

Fetal programming of sheep for production on saltbush

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Summary

Saltbush is one of the few types of forage that will grow on salt affected land but, sheep struggle to maintain weight when grazing saltbush mainly because of its high salt content. Therefore, a strategy to improve salt tolerance of sheep would be beneficial to the profitable use of revegetated saline land. This could be done by manipulating the dietary salt load of pregnant or lactating ewes which could 'program', or permanently alter the physiology of their offspring to allow them to cope better with a high-salt diet as adults. When rat dams consume a high amount of salt during pregnancy, the salt balance mechanisms of their offspring are 'programmed' due to suppression of the offspring's renin-angiotensin system in early development. If this occurs in offspring from ewes grazing saltbush, beneficial adaptations may be programmed in these offspring which could allow them to better cope with the high-salt content of saltbush. I tested the general hypothesis that offspring born to ewes that consumed a high-salt or saltbush diet from mid-pregnancy to early lactation would have an increased capacity to cope with salt that would allow them gain weight when grazing saltbush in later life.

To test this hypothesis, I pair-fed ewes either a high-salt diet (14% NaCl) or control diet (2% NaCl) in an animal house from day 60 of gestation until day 21 of lactation.

During the same period, I also conducted a field experiment where ewes grazed on saltbush (supplemented with barley) or on pasture (supplemented with lupins). I then conducted a series of three experiments with the offspring. In the first experiment I measured the plasma renin activity of the ewes during pregnancy and their lambs at birth and at 3 weeks of age. In the second experiment, when the offspring were 8 months old, I tested their preference for a low-salt or a high-salt pellet and also measured food and water intake. The offspring were also given an oral dose of 25 g and 50 g of salt, after which renin activity and salt excretion were measured. In the final

experiment, the 10 month-old offspring grazed saltbush for 8 weeks and their weight gain and wool growth was measured to establish whether the treatments had an effect on production traits.

The renin activity of the ewe was suppressed by consuming a high-salt or saltbush diet and the renin activity of her lamb was also suppressed at 3 weeks of age. In the second experiment, I found that there was no difference in the offspring's preference for salt, but the high-salt and saltbush offspring had a lower total food intake than their respective control animals. The renin activity of high-salt offspring was less responsive to a salt dose because it did not decrease as much as control offspring in the first two hours after the salt dose. This led to the high-salt offspring retaining more salt than control animals. In contrast, the renin activity of saltbush was consistently lower than pasture offspring which allowed them to excrete salt more rapidly. In experiment three, the saltbush offspring gained tissue weight after grazing saltbush for 8 weeks, whereas the offspring in the other three treatments lost weight. High-salt and saltbush offspring also had higher greasy fleece weights at 22 months of age than their respective control groups.

Feeding saltbush to ewes from mid-pregnancy to early lactation induces physiological adaptations in their offspring that allow them to cope better with salt and gain weight when grazing saltbush as adults, supporting my hypothesis. However, contrary to expectations, the high-salt offspring did not gain weight when grazing saltbush because their physiological adaptations, such as salt retention, did not allow them to cope better with a salt load. The reason that saltbush offspring showed different adaptations to high-salt offspring is likely to be because saltbush contains not only NaCl but also high amounts of other minerals such as potassium, and other plant compounds, which may

influence the adaptive responses of the offspring. This research has direct implications for farmers because it shows they could utilize otherwise unproductive saltland by grazing pregnant ewes on saltbush to 'program' their offspring to gain weight when they graze saltbush later in life.

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Publications arising from this thesis

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Statement of student contribution

The publications on the previous page have been co-authored by my supervisors. I conducted the research under the guidance of my supervisors. I wrote the published manuscripts and incorporated suggestions for improvement from my supervisors.

Signed: Philip E. Vercoe -----

Signed: Ian H. Williams -----

Signed: Dean K. Revell -----

Signed: Megan A. Chadwick -----

CHAPTER 1

General Introduction

Exposing animals to a particular environment early in life may induce important physiological adaptations that allow them to cope better with that environment as adults (Gluckman et al., 2006). The environmental cues that the animal receives from its mother *in-utero*, and in the post-natal period help to ‘program’ or permanently alter their physiology to give them beneficial adaptations (Ross and Desai, 2005). It may be possible to use these natural adaptation mechanisms of animals to improve their performance on different types of diets or environments.

With 8.2 million hectares of agricultural land in Australia affected by salinity or at high risk of turning saline, farmers must find ways to utilize this land (NLWRA, 2001). One of the few options for farmers is to grow saltbush, a halophytic shrub that can be grazed in autumn to fill the gap in pasture availability normally experienced in southern Australia (Morcombe et al., 1996). However, the major limitation to saltbush as a forage source is its extremely high salt content (up to 30%) (Norman et al., 2002). Sheep find it difficult, physiologically, to cope with ingesting large amounts of salt, resulting in decreased feed intake. Consequently, they struggle to maintain weight when grazing saltbush without supplementation (Masters et al., 2005, Warren et al., 1990). Saltland could be better utilized by farmers if sheep had an increased ability to handle the high-salt content of saltbush, so they could gain weight when grazing it.

Grazing pregnant ewes on saltbush could induce adaptive responses in their offspring that allow them to cope better with the high-salt content of saltbush. Biomedical

research using the laboratory rat as the animal model has shown that high dietary salt during pregnancy and lactation can have long-term consequences on the offspring's physiological responses to salt. The reason for these changes in the offspring is likely to stem from alterations in the renin-angiotensin system (RAS). Renin is the rate-limiting enzyme in the RAS as it controls the formation of angiotensin II, a powerful vasoconstricting hormone that stimulates salt retention. When animals consume a high-salt diet renin levels decrease, which slows the formation of angiotensin II and aldosterone so that more salt can be excreted (Morgan, 2001). The RAS also plays a pivotal role in renal development in the early phases of life, which is why the renin activity of animals at birth is often 10-20 fold higher than that of adults (Gomez and Norwood, 1995, Guron and Friberg, 2000). Feeding a high-salt diet to a pregnant animal lowers her RAS and it may also suppress the RAS of the fetus at a critical developmental stage, which could cause renal and physiological abnormalities (Balbi et al., 2004, Ingelfinger et al., 1998). Some of these abnormalities shown in rats include: changes in basal levels of RAS activity; altered responsiveness of the RAS to the ingestion of salt; alterations in water and food intake; and an increase in salt preference (Curtis et al., 2004, da Silva et al., 2003, Vidonho et al., 2004). If similar changes occur in offspring from ewes which consume a high-salt or saltbush, their offspring may be better adapted to handling salt.

Most of the previous research with rats has involved feeding a high-salt diet from conception until weaning, but it is usually only practical in southern Australia to graze pregnant ewes on saltbush during the autumn feed gap, which usually coincides with mid pregnancy (Masters et al., 2006, Morcombe et al., 1996). Results from studies investigating the effect of RAS inhibitors on the offspring suggest that the 'programming window' for altering mechanisms involved in salt balance is from mid

pregnancy to the early postnatal period (Hilgers et al., 1997, Robillard and Nakamura, 1988, Wintour, 1997). However, effects on offspring from mothers that received a high-salt diet only during this window of neonatal development have not been tested.

Similarly, the programming effects of saltbush compared to high NaCl diets have not been investigated, nor whether the resulting changes in physiology of the offspring are beneficial or detrimental to their performance on a high-salt diet such as saltbush in later life.

In this series experiments, I aim to determine the physiological and behavioural effects on the offspring from feeding their mothers a diet high in salt, either through grazing saltbush or through the addition of NaCl to a standard ration, from mid pregnancy to early lactation. I tested the general hypothesis that offspring born to ewes that consume a high-salt diet or graze saltbush from mid-pregnancy to early lactation will have increased capacity to cope with salt that, in turn, will allow them to gain weight when grazing saltbush in later life.

CHAPTER 2

Literature Review

Introduction

“Fetal programming” is a process whereby an environmental stimulus or stress at a critical period of fetal development has lasting or life-long effects on the animal (Ross and Desai, 2005). This phenomenon enables animals to develop adaptive responses which help them cope with the challenges faced in that particular environment when they encounter it in later life (Bateson et al., 2004, Rickard and Lummaa, 2007). In this review, I investigate the role that fetal programming can play in improving the weight gain of sheep when grazing saltbush.

I will first examine the role saltbush plays in agriculture and how the high-salt content of saltbush limits its nutritional value. Then, I will explain how the physiology of the animal copes when consuming a high-salt diet, with particular emphasis on the role of the renin-angiotensin system (RAS). This knowledge is then applied to pregnant animals and whether the interactions between the fetus and the mother could be influenced by a high-salt diet. The physiological consequences for the offspring of a maternal high-salt diet are then discussed, concentrating on the possible changes to the salt handling physiology, food and water intake and salt preference. Finally, I pose four questions which remain unanswered by the literature: 1) Will the changes in physiology of sheep offspring be the same as those observed in rat offspring born to mothers that consumed a high-salt diet during pregnancy and lactation? 2) Will saltbush have the same effect as a high-salt diet on the ewe and her offspring? 3) Will feeding the ewes a

high-salt diet from mid pregnancy to early lactation affect the fetus? 4) Are the changes in physiology of the offspring from feeding saltbush to its mother beneficial or detrimental to the offspring's performance when fed saltbush in later life? I finish with a general hypothesis based on my expectations of the answers to these questions.

1. Salinity and saltbush

Salinity currently affects over 5.7 million ha of land in Australia. This figure is set to rise to 17 million by 2050 (NLWRA, 2001). Western Australia has the largest salinity problem with approximately 51% of farmland affected (ABS, 2003). Reclamation of this land is not a realistic option in many situations and even wide spread re-vegetation will not prevent the spread of salinity (Cocks, 2003). If salt-affected farms are to remain viable, farmers must find profitable ways to use their saltland.

The most common use for saline land is to grow saltbush (*Atriplex spp.*), a halophytic shrub that can be grazed by livestock. It can be a valuable protein source (18% crude protein) and has high levels of vitamin E, both beneficial for sheep (Norman, 2004, Pearce et al., 2005). However, it has several characteristics that may limit its use, namely:

- i) low digestibility and low energy – the organic matter digestibility of Oldman saltbush (*Atriplex nummularia*) is around 54% (Norman, 2004),
- ii) anti-nutritional compounds – oxalates (6.2%) and total phenols (1.36%) (Abu-Zanat, 2004), and
- iii) high salt content – contains up to 30% DM of salt (Norman, 2004).

Since its nutritional value is low, sheep have to consume large quantities if they are to maintain weight when grazing saltbush.

Wilson (1977) found that sheep could eat an average of 550 g dry matter (DM) of saltbush per day, whereas Abu-Zanat (2004) recorded average intakes 770 g DM per day. Neither of these intakes were enough to maintain liveweight of sheep weighing 35-50 kg. A 45 kg sheep must eat around 1.4 to 1.6 kg DM of saltbush per day to maintain liveweight and this equates to ingesting around 300 g of salt per day. However, sheep generally can only consume about 200 g of salt per day because it depresses their food intake. The feed intake of the sheep starts decreasing after they consume around 60 g of salt in their diet, depending on the digestibility of the organic matter (Wilson, 1966). This depression of feed intake caused by the high amounts of salt in saltbush has been suggested as the major cause of low weight gain of sheep when grazing saltbush (Abu-Zanat, 2004).

It is possible for animals to adapt to the high-salt content of saltbush, but it occurs when grazing over a long period of time, which may not be viable on Australian farms. Long-term studies with sheep grazing Oldman saltbush have shown that intake can be increased over time (Abu-Zanat, 2004, Correal and Sotomayor, 1997). Le Houerou (1992) showed that after five months of sheep grazing saltbush their intake had doubled from 0.8-1.2kg to 1.5-2.5kg DM h/d. At these higher levels of intake, mature sheep were able to maintain bodyweight without supplements. However, economic analysis for the use of saltbush on farms in Australia has shown that it is most profitable to graze saltbush over autumn for only three months, when both the quality and quantity of other more nutritious pasture is low (Morcombe et al., 1996, Rehman et al., 1999). On Australian farms, sheep do not graze saltbush for long enough for them to become adapted to its high-salt levels, and consequently they lose weight when grazing it (Abu-Zanat, 2004, Correal and Sotomayor, 1997).

On most farms, autumn coincides with the last three months of pregnancy in ewes and early lactation. Pregnant ewes have a high nutritional demand at this time so supplementary feed costs are also high. If pregnant ewes grazed on saltbush over autumn, supplementary feed costs to the farmer could be reduced. However, as previously discussed, the high salt content of saltbush means that the pregnant animals will be consuming around 200 g of salt per day. The physiology of the ewe must be able to cope with ingesting large amounts of salt if she is to graze saltbush during pregnancy.

2. Effects of a high-salt diet on the body

Salt is essential to the functioning of the body. Not only is the sodium and chloride essential, but the movement of salt across membranes creates vital osmotic gradients in the body. The kidney is the major organ that absorbs and excretes sodium, depending on the sodium balance in the body. There are many hormonal and nervous systems that instruct the kidney to retain or excrete salt so that salt balance remains constant.

The kidney

The main function of the kidney is to regulate the volume, osmolarity, mineral composition and acidity of body fluids (Vander, 1995). It does this by excreting water and electrolytes in different concentrations to maintain fluid balance in the body. The main functional unit of the kidney is the nephron. Different areas of the nephron play different roles in filtering fluid. Figure 1 illustrates a nephron and its various parts.

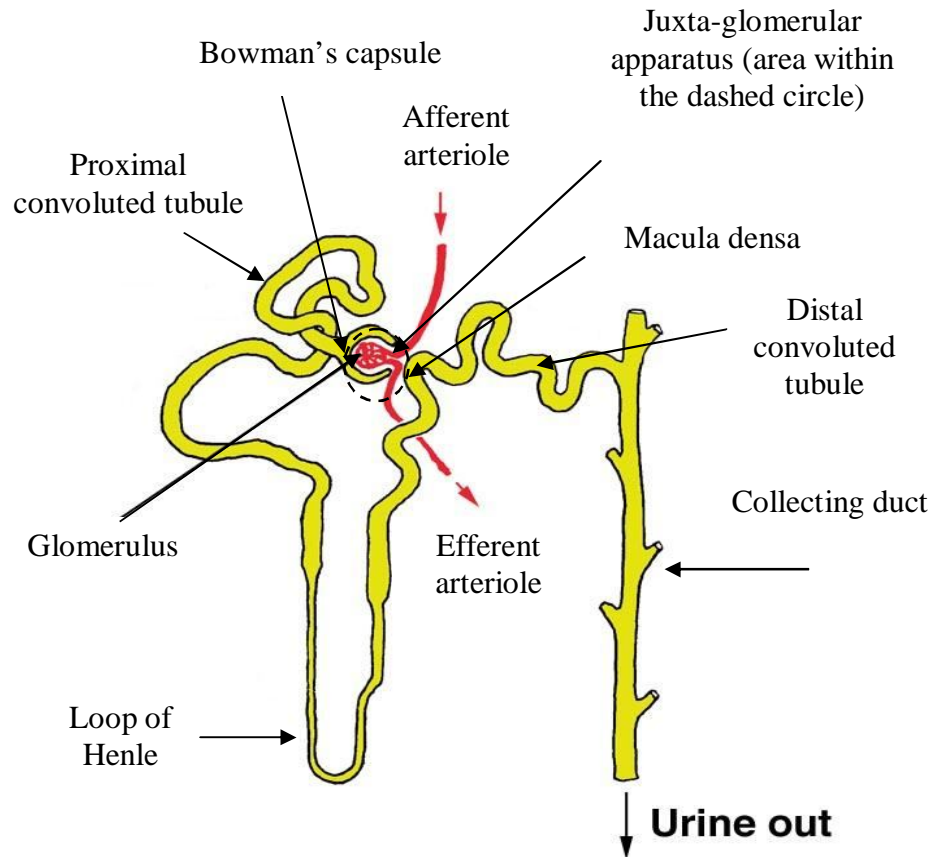


Figure 1: Structure of a nephron (adapted from Lote, 1994).

Blood enters the nephron through the afferent arteriole and enters the glomerulus which is essentially a tuft of capillaries that filter everything except cells and proteins in the blood as it moves from the capillaries into the Bowman's capsule. Molecular weight, shape and charge influence what particles are filtered through to the Bowman's capsule or remain in the capillaries to be channeled out through the efferent arteriole (Lote, 1994). From the Bowman's capsule the filtrate (blood minus cells and proteins) enters the proximal tubule and continues through the Loop of Henle. The start of the distal

tubule passes very close to the Bowman's capsule and comes into contact with the afferent and efferent arterioles. This area is known as the juxtaglomerular apparatus (JGA). Within this, there are macula densa cells in the distal tubule which respond to the composition of the fluid. Another important group of cells in the JGA are the granular cells in the afferent arteriole wall because they secrete the enzyme renin (Lote, 1994), which will be discussed in detail in a later section. After passing through the distal tubule, the remaining filtrate flows through the collecting duct on its way to the bladder to be excreted.

In normal renal function, the vast majority of the salt that passes through the tubules in the nephron is reabsorbed. Around 60% of the reabsorption occurs in the proximal tubules, 25% in Loop of Henle, 10% in the convoluted tubule and the final 2-3% in the collecting duct (O'Shaughnessy and Karet, 2006). However, when an animal is consuming a high-salt diet, such as sheep grazing saltbush, it needs to excrete large amounts of salt rather than re-absorb it.

Salt excretion

Animals consuming high amounts of salt must also excrete large amounts of salt so that the balance of ions, especially sodium and chloride in the extracellular fluid, remains within a normal range. Figure 2 shows how ingesting high amounts of salt leads to the increased excretion of salt. Each step in this process will be discussed in the following sections.

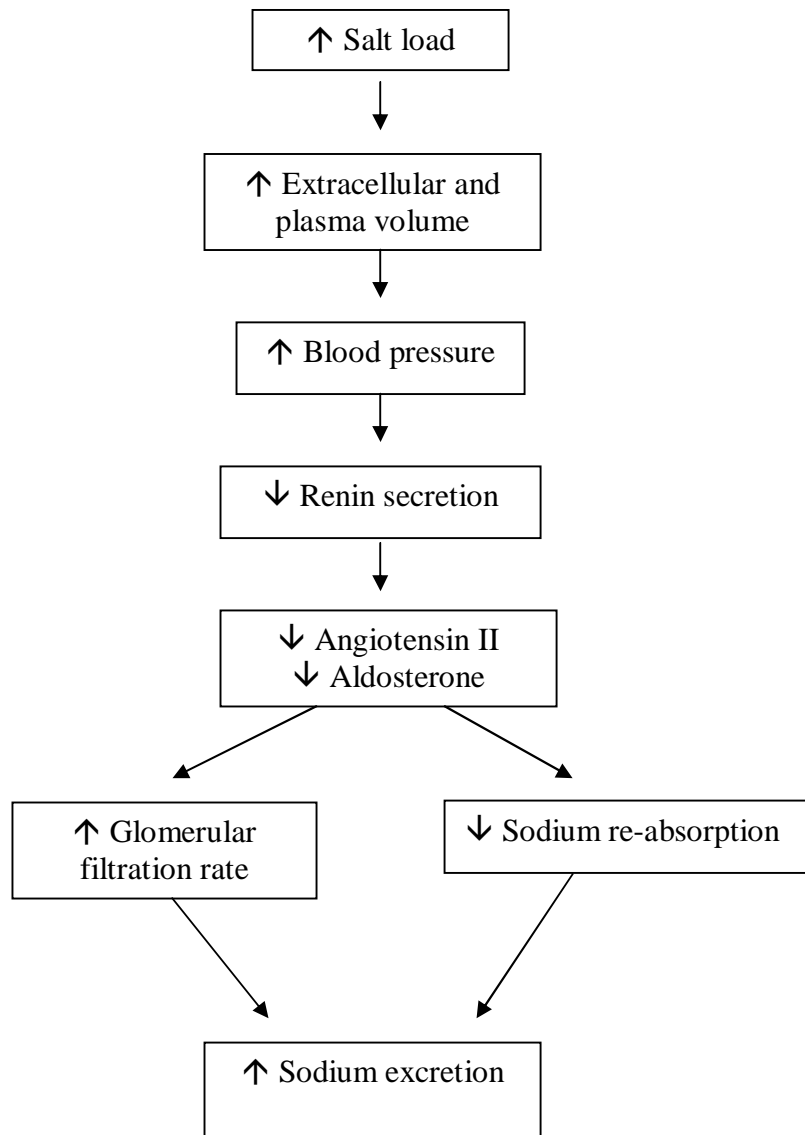


Figure 2: An overview of how sodium balance is maintained in the body.

Extracellular volume

An increase in salt (NaCl) consumption creates an osmotic gradient that causes water to diffuse out of cells and into the extracellular fluid where there is a higher concentration of NaCl. This causes an increased volume of extracellular fluid and, since plasma makes up part of the extracellular fluid, plasma volume also increases. An increase in plasma volume increases blood pressure.

Blood pressure

Blood pressure is highly regulated in the body and many systems can detect and respond to changes in blood pressure. Intra-renal baroreceptors and the macula densa sense changes in blood pressure within the kidney and there are also extra-renal baroreceptors that sense changes in blood pressure in the rest of the body. When high amounts of salt are consumed, these baroreceptors are stimulated and act to lower sodium re-absorption so more salt can be excreted. The change in blood pressure needed to elicit a natriuretic (or salt loss) response in the kidneys is known as the pressure-natriuresis relationship. Abnormalities in this relationship can lead to changes in the amount of salt an animal excretes and how quickly the animal can excrete the salt (Evans et al., 2005, Roman, 1986). Excreting salt quicker would be a valuable adaptation for sheep grazing saltbush. Once the baroreceptors have sensed an increase in blood pressure, they signal a reduction in the secretion of the enzyme renin.

Renin and angiotensin II

Renin is the rate limiting enzyme in the renin angiotensin system (RAS). The release of renin controls the formation of the powerful vasoconstricting hormone angiotensin II. The actions of the RAS will be discussed in detail in the next section but, briefly, angiotensin II stimulates the proximal tubule in the nephron to absorb more sodium. So a reduction in angiotensin II levels reduces sodium re-absorption so more salt is excreted. Because angiotensin II constricts both the afferent and efferent arterioles, it also reduces glomerular filtration rate. So, angiotensin II conserves sodium by reducing Na flow into the tubule and increasing its absorption from the tubule.

Glomerular filtration rate

Glomerular filtration rate (GFR) is the amount of fluid that flows through the glomerulus and gets filtered in the nephron. The rate that blood is filtered through the glomerulus depends on various factors such as renal plasma flow, renal arterial pressure, and dilation or constriction of the afferent and efferent arterioles (Vander, 1995). Since angiotensin II is a vasoconstrictor hormone, a reduction in its concentration allows the afferent and efferent arterioles to dilate, which means more fluid flows through the nephron so GFR is increased. An increase in GFR allows more salt to be excreted at a faster rate. However, despite large changes in sodium balance, GFR only changes a small amount. This is because GFR is auto regulated by a mechanism called tubuloglomerular feedback. This feedback stops large increases in GFR by stimulating the afferent arteriole to constrict which returns GFR to normal. Much like pressure natriuresis, alterations in the tubuloglomerular feedback mechanism can lead to changes in the response of GFR to salt which could be an important adaptation for animals consuming a high-salt diet (Majid and Navar, 2001).

Sheep, like other animals, possess highly tuned excretion mechanisms for salt that allow them to cope with consuming high amounts of salt. However, by altering these mechanisms slightly (such as pressure natriuresis or tubuloglomerular feedback), animals could be better adapted to consuming a high-salt diet which would be an advantage when grazing saltbush. In order to understand how these salt excretion mechanisms may be changed, it is important to look in more detail at the role of the renin-angiotensin system because it helps to mediate these mechanisms.

Renin angiotensin system

The RAS was originally defined as an endocrine system, but localized systems are also present in a variety of tissues including the kidney and the brain (Guron and Friberg, 2000). The main role of the RAS is in cardiovascular homeostasis (Guron and Friberg, 2000). The composition and volume of the extracellular fluid is controlled by the RAS, as well as regulating the constriction of blood vessels. This makes the RAS a vital system for the control of one of the most regulated and finely-tuned factors in the body, blood pressure. Renin is the rate limiting enzyme in the RAS so it controls the production of the most active peptide, angiotensin II. In the context of this review, the components of the RAS (Figure 3) will now be discussed with particular emphasis on the control of renin release and effects of angiotensin II.

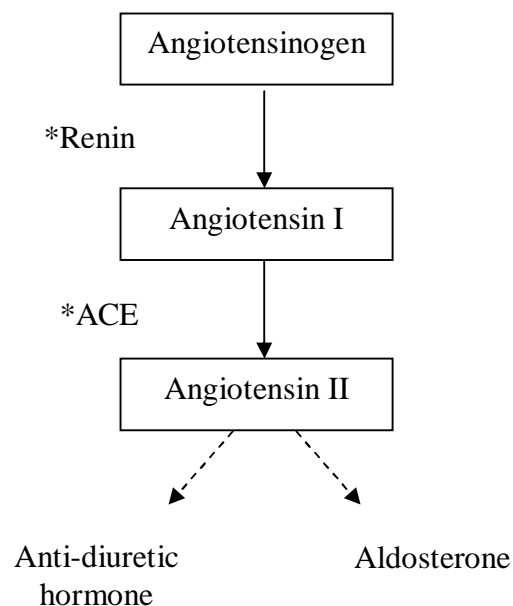


Figure 3: The components of the renin-angiotensin system. * Enzymes. ACE- angiotensin converting enzyme

Angiotensinogen is a glycoprotein that is the only known substrate for renin and is the precursor for all the angiotensin peptides (Gomez and Norwood, 1995). The largest source of angiotensinogen is in the liver where it is secreted into the blood stream at a relatively constant rate. Angiotensin I has no known physiologic function (Koeppen and Stanton, 1997) but it is converted by angiotensin converting enzyme (ACE) to angiotensin II which is the main signaling molecule in the RAS. One of its roles is to stimulate the production of anti-diuretic hormone (ADH) and aldosterone. Since the amount of angiotensinogen and ACE are maintained at relatively high levels, the main factor that controls the activity of the RAS is the release of renin (Vander, 1995).

Control of renin release

Renin is secreted from the granular cells in the afferent arteriole. Angiotensin II has a negative feedback effect on renin secretion to help regulate its release (Blair-West et al., 1971). The rate of renin release is controlled by 3 main regulatory mechanisms; renal baroreceptors, the macula densa and the sympathetic nervous system (Beierwaltes, 2003, Reid and Chiu, 1995). Nitric oxide could also play a role in the regulation of renin secretion, but less is known about the mechanisms behind this. Each of these factors are discussed briefly below.

Renal baroreceptors

The granular cells in the afferent arteriole act as baroreceptors, as well as secreting renin (Reid and Chiu, 1995, Vander, 1995). They monitor the pressure within the afferent arterioles and vary their secretion of renin accordingly. As pressure decreases, they increase their secretion of renin.

