts' for pregnant ewes fed high-salt diets. Pregnancy is characterized by sodium retention and increased extracellular volume necessary for the maintenance of the mother and growth of the foetus (Davison and Lindheimer 1989). High salt consumption leads to decreased aldosterone concentration, which reduces sodium reabsorption and increases sodium excretion (Randell *et al*. 2002). Therefore pregnancy and high salt consumption lead to opposing signals that regulate sodium retention. The aims of this component of the thesis were addressed in two parts. The first part investigated if ewes fed a high-salt diet could complete a successful pregnancy with a lamb of normal birth weight. It also investigated the physiological basis for how pregnant ewes fed a high-salt diet managed the potential physiological conflict of salt retention for pregnancy and salt excretion

associated with a high-salt diet by examining the hormones involved in the renin-angiotensin system (RAS).

Two year old single-bearing ewes fed 13% NaCl in their diet appeared to cope from the time of insemination through to lambing. There was no evidence to suggest that the high salt consumption, or consequential increase in water consumption, affected pregnancy rates, which where within typical ranges for laparoscopic artificial insemination (Hill *et al*. 1998). Previous studies by Potter and McIntosh (1974) with salty drinking water (1.3% NaCl) showed that age and/or multiple births increased the susceptibility to high salt for neonatal mortalities and complicated births. As the ewes in the present study were maiden ewes, further studies are warranted to determine whether higher parity ewes and/or those bearing twins (or triplets) would be equally capable of tolerating a high-salt feed.

Pregnant ewes in this study that consumed the high-salt diet were able to complete pregnancy successfully, with gestation and lamb birth weights remaining unchanged from control ewes fed a standard (low salt) diet. The mechanisms by which the ewes tolerated the high-salt diet included an increase in water consumption and a decrease by approximately 50% in aldosterone concentrations compared to the control ewes.

An increase in AVP was not induced through the first four months of pregnancy, presumably because of the increase in water intake. Exposure to high salt was likely to have been perceived physiologically by the animal, initially, as dehydration due to an increase in osmolality of the extracellular fluid. This would have stimulated the thirst centre and triggered an increase in the amount of water consumed. As water intake increased, plasma osmolality would have begun to return to normal levels. In non-pregnant animals, a declining osmolality would normally decrease AVP release but, during pregnancy, AVP is not suppressed at the

usual levels of body tonicity (at least in humans; Lindheimer and Davison 1995) as part of the normal water-retaining mechanisms of pregnancy. It appears that the physiological 'objective' of sheep in the present experiment to increase water retention to sustain a normal pregnancy was achieved without a change in AVP concentration because their high-salt intake induced an increase in water consumption that would have led to an increase in water retention in body fluids. As gestation progresses, body water is continually assessed (via changes in osmolality and blood pressure) by the animal, and there are continual adjustments in the volume-sensing AVP release mechanisms (Lindheimer *et al*. 1989). This mechanism could possibly explain the increase in AVP in the last month of pregnancy in both groups of sheep; i.e., an elevated AVP concentration may have resulted from a higher set point of the osmotic threshold.

The decline in aldosterone concentrations is probably the most important adaptive mechanism that allowed the ewes to successfully complete pregnancy whilst consuming high salt. When pregnant ewes were fed the control diet, aldosterone concentrations increased as pregnancy progressed to increase water reabsorption from the kidneys, and thus extracellular fluid increased as part of a normal pregnancy. When pregnant ewes were fed the high-salt diet, the mechanism of increased aldosterone concentration was apparently not required as the high salt ingestion triggered an increased water intake, which may have sufficiently increased extracellular volume to reach the 'target' level for a pregnant ewe. Therefore pregnant ewes fed high salt were likely able to avoid complications such as hypertension (Rafestin-Oblin *et al*. 1991) or neonatal mortalities (Potter and McIntosh 1974) by reducing their plasma aldosterone below the concentration in control animals and increasing water intake.

The implications of the research reported in this thesis are limited to salty feed as previous studies have shown different implications when pregnant animals drink salty water (Potter

and McIntosh 1974). Wilson (1966) also stated that the means of ingestion of salt (feed or water) alters the effects that salt may have on food intake and the health of the animal and that the acceptability or tastes of food or water containing high levels of salt is a factor in determining the salt tolerance of sheep.