Macula densa

The macula densa cooperates with the renal baroreceptors to increase renin secretion when blood pressure decreases. The macular densa senses a decrease in GFR and/or an increase in fluid reabsorption, which are signals of decreased blood pressure. In response to this, the macular densa stimulates the granula cells to release renin (Vander, 1995).

Sympathetic nervous system

Renal sympathetic nerves release norepinephrine, that stimulates renin secretion. Catecholamines circulating in the blood also stimulate renin secretion by way of beta adrenoreceptors, which are thought to be located on the juxtaglomerular apparatus. Other situations where the activity of the sympathetic nervous system is increased, during stress for example, also stimulates renin release (Richardson et al., 1995, Van de Kar and Blair, 1999).

Nitric Oxide

Nitric oxide, an endothelium-derived relaxing factor, is a unique signaling molecule. It is involved in cell-to-cell communication in the cardiovascular, nervous, renal and immune systems. It is present in the macula densa which suggests it may have a role in renin secretion. Reid and Chiu (1995) state that nitric oxide not only contributes to the basal regulation of renin secretion, but it also influences the other renin regulators such as the renal baroreceptors, macula densa and the sympathetic nervous system. The functions of nitric oxide and its interactions with the renin-angiotensin system are discussed further in a later section.

The release of renin is tightly controlled because changes in renin secretion directly affect how much angiotensin II is formed in the body. Angiotensin II has many functions in the body and affects a variety of systems, not just the kidney.

Angiotensin II

Angiotensin II is one of the most potent vasoconstrictors in the body. It has a variety of functions revolving around maintenance of blood pressure and, consequently, salt balance in the body (Vander, 1995). It acts via two main types of receptors, angiotensin type 1 receptor (AT1) and angiotensin type 2 receptor (AT2). The AT1 predominates in the adult and is used in most functions of angiotensin II (Hilgers et al., 1997). These functions can be categorized into five main groups: sodium and water retention, water and salt intake, blood flow, cell growth and renal development.

Sodium and water retention

Angiotensin II controls how much sodium is absorbed by the kidney and it acts on the nephron in two main ways; it constricts the afferent and efferent arterioles and lowers glomerular filtration rate and it stimulates the proximal tubule to absorb more sodium and water. It makes the proximal tubule in the nephron more permeable to sodium so more is retained in the body. When blood pressure is low, angiotensin II increases so more salt can be retained to bring blood pressure back to normal levels. Angiotensin II also increases salt and water retention indirectly through stimulating the release of aldosterone and anti-diuretic hormone

Aldosterone is a vasoconstricting hormone produced by the adrenal cortex. It acts on the distal tubule and the cortical collecting duct of the nephron to increase sodium absorption. Angiotensin II is not the only factor that stimulates aldosterone release.

Adrenocorticotrophic hormone (ACTH) and plasma potassium concentration also regulate aldosterone levels in the body (Lumbers, 1999).

Anti-diuretic hormone (ADH), otherwise known as arginine vasopressin (or AVP) is produced in the hypothalamus and helps to regulate the volume and osmolarity of the urine (Koeppen and Stanton, 1997). The main function of ADH is to regulate water retention in the body. ADH stimulates the membrane in the collecting duct of the nephron to be more permeable to water. This allows more water to be retained resulting in urine being more concentrated in sodium as well as other ions.

Salt and water intake

As well as influencing the amount of sodium and water retained in the body, angiotensin II can also influence the intake of salt and water. Angiotensin II works via AT1 receptors in the brain to increase water and sodium intake (Lienard et al., 1996, Weisinger et al., 1997). Osmoreceptors and sodium sensors detect cellular dehydration and extracellular fluid volume depletion. This increases angiotensin II levels in the brain and thirst is stimulated (Weisinger et al., 1997, Stricker and Verbalis, 1996). Infusion of angiotensin II into sheep causes an increase in water intake in a dose-dependant manner (Breuhaus and Chimoskey, 1990, Zhu and Herbert, 1997), but other authors have found that suppression of angiotensin II levels did not influence water intake of sheep after water deprivation (Abraham et al., 1976). It may be that the angiotensin II alters the stimulation of water intake and the threshold at which this occurs (Andersson et al., 1982). However, the control of thirst by angiotensin II is complex and not well understood (Weisinger et al., 1996).

Bodyweight and food intake

Angiotensin II can also regulate bodyweight and food intake independent of alterations in blood pressure (Cassis et al., 1998). Infusions of angiotensin II in rats for 14 days did not alter food intake, but lowered bodyweight and white adipose mass, which was possibly due to an increase in peripheral metabolism (Cassis et al., 1998). However, other authors have reported a decrease in food intake with angiotensin II infusion (Porter et al., 2003, Sunagawa et al., 2001). Some chronic, rather than acute studies of increased angiotensin II concentration show further conflicting results. Renin transgenic mice have additional renin genes so they exhibit a chronic activation of their RAS; specifically, higher levels of angiotensin II in their brain. These rats have a higher basal food and water intake than normal rats (Szczepanska-Sadowska et al., 2003). However, when rats have a chronic suppression of their circulating angiotensin II levels, there is no change in food intake (Jayasooriya et al., 2008). The exact effect of long or short term changes in angiotensin II on food intake are not clear, but it may be influenced by whether circulating angiotensin II levels are altered, or whether brain angiotensin II levels are altered.

Renal development

Angiotensin II stimulates cell growth and has a special role to play in fetal development (Millan et al., 1989). The role of angiotensin II and the RAS in renal development will be discussed in detail in a later section.

Other effects of angiotensin II

The numerous effects of angiotensin II are still emerging as research into the RAS continues. Some other effects of angiotensin II include:

- Mediating the baroreflex (response of heart rate and cardiac output to changes in blood pressure) (Breuhaus and Chimoskey, 1990)
- Increasing the activity of the sympathetic nervous system (Monument and Smith, 2003)
- Increasing blood flow to the periphery of the body (Iwamoto and Rudolph, 1979)
- Temperature regulation in the body (Cassis et al., 1998, Katovich et al., 2001)

It is clear that angiotensin II has far-reaching effects in the body and is not just confined to its classic salt-retaining effects in the kidney. Animals consuming a high-salt diet will have suppressed angiotensin II levels in order to excrete more salt, but the RAS closely intertwines with other factors that help regulate it. Since the primary role of the RAS is in vasoconstriction it stands to reason that other factors, whose role is to vasodilate, also help control sodium balance.

Other factors that influence sodium balance

There are several hormones in the body that interact with the RAS to maintain sodium balance and blood pressure. Some of these systems include kinins, nitric oxide, atrial natriuretic peptide and prostaglandins.

Kinins

Kinins are vasodilator peptides implicated in the regulation of blood pressure, renal plasma flow and renal salt and water handling (Weinberg et al., 1987). They are part of the kallikrein-kinin system that consists of the substrate kinogen which is acted on by the enzyme kallikrein to create kinins (Margolius et al., 1974). Kinins contain the peptide bradykinin as part of their sequence (Campbell, 2000). The exact role of the kallikrein-kinin system as well as its relationship with salt intake and the RAS are a

subject of conjecture. The results from several studies show that the activity of the kallikrien-kinin system decreased with a high salt intake (El-Dahr et al., 1996, Margolius, 1996, Margolius et al., 1974, Weinberg et al., 1987). Since the main action of the kallikrien-kinin system is natriuretic (salt losing) this seems counterintuitive. However, these results imply that it may act as a positive feedback loop to the RAS because it also increases with low salt intake. More recently, Hettinger et al. (2002) and Murphey et al. (2004) showed that while urinary kallikrien decreases during high salt intake, kallikrien and bradykinin in plasma actually increases with high salt intake, which is more conducive to its vasodilating actions (Hettinger et al., 2002, Murphey et al., 2004). Part of the potency of kinins is that they release other vasodilators such as prostaglandins and nitric oxide (Farhy et al., 1993). The kallikrien-kinin system helps to mediate the sodium retaining RAS by encouraging sodium excretion by the kidney.

Nitric Oxide

Nitric oxide, an endothelium-derived relaxing factor (Zou and Cowley, 1999) is vital in the control of many biological processes, among them are its effects on renal function. It plays a crucial role in the control of renal and glomerular hemodynamics, feedback responses, release of renin and sympathetic transmitters, ion transport and renal water and sodium balance (Zou and Cowley, 1999). Like most vasodilators, nitric oxide is increased with a high salt intake (Shultz and Tolins, 1993, Wilcox, 1995) and it works to increase salt loss from the body by increasing glomerular filtration rate and lowering tubular sodium re-absorption (Evans et al., 2005, Zou and Cowley, 1999). It is involved in the long-term control of blood pressure and it may also play a role in long-term adaptation to high salt intake (Shultz and Tolins, 1993, Wilcox, 1995, Zou and Cowley, 1999). Since the RAS is also involved in many of these factors, it is not surprising that the vasodilating action of nitric oxide and the vasoconstricting action of angiotensin II

combine to control salt balance and blood pressure (Fernandez-Alfonso and Gonzalez, 1999).

Atrial natriuretic peptide

Atrial natriuretic peptide is another vasodilator that interacts with the RAS. As its name suggests, the main action of atrial natriuretic peptide is in natriuresis and sodium balance (Sagnella et al., 1985). Atrial natriuretic peptide is produced mainly in the cardiac atria and is released into the circulation in response to volume expansion and high blood pressure (Ballerman et al., 1985, Campese et al., 1996). Atrial natriuretic peptide helps to excrete sodium and oppose the vasoconstricting actions of hormones such as aldosterone and angiotensin II (Deloff et al., 1992, Schielbinger et al., 1988).

Prostaglandins

Prostaglandins can also act as vasodilators. Prostaglandin release from cells is stimulated by increased activity of the renal nerves and angiotensin II. Much like kinins and nitric oxide, prostaglandins help to counteract the vasoconstrictors so that sodium balance can be maintained (Vander, 1995).

The review until this point has focused on the structure of the kidney and some of the basic hormonal mechanisms involved in maintaining salt balance. This is important to understand when considering animals consuming diets high in salt, especially if the animals are pregnant. As previously explained, grazing pregnant ewes on saltbush during autumn could reduce supplementary feeding costs to the farmer, but the ewes would be consuming large amounts of salt. The diet of ewes grazing saltbush, with a small amount of understory and some supplementary feeding, would be around 15% salt, which equates to around 200 g of salt per day. Ingesting this large amount of salt

has major effects on the salt balance systems in their body, particularly the RAS, as explained in this section. We need to establish whether pregnancy alters the ability of the animal to cope with a high-salt diet.

3. Salt balance during pregnancy

Pregnant animals normally retain some salt and water throughout gestation to supply the growing fetus, but pregnant ewes grazing saltbush have to excrete large amounts of salt rather than retain it. In humans, the state of pregnancy is normally accompanied by a gain of 500-900 mmol of NaCl that is distributed between the mother and the fetus (Vollotton, 2003). Despite pregnancy being a state of sodium retention, when the animal is faced with a salt load its salt-excretion mechanisms can over-ride this to enable the animal to excrete salt. Studies on salt tolerance by Peirce (1966, 1968) found that pregnant ewes could cope with 1% salt in their drinking water without any adverse effects, this is only slightly less than wethers that could tolerate 1.3%. Meyer and Weir (1954) fed ewes diets containing different amounts of salt (0.5, 4.8, 9.1 and 13.1% salt) from 71 days before conception until 28 days after lambing. The salt content of the diet did not lower the growth rate of the ewes before or during pregnancy, but during lactation the ewes on the 13.1% salt diet had a lower food intake and lost more weight during this period. The high-salt diets did not influence the ewe's fleece weights, lambing percentage, or growth rate of the lambs. More recently, Digby et al. (2008) showed that pregnant ewes fed 13% salt in their diet were able to adequately regulate salt balance and there were no adverse effects on the health of the ewe or her lamb. Despite the body retaining sodium during pregnancy, the blood pressure of the mother normally drops (Atherton et al., 1982, Beausejour et al., 2003). However, Beausejour et al. (2003) found that if pregnant rats were ingesting large amounts of salt (1.8% NaCl in

water), their blood pressure did not drop during pregnancy and was higher than normal. The plasma renin activity and aldosterone level of these pregnant rats was also lower. Similarly, plasma renin activity was lower in pregnant ewes that had salt added to their drinking water (0.17 mol L NaCl) from day 122 to day 128 days gestation (Gibson et al., 2003). These experiments show that pregnant animals cope with a high-salt diet in the same way that non-pregnant animals do, despite being normally in a salt-retaining state.

A pregnant ewe can cope with a high-salt diet, but it may affect her fetus whose salt balance mechanisms are not fully developed. In order to assess whether the ewe's high-salt diet will affect her offspring, we must review how the systems involved in salt balance of the ewe, and those of her lamb, interact.

4. Salt balance in the fetus

The fetus depends on its mother to maintain salt balance in early and mid gestation but, by late gestation as its kidney matures, it is capable of acting independently of its mother. Before day 105 of gestation, plasma sodium concentration of the fetal lamb is similar to that of the ewe (Carver and Mott, 1974), but from days 111 to 144 of gestation, the plasma sodium concentration of the fetal lamb is lower than that of the ewe (Broughton Pipkin et al., 1974). However, the intake of salt may affect plasma sodium of the fetus, but not the mother. In rats, Deloof et al., (2000) found that if the salt was increased to 0.43 M in the drinking water of the dam between 11 and 21 days gestation (rats have a 25 day gestation length), the mothers were able to maintain their plasma sodium, whereas the plasma sodium of their fetuses rose. The authors suggested that this was due to the immaturity of the developing kidney that could not excrete

enough sodium. In contrast, Stevens and Lumbers (1986) showed that neither maternal nor fetal plasma sodium concentrations in sheep were altered by replacing “fresh” drinking water of ewe with 0.17 M NaCl solution for 9 days from 118-136 days gestation. Fetal sheep between 110 and 135 days gestation are capable of responding to volume expansion with saline infusion by increasing their GFR and decreasing sodium re-absorption (Hurley et al., 1977). However when saline is infused into the ewe, there is no change in the fetal kidneys (Hurley et al., 1977). These results suggest that the fetus is buffered from changes in maternal sodium balance to a degree, but when large changes in sodium balance occur, the late-gestation fetus is able to cope by excreting more salt.

As previously explained, the renin-angiotensin system (RAS) is a major contributor to maintaining sodium balance in the adult, and the fetus is dependant on this as it develops its own kidney and salt handling mechanisms in the uterus. The RAS in the fetus also contributes to salt balance, but it also plays additional roles in the maturation of renal function.

The fetal renin angiotensin system

A major function of the RAS in the fetus is to modulate growth processes to develop the primitive kidney into a fully functional organ that is prepared for extra-uterine life (Gomez and Norwood, 1995). The RAS in the fetus has some similar functions to the mother such as maintenance of blood pressure, but it also plays a large part in the development of the kidney. The activity of the RAS greatly increases during fetal and early-postnatal life, which signifies its important role in renal development (Hilgers et al., 1997, Robillard and Nakamura, 1988). All components of the RAS can be detected in the sheep fetus from as early as day 40 of pregnancy. The RAS helps stimulate

nephrogenesis which is completed at day 130 in sheep, although tubular growth still occurs after this time (Moritz et al., 2000). Even though components of the RAS can be detected early in pregnancy, the RAS exerts most of its influence during the maturation of the kidney which occurs in late gestation in sheep and in the early postnatal period in rats (Matsusaka et al., 2002). A functional renin-angiotensin system is essential to normal kidney development in the fetus (Gomez and Norwood, 1995).

Fetal renin

Plasma renin activity (PRA) of the fetus is similar to that of the ewe for the first two thirds of pregnancy, but after day 104 renin activity starts to increase. At day 130 it is 10-20 times higher than that of adults, a level that is maintained until birth. This increase in renin concentration signifies its important role in the development of the kidney (Zhang et al., 2000).

Although renin activity is high in the fetus during late gestation, it can change in response to environmental factors such as plasma sodium and plasma osmolarity (Broughton Pipkin et al., 1974, Lumbers, 1995, Siegel and Fisher, 1979, Smith et al., 1974). Increasing the plasma osmolarity of the ewe in late gestation lowers the PRA of ewe and her fetus (Lumbers and Stevens, 1983). However, PRA of the fetus was not affected when ewes were given 0.17 M NaCl in their drinking water (Stevens and Lumbers, 1986). The treatment did lower the PRA of the ewe, but plasma sodium was not altered in either the ewe or the fetus which suggests that the fetus was buffered from the increase in salt intake by the ewe. When plasma sodium of the fetus is altered, there is a significant negative relationship between plasma sodium level and PRA in the fetus (Lumbers and Stevens, 1983, Stevens and Lumbers, 1986). If the salt content of the diet is high enough during late gestation to significantly increase the plasma sodium of the

ewe (e.g. a 5 mmol/l rise), the plasma sodium of the fetus will also increase and to maintain osmotic balance it will be able to suppress its PRA to excrete more salt, similar to adults.

Fetal Angiotensin II

Angiotensin II levels in the fetus are similar to that of the ewe (Broughton Pipkin et al., 1974). The placenta is a major site of removal of angiotensin II which helps to buffer the fetus from maternal changes in this vasodilating hormone (Lumbers, 1995). The actions of angiotensin II change as the fetus matures. During mid gestation, angiotensin II is essential for normal kidney development but, as the fetus matures, it also takes on similar roles to that in the adult such as modulating blood pressure, renal blood flow, and sodium excretion in the fetus (Edwards et al., 1999, Iwamoto and Rudolph, 1979, Robillard and Nakamura, 1988). This change in the function of angiotensin II in the fetus probably relates to the relative abundance of its two different receptors.

The abundance and types of angiotensin II receptors in the fetus strongly supports the important role for angiotensin II in fetal development. Angiotensin II receptors are present in many organs in the fetus, including the kidney, brain, liver, adrenal gland, skin, gastrointestinal tract as well as blood vessels, connective tissue and skeletal muscle, especially the tongue (Hu et al., 2004, Millan et al., 1989). These receptors are present in the fetal lamb from day 41 of gestation and increase in number in the last third of gestation (Hu et al., 2004, Wintour, 1997). The two major types of angiotensin II receptors (AT1 and AT2) change in abundance relative to each other as the fetus matures. Early in development, the AT2 receptor predominates in the fetus. As nephron maturation is completed, AT2 is not longer expressed. In the mature nephrons, soon after birth in the sheep, AT1 receptor expression persists and continues throughout adult

life (Cox et al., 2005, Hilgers et al., 1997). This changing of receptor types in the fetus mirrors the changing role of angiotensin II from kidney development in the fetus to its salt-retaining function in the adult.

In summary, fetal renin activity greatly increases in late gestation which signifies the crucial role the RAS plays in kidney development. The RAS of the fetus can respond to changes in salt balance so that if maternal and fetal plasma sodium increase, renin activity of the fetus can decrease so more sodium is excreted. If salt levels in the mother are maintained at a high level for an extended period of time during late gestation, RAS activity in the fetus may be suppressed. Lower-than-normal RAS levels in the fetus could interfere with its kidney development. But how does the high-salt diet of the mother suppress the RAS of the fetus and what effect would this have on the renal function of the offspring? This is the subject of the next section.

5. Suppression of fetal RAS and consequences for the offspring in the perinatal period

As previously explained, the fetus is generally buffered from small (below about 3 mmol/l) changes in mother's RAS. However, fetal renin and angiotensin II levels can change in response to similar stimuli as in the mother such as increased blood pressure. Stevens and Lumbers (1986) gave pregnant ewes 0.17 M NaCl in their drinking water for 9 days at 118 days gestation and although this altered the plasma renin activity of the ewe (by 65%) it was not sufficient enough to lower the renin activity of the offspring. The ewes in this experiment were consuming around 26g of salt per day which was not enough to alter their blood pressure. Pregnant ewes eating saltbush would consume around 200g per day over a longer time period. This higher amount is

much more likely to alter the blood pressure of the ewe and cause a much larger decrease in the RAS of the mother and subsequently the RAS of the fetus. Newborn rat offspring from dams that consumed a high-salt diet (3%) during pregnancy showed suppressed renin mRNA levels (Ingelfinger et al., 1998). Similarly, when pregnant rats were given 0.15 mol/l solution instead of water, their one day-old offspring had suppressed angiotensin II expression in their kidneys (Balbi et al., 2004). Therefore, it is likely that if pregnant ewes consume a large amount of salt (about 14% of dry matter intake) during pregnancy, their offspring will have suppressed RAS activity during late gestation and the early post-natal period.

As previously explained, the perinatal period is a critical time for normal kidney development and setting of salt-handling mechanisms so suppressing the RAS of the new-born could effect their physiology, particularly the structure of their kidneys.

Kidney structure

Suppressing the RAS of the fetus through a high-salt diet can cause structural changes to the kidney (Hilgers et al., 1997, Matsusaka et al., 2002). These changes include tubular atrophy and enlarged glomeruli, but there seems to be no change in glomeruli number (Rasch et al., 2004). Balbi et al. (2004) gave rats a high-salt diet (0.15M NaCl drinking water) throughout pregnancy and found no difference in glomerular area or glomerular number, but the glomerular filtration rate was decreased in the high salt treatment group at 30 days of age. The high salt treatment also affected cell proliferation, as indicated by proliferating cell nuclear antigen, and some of the components of the extracellular matrix in the kidney, such as fibronectin and α -smooth muscle-actin. However, when female rats were fed a high-salt diet (8%) from weaning, through pregnancy and lactation, their offspring showed no difference in size or number

of glomeruli (da Silva et al., 2003). These results show that suppression of the fetal RAS can cause structural changes in the kidney, but the extent of these changes may depend on the amount of salt consumed and the period of time it was consumed.

Suppression of the RAS early in life can cause permanent changes to the structure and function of the kidney. In addition to this, it may also cause other changes in physiology that can affect the salt balance mechanisms of the animal, which will be discussed below. These changes in physiology may alter the way the animal copes with consuming salt when they are adults.

6. Consequences for adult offspring born to mothers consuming high amounts of salt during pregnancy

The majority of the literature on how suppression of the RAS in early life can influence the adult offspring's physiology has been conducted on rats for extrapolation to humans. The main focus of these studies has been on blood pressure of the offspring. This is because hypertension is one of the major contributing factors to cardiovascular disease in humans, which accounts for nearly 30% of all human deaths in the world (Bogdarina et al., 2007, WHO, 2003). However, the purpose of my study is quite different. Rather than focusing on blood pressure, I am focusing on the physiological changes that the offspring may possess which would help them to cope better when consuming a high-salt diet such as saltbush. For the purposes of this review, these changes fall into two main categories. First, those changes that directly involve the RAS and how efficiently salt is excreted from the body. This includes changes the basal activity of the RAS, how sensitive the RAS is to ingestion of salt and the ability of the

animal to excrete salt. The second category is the changes that influence the offspring's intake of food and water and salt preference. These two categories will now be discussed separately, but they are by no means independent of each other.

Possible changes to the RAS and salt excretion in the adult offspring

Suppression of the RAS early in life has been shown to cause changes in blood pressure, basal activity of the RAS and responsiveness of the RAS to salt intake. All of these factors then influence how efficiently salt can be excreted from the body, which is imperative to sheep being able to consume a high-salt diet such as saltbush. Figure 4 gives an overview of some of the possible changes in the offspring during the perinatal period and the alterations to the adult offspring's physiology. The changes in the diagram are classified into four groups: 1) the results which are known to happen (in bold), 2) those results which I predict are likely to happen (in circles), 3) alternative results which are possible (in squares) 4) those results which have not been tested before in rats or sheep (in italics). The "fork in the road" represents the two possible pathways where there are conflicting results. The changes to the offspring in the perinatal period, such as suppression of the RAS and kidney structure, were discussed in the previous section so I will now focus the review on the possible changes in the adult offspring's physiology and the changes that I expect.

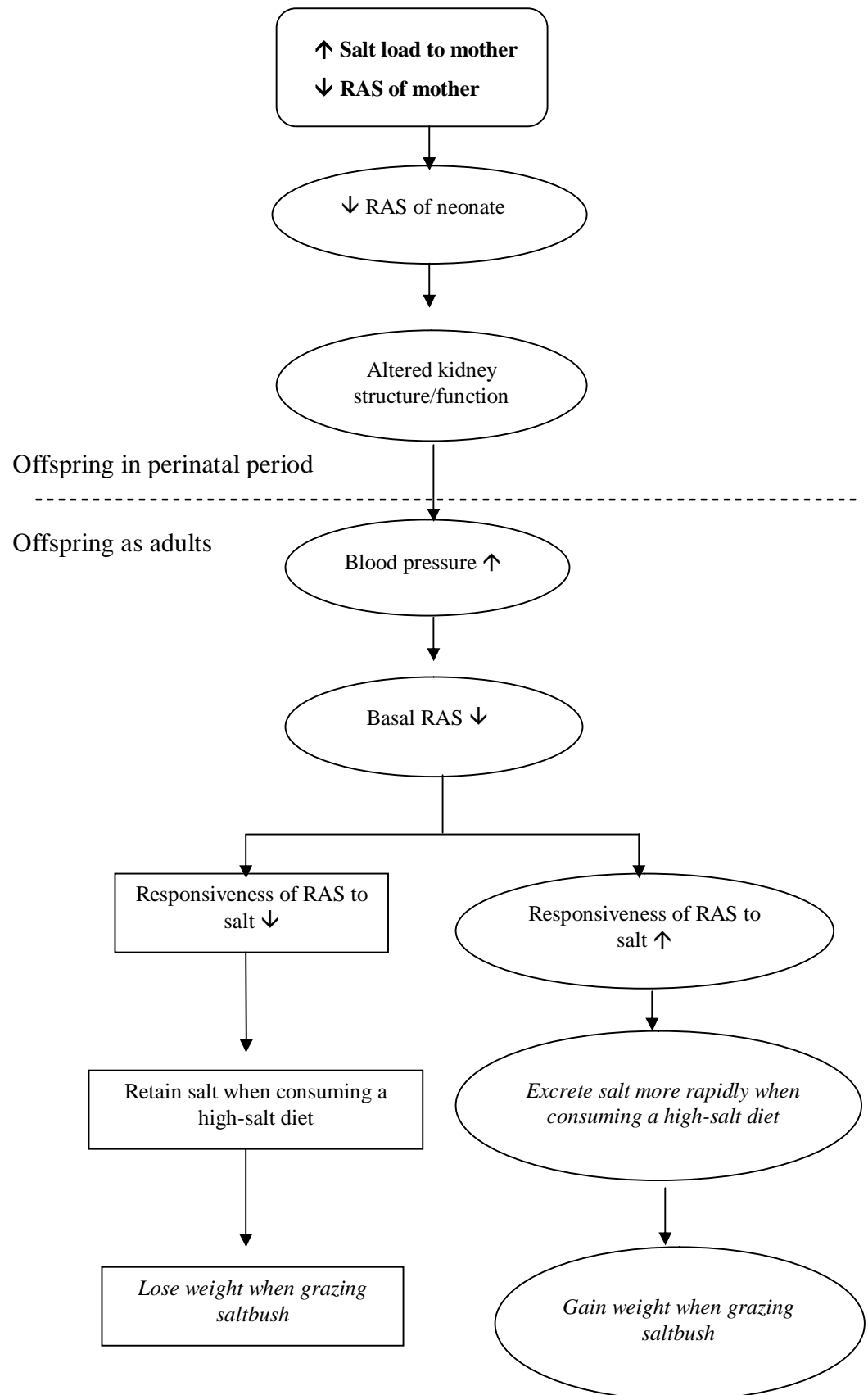


Figure 4: Possible alterations in physiology of offspring from mothers consuming a high-salt diet based on rat studies. Results in **bold** are those which are known to occur. Results in circles are those which I think are likely to happen. Results in squares indicate alternative changes that are also possible. Responses in *italics* have not been tested before, but I have developed hypotheses. The “fork in the road” represents conflicting results which could lead to two different physiological pathways.

Blood Pressure of adult offspring

Since the RAS is important for blood pressure control, it is not surprising that suppression of the RAS early in development can re-set the normal blood pressure of these animals to higher levels. Contreras (1993) fed pregnant rats a diet containing 3% salt throughout gestation and lactation and found that their offspring at adulthood had higher systolic blood pressure than rats raised on a normal salt diet. These results are consistent with those from many other studies in rats when mothers were fed a high-salt diet throughout pregnancy and lactation (Balbi et al., 2004, Contreras et al., 2000, da Silva et al., 2003, Vidonho et al., 2004). In contrast, when rats were fed a high-salt diet (8%) only during pregnancy, and not lactation, there was no difference in blood pressure between groups, but the offspring's blood pressure and heart rate were more responsive to stress (one hour of restraint) (Porter et al., 2007). An increased blood pressure from animals raised on a high-salt diet may also be influenced by other factors, most likely the kallikrein-kinin system and nitric oxide (El-Dahr et al., 1996, Katori and Majima, 2006, Lahera et al., 1997). Based on the majority of studies on rats (Balbi et al., 2004, Contreras et al., 2000, da Silva et al., 2003, Vidonho et al., 2004), feeding a high-salt diet to ewes during pregnancy and early lactation is likely to increase their offspring's blood pressure.

Basal RAS in adult offspring

Suppression of the RAS during critical developmental stages in the fetus is likely to permanently suppress the basal circulating RAS activity as adults. da Silva et al. (2003) fed female rats a high-salt diet (8%) and found an increase in angiotensin II levels in the kidneys of these offspring as adults but there was no difference in circulating renin activity. However, a decrease in angiotensin II and renin mRNA has been found in offspring born to rat dams that consumed a high-salt diet during pregnancy and

lactation. This suggests that if renin activity is suppressed during a critical time during development, they are likely to stay suppressed in adulthood. I believe that suppression of the RAS early in life will lower the set-point for basal RAS activity. Therefore, offspring from ewes that consume a high-salt or saltbush diet during pregnancy will have a suppressed RAS as adults.

Responsiveness of the RAS to salt

Much of the literature on the effects of perinatal RAS suppression focuses on salt sensitivity of blood pressure. This is described as the change in blood pressure in response to changes in salt intake (Dichtchekian et al., 1989). The sensitivity of the blood pressure to salt is determined by the responsiveness of the RAS to salt (He and MacGregor, 2003). For the purposes of this review, I will focus on the responsiveness of the RAS to salt intake (rather than blood pressure) because it is directly related to salt excretion. Figure 5 shows the normal response of RAS activity to an increase in salt content of the diet. It also shows the responses of animals whose basal RAS is suppressed (as expected in offspring born to ewes grazing saltbush) and whether their RAS is more, or less, responsive to an increase in salt intake.

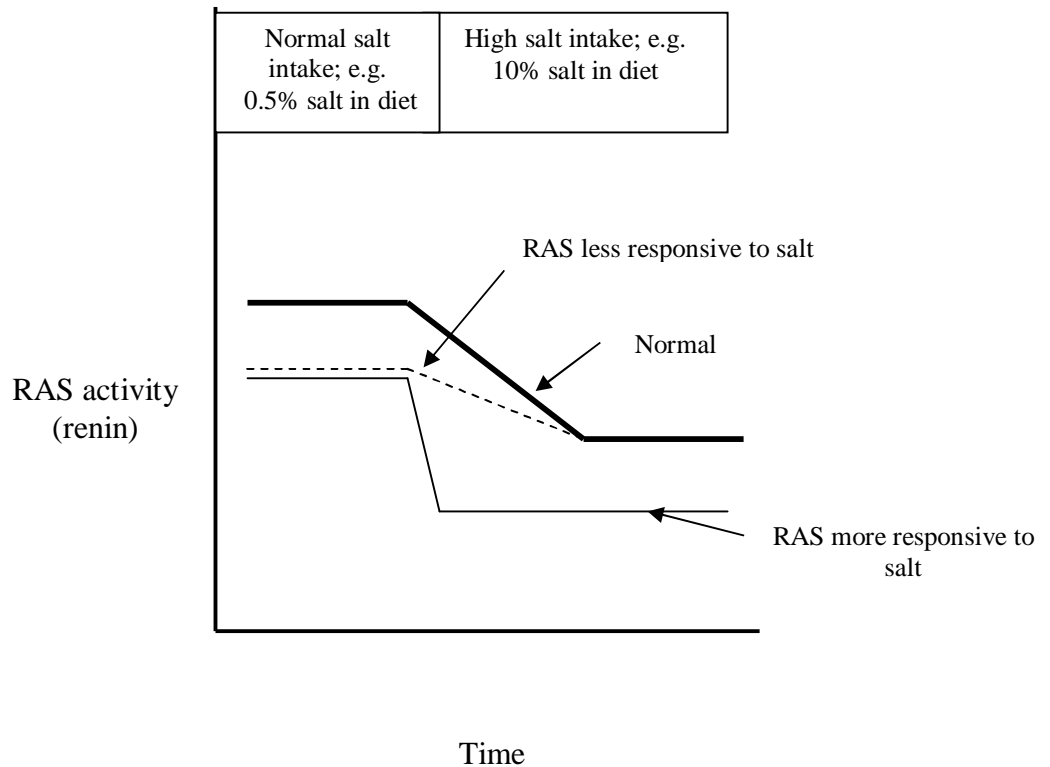


Figure 5: A simple diagram of the responsiveness of RAS activity to a change in salt content of the diet from normal salt content (e.g. 0.5% NaCl) to high-salt content (e.g. 10% NaCl). The bold line represents a normal animal, the dashed line represents an animal with a lower basal RAS activity that is less responsive to salt and the thin solid line represents an animal that also has a lower basal renin activity, but is more responsive to salt (adapted from Osborn et al. 2003).

There are conflicting reports on whether feeding a high-salt diet to a pregnant animal makes the RAS of her offspring more or less responsive to salt. da Silva et al. (2003) fed female rats an 8% salt diet from weaning, through pregnancy and lactation until their offspring were weaned. The authors found the adult offspring's RAS was less responsive to salt because their plasma renin activity did not change from a low to a high-salt diet. However, the rat dams in this experiment were consuming a high-salt diet almost life-long (from weaning). In contrast, ewes grazing saltbush would only be on a high-salt diet for the last half of pregnancy and early lactation because that often coincides with the period of pasture shortage in southern Australia (see first section).

Therefore, the changes in responsiveness of the RAS of the offspring may be less severe than those seen in the experiment by da Silva et al. (2003). To support this, Porter et al. (2007) found no difference in the responsiveness of the RAS to salt in offspring from rat dams that were fed an 8% salt diet only during pregnancy. Other authors have reported that adult rats raised by mothers consuming a high-salt diet showed increased blood pressure responsiveness to infusions of angiotensin II, which may suggest they are more responsive to salt (Arguelles et al., 1996, Contreras and Oparil, 1992, Osborn and Camara, 1997). Whether a maternal diet high in salt makes the RAS of the offspring more, or less, responsive to salt may depend on the amount of salt consumed and how long the mother consumes the high-salt diet, i.e. life-long or just during pregnancy.

Offspring born to ewes that consume saltbush during pregnancy may be more likely to have an increased responsiveness of the RAS to salt because it confers an adaptive advantage to them when grazing saltbush in later life. Animals raised in a particular environment develop adaptive responses which enable them to cope with the challenges faced in that environment (Bateson et al., 2004, Rickard and Lummaa, 2007).

Therefore, offspring whose mothers consumed saltbush while pregnant and during early lactation could be better prepared, physiologically, to cope with the high-salt content of saltbush in later life. If the offspring of pregnant ewes grazing saltbush have more responsive RAS, salt would be excreted more rapidly which would be an adaptive advantage to animals consuming a high-salt diet such as saltbush.

Alterations in salt excretion of offspring

The responsiveness of the RAS to salt determines how much salt is excreted and how rapidly it is excreted. Lack of responsiveness of the RAS to salt (the dotted line in

Figure 5) can lead to less efficient salt excretion and the retention of salt (Campese et al., 1996, Simchon et al., 1999). This is because after the ingestion of salt, the decrease in renin levels determines how much angiotensin II and aldosterone is formed and, in turn, how much salt is excreted. Therefore, if renin activity is not responsive to salt and does not decrease as much, less salt will be excreted and more salt will be retained. This salt retention can lead to increased blood pressure and in the case of humans, can lead to cardiovascular disease in later life (Bogdarina et al., 2007, Simchon et al., 1999). If these changes occur in sheep it is not known how this will affect production levels, especially when consuming high-salt diets such as saltbush. However, it is most likely that the retention of salt will not be a beneficial adaptation for sheep when grazing saltbush; it could even be detrimental to their performance if salt retention continues in the long-term.

The more likely possibility is that the RAS of offspring from ewes grazing saltbush will become more responsive to salt (the thin solid line on Figure 5) and their salt excretion capabilities are improved. There have been no studies on salt excretion of offspring whose RAS is more responsive to salt, but it is logical to suggest that salt excretion would improve. This is because when salt is ingested, renin activity decreases and more salt is excreted. If this decrease happens more rapidly, and renin is decreased to a lower level, more salt will be excreted at a faster rate. More efficient salt excretion mechanisms would be an advantage to sheep grazing saltbush, since the ability of the animal to excrete salt is the main factor limiting feed intake.

The physiology behind how RAS responsiveness and salt excretion mechanisms can be altered is very complex and not fully understood, but much of the literature points towards alterations in the pressure-natriuresis and the tubuloglomerular feedback

mechanisms to be the cause. The pressure-natriuresis mechanism is the amount of change in blood pressure required for the body to alter its excretion of salt (see section 2). The pressure-natriuresis “curve” can be shifted to the right so that it takes higher blood pressures to excrete the same amount of salt, or it can be shifted to the left where lower blood pressures are required to elicit a salt loss (or natriuretic) response (Dukacz et al., 1997). A shift in this “curve” can be caused by alterations in the RAS where a lower activity of the RAS can shift the curve to the left, and higher activity can shift the curve to the right (Evans et al., 2005, Gouldsborough and Aston, 2001, Madeddu et al., 1995). If we assume that the basal RAS of the offspring from mothers consuming a high-salt or saltbush diet have a suppressed basal renin activity, their pressure natriuresis curve may be shifted to the left which would allow them to excrete salt at lower blood pressures. The slope of the curve can also change where animals show a “blunted” pressure-natriuresis relationship where it takes a much higher change in blood pressure to initiate salt excretion, which would occur if the offspring’s RAS is less responsive to salt (Roman, 1986).

Much like pressure-natriuresis, the tubuloglomerular feedback (TGF) mechanism, which controls the autoregulation of glomerular filtration rate, can also be re-set. If TGF is re-set to a lower sensitivity, this may allow glomerular filtration rate to increase so more salt can be excreted (Persson et al., 2000). Nitric oxide activity and the kallikrein kinin system are likely to also be factors interacting with the renin-angiotensin system to control TGF and pressure natriuresis (Gouldsborough and Aston, 2001, Madeddu et al., 1995). Both TGF and pressure natriuresis are mechanisms that can be re-set to change how the animal responds to the ingestion of salt, but the exact nature of these changes and how they relate to offspring with perinatal exposure to a high-salt diet are

yet to be determined (da Silva et al., 2003, Hua et al., 1990, Manning et al., 2002, Roman, 1986).

Another uncertainty, which is crucial to this study, is whether these changes in the salt handling mechanisms of the offspring will allow them to cope better with the high-salt content of saltbush and gain weight when grazing it in later life. I will now focus on how the physiological changes in the offspring may influence their production on-farm.

Possible implications for performance when grazing saltbush

As Figure four illustrates, there are two likely “pathways” for the changes that could occur in offspring whose mothers consume a high-salt diet during pregnancy and the ability of the offspring to gain weight on saltbush may depend on which “pathway” they take. If the offspring’s RAS becomes less responsive to salt, they are likely to retain salt. This would be a disadvantage when consuming a high-salt diet because continuous salt retention would cause physiological problems such as increased extracellular volume if the high-salt diet was fed long-term. These adaptations would not be conducive to the offspring gaining more tissue weight when grazing saltbush. However, if the other “pathway” was taken, where the offspring’s RAS was more responsive to salt, they may be able to excrete salt more rapidly than normal animals. Excreting salt faster may enable the animals to consume more saltbush and gain more weight than normal sheep. Therefore, if offspring born to ewes that consume a high-salt diet or saltbush have an RAS that is less responsive to salt, it is unlikely that they will gain weight when grazing saltbush, but if the RAS of the offspring is more responsive to salt, they may be able to excrete salt faster which may enable them to gain weight when grazing saltbush.

In summary, a high-salt diet during the perinatal period has the potential to re-set not only basal RAS levels but also many of the mechanisms under the influence of the RAS that control salt balance and handling. As a consequence of its mother's high-salt diet, the RAS of the offspring may be suppressed early on in life and this suppression may continue into adulthood. There is evidence to suggest that the high-salt diet of the mother could make the offspring either more, or less responsive to salt, but I believe it will make them more responsive to salt because it provides an adaptive advantage for the offspring. This adaptation will cause the offspring to excrete salt more rapidly which may allow them to cope with the high-salt content of saltbush better, so they may be able to gain weight when grazing saltbush. However, the offspring's performance on saltbush could also be influenced by other factors, such as their intake of fluid and food that may also be altered by their mother's high-salt diet, this will now be discussed.

Possible changes to food and water intake and salt preference of the adult offspring

As well as altering the physiology involved in salt balance, feeding the pregnant mother a high-salt diet can also lead to changes in food and water intake and salt preference of the offspring. Exposure to the taste of salt early in development can alter taste signaling mechanisms and can also change what the animal perceives as a normal salt taste. In addition to this, the RAS plays a role in the regulation of water and food intake, as discussed in section 2. Therefore, any changes in the basal level of RAS activity could lead to changes in water and food intake. The possible alterations in food intake, water intake and salt preference of the offspring will now be discussed.

Food intake

Offspring born to mothers that consume a high-salt diet during pregnancy could have an altered food intake because of their altered RAS activity. As discussed in section 2,

there are conflicting results on the effect that angiotensin II has on appetite and the long-term effects of altered RAS seem to be different from the short term effects. In acute experiments, infusion of angiotensin II for 4-10 days has been shown to decrease food intake and bodyweight in rats (Cassis et al., 2002, Porter et al., 2003) and in ewes (Sunagawa et al., 2001), whereas Cassis et al. (1998) found no difference in food intake but a lower bodyweight in rats after angiotensin II infusions. However, chronic studies would be a better comparison for the long-term alterations in the RAS that could be seen in offspring from mothers that consumed a high-salt diet during pregnancy.

Chronic up-regulation of the RAS in transgenic rats caused them to have a higher food and water intake (Szczepanska-Sadowska et al., 2003). Therefore, we might expect that offspring with a suppressed basal RAS, such as if their mother consumed a high-salt diet, may have a lower food and water intake. However, results of Jayasooriya et al. (2008) show that transgenic mice with between 70-97% suppression in their RAS have no change in food intake, but have a lower bodyweight. Interestingly, both decreased (Jayasooriya et al., 2008) and increased angiotensin II levels (Cassis et al., 2002) have been associated with increased energy expenditure. No changes in food intake were reported in offspring from rat dams that received a high salt diet from conception until weaning (Contreras et al., 2000, Curits et al., 2004). However, they did not measure whether the offspring had changes in the basal level of RAS activity. It is possible that if the basal RAS of offspring from mothers consuming a high-salt diet is altered, food intake and bodyweight could also be changed, though the direction of the change is not clear.

Food intake may also be affected by other factors in the body that are influenced by a high-salt diet, such as fetal swallowing and hormones like insulin and leptin. Fetal swallowing of amniotic fluid is essential for many aspects of development, especially

gastrointestinal maturation, and may program thirst and appetite responses in animals in their adult life (El-haddad et al., 2004). Swallowing responses develop quickly in sheep at about day 116 of gestation and can be influenced by the fetal RAS. Blockade of angiotensin II receptors or ACE inhibitors can markedly reduce fetal swallowing (El-haddad et al., 2004). Since fetal swallowing can program water and food intake in the fetus, factors that reduce the activity of the RAS, such as high-salt diet, may permanently alter water and food intake of the animal (Vidinho et al., 2004). Altering the diet of the mother can also change the regulation of hormones such as insulin and leptin that control appetite (Blache et al., 2007, Digby et al., 2008). Exposure to a high-salt diet during pregnancy and lactation tended to increase the insulin sensitivity on adult male offspring (Vidinho et al., 2004). Little work has focused on alterations in food intake from animals receiving high perinatal salt intake, but it is likely that a high-salt diet could affect fetal swallowing, insulin sensitivity and basal RAS levels which could in turn alter food intake.

Water intake

Only a small number of studies have looked at the fetal programming of thirst, but they show that it can be altered. Increasing plasma sodium concentration of pregnant ewes through dehydration from day 110 to birth increased the plasma osmolarity of their offspring as well as increasing the thresholds for AVP secretion (Desai et al., 2003). Having an altered osmolarity set-point could influence the amount of water needed to maintain osmolarity and thus influence thirst. Curtis et al. (2004) fed a high-salt diet (3%) to rats during pregnancy and lactation and observed the water intake of their offspring. They found that rats with perinatal exposure to a high-salt diet drank less water and took longer to start drinking only when they had ingested a concentrated salt solution, but not after the salt was infused intravenously. The authors attributed this to

alterations in pre-absorptive signals associated with NaCl taste in the offspring. These include signals from the oral cavity such as taste and swallowing, the stomach, as well as by local osmoreceptor signals arising from the liver. Another, recently discovered, pre-absorptive signal that could be altered are visceral osmoreceptors (or Na⁺ receptors), which provide an early stimulation of thirst in rats given a high-salt diet (Manesh et al., 2006). Curtis et al. (2004) propose that a high-salt diet early in life could also alter the set point for what is perceived as normal NaCl taste, thus altering water intake. Offspring born to ewes that consume large amounts of salt during pregnancy may have altered water intake due to changes in pre-absorptive signals associated with the taste of salt.

Salt preference

A high-salt diet during pregnancy and/or lactation can alter the salt preference of the offspring. Contreras and Kosten (1983) fed a high-salt diet (3%) to pregnant rats from conception and continued to feed the offspring the high-salt diet until they were 30 days old. These offspring were given a preference test at 90 days of age with the choice of water or NaCl solution at different concentrations. Both males and females that received a high-salt diet from an early age showed a higher preference for NaCl solutions of 0.1, 0.2 and 0.3 M compared to offspring raised on a normal or low salt diet. These results were supported by results from later studies where there was a similar increase in preference for solutions high in NaCl by rats raised on a high-salt diet (Contreras and Ryan, 1990, Curtis et al., 2004). Similar results have also been found in dairy cows. Mohamed and Phillips (2003) supplemented dairy cows in the last 2 months of pregnancy with salt (extra 70 g/per day) then gave their calves a choice of concentrates with normal (5.8 g/kg DM) or high sodium concentrations (10.8 g/kg DM). Calves from the cows that received supplementary sodium during pregnancy preferred the high

sodium concentrate more than calves from cows that received a normal sodium intake during pregnancy. An increase in salt preference is also seen when animals are exposed to mineralofluid loss (maternal vomiting), extracellular dehydration and excessive salt and water intake in the perinatal period (Arguelles et al., 2000, Leshem, 1998, Nicolaidis et al., 1990). Salt preferences can also be altered by exposure to salt only in the early postnatal period. Phillips et al. (2002) increased the level of sodium in the concentrate diet of calves from 4 to 9g/kg DM from birth through to weaning at 42 days. The authors found that at 6 months of age, the calves' preference for sodium in silage increased from 3 g/kg DM of sodium in controls to 9 g/kg DM if they had previous exposure to the high-salt feed. An increase in salt preference can be seen even after a very brief exposure to salt in the perinatal period. Smriga et al. (2002) placed salt enriched milk (85 mmol/l) on the tongue of rat pups (7 days old) nine times for 15 minutes each time. This was repeated every four hours for 32 hours. The authors found that this short period of exposure increased the adult rat's preference for salt. Salt preference of an animal can be increased by exposure to the taste of salt early in life.

Salt preference may only be altered if animals are exposed to the taste of salt during a sensitive period of development. In the same experiment as above, Smriga et al. (2002) found no difference in salt preference in rats exposed to salt 14-15 days after birth. There is also no difference in salt preference if rats are exposed to a high-salt diet between weaning and adulthood (Midkiff and Bernstein, 1983). There seems to be a sensitive period in development where salt preference can be modified (Bradley and Mistretta, 1980, Hill and Przekop, 1988, Phillips et al., 1999). Neurons of fetal sheep first start responding to salt at day 114 of gestation and, as seen in cows and rats, salt preference can be altered with only postnatal exposure to salt (Bradley and Mistretta, 1980, Phillips et al., 1999, Smriga et al., 2002). This suggests that most critical time for

altering salt preference in sheep could be between the last third of gestation and in the post-natal period until weaning.

Alterations in salt preference could be caused by the renin-angiotensin system (RAS) and the responsiveness of the chorda tympani (CT) nerve to salt. The blockade of the AT1 receptor during gestation leads to an increased thirst and sodium appetite in the offspring (Butler et al., 2002). This suggests that suppression of the RAS of the fetus could cause the increase in salt preference. It may do this by up-regulation of AT1 receptors as adults, which may increase the effect of angiotensin II on salt appetite (Hill, 1986). An increased salt preference could also be a function of CT nerve responsiveness. Pittman and Contreras (2002) found that rearing rat pups on a high-salt diet could decrease the CT nerve response to salt by altering the amount of sodium channels in the membrane of taste receptor cells. However, an earlier study by Bird and Contreras (1987) found no change in CT nerve responsiveness from exposure to a high-salt diet early in life. These authors concluded that the reason for the increased salt preference in animals raised on a high-salt diet is that salt simply “tastes better” to them. An increase in salt preference due to early exposure to a high-salt diet could also be attributed to a long-term decrease in the amount of the hormone norepinephrine in the amygdala in the brain (Smruga et al., 2002). The increase in salt preference seen in animals raised on a high-salt diet could be due to hormones, nerves, previous experience with salt or a combination of all of these factors.

Most of these studies on salt preference have included giving lactating animals a high-salt diet. This may mean that a high-salt diet can change the sodium concentration of

their milk which, may contribute to their offspring's altered salt preference. I will now discuss how a high-salt diet during lactation could affect the ewe and her lamb.

7. High-salt diet during lactation

There is conjecture about whether an increased sodium intake of the mother increases the sodium content of her milk. Vijande et al. (1973, 1996) found that when lactating rats consume excessive amounts of salt and water (due to partial ligation of their abdominal aorta) the sodium content of their milk increases. Dlouha et al. (1973) found that lowering the salt content in the diet of lactating rats from 175 to 51.4 mEq of Na/kg also lowered the Na content of their milk from 7 to 20 days lactation. However, this was not the case at birth where the low salt group actually had higher concentrations of Na in their milk. Results from ruminant animals show no change in sodium content of the milk when a high-salt diet is fed. Supplementation of sodium deficient cows with salt failed to consistently alter the sodium content of their milk (Murphy and Plasto, 1973). When ewes were fed a high-salt diet (13% NaCl) the concentration of sodium and potassium in their milk did not change, but the concentration of chloride in the milk increased (Meyer and Weir, 1954). This suggests that concentrations of other cations, such as magnesium, may have increased in order to balance the negative charge of the chloride. Even when ewes are fed large amounts of salt, it is unlikely that the sodium concentration in their milk will increase, but there may be changes in the concentrations of other minerals, such as chloride.

Studies involving the cross-fostering of rat pups from hypertensive mothers to normal mothers have shown there could be vasoactive factors in milk. Blood pressure of a spontaneously hypertensive rat can be permanently lowered by 20-30 mmHg if they are fostered to a normotensive mother from birth (Gouldsbrough and Aston, 2001). However, the sodium content of the milk of hypertensive rats and normotensive rats does not differ, so there must be another factor in milk that can permanently alter blood pressure (McCarty and Tong, 1995). Milk proteins contain bioactive peptides, some of which can inhibit angiotensin converting enzyme (ACE) (Park et al., 2007). Many peptides that inhibit ACE have been found in milk, these are used in the treatment of high blood pressure to lower the activity to the RAS (Park et al., 2007). Lactating animals consuming a high-salt diet may produce more ACE inhibitors which suppress their RAS to allow more salt to be excreted. Therefore, there is a possibility that this may also increase the abundance of ACE inhibitors in their milk, which would lower the RAS of the suckling offspring. The high-salt diet of the mother could suppress the RAS of her offspring during lactation by increasing the sodium content of the milk and/or increasing the amount of ACE inhibitory proteins in milk.

In summary, feeding a high-salt diet to animals during pregnancy and lactation has the potential to change their offspring's physiology. Some of the salt-regulating mechanisms of the offspring could be altered such as the responsiveness of their RAS to salt and how efficient they are at excreting salt. The food and water intake of the offspring could also be altered due to changes in basal RAS activity and salt preference could also be changed due to the exposure of the offspring to salt early in life. However, there are gaps in the literature that need to be addressed.

8. Key gaps in our knowledge

There are four main questions of critical importance to this study that remain unanswered by the literature:

- 1) Will the physiological changes in sheep offspring be similar to those observed rat offspring born to mothers that consumed a high-salt diet during pregnancy?
- 2) Will saltbush have the same effect as a high-salt diet on the ewe and her offspring?
- 3) Will feeding the ewes a high-salt diet only from mid pregnancy to early lactation have the same effect as feeding from conception until weaning like most rat studies?
- 4) Are the resultant changes in physiology of the offspring beneficial or detrimental to their performance on saltbush in later life?