The initial conclusions provide evidence that pregnant ewes can tolerate 13% NaCl in their feed whilst they have access to fresh drinking water. This provides the initial step to further investigate whether pregnant ewes can graze halophytic forages. It is apparent that they could withstand the high sodium chloride content typical, for example, of a saltbush-based pasture (Wilson 1975), but saltbush and other halophytes contain a secondary compounds that were not evaluated in the present study. Such compounds found in significant quantities in saltbush are oxalates and nitrates (Norman *et al*. 2004). Research has shown that oxalates cause calcium deficiency and causes the precipitation of insoluble calcium oxalate in the rumen and kidney and could ultimately lead to death (Dynes and Schlink 2002). The mechanisms by which high levels of nitrates becomes toxic to livestock is through the conversion in the rumen to nitrites. Absorption of nitrites results in the conversion of haemoglobin to methaemoglobin which is unable to bind to oxygen. This then leads to anoxia, which is accompanied by increased pulse and respiration rates (Dynes and Schlink 2002). Nitrates also result in depressed feed intake.

Effects of high dietary salt during pregnancy on offspring

Previous research in rats (Arguelles *et al*. 1996; Alves da Silva *et al*. 2003) have shown high-salt diets during pregnancy have been associated with lowering the RAS in the dam, which may possibly alter kidney development/function of the offspring. Also high-salt diets during pregnancy and postnatally have been associated with an increased preference for salt in the offspring (Smriga *et al*. 2002; Curtis *et al*. 2004).

The sequence of experiments reported in this thesis on the offspring found for the first time that there are physiological differences between lambs born to ewes fed a high-salt diet during pregnancy (S lambs) and lambs born to ewes fed a control (standard salt) diet (C lambs). When lambs were administered an oral dose of salt (40 g NaCl in solution), S lambs drank 400 mL less during the following two-hour period than C lambs, suggesting that S lambs had an altered thirst threshold. S lambs also had a lower concentration of aldosterone following the control challenge and exhibited a smaller decline in aldosterone concentration following a salt challenge. This difference possibly reflects a lowered responsiveness to aldosterone.

When water was restricted, S lambs were able to increase their rate of sodium excretion significantly more than C lambs. These data suggest that (i) when sheep are consuming a high-salt diet and not able to regularly consume fresh water immediately after ingesting the salt, S lambs may be better able to cope; and (ii) if fresh water is freely available over time and in close proximity to the salty feed (or forage), then the differences between C and S sheep may not be apparent.

A second challenge of salt after 8 hours of the first, indicates that a more continual exposure to salt may influence the ability to excrete the entire salt load and suggests that animals are able to adapt to high-salt feeds. In the first 4 hours after the first dose of NaCl, S lambs were able to excrete more sodium than C lambs, however after the second dose, excretion was identical across both groups. This suggests that any benefit conferred to S lambs in terms of adaptability to salt ingestion will only be evident in the immediate short-term, and that under conditions of repeated (or continual) oral salt 'challenge', all sheep (C and S lambs) have virtually the same capacity to excrete excess sodium. A practical implication of this finding is that S lambs may more readily adapt to high salt ingestion when first moved onto a high-salt forage such as saltbush. However, over time, this advantage may be lost as all sheep adapt to

the salty feed. This was confirmed in the last experiment in which a continual salt challenge was administered via drinking water. This experiment showed that there was no difference in sodium excretion between C and S lambs. There were however, changes in the differences for water consumption and aldosterone concentrations for C and S lambs, consistent with the earlier experiments in this thesis.

Collectively, the experiments reported in this thesis provide evidence to support the hypotheses that S lambs have a higher thirst threshold and a lowered responsiveness to aldosterone. Thus they excrete sodium at the same rate as C lambs but do so without the initial drive of water consumption and without lowering their aldosterone concentrations to the same degree. Whether this adaptation is beneficial in the field remains to be determined. For example, are there advantages for S lambs in increased liveweights or increased feed intakes? Data in the final experiment showed, for the first time, that feed intake in S lambs was actually reduced compared to C lambs. The mechanisms for this remain unknown, but it is a potentially important result as it may override any of the adaptive advantages described above. That is, the net effect of high-salt feeding during pregnancy on the offspring may be negative because of a reduced voluntary feed intake. This needs to be confirmed with animals offered forage (pasture) rather than a concentrate-based diet as used in the studies reported here.

Future research

A next step in research is to identify the exact mechanisms that account for the effects on the offspring. It would be useful to identify if there is a window of time during pregnancy that feeding high salt could increase the capacity of the offspring to deal with high salt, instead of feeding high dietary salt through entire pregnancy.

There are many factors that are involved in sodium excretion, urinary output and water intake, including mechanisms that were not measured within this thesis. These include possible changes in glomerular filtration rates (Potter 1968), the expression of aquaporins (Ohara *et al*. 1998), the length of the glomerulus and the loop of Henle (Shanas and Haims 2004), the sensitivity to salt (i.e. salt receptors), altered renin and renin activity and angiotensin II concentrations (Alves da Silva *et al*. 2003). In addition, the finding in the last experiment of this thesis that S lambs had a markedly lower voluntary feed intake needs to be confirmed. This response was observed with the sheep being exposed to high salt via drinking water. We need to determine if the same result occurs with animals exposed to salty feed and/or with animals consuming low (normal) salt levels with fresh drinking water. After investigation of these parameters, further research will need to determine the net benefits or costs to the offspring in terms of feed intake, liveweight and feed conversion ratios.

This thesis has provided new insights into the physiological consequences of high-salt diets during pregnancy on ewes and their offspring. Further research will determine if the differences observed in C and S lambs translate to differences under field conditions.

APPENDIX

Catheter preparation

Three metres of tygon microbore tubing (ID 0.04”; OD 0.070”; Wall 0.015”; Scientific Supplies Ltd, Australia) was measured and cut. Knotting both ends of the tubing it was then folded in the middle to make two catheters (1.5m long each). The catheters were then placed in a 2% Trition solution (Solution A) and left on an agitator over night. The following day the catheters were rinsed with deionised water and placed in 1% Norocillin solution (Solution B) and left on an agitator over night. The catheters were then removed from the solution and were hung in a drying area where disturbance was minimal (One day was sufficient for drying). The cuffs were then made (**Figure A**). To insert the cuffs onto the catheter the 3m tubing was cut at the fold in the middle and the knots were cut off. A marking was made 20cm from one end where the catheter would enter the artery and the cuff would be placed on top of the artery wall. A drop of Norocillin was used as a lubricant to push the cuff onto the tubing to the 20cm mark on the catheter. It was then left overnight to dry and a drop of glue was placed in between the cuff and catheter the following day to ensure the cuff was securely attached to the catheter. After the glue had dried a number of catheters were placed into a measuring cylinder with approximately 3ml of Tridodecylmethyammonium chloride heparin (TDMAC; Bioscientific Pty., Ltd., Gymea, NSW). Using a 20mL syringe the TDMAC heparin was drawn into the catheter in order to coat the inside of the catheter. The TDMAC heparin was then allowed to drain out of the catheter by gravity. A 20mL syringe was used to expel the remaining TDMAC heparin from the catheter. The catheters were then left to dry and then sterilised in ethylene gas at 55°C for 2.5 hours, with an 8-hour aeration cycle (King Edward Memorial Hospital, Subiaco, WA).

Solution A:

Tare 2L bottle

Add 40 mg of Trition (using pipette tip that has been cut off).

Make up 2000g with deionised water

Leave to mix in a tub of hot water.

Solution B:

Tare 2L bottle

Add 20mg of Norocillin (1% procaine penicillin; Western Drug Holdings Ltd., Balcatta, WA)

Make up 2000g with deionised water

Mix

Making cuffs

Approximately 8mm of peristaltic pump tubing (Green/Green Tygon tubing; 2.0 mL min^{-1}; 0.073"ID; TAC's Australia Pty., Ltd., NSW) was cut for each catheter. 15cm suture (Braun supramid suture; size 3 metric; Western Drug Holdings Ltd., Balcatta, WA) was then threaded through the tubing and back out the same side (**Figure A**) using a size 16 curved needle. This was repeated for the other side of the cuff.