I expect that offspring born to ewes fed a high-salt diet during pregnancy will show similar changes in physiology as rat offspring born to dams that consume a high-salt diet during pregnancy. Rats show all the same salt-handling physiology as sheep, the only difference between the species may be in the type of placenta and stage of development at birth. Rats have a discoid, hemochorial placenta, whereas sheep have a cotyledonary epitheliochorial placenta (Carter and Enders, 2004). The size and the structure of these placentas differ. For example, in rats, the placenta has direct contact with maternal blood, whereas in the sheep placenta there is a barrier of capillary walls and connective tissue between the placenta and maternal blood (Stulc, 1997). There are also some differences in the transport properties such as diffusion permeability's and differences in the rate of fetal growth compared to placental weight (Stulc, 1997). When rats are born, their kidney and other organs are less developed than that of a newborn

lamb, so rats continue more organ development post-natally. However, the processes of development are essentially the same between the species. Recent work has focused on both the effects of a high-salt diet on the ewe (Digby et al., 2008) and the physiological implications for her offspring when the ewes are fed a high-salt diet during pregnancy (S. Digby and D. Revell, personal communication). The results from this work suggest that sheep offspring show similar physiological changes to rat offspring. In light of this, I expect that the physiological changes in the offspring from mothers that consume a high-salt diet during pregnancy will be similar between sheep and rats.

It is not clear if a saltbush diet will have the same effect on the ewe, and her offspring, as a high-salt diet. The high salt content of saltbush is thought to be the main challenge to the physiology of the animal, so the physiological effects of salt are often examined in isolation (Masters et al., 2006). However, a high-salt diet is not the same as saltbush because saltbush also contains high concentrations of other minerals such as potassium which can have an added effect on the mineral balance of the animal (Masters et al., 2005). Saltbush also contains secondary compounds such as oxalates (Abu-Zanat et al., 2003), but nothing is known about how these compounds effect the fetus. Even though saltbush contains other compounds and minerals, a high amount of NaCl is the main factor which exerts physiological pressure on the animal (Masters et al., 2006).

Therefore, it is likely that the fetal programming effects on the offspring from pregnant ewes consuming saltbush will be similar to those observed when high-salt diet is fed to the pregnant ewe.

Feeding saltbush to the ewe rather than a high-salt diet can have an advantage on the performance of the offspring because it exposes them to saltbush, not just salt, from an early age. Exposure to a particular food early on in life increases the animal's

consumption of that food in later life. Lambs prefer to eat a shrub if they are exposed to it between 7 weeks and 4 months of age (Nolte et al., 1990). Squibb et al. (1990) also found that when lambs were exposed to a forage shrub (*Cercocarpus montanus*) from 4-8 weeks of age, they consumed more of it when they were older. This period of time corresponds to the transition from non-ruminant to ruminant and is a sensitive period for introducing the digestive system to a particular forage. Distel et al. (1996) fed a low quality roughage (weeping lovegrass) to wethers or high quality roughage (fresh oats) from one to five months of age then tested their intake of roughage when they were nine months of age. The animals with prior exposure to low quality roughage consumed 15% more low quality roughage (sorghum) than animals exposed to high quality roughage at an early age. These animals were also able to retain more nitrogen when consuming the low quality roughage. Similarly, when lambs are fed blackbush (a tannin-rich browse) from 6-26 weeks of age, they consume 27% more blackbush when tested nine months later (Distel and Provenza, 1991). This was further supported by Glasser et al. (2009) who found that rearing environment, rather than genetics, had more of an influence on the preference of goat kids for a tannin-rich browse. Wiedmeier et al. (2002) exposed suckling calves to ammoniated wheat straw for 66 days then looked at their performance 5 years later for 3 consecutive years when they were fed ammoniated wheat straw supplemented with alfalfa during winter from December to May (and grazed irrigated meadow pastures for the remainder of the year). Cows exposed to ammoniated wheat straw as calves had an increased bodyweight and body condition score for all of the 3 years studied and had increased milk production in 2 out of the 3 years. Results from these studies clearly show that exposure to low quality roughage or forage can increase the animal's preference for, and performance on, that food in adult life. Therefore, offspring born to ewes consuming saltbush during pregnancy and early

lactation may have a preference for saltbush, and perform better on it, simply because they were exposed to the forage early in life.

Even though most experiments with rats have involved the dam being fed a high-salt diet from conception until weaning, I expect that feeding a high-salt diet only from mid pregnancy to early lactation will have a similar effect on the offspring. I have chosen to concentrate on this developmental period for two reasons. As discussed in section 1, autumn is the time when it is most profitable to graze sheep on saltbush and it also coincides with mid pregnancy to early lactation in sheep. Therefore, this period of time is when it would be most practical to graze pregnant ewes on saltbush. The second reason is that mid to late pregnancy and early in the postnatal period is thought to be the “developmental window” for altering the RAS and salt-handling physiology of the offspring (Hilgers et al., 1997, Konje et al., 1996, Robillard and Nakamura, 1988, Wintour, 1997, Woods et al., 2001). Evidence for this has involved using RAS inhibitors at different stages of pregnancy then observing the resultant changes in the physiology of the offspring. The effect of a high-salt diet only during mid-late pregnancy and early lactation has not been tested before, but results from the RAS inhibitor studies suggest that suppression of the RAS during this period will alter the physiology of the offspring.

If changes in physiology occur in offspring born to ewes consuming a high-salt or saltbush diet, I expect these changes to be beneficial to the offspring’s performance on saltbush in later life. The combination of *in-utero* programming of salt balance and the post-natal exposure to saltbush may produce an animal that is physiologically, and behaviorally, adapted to consuming a high-salt diet. This may increase the offspring’s consumption of saltbush when they graze it in later life, which will allow it to gain more

weight. Therefore, performance of sheep on saltbush may be increased if their mothers grazed saltbush while pregnant and in early lactation.

9. Objectives

The objectives of this study are to investigate the physiological and behavioral effects on the offspring from feeding a high-salt diet or saltbush to their mothers from mid pregnancy to early lactation. My general hypothesis is that offspring born to ewes that consumed a high-salt or saltbush diet from mid-pregnancy to early lactation will have increased capacity to cope with salt that would allow them gain weight when grazing saltbush in later life.

CHAPTER 3

Feeding pregnant ewes a high-salt diet or saltbush suppresses their offspring's postnatal renin activity

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Abstract

If ewes consumed a high-salt diet or saltbush during the last 3 months of pregnancy and for 3 weeks after birth, I expected the renin activity of their lamb to be suppressed at birth and at 3 weeks of age. I also expected an increase in the concentration of cations other than sodium in the ewe's milk and an increase in the plasma Na concentration of the lamb at birth. To test these hypotheses, Merino ewes were fed a high-salt diet (14% NaCl) in an animal house and compared to control ewes eating a control diet (2% NaCl). In addition, I compared ewes grazing saltbush (about 13% salt in diet) to ewes grazing pasture from day 60 of pregnancy to 3 weeks after birth. Lambs born to ewes consuming saltbush had 85% lower ($P<0.001$) renin activity than lambs from ewes consuming pasture at 3 weeks of age. Similarly, lambs born to ewes consuming a high-salt diet had 20% lower renin activity at birth and 3 weeks ($P=0.07$). Feeding ewes a high-salt diet or saltbush altered the mineral composition of the milk, the largest change was a 10% increase in K levels ($P\leq 0.05$). Consuming a high-salt diet or saltbush lowered the plasma Na of ewes at 130 days of gestation (by 3-5 mmol/l; $P<0.001$), but only lambs from ewes fed the high-salt diet had a lower plasma Na at birth ($P<0.05$).

Suppression of the renin activity of lambs could lead to permanent physiological changes in salt balance in later life.

Introduction

When animals consume diets high in salt (NaCl), their rate of salt excretion increases because they have lower renin activity in their kidney. When less renin is released from the kidney, the formation of angiotensin II and aldosterone is slowed which signals the kidney to retain less salt (Morgan, 2001). The renin-angiotensin system (RAS) plays a critical role in the fetus and newborn by regulating the maturation of the kidney and processes that control salt balance (Gomez and Norwood, 1995, Matsusaka *et al.*, 2002). Suppression of the RAS during development can lead to physiological changes in the animal that can alter permanently the animal's salt and water handling capability, blood pressure, and salt preference (Curtis *et al.*, 2004, da Silva *et al.*, 2003). When pregnant rats are fed a high-salt diet, their offspring's angiotensin receptors are downregulated and the renin mRNA levels in their kidney are also suppressed in the first few days after birth (Balbi *et al.*, 2004; Ingelfinger *et al.*, 1998). Therefore, if pregnant animals consume large amounts of salt, the renin activity of their offspring may be suppressed which could alter their ability to cope with a high-salt diet as adults.

Pregnant ewes consume high amounts of salt (mainly NaCl) when they are grazing saltbush, a halophytic plant that is grown on salt land and is used widely in agriculture (Masters *et al.*, 2001). Lambs born to ewes consuming saltbush may have suppressed renin activity because of their mother's high-salt diet. Lambs receive almost all their nourishment from ewe's milk for the first three weeks of their life and, if the mineral (Na as well as other minerals) content of the milk is increased by the high-salt diet of the ewe, the lambs would ingest more minerals which would depress their RAS. Hence,

any postnatal alteration to the RAS activity in lambs may be linked to either their development *in-utero* or an increased mineral intake from their mother's milk.

Therefore, my first hypothesis is that feeding pregnant ewes a high-salt diet or saltbush will suppress their renin levels during pregnancy and those of their lambs during the early postnatal period (birth and at 3 weeks of age).

Although highly protected, milk composition can be changed by the diet of the ewe.

Both sodium and potassium levels in the milk of rats can be altered by feeding them a low-salt or a high-salt diet (Dlouha et al., 1973, Vijande et al., 1996). However, when a high-salt diet (about 13%) was fed to pregnant ewes there was no change in the sodium concentration in their milk, but chloride levels in the milk increased (Meyer and Weir, 1954). It is possible that consuming excessive amounts of sodium and chloride could alter the regulation of other minerals in the milk because, if chloride increases and sodium remains the same, concentrations of other cations such as potassium, calcium or magnesium would be expected to increase to balance the charge. If the mineral content of the milk can be altered by the diet of the ewe, it is likely that the lamb's consumption of minerals will increase and its RAS will be depressed when consuming this milk. The second hypothesis tested was that feeding ewes a high-salt diet or saltbush would increase the concentration of cations, particularly potassium, in their milk.

The high-salt diet of the ewe should not change her plasma sodium concentration, but may increase her plasma chloride concentration, as shown in previous studies with sheep (Meyer and Weir, 1954, Potter and McIntosh, 1974). However, results from rat dams consuming large amounts salt have shown that although the plasma sodium of the mother may not change, the plasma sodium of the offspring may increase because of the immaturity of their kidney (Deloof et al., 2000). Hence my third hypothesis is that a

high-salt diet or saltbush will increase plasma concentrations of chloride and keep sodium the same in ewes but increase plasma concentrations of both chloride and sodium in lambs at birth.

To test these hypotheses I conducted an animal house experiment where ewes were fed a high-salt diet (14%) or a control diet (2%). I also conducted a field study where pregnant ewes grazed saltbush or a control diet of pasture based on sub-clover grown on non-saline land. These two experiments were analysed separately although the data appear in the same figures and tables.

Materials and Methods

Animals and diets

Two-year-old Merino ewes were artificially inseminated using semen from the same ram. They grazed the same clover-based pasture until day 55 of gestation when they were pregnancy scanned. Sixty single-bearing ewes were housed in individual pens and were termed “animal house ewes”. They were assigned to either a high-salt diet (14%) or control diet (2%) and the control ewes were pair-fed to the salt ewes. Another 70 ewes were placed in either the saltbush or pasture treatments. These ewes are referred to as “field ewes” and they grazed saltbush or grazed the control diet of dry pasture based in subterranean clover. Treatments commenced on day 60 of pregnancy and concluded when the lambs were an average of 3 weeks old. Plasma renin activity of the ewes was measured before the treatments at 60 days gestation, then at 90 and 130 days gestation and at 21 days lactation. Plasma renin activity of the lambs was measured at birth (within 24 hours), 21 days of age and at weaning at 15 weeks. Plasma sodium and chloride concentration was measured in the ewes at 130 days gestation and in the lambs at birth. Milk samples were taken on day 21 of lactation. After the lambs were 3 weeks

old, ewes were taken off their treatment diets and all ewes and their lambs grazed together on pasture.

Animal house ewes

Thirty ewes were fed a diet containing approximately 14% salt and termed “high-salt ewes”. Another 30 ewes were fed the control diet (termed “control ewes”) and were pair fed to ewes on the high-salt diet in such a way that each control ewe consumed the same daily amount of organic matter as its salt-fed partner. Ewes were fed to maintain conceptus-free weight throughout pregnancy (SCARM, 1990) (Table 1). Water intake of the ewes was not measured.

Field ewes

Thirty-five ewes were allocated to the saltbush treatment and termed “saltbush ewes” and another 35 were allocated to graze the control diet of pasture, termed “control pasture ewes”. Ewes from each treatment were divided into three groups (replicates), two of single-bearing ewes (12 ewes in each) and one group of twin-bearing ewes (11 ewes). The three groups of saltbush ewes were rotationally grazed on 12, one-hectare, saltbush plots. From day 60 to day 108 they were grazed on River Saltbush (*Atriplex amnicola*) and, from day 108 to week 3 of lactation, they grazed Oldman Saltbush (*Atriplex nummularia*). Ewes grazing saltbush were supplemented with barley to meet their energy requirements to maintain their conceptus-free weight throughout pregnancy, with twin-bearing ewes fed proportionally more supplement than single-bearing ewes to achieve this (SCARM, 1990). The two groups of single-bearing ewes in the pasture treatment grazed a pasture based on subterranean clover (80%), annual ryegrass (10%) and cape weed (10%) in two separate 10ha plots. These ewes were supplemented with lupins to match the higher crude protein intake of the saltbush ewes

and these were fed to maintain the ewe's conceptus-free weight (SCARM, 1990) (Table 1). The twin group grazed barley crop residue (stubble) and were supplemented with lupins and barley at a higher rate than single-bearing ewes in order to maintain their conceptus-free weight (SCARM, 1990). Ewes were weighed every two weeks and the level of supplementary feeding was adjusted according to weight change so all ewes maintained their conceptus-free weight (Wheeler et al., 1971).

Offspring

Lambs were weighed within 24 h of birth, at 3 weeks of age (when the treatments stopped), and at 15, 20 and 24 weeks of age to determine if there was a longer-term effect of treatment on liveweight. Blood samples were assayed from 23 high-salt offspring (13 female and 10 male) and 21 control offspring (10 female and 11 male). In the field experiment blood samples were assayed from 25 saltbush offspring (11 female and 14 male) and of these offspring, 19 were single-born and 6 were twin-born. Blood samples were assayed from 23 pasture offspring (12 female and 11 male) and of these, 17 were single-born and 6 were twin-born. In the case of twins, both lambs were studied.

Table 1: Composition of diets of ewes in the animal house and ewes in the field. The diet of the ewes grazing pasture was mainly dry subterranean-clover plus lupin supplement. Diet of ewes grazing saltbush consisted of saltbush, dry inter-row and barley supplement.

% DM	Animal house experiment		Field experiment				
	Control	High-salt	Control pasture ewes		Saltbush ewes		
			Pasture	Lupin	Saltbush	Inter-row	Barley
Organic matter	93.5	84.2	95	98.8	75.3	96.6	98.7
Crude protein	18.6	16.1	11.4	29.1	18.4	4.4	12.5
Estimated ME¹ (MJ/kg DM)	9.96	8.55	6.50	13.0	7.40	5.80	12.0
P	0.27	0.26	0.14	0.30	0.20	0.07	0.44
K	1.23	1.07	0.75	1.30	2.29	0.10	0.53
S	0.23	0.21	0.13	0.21	0.40	0.07	n.a. ²
Na	0.62	5.26	0.23	0.05	7.90	0.07	0.027
Ca	0.47	0.44	0.71	0.23	0.70	0.30	0.14
Mg	0.16	0.14	0.15	0.16	0.65	0.09	0.19
Cl	1.39	9.00	0.53	n.a.	10.53	0.24	n.a.
mg/kg DM							
Cu	11	5	9	3	4	3	8
Zn	46	40	21	32	31	14	48
Mn	40	34	85	22	356	116	12
Fe	192	192	1531	400	91	331	n.a.
B	7	6	11	n.a.	41	4	n.a.
% content of diet as gestation progressed:							
Day 60	100	100	90	10	70	20	10
Day 130	100	100	70	30	55	15	30
Day 21 of lactation	100	100	63	37	47	13	40

¹ ME= Metabolisable energy, estimated from ingredient composition of animal house diets and estimated for saltbush (D.G. Masters, personal communication). ² n.a. Not available.

Blood samples

Ewes were blood sampled before treatments commenced at day 60, then at days 90 and 130 of gestation and approximately 3 weeks into lactation before ewes were taken off their treatment diets. Within 24 hours after birth lambs were weighed and 5-10 ml of blood was taken from the jugular vein of the lambs. Blood samples from the animal house lambs were often taken immediately after birth, before the lamb had suckled. In the field experiment where ewes were not confined with their lamb, the lamb suckled before the blood sample was taken so as not to disturb the ewe-lamb bonding process. Lambs were blood sampled again at 3 weeks of age. However, the field lambs were blood sampled 3 days later than the animal house lambs due to logistical reasons. A

blood sample was also taken at weaning when offspring were 15 weeks of age. All blood samples were kept at room temperature for no longer than 10 minutes before centrifugation for 15 min at 1500 x g and frozen at -20°C until analysed. Plasma renin activity was measured as the rate of formation of angiotensin I using a commercially available kit (Gamma Coat Plasma Renin Activity RIA kit, DiaSorin, Stillwater, Minnesota, USA). The inter-assay coefficient of variation was 7.2% and the intra-assay coefficient of variation was 5%. The detection limit after 18 hour incubation was 0.01 ng/ml/hr. Plasma concentration of sodium and chloride was measured using Ion Selective Electrode and was run on an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd. Tokyo, Japan).

I attempted to minimize stress on ewes and lambs when blood samples were collected because stress can increase renin activity. I did this by having the ewes and their lambs in close proximity in pens for at least an hour before, and during sampling. An exception was the sample collected from lambs at birth in the field because I did not confine ewes prior to lambing so they could continue to graze their treatment diets.

Milk samples

Milk samples were collected before the ewes were taken off their treatment diets at week 3 of lactation. Lambs were separated from the ewes for 3 hours before the foremilk was collected (20 ml) at 11 am. Samples were frozen at -20°C until analysis. Mineral composition of milk was determined using an Inductively Coupled Plasma Optical Emission Spectrometer, performed on an ARL 3580 B machine.

Statistical analysis

The statistics program Genstat (10th Edition, VSN International Ltd) was used to analyse the data. Plasma renin activity results were analysed using residual maximum likelihood (REML) linear mixed model with diet of the ewe and time as fixed effects and tag number as a random effect. Lamb renin values were transformed by natural log to obtain a normal distribution before analysis using REML. Gender of the offspring was also incorporated into the fixed effects as well as birth status of the lamb (single or twin-born) for the offspring born in the field experiment. Simple linear regression was used on the natural log of renin activity to calculate the relationship between the lamb's renin concentration and that of its mother. All other results were analysed using ANOVA.

All procedures were approved by the CSIRO Animal Ethics Committee (Floreat, Western Australia).

Results

Plasma renin activity of ewes

Renin activity was lowered ($P < 0.001$) by an average of two thirds in ewes consuming a high-salt diet or grazing saltbush during pregnancy and early lactation (days 90 and 130 days of gestation and at 3 weeks lactation) compared to their counterparts consuming the control diet or pasture. All ewes had similar renin activity at day 60 of gestation before treatments commenced (Figure 6).

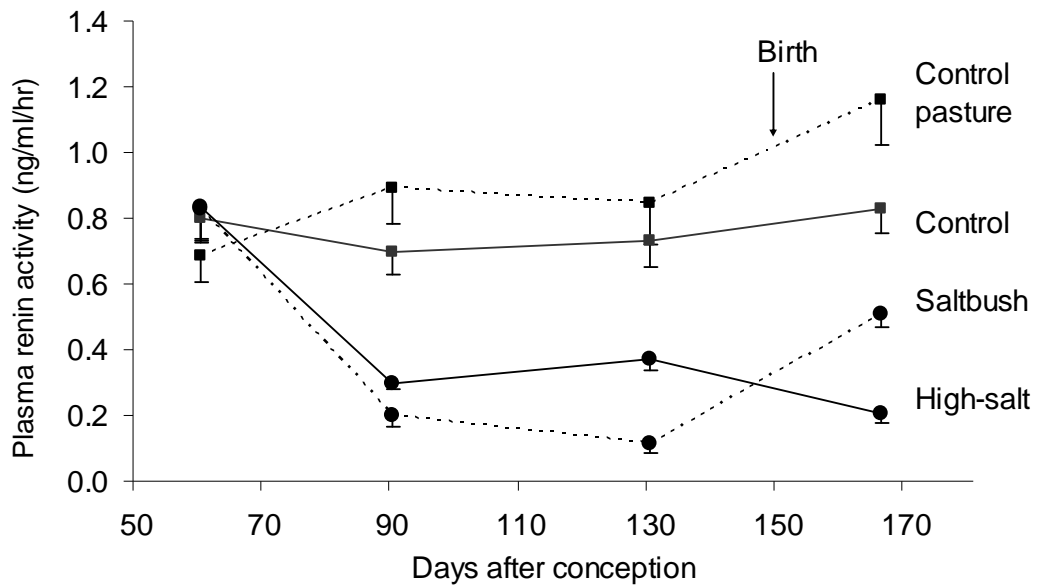


Figure 6: Plasma renin activity (mean value \pm s.e.) of pregnant ewes consuming a high-salt or control diet in the animal house and ewes grazing saltbush or dry pasture in the field. Significant differences existed between the salt and saltbush treatments and their controls ($P < 0.001$).

Plasma renin activity of offspring

In the animal house study, lambs from ewes fed a high-salt diet had 20% lower renin activity ($P = 0.07$) than control lambs in the early postnatal period from birth to 3 weeks of age. There was no age by treatment effect because the difference in renin activity of the high-salt and the control lambs was unchanged between birth and 3 weeks (Table 2). At 15 weeks of age (weaning) plasma renin levels of high-salt lambs were similar to control lambs (control 1.5 ± 0.25 vs high-salt 1.5 ± 1.33).

There was an interaction between age and treatment for renin activity in lambs in the field experiment. Feeding saltbush to ewes during the last 3 months of pregnancy and 3 weeks after birth suppressed the renin activity of their lambs at 3 weeks of age to one-sixth of the renin activity of the control pasture lambs ($P < 0.001$). No difference existed at birth (Table 2). Saltbush lambs had similar renin activity to control pasture lambs at

weaning (pasture 1.4 ± 0.06 vs saltbush 1.3 ± 0.08 ; $P=0.1$). Gender, birth status (single or twin) or birthweight had no significant effect on any of the parameters measured ($P>0.05$) with the exception of twins having a lower birth weight, which is reported later.

Table 2: Plasma renin activity (ng/ml/hr) (\pm s.e.) of lambs during the early postnatal period of birth and three weeks of age.

	Animal house offspring		Field offspring	
	Control	High-salt	Pasture	Saltbush
Birth	17.5 ± 1.48	12.8 ± 1.44	19.7 ± 1.58	19.1 ± 2.28
3 wk	10.5 ± 1.13	8.5 ± 1.22	4.6 ± 0.49	0.7 ± 0.12
	$P=0.07^t$		$P<0.001^{ta}$	

^tTreatment effect. ^{ta}Treatment by age effect.

In the animal house experiment, renin activity of lambs at birth was correlated to that of their mothers at 130 days of pregnancy ($y = 13.79x + 6.65$; $P=0.002$, $R^2 = 0.4$).

However, this relationship was not found in the field experiment. When data from both experiments were combined, plasma renin activity of the lambs at three weeks old was not related to that of their mothers at the same time (i.e., week three of lactation) but there was a significant correlation between renin activity of the lamb at three weeks of age and that of their mothers at 130 days of pregnancy ($y = 6.282x + 1.53$; $P<0.001$, $r^2 = 0.4$; Figure 7).

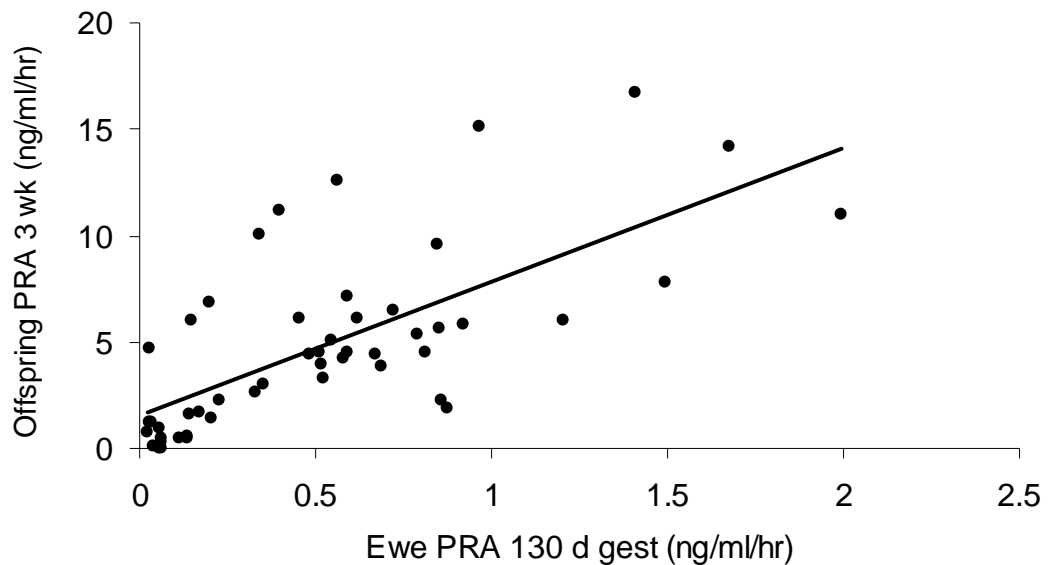


Figure 7: Relationship between plasma renin activity (PRA) of the ewe at day 130 of gestation and the PRA of her lamb at 3 weeks of age. Data from both the animal house experiment and the field experiment were combined. ($y=6.28x + 1.53$, $R^2= 0.48$, $P=0.002$).

Mineral concentration in milk

Feeding lactating ewes a high-salt diet increased (P values varying from $P \leq 0.05$ to $P < 0.001$) the concentration of K, Mn and B in the milk compared to control animals. However, total mineral content of the milk was not significantly different between ewes consuming the high-salt diet and ewes consuming the control diet.

Ewes grazing saltbush also had higher K, Mn, B and P concentration in their milk ($P < 0.001$). In addition, they had higher Zn levels ($P \leq 0.05$) but lower Al and Fe concentrations ($P \leq 0.05$). Ewes grazing pasture had lower total mineral content in their milk than saltbush ewes ($P \leq 0.01$) (Table 3). Unfortunately, repeatable results for the concentration of chloride in milk samples could not be obtained.

Table 3: Comparison of mineral content of milk (mean value in mg/kg, with s.e.) at week three of lactation from ewes fed control or high-salt diet in an animal house or ewes grazing pasture or saltbush in the field.

	Animal house ewes				Field ewes			
	Control	S.E.	High-salt	S.E.	Pasture	S.E.	Saltbush	S.E.
Na	421	25	372	27	353	12	337	21
K	1472	33	1626*	70	1452	30	1609***	25
Ca	2217	91	2161	101	2031	61	1962	60
P	1407	34	1437	38	1275	36	1541***	50
S	391	13	402	14	382	10	439**	14
Mg	177	5	189	9	157	4	139**	5
Zn	5.4	0.50	4.9	0.42	4.9	0.25	6.0*	0.40
Al	1.9	0.72	2.1	0.39	0.8	0.17	0.3*	0.13
Fe	1.1	0.07	1.5	0.21	0.7	0.08	0.4*	0.07
Cu	0.6	0.06	0.6	0.06	0.4	0.04	0.4	0.03
Mn	0.1	0.01	0.2**	0.03	0.1	0.02	0.2***	0.02
B	0.05	0.004	0.08***	0.01	0.2	0.02	0.3***	0.02
Total	6056	158	6196	175	5657	85	6034**	109

* $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ for comparison with their respective control treatments.

Plasma sodium and chloride concentration

The high-salt diet of the ewes in the animal house lowered their plasma sodium ($P < 0.001$) and increased their plasma chloride concentration ($P < 0.001$) at 130 days gestation. Lambs from ewes that received the high-salt diet also had lower plasma sodium at birth ($P \leq 0.05$), but their plasma chloride concentration was not significantly different from control lambs.

Saltbush ewes followed the same trend and had lower plasma sodium ($P \leq 0.01$) and higher plasma chloride concentration ($P < 0.001$) than control pasture ewes. However, this did not influence the plasma sodium or chloride concentration of their lambs (Table 4).

Table 4: Concentration of sodium and chloride in plasma (mean value in mmol/L, \pm s.e.) of ewes at 130 days gestation and their lambs at birth. The ewes were consuming a control or high-salt diet in an animal house, or grazing pasture or saltbush in the field during the last three months of pregnancy and three weeks after birth.

			Plasma Na	Plasma Cl
Animal house	Ewes	Control	149 \pm 0.3	107 \pm 0.4
		High-salt	144 \pm 0.4 ^{***}	111 \pm 0.6 ^{***}
	Lambs	Control	149 \pm 0.5	100 \pm 0.7
		High-salt	146 \pm 0.9 [*]	98 \pm 0.7
Field	Ewes	Control pasture	149 \pm 0.3	109 \pm 0.5
		Saltbush	147 \pm 0.5 ^{**}	112 \pm 0.5 ^{***}
	Lambs	Control pasture	141 \pm 0.8	96 \pm 0.9
		Saltbush	143 \pm 0.7	97 \pm 0.7

^{*} P \leq 0.05 ^{**} P \leq 0.01 ^{***} P $<$ 0.001 for comparison with their respective control animals.

Birthweight and growth

Birthweight and growth rate of lambs from ewes that received the high-salt or control diets were similar. Lambs from ewes grazing saltbush or pasture also had similar birthweights and growth rates up to six months of age. Twin-born lambs had a lower birthweight than single-born lambs (P $<$ 0.05), but there were no differences between treatments in birthweight (P $>$ 0.05) (Table 5).

Table 5: Change in conceptus-free weight of ewes from day 60 of pregnancy to three weeks lactation and birth weight and growth rate of their lambs up to six months of age (\pm s.e.). Ewes were consuming a control or high-salt diet in an animal house or grazing pasture or saltbush in the field.

	Animal house		Field	
	Control	High-salt	Pasture	Saltbush
Ewe wt change (kg)	-2.4 \pm 0.1	-2.5 \pm 0.6	-2.3 \pm 0.6	-2.9 \pm 0.4
Birth weight (kg):				
Single-born	5.1 \pm 0.51	5.3 \pm 0.42	5.5 \pm 0.9	5.5 \pm 0.37
Twin-born			4.3 \pm 0.17*	4.0 \pm 0.16*
Growth rate (g/d)	200 \pm 4.6	197 \pm 3.3	206 \pm 5.4	207 \pm 6.0

*P<0.05 Twin born lambs compared to single-born lambs in the field experiment.

Discussion

Plasma renin activity was suppressed in ewes, from mid gestation through to 3 weeks of lactation, and in their lambs at 3 weeks of age by feeding a high-salt diet or saltbush to the ewe from mid pregnancy to early lactation, a finding that supported my first hypothesis. The reason for the changes in circulating renin activity of the lamb in the first 3 weeks of life could be due to either an *in-utero* effect, or the mineral composition of the mother's milk, but the data reported here suggest the former is more likely. A linear regression showed that the lamb's renin levels at 3 weeks were related to its mother's renin at day 130 of gestation, but not at 3 weeks lactation. This suggests that the renin activity of lambs in the early postnatal period is influenced mainly by the salt that the mother consumes during gestation, rather than lactation. However, this could also be due to alterations in the ewe's RAS during the transition from pregnancy to lactation. It is unlikely that the mineral composition of the mother's milk was a key determinant of neonatal renin activity because the difference in renin activity across the four groups was not always associated with differences in the mineral content of milk.

For example, ewes in the animal house experiment and ewes grazing saltbush had similar mineral content in their milk, but their offspring had different renin activity. The postnatal renin activity of the offspring could be influenced by substances other than minerals in the milk that may inhibit the lamb's renin-angiotensin system (Contreras, 1993, Park *et al.*, 2007). My experimental design could not differentiate between prenatal and postnatal effects of the mother's diet on the renin activity of the lambs because lambs were not cross-fostered after birth. However, the linear regression data support the theory that the lamb's *in- utero* environment, rather than its postnatal environment, has suppressed its renin activity in the high-salt and saltbush treatments.

The renin activity of saltbush and pasture lambs was different at 3 weeks of age as anticipated, but it was similar at birth, a curious and unexpected result. One explanation for this result is stress. Stress increases the activity of the sympathetic nervous system, a potent stimulant of renin secretion (Richardson Morton *et al.*, 1995). In the field, lambs were born in a large paddock and had to be chased before they were caught for blood sampling. Hence, catching and separating lambs from their mothers in the field represented a stress that may have swamped any effects on their renin activity that were caused by the treatments. This could explain why the renin activity of the field lambs at birth was not related to that of their mothers. The correlation existed at 3 weeks, possibly because lambs were in confined pens in close contact with their mothers for this sampling and, consequently, under less stress. By contrast, ewes and lambs in the animal house were in confined pens with their mothers at birth and 3 weeks so that taking blood samples from these lambs were likely to be less stressful than collecting blood from the field lambs. Accordingly, in the animal house experiment, renin activity was consistently lower in the high-salt lambs at birth and 3 weeks and renin levels of lambs at birth reflected those of their mothers as was expected. However, the renin

activity of the field offspring was not higher than the control offspring in the animal house experiment. This may be because the renin activity of some of the field animals reached its maximum value, which is thought to be around 25 ng/ml/hr (Fleischman et al., 1975), and even when stressed, renin could not exceed this level of activity. If field offspring were blood sampled in confined pens and under less stress at birth, I may have seen a difference in renin activity between saltbush and pasture offspring.

Consuming high amounts of salt suppressed the renin activity of the ewe and it also suppressed the renin activity of their lamb at 3 weeks of age. All offspring had very high renin activity at birth which then declined with age, a result that has been shown in other studies in sheep and rats (Broughton Pipkin et al., 1974, Hilgers et al., 1997). Since the field lambs were blood sampled 3 days later than the animal house lambs, this could be why the field offspring tended to have a lower renin activity than the animal house offspring at 3 weeks of age. Suppression of the lamb's renin activity in the early postnatal period most likely originates from changes at a molecular level. Feeding pregnant rats a high-salt diet has been shown to down regulate angiotensin receptor (AT1) protein expression in the offspring and lower the number of cells in the renal cortex expressing angiotensin II in the offspring (calculated by immunohistochemical analysis) (Balbi et al., 2004). Feeding a high-salt diet to pregnant rats has also been shown to suppress renin mRNA levels in the kidney of the offspring (Ingelfinger et al., 1998). The high-salt diet of the ewe could have down regulated renin mRNA in their lambs leading to lower activity of circulating renin. These molecular changes could have had an effect on the lamb throughout the early perinatal period. However there was no difference in renin activity between treatment groups at 15 weeks of age. This could suggest that the lower renin levels of the lambs from the salt treatments is not permanent, although this needs to be confirmed when the offspring reach adulthood.

My second hypothesis was supported as the concentration of cations in milk, especially potassium, increases in ewes receiving a high-salt diet. Unfortunately, repeatable results were not obtained for the chloride concentration in milk, but plasma concentration of chloride increased in ewes consuming a high-salt diet, so it is possible that the chloride concentration in their milk also increased. If this occurred, the negative charge of the chloride would have to be balanced by an increase in the concentration of cations. The low activity of the renin-angiotensin system decreases aldosterone secretion, which compromises the ability of the kidney to excrete potassium (Giebisch and Stanton, 1979, Potter and McIntosh, 1974). This could be why potassium concentration showed the largest increase in milk in both the high-salt and saltbush ewes. The increased P, S and Mg concentrations in the milk of the saltbush ewes compared to the pasture ewes may be a reflection of the increased levels of these minerals in their diet. Excreting large amounts of NaCl during lactation can interact with the regulation of other minerals in the body, such as potassium, which alters the concentration of cations other than sodium in the ewe's milk.

My third hypothesis was partly supported as the high-salt diet of the ewe increased the chloride concentration in the plasma, but plasma sodium concentration was lower, perhaps due to the increased water intake of animals consuming the high-salt diet. Although I did not measure water intake of the ewes, it is well known that consuming salt proportionally increases water intake (Gamble *et al.*, 1929, Stricker *et al.*, 2003). Furthermore, based on results from Digby *et al.* (2008), who fed a similarly high salt diet to ewes during pregnancy, we can assume water intake would have increased by at least two-fold in the ewes fed a high-diet or saltbush. Consuming large amounts of water have been shown to decrease plasma sodium concentration proportionately (Nose

et al., 1987). However, my results differ from those of Meyer and Weir (1954) who found no difference in the sodium concentration of plasma of ewes when they were fed a high-salt diet (13.1%) throughout pregnancy. Ewes in the study by Meyer and Weir (1954) lost more weight during pregnancy so they may not have been consuming as much of the diet as the ewes in my experiment that were fed 14% salt. Therefore the ewes in my experiment would have been consuming more salt, and presumably, more water. Consuming high amounts of salt over an extended period of time (3 months) could lower plasma sodium concentration, mainly due to an excessive intake of water.

The plasma sodium concentration of the high-salt offspring was lower than control offspring, which could influence their physiology in later life. However, unlike the high-salt offspring, the saltbush offspring did not have a lower plasma sodium concentration at birth. This could be due to the buffering capacity of the mother (Dancis and Springer, 1970) or the time after birth that the blood sample was taken. The saltbush lambs may have been well buffered from the 2 mmol/l decrease in their mother's plasma sodium but, the 5 mmol/l decrease in plasma sodium seen in the ewes consuming the high-salt diet in the animal house may have been too much to buffer, so the plasma sodium of the high-salt lambs decreased by 10 percent. However, the plasma sodium of the lambs in the animal house experiment seemed to closely resemble that of their mothers, whereas the plasma sodium of the field lambs are much less than their mothers. A reason for this could be that blood samples were often taken immediately after birth in lambs born in the animal house experiment. But, in the field experiment where ewes and lambs were not confined, blood samples were taken later after the lamb had suckled. Therefore, the plasma sodium of the lambs in the field may have been influenced more by their mother's milk rather than their mother's plasma sodium concentration. The lower plasma sodium of the high-salt offspring could have long-term

consequences for the animal. Alterations in plasma sodium and plasma osmolarity early in life have been shown to re-set thresholds for the release of AVP (Desai et al., 2003). Therefore, the high-salt offspring may have altered regulation of water balance as adults. The reason that the plasma sodium of the high-salt offspring, but not the saltbush offspring, was lower than controls could be due to either the buffering capacity of the mother, or whether the lamb had suckled before the blood sample was taken.

The high-salt or saltbush treatments did not affect birth weight or growth of the lambs up to six months of age, a result consistent with other studies (Digby et al., 2008, Meyer and Weir, 1954). Lambs born as a twin had a lower birthweight than single-born lambs which is to be expected (Baharin and Beilharz, 1977).

In conclusion, lambs born to ewes consuming high amounts of salt have suppressed renin activity in the early postnatal period, a time when renin activity would normally be high. This has implications for the animal as it could cause changes in the mechanisms by which these sheep balance salt in their body. These changes may include alterations in blood pressure, salt and water regulation and preference for salt in later life. If these changes occur they may influence the ability of the offspring to cope with a high-salt diet, such as saltbush, when they consume it as adults.

CHAPTER 4

Dietary exposure of pregnant ewes to salt dictates how their offspring respond to salt

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Abstract

I investigated changes in salt preference, food and water intake, renin activity and salt excretion in adult offspring from ewes that were fed a high-salt diet (14% NaCl) or grazed saltbush from day 60 of pregnancy until day 21 of lactation. High-salt offspring were compared to offspring born to ewes consuming a control diet (2% NaCl) and saltbush offspring were compared to offspring from ewes that grazed a control diet of dry pasture. All offspring were weaned at 3 months of age and grazed the same clover-based pasture until testing started at 8 months of age. The preference for a low-salt diet (0.5% NaCl) when offered with an alternative (7% NaCl) did not differ between the offspring groups. High-salt offspring and saltbush offspring had a lower food intake (14% and 27% respectively) and lower water intake (35% and 20% respectively) than their control offspring. Both high-salt offspring and saltbush offspring had lower basal renin activity than their respective controls. After consuming salt, the renin activity of the saltbush offspring continued to be lower than controls whereas the renin activity of the high-salt offspring became similar to controls. In general, the saltbush offspring excreted an oral salt load more rapidly, though this depended on the extent of the salt load. This important adaptation of offspring born to ewes that consumed saltbush during

pregnancy, may improve their ability to cope with high-salt diets such as saltbush when they consume it as adults. However, the high-salt offspring did not possess such beneficial adaptations.

Introduction

Grazing pregnant ewes on saltbush (a halophytic shrub containing about 20% salt) has tactical benefits in the management of feed resources on farms in Australia (Masters et al., 2006). When a pregnant ewe grazes saltbush, the salt content of the diet has no adverse effects on her health (Digby et al., 2008, Meyer and Weir, 1954). However, the consequences of this high-salt diet for her offspring have not been investigated.

When pregnant rats are fed a high-salt diet (NaCl), their offspring have altered kidney development and salt handling mechanisms (da Silva et al., 2003, Balbi et al., 2004, Contreras and Ryan, 1990). The renin-angiotensin system (RAS) is likely to be involved in initiating these changes because it is essential for the functioning of both the salt balance mechanisms of the mother and the kidney development of the offspring (Gomez and Norwood, 1995, Morgan, 2001). I showed that feeding ewes a high-salt diet (14% NaCl) or grazing ewes on saltbush from day 60 of pregnancy to day 21 of lactation not only lowers the RAS of the ewe, but also lowers the renin activity in their lambs at 3 weeks of age (Chapter 3). In rats, suppression of the RAS or exposure to high dietary salt early in development causes permanent physiological changes in the animal in later life. These changes include an increased preference for salt, altered water intake, changes in responsiveness to salt and RAS activity (da Silva et al., 2003, Arguelles et al., 1996, Contreras, 1993, Osborn and Camara, 1997, Woods and Rasch, 1998). If similar changes occur in offspring from ewes consuming a high-salt diet or saltbush,

they could be caused by suppression of the RAS early in development, exposure to high amounts of salt *in-utero*, or both.

Some of the physiological changes that could take place in these offspring include altered sodium excretion, food and water intake and salt preference. However, not all studies agree on the nature of these changes. If offspring exposed to a high-salt diet *in-utero* have suppressed RAS as adults they may show an increased ability to excrete salt. This is because lower renin activity allows a higher concentration of sodium in urine by reducing secretion of angiotensin II and aldosterone (Lumbers, 1999, Morgan, 2001). However, some studies in rats suggest that a high-salt diet early in life could be detrimental to the animal's renal function by causing structural changes in the kidney and a lack of responsiveness of the RAS to changes in salt intake (da Silva et al., 2003, Balbi et al., 2004, Ingelfinger et al., 1998). Alterations in renin activity could also influence ingestion of food and water (Weisinger et al., 1997). Rats with a long-term increase in renin activity have been shown to increase their food and water intakes (Szczepanska-Sadowska et al., 2003). In contrast, rats with suppressed RAS and poor kidney development (caused by a genetic deletion of angiotensin converting enzyme) did not have altered food intake, but did show increased water intake and energy expenditure (Campbell et al., 2004, Jayasooriya et al., 2008). The salt preference of an animal can also be influenced by their exposure to a high-salt diet early in life (Arguelles et al., 2000, Contreras and Kosten, 1983, Contreras and Ryan, 1990). The mechanisms behind this change could be due to an altered activity of the RAS, but it could also be influenced more directly as a result of developmental changes in the gustatory system that affect the responsiveness to the taste of salt in the offspring (Contreras and Ryan, 1990, Curtis et al., 2004, Pittman and Contreras, 2002). I expected that adult offspring born to ewes that consumed a high-salt diet or saltbush during mid

pregnancy to early lactation would have, first, increased preference for salt and altered food and water intake, second, reduced activity of renin that would be associated with increased sodium excretion following the ingestion of salt.

Materials and Methods

Experimental design

I compared offspring born to ewes that received a high-salt diet (14% NaCl, high-salt offspring) to offspring born to ewes that received a control diet (2%, control offspring) in an animal house from day 60 of pregnancy until day 21 of lactation. I also compared offspring born to ewes that had grazed saltbush (saltbush offspring) to offspring born to ewes that grazed a control diet of dry pasture (pasture offspring). When the offspring were eight months old, I compared their preference for salt and measured food and water intake after offering them a low-salt (0.5%) diet, a high salt diet (7%) or a choice between the two. To test the mechanisms involved in salt balance of the offspring I gave them an oral dose of 25 g of NaCl and a 50 g dose of NaCl and compared their renin activity, urine volume and sodium concentration, water and food intake. These experiments have been approved by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) animal ethics committee.

Maternal treatments

A full explanation of maternal treatments including nutritional content of the diets has been described elsewhere (Chapter three). Briefly, Merino ewes aged three years were artificially inseminated to the same ram and grazed pasture until day 60 of pregnancy when they were randomly assigned to treatment groups. Average length of gestation was 150 days (± 2). In the animal house experiment there were two treatments of “high-salt” and “control”. In a separate field experiment conducted at the same time, there

were also two treatments of “saltbush” and “control pasture”. In the animal house experiment, thirty ewes received a pelleted control diet (2% NaCl) and 30 received a pelleted high-salt diet (14% NaCl). These ewes were kept in individual pens and were pair fed so both groups had identical organic matter intakes. All ewes were fed enough to maintain conceptus-free liveweight (the weight of the ewe minus the weight of the fetus and placenta, estimated from the day of gestation (SCARM, 1990, Wheeler et al., 1971)). In the field experiment, 35 ewes grazed a control diet of dry pasture based on subterranean clover, supplemented with lupin grain and another 35 ewes grazed saltbush (mainly *Atriplex nullumaria*) with a small amount of understory and supplemented with barley grain. The ewe’s diet needed to be supplemented with grain because the nutrient content of the saltbush and pasture diets were not enough to maintain conceptus-free liveweight of the ewes. The NaCl content of the saltbush ewe’s diet decreased during late pregnancy and lactation, when more supplement was fed. Ewes grazing saltbush had approximately 13% NaCl in their diet at 60 days gestation, 10% at day 130 of gestation and 8.7% NaCl in their diet at day 21 of lactation. Ewes grazing dry pasture had approximately 0.7% NaCl in their diet. Twelve ewes from each of the saltbush and pasture treatments were carrying twins.

All ewes consumed their treatment diets from day 60 of pregnancy until day 21 of lactation. This period of time was chosen for two reasons. First, mid-late pregnancy and the early postnatal period have been shown to be the “developmental window” for altering the RAS (Konje et al., 1996, Robillard and Nakamura, 1988, Wintour, 1997). Second, grazing sheep on saltbush during this time in autumn is most cost-effective for farmers (Morcombe et al., 1996). After the treatments ended, all ewes were grazed together on pasture based on subterranean clover until weaning. Offspring were weaned at 15 weeks of age in accordance with normal farm practices. Weaned lambs continued

to graze the clover pasture together until they were eight months old before being brought into the animal house for testing. Birthweight and growth rate of the animals was similar up until eight months of age so all animals were similar bodyweights at the start of the experiments (Chapter 3).

Experiment 1- Intake experiment

Twenty sheep from each treatment group were placed in individual pens in an animal house and offered introductory pellets from day one to ten. To accustom them to the preference test procedures, two buckets containing the introductory pellet were placed in the feed bin of each sheep. One of the buckets also contained a small amount of lupin seed to train the sheep that the contents of the two buckets were different. From days 11 to 14, basal food and water intake were measured while animals consumed this introductory diet containing low amounts of salt (0.5%). From days 15-18, the salt preference of the offspring were tested by offering them a pelleted low-salt diet (0.5%) in one bucket and a pelleted high-salt diet (7% salt added) in the other bucket (Table 6). Preferences were calculated as the percentage of the high-salt diet that animals chose to include in their overall feed intake (i.e. 15% salt preference means the animal's total feed intake consisted of 15% high-salt diet and 85% of the low-salt diet). Seven percent salt in the diet was chosen because it is about the point at which the salt content of the diet starts to have an immediate limiting effect on food intake (Thomas and Masters, 2008). The amount of feed consumed was measured five hours and 24 hours after receiving fresh pellets. The type of pellet in each bucket was switched daily and water intake was measured daily. From days 19 to 22 animals were fed only the high-salt diet (7% salt) and their food and water intake was measured. The different diets were offered sequentially so that all animals were offered the same dietary treatments at any given time (and age). Short periods (four days) of feeding the high or low-salt diets

were used because the effect of dietary salt on feed intake is apparent immediately (day one) and the majority of the effect occurs within four days (Thomas and Masters, 2008).

Blood samples (8 ml) were taken from the jugular vein by venipuncture to measure renin activity before the preference test on day 14 and then on days 18 and 22. After day 22, sheep were fed a low salt pellet for one week before experiment two commenced.

Table 6: Composition (analyses of dry matter) of diets fed to offspring.

	Introductory pellet	Low-salt pellet	High-salt pellet
Organic matter (%)	95.8	96.2	89.3
Inorganic matter (%)	4.2	3.8	10.7
Crude protein (%)	18	15	14.8
Metabolisable energy (MJ/kg DM)	10.5	9.1	9.0
NaCl content (%)	0.5	0.5	7

Experiment 2 – Salt dose experiment

Fifteen sheep from each of the four treatments were used in this experiment. Catheters were inserted into the jugular vein of each sheep before they were placed in metabolism crates. They were allowed to adjust to the crates for one day before testing started.

Sheep were given 25 g of NaCl as an oral dose (83 ml of 30% salt solution) administered by a syringe with a plastic tube placed in the mouth of the sheep and, four days later, they were given 50 g of salt (167 ml in 30% solution).

Measurements

Basal urine volume, urine concentration, and water intake were measured over 16 hours before the salt doses. Blood samples (8 ml) were taken one hour before the salt doses. After both salt doses, urine volume, urine concentration and plasma renin activity were

measured at 2, 4, 6, 8, 10, 24 and 30 hours after the salt doses. Water intake was measured at 1, 2, 4, 6, 10 and 24 hours after the salt doses. Two hours after the salt doses, sheep were offered a normal-salt pellet (0.5%) *ad-libitum*. Feed intake was measured 6, 10 and 24 hours after the salt doses. Feed intake during this experiment was expressed as a percentage of basal food intake, which was averaged over 12 days prior to the salt doses.

Analyses of samples

Blood samples were placed in tubes containing EDTA and kept at room temperature before centrifugation for 15 minutes at 1500 x g. They were then frozen at -20°C until analysed. A commercially available kit was used to measure activity of plasma renin as the rate of formation of angiotensin I (Gamma Coat Plasma Renin Activity RIA kit, Dia Sorin, Stillwater, Minnesota, USA). The inter-assay coefficient of variation was 7.2% and the intra-assay coefficient of variation was 5%. The detection limit after 18 hour incubation was 0.01 ng/ml/hr. Sodium concentration in urine was measured using an ion selective electrode and was run on an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd).

Statistics

The statistics program Genstat (10th Edition, VSN International Ltd) was used to analyse the data. All results were analysed using residual maximum likelihood (REML) linear mixed model. In experiment one I used fixed effects of maternal diet, type of diet consumed by the offspring (low salt, high salt or choice of diet) and during the salt preference test I also used time (5 hrs or 24 hrs). Individual animal identification was used as a random effect. For experiment two I used treatment, salt dose, and time as fixed effects and animal identification as a random effect. Data that did not have a

normal distribution were transformed using natural logarithms. The data from offspring born to ewes in the animal house experiment and data from offspring born to ewes in the field experiment were analysed separately although they are presented in the same figures and tables.

Results

Experiment 1- Intake experiment

Salt preference

The salt content of the diet of the ewe had no effect on their offspring's preference for salt ($P>0.05$). The total food intake by the high-salt offspring in the first 5 hours consisted of 15% high-salt diet (7% NaCl) and 85% low-salt diet (0.5% NaCl). The control offspring chose a similar proportion of the high-salt diet in their total food intake (17% high-salt diet and 83% low-salt diet). There was also no difference in salt preference at 24 hours. Saltbush and pasture offspring also showed the same preferences for the low and high-salt pellets (Table 7).

Table 7: Percentage (\pm s.e.) of a pelleted high-salt diet (7%) that sheep selected in their total food intake at 5 and 24 hours after being presented with fresh food. Sheep were offspring born to ewes fed a control or high-salt diet in an animal house or grazed on pasture or saltbush in the field during mid pregnancy to early lactation.

	Maternal diet		Maternal diet	
	Animal house offspring		Field offspring	
	Control	High-salt	Pasture	Saltbush
5 hours	17 \pm 1.8	15 \pm 1.7	14 \pm 1.7	16 \pm 2.4
24 hours	9 \pm 1.3	8 \pm 1.8	11 \pm 1.8	11 \pm 2.5

Water and food intake

High-salt offspring consumed about 14% less food than control offspring ($P < 0.05$) and drank 35% less water than control offspring when consuming the low salt pellets, or when given a choice between the low salt and the high salt pellets ($P < 0.01$). This means that, on average, high-salt offspring drank about 20% less water per unit of feed intake than control offspring. The high-salt offspring tended to drink less water per unit of food intake when consuming the low salt diet rather than the high-salt diet, but this was not a significant difference.