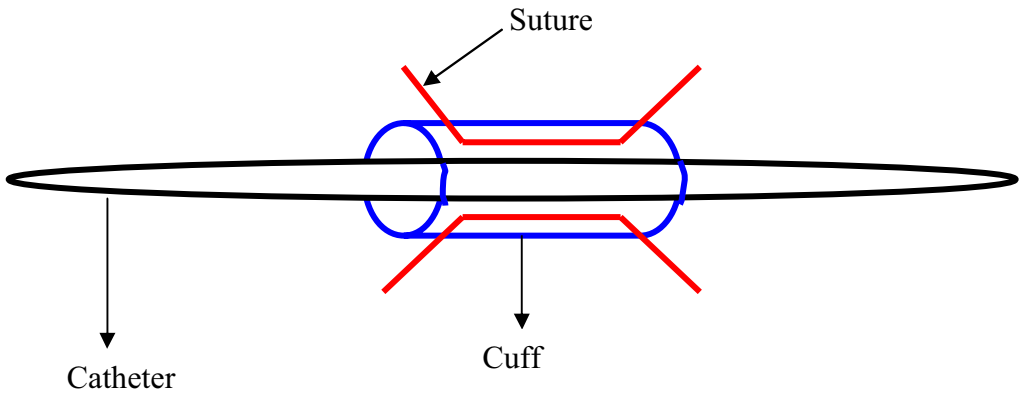

Figure A: Cuff placement on catheter

Surgical procedure

The animals were fasted for 24 hours prior to surgery, with access to clean drinking water during the fasting period. The animals were anaesthetised using 20-25mL (depending on the size of the animal) of either Nembutal or Thiobutal (Independent Vet Supplies, S.A.). After the induction of anaesthesia an endotracheal tube was inserted into the trachea and anaesthesia was maintained with isoflurane via the endotracheal tube. The right side of the neck was finely clipped and swabbed with betadine ready for catheter placement.

A 10 cm incision was made dorsal to the jugular vein and the m.sternocephalous and m.sternothroideus were dissected to expose the carotid artery running in the same sheath of connective tissue as the vagus nerve. The artery was carefully separated by blunt dissection away from the nerve and two ribbons were placed under the artery about 5cm apart to help raise the artery and temporarily constrict blood flow (**Figure B**). A purse string suture (0.5 cm x 0.5 cm) was placed in the artery using Ethibond 4-0 Excel suture and 17mm round curved needle (Independent vet supplies, Adelaide SA). A small incision was made in the artery in the middle of the purse suture. A 20 cm length of catheter was placed into the artery and the purse string was pulled to hold the catheter in place and tied around the cuff of the catheter.

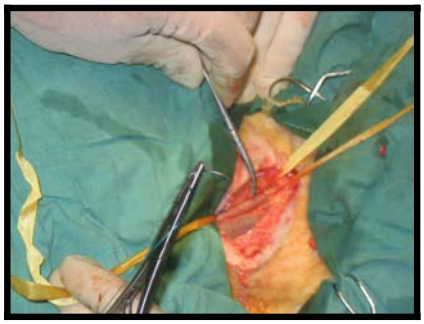

Figure B: Exposed carotid artery

Suture material on the cuff of the catheter was looped around the artery and tied off to help secure the catheter in place. The ribbon was released and blood flow in the catheter was confirmed. 5mL of heparin/saline solution (100 IU/mL heparin) was flushed through the

catheter and the end was closed using a blunt 18-gauge needle, plugged with a tip of a syringe filled with glue. A small amount of benzyl-penicillin was sprinkled on the wound to prevent infection. A further incision was made in the skin near the back of the neck through which the catheter was exteriorised. The wound was closed with an interrupted mattress suture using Vetafil (IVS, S.A. Australia). Once closed, another small amount of benzyl-penicillin powder was sprinkled on the exterior of the wound and the catheter was coiled up and placed on the back of the neck. A bandage covered the catheter and tape was used to hold the bandage in place (**Figure C**). The animal was then allowed to gain a semi-conscious state, so the endotracheal tube could be removed. The ewes was then placed back in her pen to recover. Each animal was treated intramuscularly with 2mL of Norocillin for three day post-surgery and catheters were flushed daily with heparin/saline solution (100IU heparin/ml).

Figure C: Final result if indwelling arterial catheters.

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