The food intake of the saltbush offspring was depressed by 27% compared to pasture offspring ($P < 0.001$). The saltbush offspring also drank less water than pasture offspring, especially when they were consuming the high-salt pellet ($P < 0.05$). However, there was no difference between treatments in the amount of water consumed per unit of food intake (Table 8).

Table 8: Average daily feed intake (kg), water intake (l) and water intake per unit food intake (l/kg) of offspring when consuming a low-salt diet (0.5%), a high-salt diet (7%) or a choice between the two (\pm S.E.). Sheep are offspring from ewes that consumed a control or high-salt diet in an animal house or grazed a control diet of pasture or saltbush in the field. Significance values refer to effect of maternal diet and offspring diet. Animal house and field offspring were analysed separately.

		Maternal diet		Maternal diet	
		Animal house offspring		Field offspring	
Offspring	Diet	Control	High-salt	Pasture	Saltbush
	Feed intake (kg)	Normal	1.07 \pm 0.06	0.92 \pm 0.07	1.16 \pm 0.06
Choice		1.56 \pm 0.06	1.32 \pm 0.09	1.65 \pm 0.06	1.19 \pm 0.07
High-salt		1.69 \pm 0.07	1.53 \pm 0.09	1.85 \pm 0.06	1.43 \pm 0.10
Significance		M*, O**		M***, O***	
Water intake (l)	Normal	3.90 \pm 0.40	2.56 \pm 0.38	3.68 \pm 0.46	3.39 \pm 0.39
	Choice	4.40 \pm 0.38	2.80 \pm 0.36	4.47 \pm 0.40	3.29 \pm 0.28
	High-salt	7.88 \pm 0.50	6.69 \pm 0.51	9.06 \pm 0.71	7.12 \pm 0.56
Significance		M**, O***		M*, O***	
Water per kg feed	Normal	3.78 \pm 0.34	2.55 \pm 0.32	3.51 \pm 0.58	4.87 \pm 0.41
	Choice	2.85 \pm 0.24	2.09 \pm 0.20	2.70 \pm 0.21	2.97 \pm 0.29
	High-salt	4.65 \pm 0.22	4.34 \pm 0.19	4.93 \pm 0.36	5.07 \pm 0.31
Significance		M**, O**		O**	

M= effect of maternal diet, O= effect of offspring diet. There were no interactions between maternal and offspring diet. *P<0.05 **P<0.01 ***P<0.001.

Plasma renin activity

Plasma renin activity (PRA) of the high-salt offspring was 40% lower than control offspring when fed the low salt pellet (0.5% salt). However, their PRA was similar to control offspring when they were given a choice between the low-salt and the high-salt pellet, or when they consumed only the high-salt pellet so there was a treatment by diet effect (Figure 8).

Saltbush offspring had a consistently lower renin activity than the pasture offspring, regardless of the salt content of their diet ($P < 0.01$). Like the high-salt offspring, saltbush offspring had a 40% lower PRA than pasture offspring when consuming a low salt diet. However, unlike high-salt offspring, their PRA continued to be 40% lower when they had a choice between diets ($P < 0.01$). The PRA of saltbush offspring was half that of the pasture offspring when consuming the high-salt diet (Figure 8). When data from all treatments were combined, average feed intake of the three diets (control, high salt, or combination of both) was positively correlated to average basal renin activity ($y = 180x + 993$; $R^2 = 0.2$, $P < 0.001$).

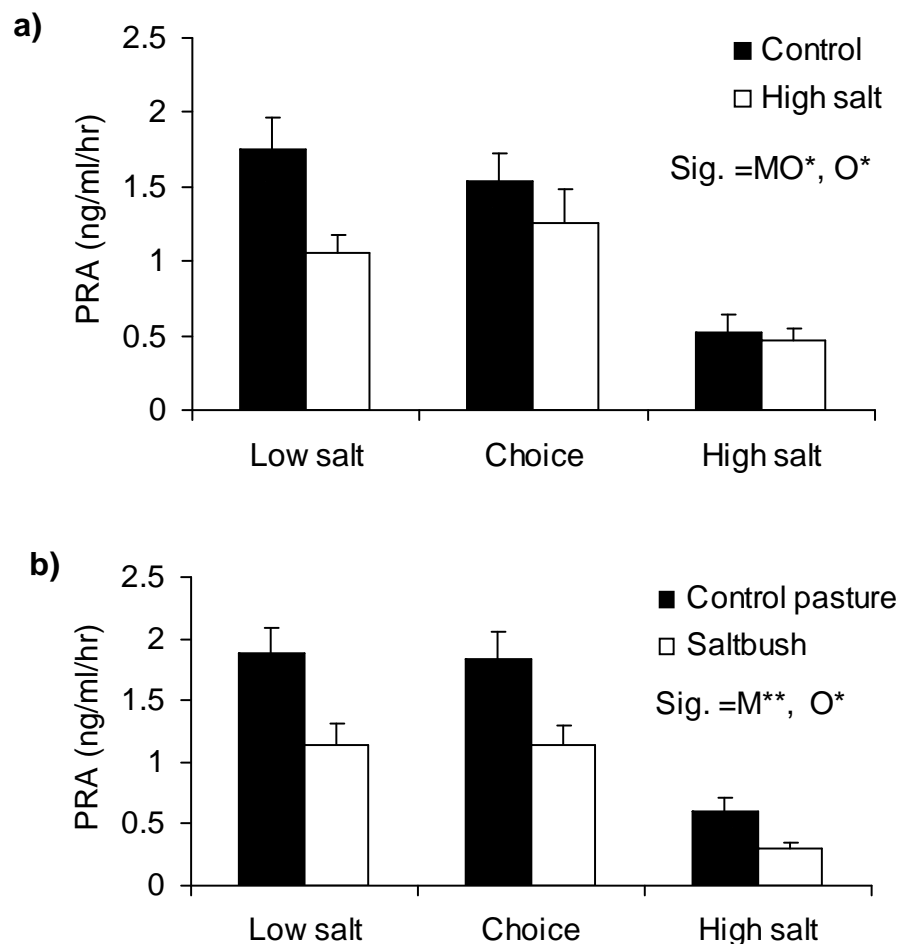


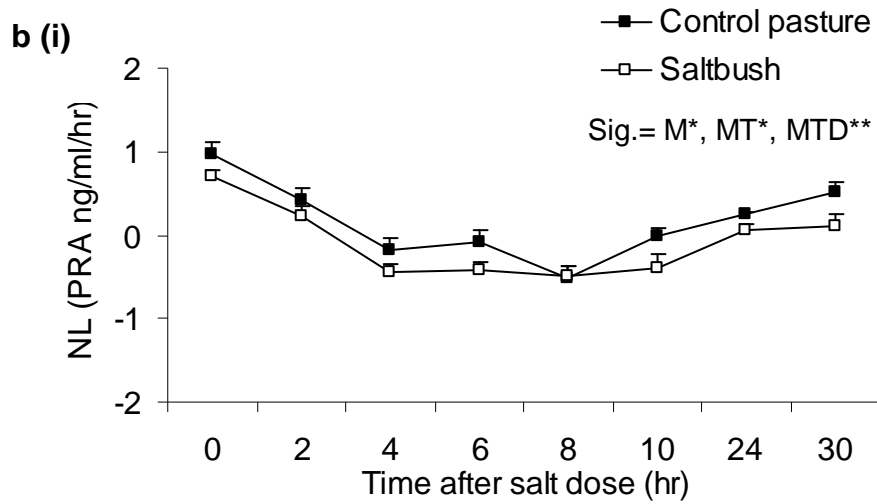
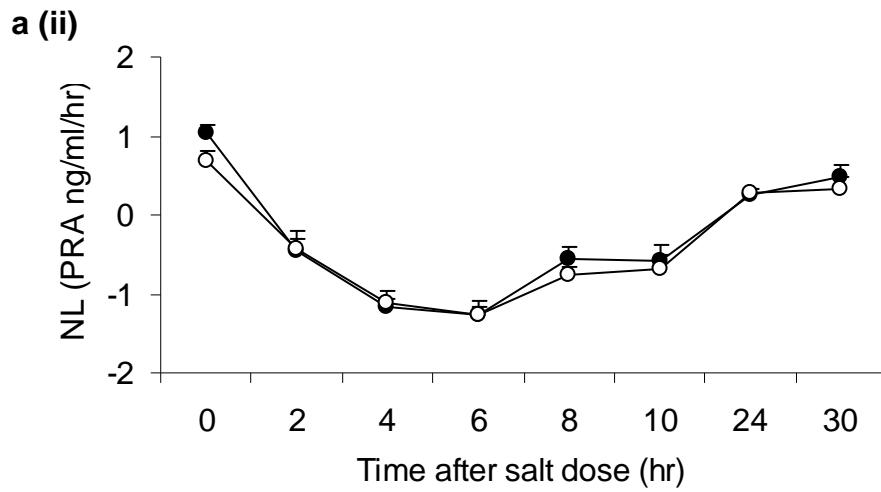
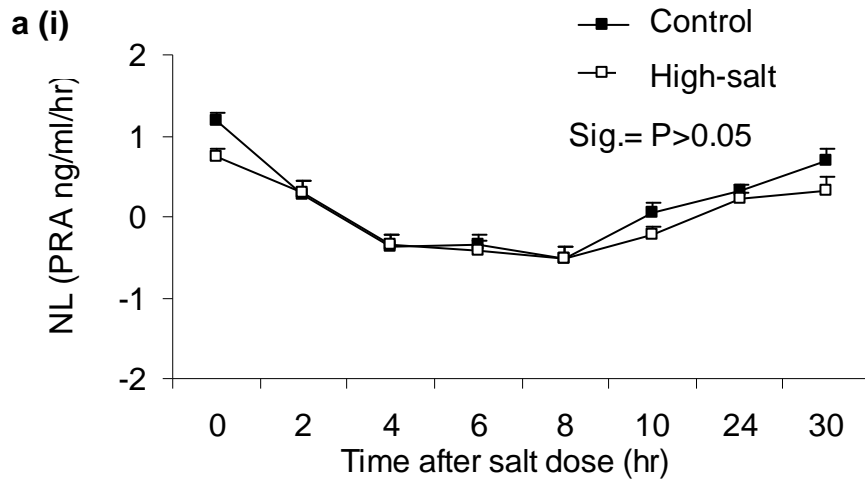
Figure 8: Plasma renin activity (PRA) of offspring offered a low-salt diet, a high-salt diet or a choice between the two. Sheep are offspring born to ewes who received a control or high-salt diet (a) or grazed pasture or saltbush (b) during mid pregnancy to early lactation. Sig.= Significance, M= effect of maternal diet, O= effect of offspring diet. MO= Interaction between maternal and offspring diet. * $P < 0.05$ ** $P < 0.01$.

Experiment 2- Salt dose experiment

Plasma renin activity

Basal renin levels of the high-salt offspring were lower than the controls by about 38%. However, two hours after the 25 g salt dose, the renin activity in both groups of offspring was the same; the high-salt offspring decreased only half as much as the control offspring. Between two and 24 hours the renin activity in high-salt and control lambs was similar but, by 30 hours, the renin activity in high-salt lambs was once again lower than controls. A similar pattern of renin activity was shown after the 50 g salt dose, with renin activity in the high-salt offspring being lower than the control offspring before the salt dose and then decreasing less than the control offspring in the first two hours so that renin activity was similar between treatments thereafter (Figure 9a).

Saltbush offspring had a lower renin activity than pasture offspring before the salt dose and it continued to be lower during the 25 g salt dose and during the first 4 hours of the 50 g dose ($P < 0.05$). There was also an interaction between treatment and time for the two salt doses because saltbush offspring had lower renin activity than pasture offspring in the first 4 hours after the salt doses. At 4 hours, renin activity in saltbush offspring was at its lowest and then began to rise. In contrast, it took 8 hours after the salt dose for the renin activity in the pasture offspring to start to rise. This treatment by hour interaction was also influenced by the salt dose (i.e. there was a treatment by time by salt dose interaction ($P < 0.01$)). In the 25 g salt dose, renin activity in saltbush offspring was constant between 4 and 10 hours before increasing. In contrast, after the dose of 50 g of salt, renin activity in saltbush offspring dropped rapidly by about 90 % in the first four hours but, unlike the 25 g dose, it started to rise again at six hours (Figure 9b).



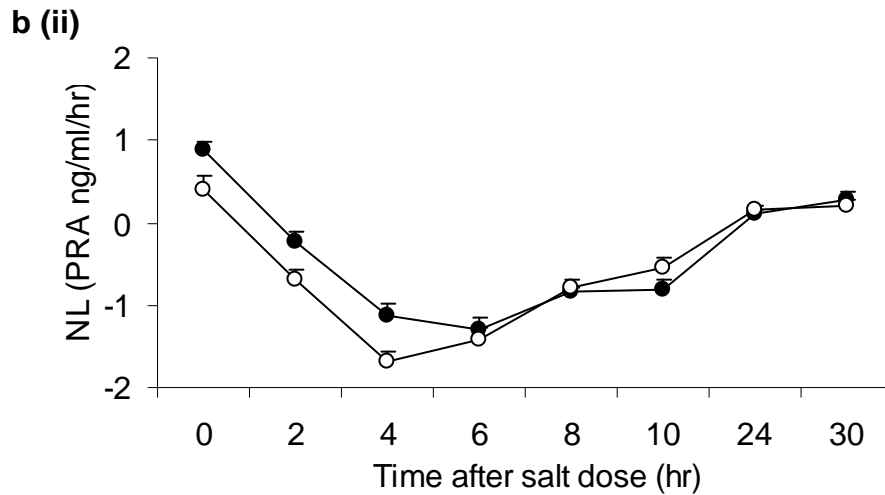
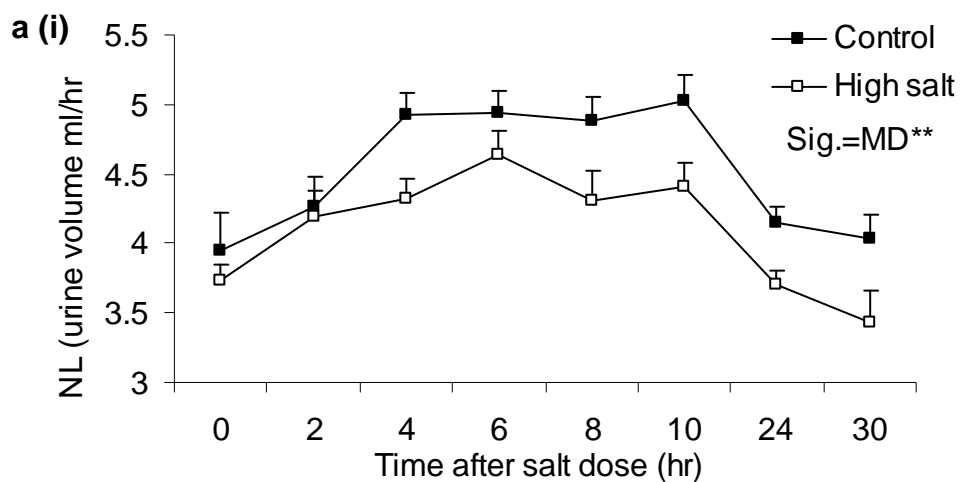


Figure 9: Natural logarithm (NL) of plasma renin activity (PRA ng/ml/hr) in response to a salt dose of 25 g (i) or 50 g (ii). Sheep were born to ewes consuming a control or high-salt diet (a) or ewes grazing pasture or saltbush (b) during mid pregnancy and early lactation. Sig.= Significance, M= effect of maternal diet, T= effect of time, D= effect of salt dose (25 g or 50 g). * $P<0.05$ ** $P<0.01$.

Salt excretion

The high-salt offspring excreted less urine overall after the 25 g salt dose but urine volume was similar to controls after the 50 g salt dose ($P<0.01$). Urine concentration was similar between the high-salt and the control treatments for the 25 g and the 50 g salt dose (Figure 10). After the 25 g salt dose, high-salt offspring excreted a lower volume of urine at the same concentration as controls, which meant that during the 30-hour period, they excreted less salt than controls ($P<0.01$).



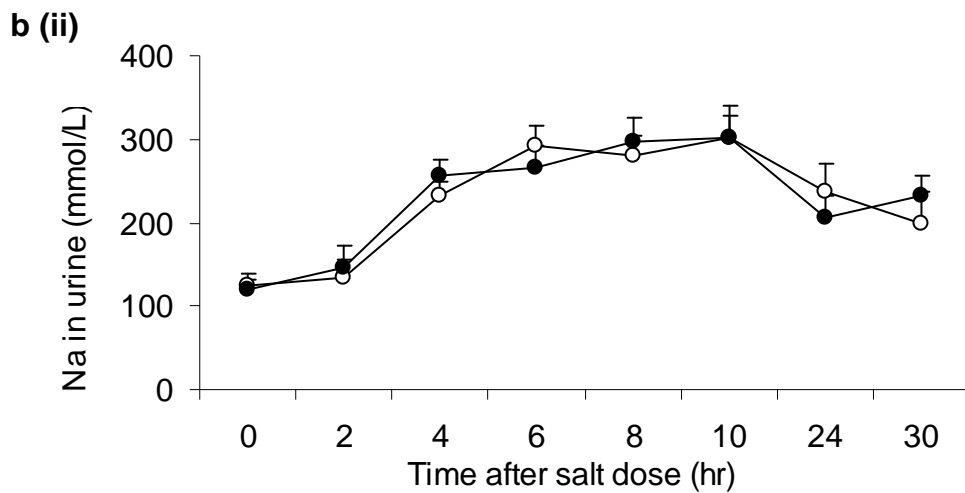
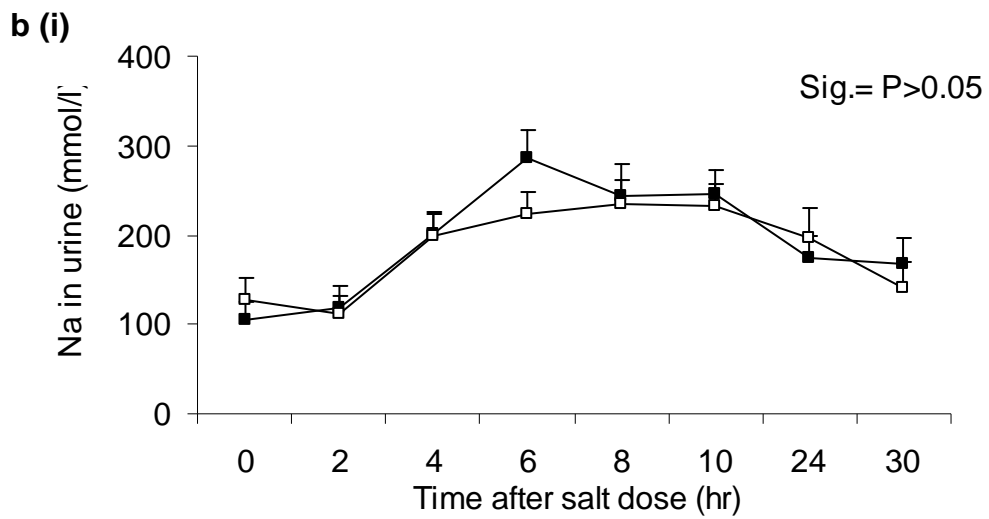
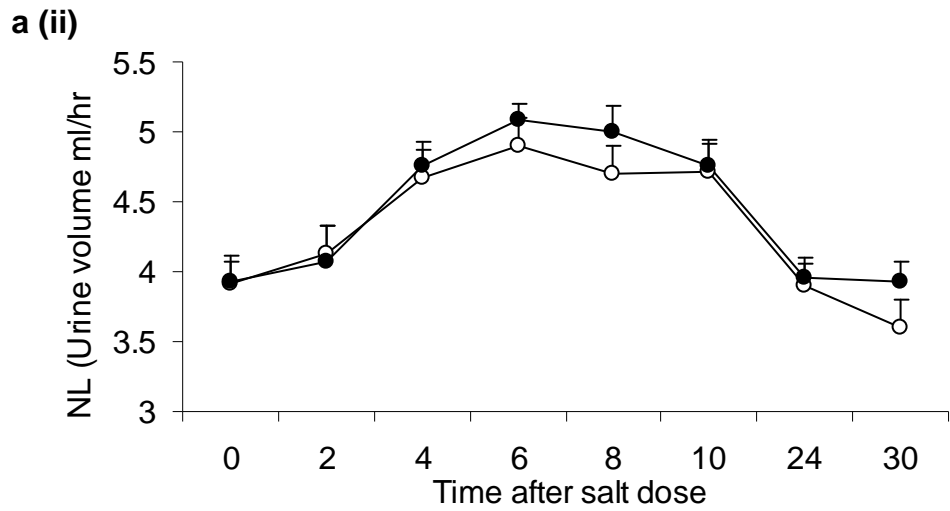
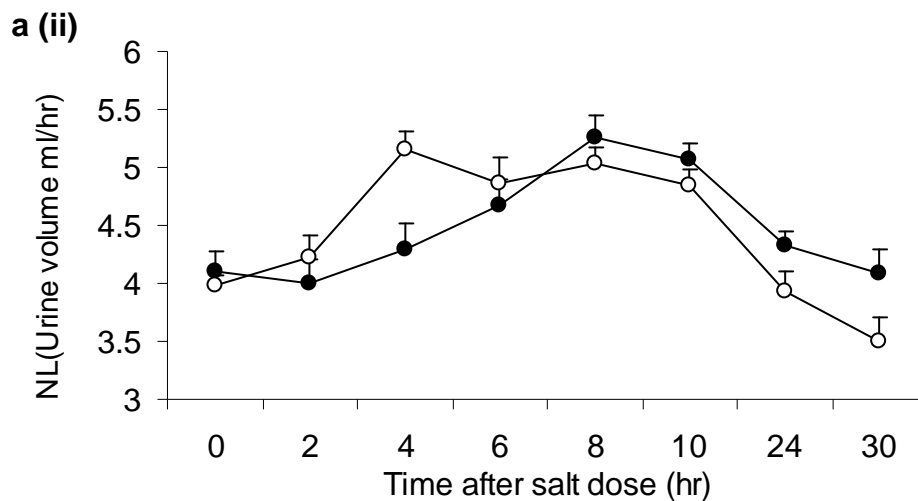
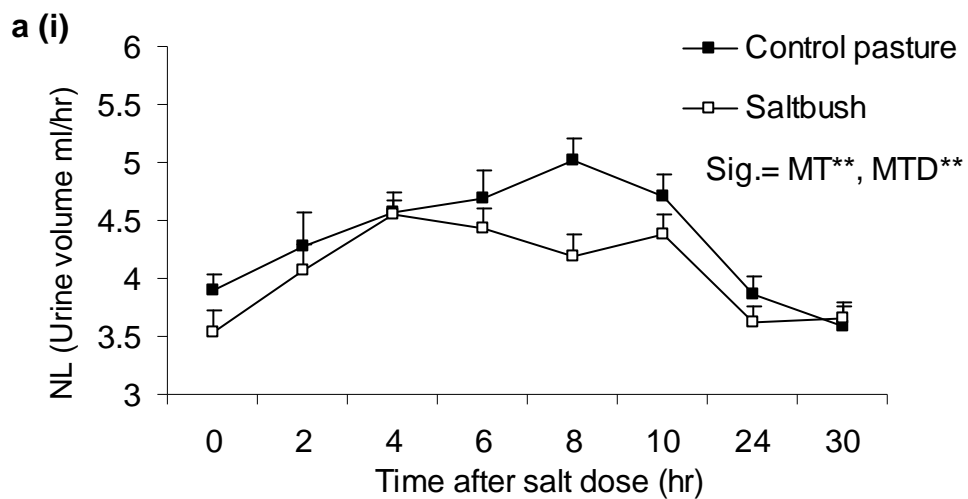


Figure 10: Natural log (NL) of urine volume (a) and concentration of Na in urine (b) of sheep after a 25 g oral salt dose (i) or a 50 g salt dose (ii). Sheep were born to ewes that consumed a control diet (filled shapes) or a high-salt diet (open shapes) during mid pregnancy to early lactation. Sig.=Significance, M= effect of maternal diet, D= effect of salt dose (25 g or 50 g). ** P<0.01.

The pattern of salt excretion in the saltbush offspring was also altered by their mothers' diets. During the 25 g salt dose, urine volume of the saltbush offspring was lower than the pasture offspring, but this was compensated for by an increase in urine concentration ($P<0.05$). Therefore, salt excretion per hour was similar between the saltbush and the pasture treatments for the 25 g salt dose. However, for the 50 g dose, saltbush offspring excreted the salt more rapidly than pasture offspring ($P<0.01$) by increasing their urine volume so they reached their maximum rate of urine output 4 hours after the salt dose. In contrast, it took control offspring 8 hours to reach their maximum rate of urine output per hour after the 50 g salt dose (Figure 11).



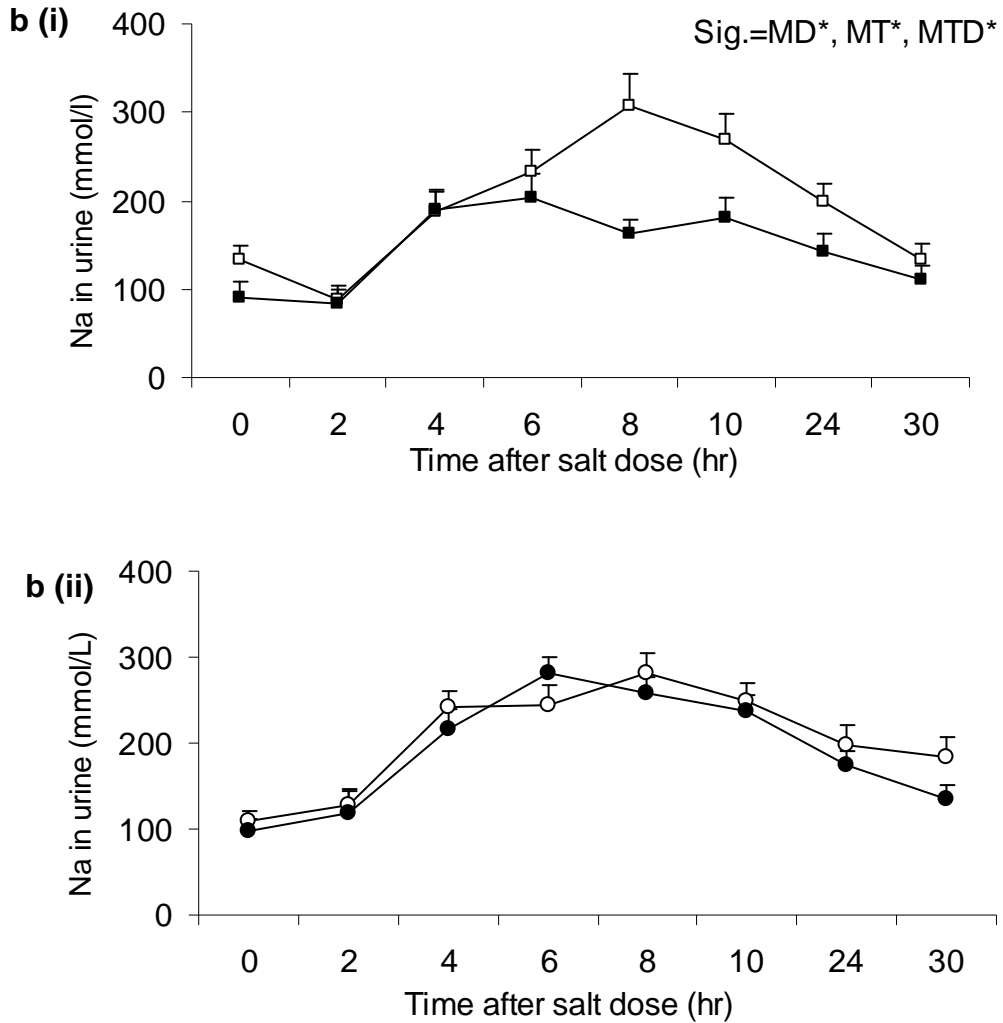


Figure 11: Natural log (NL) of urine volume (a) and Na concentration in urine (b) of sheep after a 25 g salt dose (i) or a 50 g salt dose (ii). Sheep were the offspring of ewes that grazed either pasture or saltbush during mid pregnancy to early lactation. Sig.= Significance, M= effect of maternal diet, T= effect of time, D= effect of salt dose (25 g or 50 g). *P<0.05 **P<0.01.

Water intake

There was no difference in water intake between the high-salt and the control offspring after the 25 g or 50 g salt dose (data not shown). Saltbush offspring drank a total of 5 L less water than the pasture offspring for the two salt doses (P<0.05). There was also a treatment by time interaction because saltbush offspring had lower water intake at 6 and 8 hours after both the 25 g and the 50 g salt dose (P<0.05) (Figure 12).

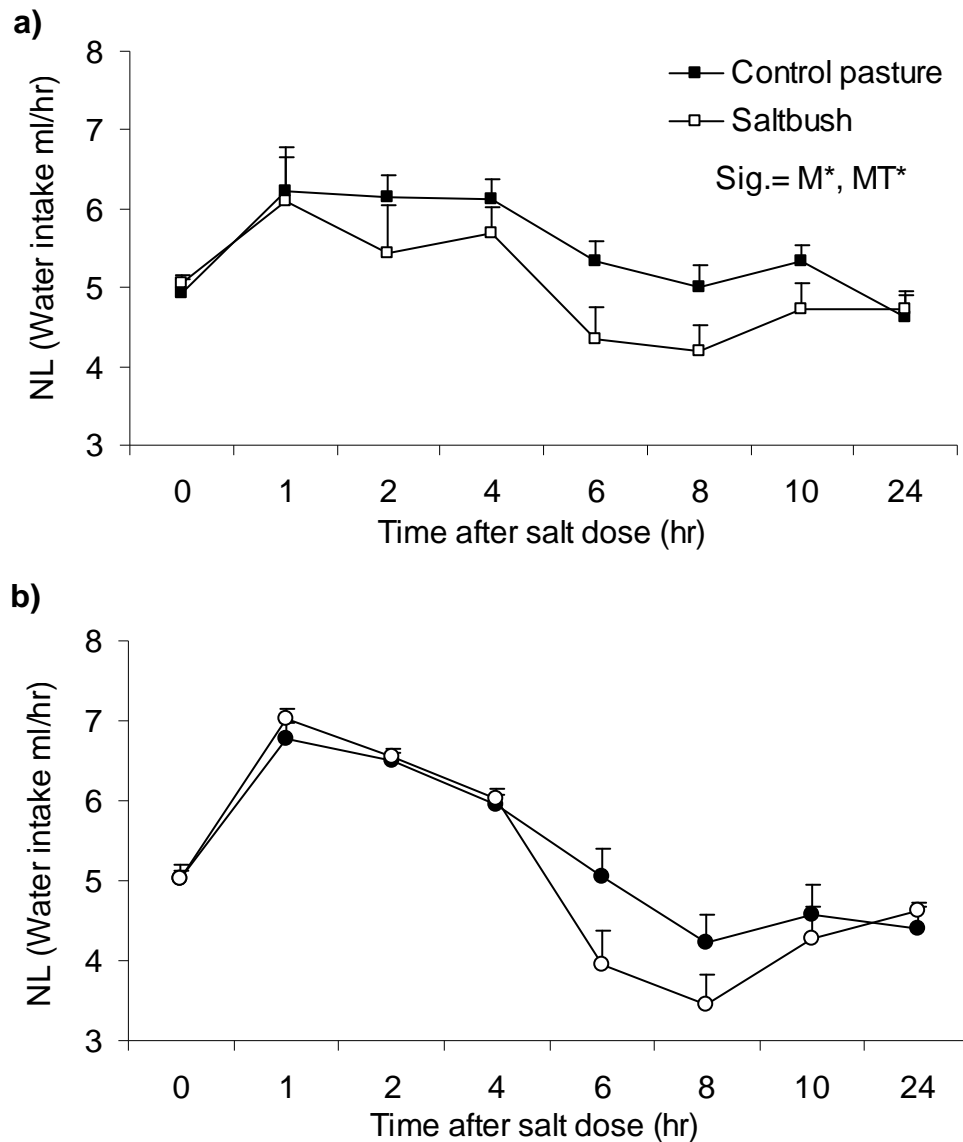


Figure 12: Natural log (NL) of water intake (ml/hr) of sheep after an oral 25 g salt dose (a) or a 50 g salt dose (b). Sheep were born to ewes that grazed either pasture or saltbush during mid pregnancy to early lactation. Sig.=significance, M= effect of maternal diet, T= effect of time. *P<0.05.

Food intake

Food intake by the offspring after the salt doses has been expressed as a percentage of their usual daily consumption (averaged over 10 days) because of the difference between treatments in their basal food intake as shown in experiment one (Table 8). The food intake by the high-salt offspring was depressed to the same degree as the control offspring after the salt doses ($P>0.05$, data not shown). However, there were differences

in food intake between treatments in the field experiment. The food intake by the saltbush offspring was not depressed by the salt doses, whereas the salt depressed the food intake by the pasture offspring by approximately 40% after the 50 g salt dose ($P<0.01$). There was also an hour by treatment interaction as the groups ate similar amounts 6 hours after the salt dose but then the saltbush offspring ate a higher percentage of their daily food intake at 10 and 24 hours after the salt doses ($P<0.01$)(Figure 13).

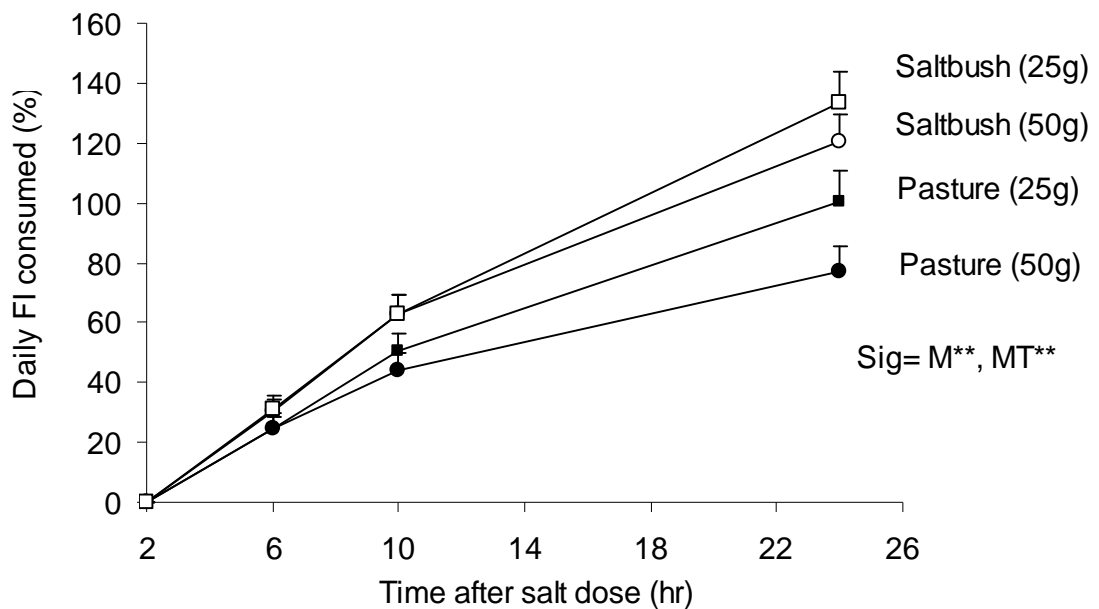


Figure 13: Percentage of average daily food intake consumed over time after a 25 g salt dose (squares) and 50 g salt dose (circles) by sheep whose mothers grazed pasture (filled shapes) or saltbush (open shapes). Sig.= Significance, M= effect of maternal diet, T= effect of time. ** $P<0.01$.

Bodyweights

Bodyweights of the offspring from all treatments were similar at the start of the experiments. High-salt and control offspring gained similar amounts of weight during the experiments, whereas saltbush offspring gained less weight than did pasture offspring ($P<0.001$) (Table 9).

Table 9: Bodyweights (kg) of offspring at the start of the experiment and weight gain (g/d) of offspring during the two experiments.

	Maternal diet		Maternal diet	
	Animal house offspring		Field offspring	
	Control	High-salt	Pasture	Saltbush
Bodyweight (kg)	33.7 ± 0.79	33.4 ± 0.79	35.1 ± 1.11	35.4 ± 0.96
Weight gain (g/d)	80 ± 22.4	43 ± 26.7	106 ± 21.9	1 ± 26.0***

***P<0.001

Discussion

I found that adult offspring born to ewes that consumed high amounts of salt during mid pregnancy to early lactation had altered renin activity and sodium excretion as well as altered food and water intakes. Basal renin activity was lower in high-salt and saltbush offspring than their respective controls, which suggests that they would be better able to excrete salt than their counterparts that received no exposure to salt or saltbush *in-utero* and in the first three weeks of life. However, there were differences between high-salt and saltbush offspring following a salt load. Rather than excreting salt faster, the high-salt offspring retained more of the 25 g salt dose than control offspring. In contrast, the saltbush offspring excreted more concentrated urine when given 25 g of salt and they excreted the 50 g salt dose more rapidly than their controls, the pasture offspring. Thus, the hypothesis that a maternal high-salt or saltbush diet would increase the ability of the offspring to excrete sodium was supported for the saltbush offspring but not for the high-salt offspring. There was no difference in preference for salt between any of the treatments, which was unexpected. However, the results for food and water intake were as predicted. That is, high-salt and saltbush offspring ate less food and drank less water than their respective control offspring. In addition, an interesting result is that the high-salt offspring drank less water per unit of food consumed relative to the control

offspring. These results show that feeding a high-salt or saltbush diet to ewes from mid pregnancy to early lactation can alter their offspring's salt balance mechanisms and also alter their intake of food and water.

In the salt dose experiment, I found that high salt or saltbush intake during pregnancy affected the adult offspring's renin activity and salt excretion in response to the ingestion of salt. The high-salt or saltbush diet of the ewe suppresses her renin activity, and this in turn can suppress the renin activity in her offspring early in life (Chapter 3). The renin angiotensin system (RAS) is essential for normal renal development, so suppression of the RAS at a critical time when salt handling mechanisms are being set could result in permanent changes to these set-points (Guron and Friberg, 2000, Hilgers et al., 1997). This is why, as adults, the high-salt and saltbush offspring show altered renin activities, which result in altered patterns of salt excretion. However, the changes seen in saltbush offspring seemed to be more profound than those seen in the high-salt offspring. A reason for this could be because of the degree of suppression of their renin activity early in life. The high-salt offspring had 20% lower renin activity than controls at 3 weeks of age, whereas the renin activity in the saltbush offspring was suppressed by a much larger 84% compared to the pasture offspring at 3 weeks of age (Chapter 3). The suppressed renin activity early in life had a lasting effect as both the high-salt and saltbush offspring had a lower basal renin activity at 8 months of age. However, when large amounts of salt were ingested, the renin activity in the high-salt offspring was the same as the control offspring which shows that only basal, not suppressed renin activity was altered compared to control animals. The attenuated response of renin activity in the high-salt offspring was associated with abnormal salt retention. The renin activity in the saltbush offspring was suppressed more than the high-salt offspring early in life, which appears to have re-set both the basal and suppressed levels of renin activity since

the saltbush offspring showed consistently lower renin activity than pasture offspring regardless of the amount of salt ingested. This lower renin activity was associated with increased concentration of Na in urine and more rapid salt excretion in the saltbush offspring. The degree of suppression of renin activity early in life could determine what physiological changes are shown in the offspring as adults.

One of the physiological changes seen in high-salt offspring is the responsiveness of their RAS to changes in salt intake. During the first two hours after the 25 g salt dose, the renin activity in high-salt offspring decreased only half as much as the control offspring, which suggests that the renin activity in the high-salt offspring was less responsive to salt. This attenuated response of renin to salt has been shown in rats where a high-salt diet (8%) was fed to female rats from when they were weaned, through pregnancy and lactation (da Silva et al., 2003). The authors found that the plasma renin activity in the offspring did not change when fed a low salt compared to a high-salt diet (da Silva et al., 2003). My results show only a slightly attenuated response of renin activity to salt, possibly because the period of salt exposure in my experiment was much shorter. Since renin activity was less responsive to salt, salt excretion was also less responsive, which led to the high-salt offspring retaining salt after the 25 g salt dose. This type of salt retention has been shown in salt-sensitive rats, which also show a attenuated release of renin in response to saline infusion (Dichtchekian et al., 1989, Simchon et al., 1999). Feeding a high-salt diet to rat dams has also been shown to impair renal function of offspring (Balbi et al., 2004), but renal function of the high-salt offspring in the current study did not appear to be impaired because when given the 50 g salt dose, they were able to excrete the salt as well as the control offspring.

It is possible that osmoregulation is slightly impaired in the high-salt offspring, which causes their RAS to be less responsive to changes in sodium balance. However, there was no difference in basal plasma sodium concentration between the control and the high-salt offspring (Chapter 5). Furthermore, changes in plasma sodium concentration are generally small even in salt-sensitive rats when they are maintained on a high-salt diet (Orlov and Mongin, 2007). Although I did not measure blood pressure of the offspring, results from studies in rats have shown that a maternal high-salt diet can increase the blood pressure of the adult offspring (da Silva et al., 2003, Contreras, 1993, Contreras et al., 2000, Vidonho et al., 2004). If this occurred in the high-salt offspring, it may have affected pressure-natriuresis (Evans et al., 2005, Mervaala et al., 1999, Roman, 1986) and an altered sensitivity to tubuloglomerular feedback (Persson et al., 2000). In short, the perinatal environment of the high-salt offspring may have caused a slight re-setting of these mechanisms so they retain salt when ingesting a small amount (25 g). However, renal function is not severely impaired in the high-salt offspring because they can still excrete salt when given higher amounts (50 g).

In contrast to the salt retention of the high-salt offspring, the saltbush offspring were able to excrete salt more rapidly than the pasture offspring, possibly because of their altered renin activity. Like the high-salt offspring, saltbush offspring also showed lower basal renin activity but, unlike the high-salt offspring, their renin levels continued to be lower when more salt was ingested. Having a lower renin activity allowed the saltbush offspring to increase the sodium concentration in their urine after the 25 g salt dose. Because their urine was more concentrated, the saltbush offspring did not need to drink as much water as the pasture offspring to restore osmotic balance. During the 50 g salt dose, saltbush offspring also had a lower renin activity, particularly in the first 4 hours after the salt dose, and this allowed them to excrete salt more rapidly than pasture

offspring. It is possible that the renin activity in the saltbush offspring was so low that a “floor effect” prevented further decrease. However, with the 2 hour intervals between sampling I did not see a plateau in renin activity after the 50 g dose. Saltbush offspring were also able to maintain their feed intake after the salt doses whereas the feed intake of the pasture offspring decreased by 40%. This could be because the basal food intake by the saltbush offspring was lower than the pasture offspring so food intake was not lowered any further by consuming salt. However, it could also be because saltbush offspring were able to excrete salt more rapidly so food intake was not compromised. The changes in salt excretion of the saltbush offspring may mean that they do not cope well with salt deficiency but I did not test the effects of salt deficiency on these animals. The increased capacity of the saltbush offspring to excrete salt seems to be conducive to them coping well with a high-salt diet, which is in contrast with the salt retention tendencies of the high-salt offspring.

The saltbush and high-salt offspring were both born to ewes consuming high amounts of salt during pregnancy, but the resultant changes in physiology and behaviour are different. Both groups have a suppressed basal renin activity, but the responsiveness of their renin activity to salt is different, resulting in different patterns of salt excretion. The results obtained from the high-salt offspring are in accordance with those of rats (da Silva et al., 2003), but my results from the saltbush offspring suggest that characteristics of the plant besides its high NaCl content influence the salt-handling mechanisms of the offspring. Saltbush contains not only NaCl, but also high levels of other minerals such as K, Mg, P, S and others (Chapter 3, Norman et al., 2004). It is possible that the interactions of other minerals in conjunction with NaCl caused different adaptive responses in the saltbush offspring. Saltbush also contains secondary compounds such as oxalates and tannins (Abu-Zanat et al., 2003) that the offspring may have been

exposed to through the diet of their mother which could influence their physiology. However, nothing is known about these compounds in relation to the effects of their consumption during pregnancy. Another influence could be the timing of the salt intake. Ewes consuming the high-salt diet had a constant 14% NaCl in their diet whereas the percentage of salt in the diet of saltbush ewes decreased as pregnancy progressed because the amount of barley supplement they were given increased. The differences in diets as well as differences between the amounts of salt that the mothers consumed at different stages of pregnancy, could account for some of the differences in physiology observed between the high-salt and saltbush offspring.

In the intake experiment, the salt preference of the offspring did not differ between treatments, which is in agreement with other research on sheep (SN Digby and DK Revell, personal communication), but contrary to what has been shown in rats. The rat studies showed an increased preference for salt in solution in offspring from rat dams offered a high-salt diet (3%) throughout pregnancy until weaning (Contreras and Kosten, 1983, Contreras and Ryan, 1990, Curtis et al., 2004). In contrast, my study used offspring from ewes on a much higher salt diet and tested preference for salt in food. However, the most likely reason that I did not see an increase in salt preference is because the duration of salt exposure in my experiment was restricted to the latter half of pregnancy and for the first three weeks after birth. At three weeks of age, lambs are too young to consume the same diet as their mother and since there was no difference in the salt content of their mother's milk (Chapter three) they would not have been exposed to the taste of salt. Results from other studies in both rats and cows show that exposure to the taste of salt at an early age increases the animal's salt preference in later life (Phillips et al., 1999, Smriga et al., 2002). Early exposure to the taste of salt may alter the perception of a "normal" salt taste and may alter pre-absorptive taste signals

(Curtis et al., 2004). Therefore, if I had continued the treatments until weaning, the offspring would have been old enough to consume some of their mother's diet and develop a "taste" for salt which may have increased their preference for salty foods in later life.

Offspring from ewes consuming either a high-salt diet or saltbush had a lower total food intake when consuming a diet of pellets, a result which conflicts with other studies.

Offspring born to rat dams that were fed a high-salt diet (3%) did not show any difference in total food intake (Contreras et al., 2000, Curtis et al., 2004). The marked response in my experiment could be because the salt content of the diets were over four times higher than that of the rat experiments. Food intake by all offspring in my study was positively correlated to their basal plasma renin activity. This suggests that the lower feed intake of the high-salt and saltbush offspring could be influenced by their suppressed renin levels. However, rats with suppressed RAS through a genetic deletion of angiotensin converting enzyme (ACE) show no change in food intake, but have a higher energy expenditure and a lower bodyweight (Jayasooriya et al., 2008). My animals had reduced bodyweight, which may be attributable to their lower feed intake rather than to increased energy expenditure. The renin activity in the animals in my study was suppressed by around 38% whereas a much larger suppression of the RAS was caused by the genetic deletion of the ACE gene in the rat study (plasma angiotensin II by 70% and tissue angiotensin II by up to 97%)(Campbell et al., 2004, Jayasooriya et al., 2008). The amount of suppression of the RAS could determine what physiological changes the animal possesses. Conflicting results on food intake have also been found in studies where angiotensin II has been increased. Infusions of angiotensin II can cause a decrease in food intake (Cassis et al., 2002, Porter et al., 2003, Sunagawa et al., 2001) or no change in food intake (Cassis et al., 1998). In contrast, renin transgenic mice with

a chronic increase in their RAS show an increased food intake (Szczepanska-Sadowska et al., 2003). The differing results of these studies, as well as mine, could be due to the activity of the localized RAS system in the brain, operating independently from the circulating RAS that could be influencing food intake, but this needs to be investigated further (Kumar et al., 2007, Szczepanska-Sadowska et al., 2003).

It is interesting that differences in food intake and, consequently, weight gain of the saltbush offspring were only apparent when they started consuming the concentrated pellet diet. Previously, when grazing a roughage diet there were no differences in weight gain (Chapter 3). Hence, the type of diet they consume may influence their food intake. It is possible that the high-salt or saltbush diet of the mother influenced her satiety hormones such as leptin or insulin (Cassis et al., 1998, da Costa Lima et al., 1997, Vidonho et al., 2004), which led to altered sensitivity to leptin and insulin in her offspring (El-haddad et al., 2004). Therefore, the suppressed food intake by the high-salt and saltbush offspring could be due to the direct effect of their suppressed RAS or an indirect effect through other factors that regulate food intake.

All offspring, regardless of maternal treatment, consumed more food and water as the salt content of their diet increased. This result is in agreement with results from studies in weanling rats that show a higher food intake when a high salt diet (3%) is consumed, compared to a normal (0.5%) or low salt diet (0.06%)(Coelho et al., 2006). The reason for the higher food intake on the high-salt diet could be due to higher energy expenditure (Coelho et al., 2006). It could also be simply because the animals are growing and increased their food intake over the period of 12 days. However, this is unlikely since the average growth of the animals was only 0.7 kg over 12 days and they increased their food consumption by 0.6 kg per day on the high-salt diet. The higher

intake of salt when consuming the high-salt pellet increased the water intake proportionately as shown by others (Stricker et al., 2003), but this was not the case in the high-salt offspring.

Offspring from ewes that were fed a high-salt diet drank less water per unit of food consumed. Contrary to this result, a study in rats showed increased water to food intake ratio in weanling rats whose mothers were fed a 3% salt diet, but this effect did not persist into adulthood (Curtis et al., 2004). Alterations in water intake in response to salt ingestion has been shown in offspring from ewes receiving a high-salt diet throughout pregnancy but water intake to food intake was not measured (SN Digby and DK Revell, personal communication). The renin angiotensin system could be involved in the decreased ratio of water to food intake by the high salt offspring (El-haddad et al., 2004, Weisinger et al., 1997). Suppressing the RAS of rats with an angiotensin II receptor antagonist decreases water intake in rats (Weisinger et al., 1997). Similarly, offspring from rat dams with increased renin activity show a higher water intake than normal offspring (Perillan et al., 2004). It is possible that the suppressed basal renin activity in the high-salt offspring could lower their basal water intake. However, the saltbush offspring did not have a lower ratio of basal water to food intake and they also show suppressed renin activity. Therefore, the decreased water intake of the high-salt offspring may be associated with plasma sodium and osmolarity set-points, rather than direct effect of the RAS. At birth, the high-salt offspring, but not the saltbush offspring, had a lower plasma sodium concentration than the control animals (Chapter 3), which may have influenced their water intake in later life. Offspring from ewes subjected to hypertonicity through dehydration during pregnancy show increased basal levels and altered set-points of plasma sodium and plasma osmolarity as well as changes in the threshold for arginine-vasopressin (AVP) secretion (Desai et al., 2003, Ross et al.,

2005). Thus, it is possible that the high-salt and high-water intake of the ewe during pregnancy altered the set point of plasma osmolarity in the offspring which may influence their water intake.

In conclusion, my results show that offspring from pregnant ewes that consumed a high-salt diet or saltbush from mid pregnancy until early lactation have altered renin activity that is associated with changes in salt excretion and decreased food and water intake. Saltbush offspring show salt handling adaptations that are conducive to them being better prepared to handle excess salt in their diet, such as a faster rate of salt excretion. In contrast, high-salt offspring have a tendency to retain salt which may be detrimental to them when consuming excess salt in their diet. Reasons for these differences in adaptations could be the degree and timing of suppression of their renin activity early in life. The high mineral content of saltbush (minerals other than NaCl) could also have influenced what adaptations the offspring possess. Overall, my data from sheep support previous findings in rats that consuming a high-salt diet during pregnancy can change the physiology of the offspring. However, the more intriguing finding is that the consumption of saltbush by ewes during pregnancy can cause their offspring to have beneficial adaptations that enable them to cope better with diets high in salt. This may enable them to gain bodyweight when they graze saltbush as adults.

CHAPTER 5

Programming sheep production on saltbush: adaptations of offspring from ewes that consumed high amounts of salt during pregnancy and early lactation

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Abstract

I investigated if feeding a high-salt diet (14% NaCl) or saltbush to ewes between day 60 of gestation and day 21 of lactation would allow their adult offspring to gain more weight when grazing saltbush and grow more wool than offspring from ewes that were fed a control diet (2% NaCl) or grazed pasture. At 10 months of age, offspring grazed saltbush for 8 weeks then dry pasture for 2 weeks and bodyweights, plasma renin activity (PRA) and wool growth were measured. Fleece weights and fleece characteristics were measured at 14 months of age and greasy fleece weight was taken again at 22 months after grazing pasture. Saltbush offspring were able to gain liveweight when grazing saltbush ($P \leq 0.05$) and also had lower PRA, whereas the other 3 treatment groups all lost weight when grazing saltbush ($P > 0.05$). The high-salt offspring had an 8 and 10% increased fleece weight at 14 and 22 months of age respectively ($P \leq 0.01$). Saltbush offspring also showed an 8% increase in greasy fleece weight at 22 months of age ($P \leq 0.05$). Grazing saltbush during pregnancy and early lactation induces important adaptations in plasma renin activity of the offspring, which allows them to gain weight when grazing saltbush and may also increase wool follicle

density. Grazing pregnant ewes on saltbush can profit farmers in three main ways, 1) ability to utilize saltland 2) increase weight gain of sheep when grazing saltbush 3) increase fleece weight.

Introduction

Grazing sheep on saltbush (*Atriplex spp.*) is one of the few ways that farmers can utilize salt-affected land in southern Australia and it can also reduce supplementary feeding costs during autumn (Masters et al., 2006, Morcombe et al., 1996). However, sheep do not tend to gain weight when grazing this forage without additional energy via supplementation or complementary pasture because it has a very high-salt content (about 20%)(Masters et al., 2001). The capacity to deal with excessive salt is therefore an important determinant of an animal's performance when grazing saltbush.

In previous experiments I have shown that feeding ewes a high-salt diet or saltbush from mid pregnancy to early lactation alters their offspring's physiological response to salt, especially their plasma renin activity (PRA) (Chapter four). Renin is the rate-limiting enzyme in the renin-angiotensin system (RAS) that helps control salt balance in the ewe and kidney development in the fetus (Gomez and Norwood, 1995, Matsusaka et al., 2002). The high-salt or saltbush diet of the mother influences the salt balance mechanisms of her offspring so that when they are adults they have a lower basal renin activity (Chapter four). This allows them to excrete salt more rapidly, drink less water and maintain their food intake when given an oral dose of salt compared to the offspring from ewes that consumed pasture during pregnancy (Chapter four). This suggests that offspring from ewes that consumed saltbush during pregnancy would have an adaptive advantage when grazing saltbush. However, offspring from ewes that consumed a high-salt diet, rather than saltbush, during pregnancy do not have these same beneficial

adaptations for handling salt. Instead, these sheep retain more salt than control offspring when given an oral dose of salt and this suggests that they would not have an adaptive advantage when grazing saltbush (Chapter four). As well as physiological adaptations obtained *in-utero* in offspring from ewes that consumed saltbush, these offspring may learn to graze saltbush by watching their mothers and this may increase their consumption. There are several examples where a young animal learns how to eat certain diets from its mother and this often leads to an increase in consumption of that food in later life and a concomitant increase in performance (Nolte et al., 1990, Squibb et al., 1990, Wiedmeier et al., 2002, Glasser et al., 2009). My first hypothesis is that adult offspring from ewes that consumed saltbush from day 60 of pregnancy to day 21 of lactation will gain more weight when grazing saltbush themselves, but offspring from ewes that consumed a high-salt diet will not gain weight when grazing saltbush.

Exposure to saltbush *in-utero* not only alters the lamb's ability to tolerate salt but may also stimulate wool growth by altering blood flow to the skin. The high-salt or saltbush diet of the mother from mid to late pregnancy may suppress the RAS of the fetus and this would increase blood flow to the periphery of the body including the skin at a critical time when wool follicles are being developed (Iwamoto and Rudolph, 1979, Moore et al., 1996). Increasing blood flow to the skin increases wool growth in adult sheep (Hocking Edwards and Hynd, 1994) so in the fetus, it may increase the density of wool follicles which would increase fleece weight. Therefore, my second hypothesis is that offspring from ewes that consumed a high-salt diet or saltbush during mid pregnancy to early lactation will have an increased fleece weight.

To test these hypotheses ewes were pair-fed a high-salt (14% NaCl) or control diet (2% NaCl) in an animal house from day 60 of pregnancy until day 21 of lactation. In a

separate experiment during this period, I also grazed ewes on either saltbush or pasture. At ten months of age, offspring from these treatments were grazed on saltbush for eight weeks and then on pasture for two weeks and their bodyweights, PRA and wool growth rate were measured. Their fleece weights were measured at 14 and 22 months of age. The high-salt and control treatments in the animal house were analysed separately from the saltbush and control pasture treatments in the field.

Materials and Methods

Maternal treatments

A full explanation of maternal treatments during pregnancy and early lactation has been published previously (Chapter three). Briefly, Merino ewes aged three years were artificially inseminated to the same ram and grazed pasture until day 60 of pregnancy when they were randomly assigned to treatment groups. One experiment was conducted in an animal house with two treatment groups. Another experiment was conducted in the field with two treatment groups.

In the animal house experiment, thirty ewes received a normal salt pellet (2% NaCl) and 30 received a high-salt pellet (14% NaCl). Fourteen percent NaCl was chosen because I predicted that the saltbush ewes would be consuming a similar amount in the field given that saltbush contains 20-25% salt and sheep were also consuming understorey and grain supplement (Norman et al., 2002). These ewes were kept in an animal house in individual pens and were pair fed so both groups had identical organic matter intakes and were fed enough to maintain conceptus-free liveweight (SCARM, 1990). Offspring from ewes that consumed a high-salt diet during mid-pregnancy to early lactation are referred to as “high-salt offspring” and offspring from ewes that consumed the control diet are termed “control offspring.”

In the field experiment, 35 ewes grazed a control diet of dry pasture based on subterranean-clover, supplemented with lupin grain and another 35 ewes grazed saltbush (mainly *Atriplex nummularia*) with a small amount of annual pasture understorey and supplemented with barley grain. Twelve ewes from each of the saltbush and control pasture treatments were carrying twins. As pregnancy progressed, the amount of supplement in the diet of the field ewes increased so that all ewes maintained conceptus-free weight (SCARM, 1990). Offspring from ewes that consumed saltbush from mid-pregnancy to early lactation are referred to as “saltbush offspring” and those offspring born to ewes that consumed the pasture diet are termed “pasture offspring.”

All ewes in both experiments consumed their treatment diets from day 60 of pregnancy until day 21 of lactation. After this, all ewes grazed together on pasture based on subterranean clover until weaning at 15 weeks of age. Lambs continued to graze this pasture until they were eight months old before they were brought into the animal house for preference and salt-dose testing, during which time they were fed a pelleted diet (Chapter four). After this testing was completed, the offspring grazed pasture based on subterranean clover and were supplemented with barley for three weeks before being transferred to saltbush plots.

Offspring grazing saltbush

Offspring grazed saltbush when they were approximately ten months old. Twenty high-salt offspring (13 ewes and 7 wethers) and twenty control offspring (9 ewes and 11 wethers) were used in the experiment and these were all single-born offspring. Twenty nine saltbush offspring (12 ewes and 17 wethers) were used comprising of 22 single-

born and 7 twin-born lambs. There were 25 pasture offspring (12 ewes and 13 wethers) of which, 17 were single-born and 8 were twin-born lambs. All offspring grazed together, rotating through 12 1-ha plots of saltbush with a small amount of annual pasture understorey for eight weeks. Sheep were moved onto the next plot when 70% of the edible feed on offer had been removed. For the first month they grazed river saltbush (*Atriplex amnicola*), and for the second month they grazed old man saltbush (*Atriplex nummularia*). Sheep were supplemented with 250 g/h/d of barley grain to meet their energy requirements to maintain weight (SCARM, 1990). The nutritive value of the components of their diet is shown in Table 10. Offspring had unlimited access to fresh water throughout the experiment.

Table 10: Nutritive value of the components of the offspring's diet while grazing saltbush and its estimated total nutritional content. Minerals are expressed as percentage dry matter (%DM).

	Saltbush	Inter- row	Barley	Est total in diet
Estimated % in diet	80	10	10	100
Estimated ME¹ (MJ/kg DM)	7.4	5.8	12	7.94
Crude protein (% DM)	9.19	3.31	12.00	8.21
P	0.16	0.06	0.44	0.21
K	2.54	0.14	0.53	1.40
Na	7.17	0.23	0.03	5.00
Ca	0.60	0.25	0.14	0.60
Cl	12.17	0.08	0.02	8.02

¹ME=Metabolisable energy, values estimated from previous data (D.G. Masters, personal communication).

The offspring were weighed when they started grazing saltbush, then every two weeks while grazing saltbush. After eight weeks sheep were taken off saltbush and grazed dry pasture based on subterranean-clover for two weeks from weeks 8-10 to allow body

water levels to return to normal (Casson et al., 1996) before a final measurement of bodyweight was taken. The percentage of saltbush consumed in the diet of saltbush and control pasture offspring was estimated from the ratio of C^{12} : C^{13} in the fecal samples of the sheep at six and eight weeks after they started grazing saltbush (Norman et al., 2008). The small sub-sample of fecal matter was collected *per rectum*.

Blood samples

Blood samples were taken prior to the offspring grazing saltbush, when they were grazing dry pasture based on subterranean-clover. All sheep were again blood sampled at 2, 6 and 8 weeks when grazing saltbush. Plasma renin activity was measured at all of these times, and plasma concentration of sodium and chloride was measured at 0 and after 8 weeks of sheep grazing saltbush. All blood samples were kept at room temperature before centrifugation for 15 min at 1507 x g and frozen at -20°C until analysed. Plasma renin activity was measured as the rate of formation of angiotensin I using a commercially available kit (Gamma Coat Plasma Renin Activity RIA kit, Dia Sorin, Stillwater, Minnesota, USA). The inter-assay coefficient of variation was 7.2% and the intra-assay coefficient of variation was 5%. Plasma concentration of sodium and chloride was measured using an Ion Selective Electrode and were run on an Olympus AU400 automated chemistry analyser (Olympus Optical Co. Ltd).

Wool

Offspring were first shorn at 14 months of age. Greasy fleeces were weighed and a mid-side sample was taken for analysis. The fleece characteristics measured were fibre diameter, coefficient of variation and standard deviation of fibre diameter, yield, staple strength and length, coefficient of variation of staple length and curvature. Sheep were then shorn again eight months later when they were 22 months of age and only greasy

fleece weight was measured. Dyebands were used to measure wool growth before, during and after grazing saltbush (Chapman and Wheeler, 1963). Wool growth measurements started from when offspring were seven months of age then a dye band was made when the offspring started grazing saltbush at nine months of age. Another band was made when the offspring stopped grazing saltbush at 11 months, and the dye band was removed before shearing at 14 months of age.

Management of offspring from 11-22 months of age

After the saltbush grazing experiment, the saltbush and pasture offspring were fed on high-quality pellets for five weeks, whereas the high-salt and control treatments grazed pasture based on subterranean clover. After this, all offspring from the four treatment groups grazed together on subterranean-clover based pasture and were supplemented with lupin grain when feed became limiting in order to maintain liveweight.

Statistics

Residual maximum likelihood (REML) linear mixed model was used to analyse data with treatment and time (or age) as fixed effects (Genstat, 10th Edition, VSN International Ltd, United Kingdom). Gender of the offspring was also incorporated into the fixed effects as well as birth status of the lamb (single or twin-born) for the offspring born in the field experiment. Fleece characteristics and saltbush intake data were analysed using ANOVA (analysis of variance). ANOVA was also used to analyse bodyweights of saltbush and pasture offspring after grazing saltbush (week 10). Natural log was used to transform plasma renin activity values in order to obtain a normal distribution. Greasy fleece weight at 22 months of age was regressed against renin activity of offspring as adults. In order to determine if factors early in life contributed to fleece weight, I also used data from previous experiments (Chapter three and four).

Plasma sodium of the ewe at day 130 of gestation and plasma sodium of the offspring at birth was regressed against fleece weight to see if sodium status was involved. Plasma renin activity of the ewe during pregnancy and that of her lamb at birth and at three weeks of age was also regressed against fleece weight in order to see if the renin-angiotensin system in early life was involved in programming fleece weight. Data from offspring born in the animal house experiment (high-salt and control offspring) were always analysed separately to data from offspring born in the field experiment (saltbush and pasture offspring.)

Results

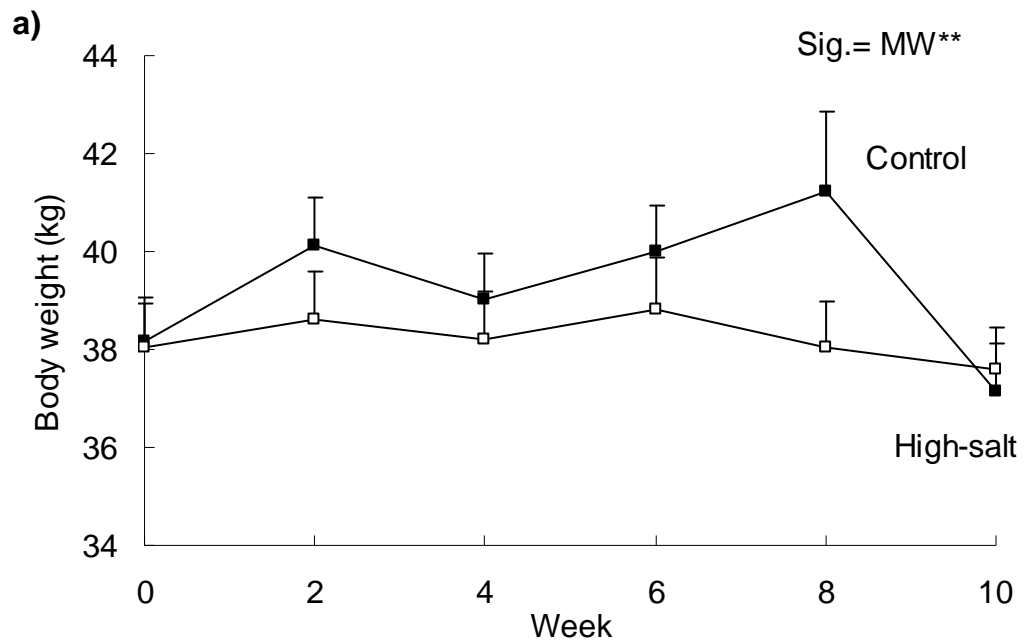
Bodyweight

Before and after grazing saltbush bodyweights of the high-salt and control offspring were similar, but there were differences in their pattern of weight change ($P \leq 0.01$). High-salt offspring maintained a reasonably constant bodyweight throughout the ten week period and overall lost approximately 0.5 kg of bodyweight. The pattern of weight gain for the control offspring was quite different. They gained 2 kg in the first two weeks when grazing saltbush and by eight weeks had gained 3 kg. However, when they returned to grazing low salt pasture they lost 4 kg so by ten weeks they had lost about 1 kg from their original bodyweight at the start of the experiment (Figure 14).

There was also a difference in the pattern of weight gain between saltbush and pasture offspring ($P \leq 0.01$). This was mainly due to the saltbush offspring being 2 kg heavier than the control offspring at the end of the ten week period ($P \leq 0.05$). Saltbush and pasture offspring maintained similar bodyweights from 0 to 8 weeks, but when the animals stopped grazing saltbush after 8 weeks, the bodyweight of the pasture offspring fell by 1.7 kg whereas the saltbush offspring only lost 0.3 kg. Therefore, at the end of

ten weeks, saltbush offspring had a higher bodyweight than the pasture offspring as they had gained approximately 1 kg while the control pasture offspring had a net loss of 1 kg of bodyweight from their bodyweight before grazing saltbush (Figure 14).

Gender and birth status (single or twin) had no significant effect on any of the parameters measured in this experiment ($P>0.05$).



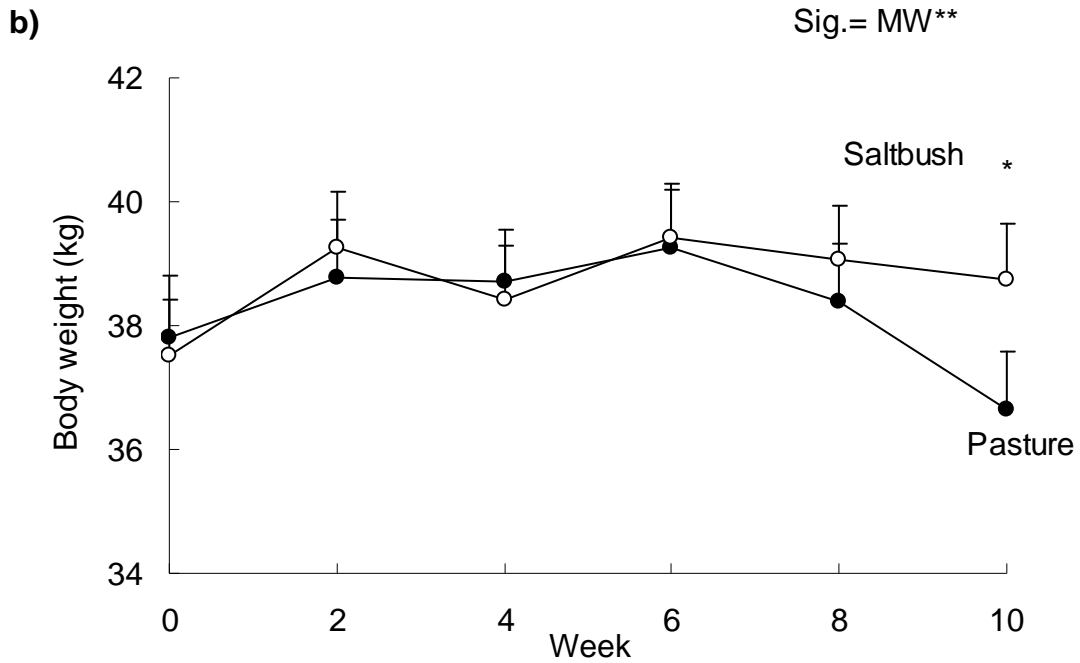


Figure 14: Bodyweight (kg) of offspring when grazing saltbush for 8 weeks, then grazing pasture from 8 to 10 weeks. Sheep are offspring from ewes that were fed (a) a control or high-salt diet in an animal house or (b) grazed pasture or saltbush in the field from mid pregnancy to early lactation. Sig.= significance, MW= effect of maternal diet by week of saltbush grazing. ** $P \leq 0.01$. At week 10, weights were significantly different in figure b ($P \leq 0.05$) (ANOVA).

Plasma renin activity

High-salt offspring tended to have higher renin activity after two weeks of grazing saltbush than control offspring, but their renin activity was similar to controls for the rest of the period such that there was no significant effect ($P > 0.05$) (Figure 15).

Saltbush offspring had a lower renin activity than pasture offspring ($P \leq 0.01$), and there were also differences between saltbush and pasture offspring in their pattern of renin activity over the eight week period ($P \leq 0.001$). Saltbush offspring showed lower renin activity before grazing saltbush but, after 2 weeks, renin activity of saltbush and pasture offspring were similar. However, at six and eight weeks, renin activity of saltbush offspring was 30% lower than control pasture offspring (Figure 15).

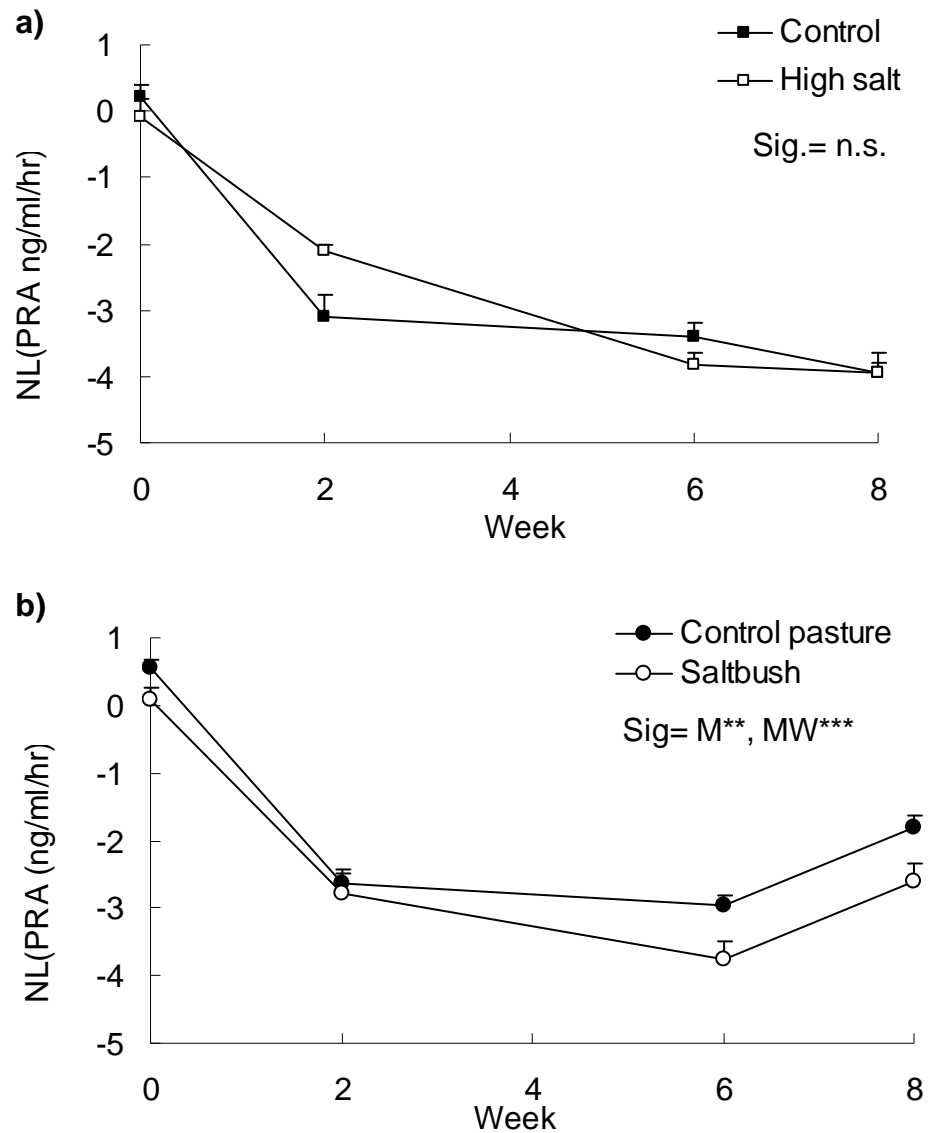


Figure 15: Natural log (NL) of plasma renin activity (ng/ml/hr) (rate of formation of angiotensin I) of offspring when grazing saltbush for 8 weeks. Sheep are offspring from ewes that were (a) given a control (2%) or high-salt diet (14%) in an animal house or (b) grazed pasture or saltbush in the field during mid pregnancy to early lactation. Sig.= significance, M= effect of maternal diet, MW= effect of maternal diet by week of saltbush grazing. ** $P \leq 0.01$. *** $P \leq 0.001$. n.s= not significant.

Wool production

At 14 months of age, high-salt offspring had an 8% higher GFW than controls and at 22 months of age they had a 10% higher GFW than control offspring ($P \leq 0.01$). Clean fleece weight of the high-salt offspring was also higher than controls at 14 months. There was no difference in bodyweights between treatments at the time of shearing. The high-salt offspring grew more wool than the control offspring regardless of whether

they were grazing saltbush or not ($P \leq 0.01$). High-salt offspring had a much greater wool production than controls before grazing saltbush (extra 2.7 g/d). However, when grazing saltbush this higher growth was reduced to a margin of 0.4 g/d, although there were no significant maternal treatment by diet interactions ($P = 0.09$) (Table 11).

Saltbush offspring grew more greasy wool at 22 months of age, but not at 14 months of age ($P \leq 0.05$). Similarly, clean fleece weight did not differ between saltbush and control pasture offspring at 14 months of age ($P > 0.05$). Bodyweights of saltbush and pasture offspring were similar at both ages. However, saltbush offspring tended to have much lower wool growth immediately before grazing saltbush than control offspring (1.1 g/d less). In contrast, during and after grazing saltbush, saltbush offspring tended to grow more wool than control offspring (approximately 0.6 g/d more) though this was not significant ($P = 0.1$) (Table 11).

GFW was related to plasma sodium of the ewe at 130 days gestation ($P < 0.02$, $R^2 = 0.17$). No other significant relationships were found between fleece weight and plasma renin activity of the ewe during pregnancy (60, 90 or 135 days gestation) and her offspring at birth or a three weeks of age ($P > 0.05$). Similarly, no relationships were found between fleece weight and any of the parameters measured in Chapter four.

Table 11: Bodyweight (kg) and greasy fleece weight (kg) of offspring at 14 and 22 months of age and wool growth up to 14 months of age before, during and after grazing saltbush (sb) (\pm s.e.). Sheep are offspring from ewes that were fed a control or high-salt diet in an animal house or grazed pasture or saltbush in the field from mid pregnancy to early lactation.

	Maternal diet		Maternal diet	
	Animal house offspring		Field offspring	
	Control	High-salt	Pasture	Saltbush
	<i>Bodyweight (kg)</i>			
14 months	42.9 \pm 1.21	42.2 \pm 1.16	44.1 \pm 1.11	44.7 \pm 0.92
22 months	49.7 \pm 1.13	48.1 \pm 1.09	48.6 \pm 1.39	51.0 \pm 1.38
Significance	n.s		n.s	
	<i>Greasy fleece weight (kg)</i>			
14 months	3.32 \pm 0.08	3.60 \pm 0.07	3.57 \pm 0.10	3.56 \pm 0.08
22 months	4.77 \pm 0.14	5.25 \pm 0.13	4.93 \pm 0.14	5.31 \pm 0.16
Significance	M**		MA*	
	<i>Clean fleece weight (kg)</i>			
14 months	2.6 \pm 0.06	2.9 \pm 0.06	2.8 \pm 0.07	2.8 \pm 0.07
Significance	M*		n.s.	
	<i>Wool growth (g/d)</i>			
Pre-saltbush grazing	16.3 \pm 0.91	19.02 \pm 0.76	17.87 \pm 0.59	16.72 \pm 0.55
Saltbush	9.89 \pm 0.50	10.28 \pm 0.31	9.55 \pm 0.43	10.18 \pm 0.68
Post-saltbush grazing	12.98 \pm 0.50	13.55 \pm 0.38	14.04 \pm 0.5	14.61 \pm 0.46
Significance	M**		P=0.1	

M= effect of maternal diet, MA= interaction between maternal diet and age of offspring
* $P \leq 0.05$, ** $P \leq 0.01$. n.s.= Not significant

In the offspring from the animal house experiment, some fleece characteristics at 14 months of age differed between the high-salt and the control offspring. Fibre diameter was not different between treatments ($P > 0.05$), but the standard deviation of fibre diameter was higher in high-salt offspring ($P \leq 0.05$). High-salt offspring had lower curvature values than control offspring ($P \leq 0.05$). There was no difference in staple

length or strength between the high-salt and control offspring. There was also no difference in any of the other characteristics of the fleece such as fibre diameter, staple length or staple strength ($P>0.05$) (Table 12).

Table 12: Fleece characteristics (\pm s.e.) of offspring at 14 months of age.

Offspring are offspring from ewes that were fed a control (2%) or high-salt diet (14%) in an animal house or grazed pasture or saltbush in the field from mid pregnancy to early lactation.

	Maternal diet		Maternal diet	
	Animal house offspring		Field offspring	
	Control	High-salt	Pasture	Saltbush
Fibre diameter (μm)	17.1 \pm 0.31	17.5 \pm 0.27	17.3 \pm 0.26	17.2 \pm 0.23
CV of FD (%)	21.0 \pm 0.54	22.2 \pm 0.54	21.1 \pm 0.49	21.6 \pm 0.56
SD of FD	3.6 \pm 0.08	3.9* \pm 0.10	3.6 \pm 0.08	3.7 \pm 0.10
Yield (%)	78.8 \pm 0.89	79.1 \pm 0.49	77.9 \pm 0.71	78.6 \pm 0.67
Staple strength				
(n/ktex)	22.6 \pm 1.98	23.3 \pm 1.39	19.5 \pm 1.66	21.3 \pm 1.75
Staple length (mm)	80.7 \pm 2.50	81.4 \pm 1.92	83.9 \pm 1.29	82.7 \pm 2.35
CV of staple length				
(%)	6.6 \pm 0.75	6.2 \pm 0.78	6.0 \pm 0.55	5.4 \pm 0.58
Curvature (deg/mm)	94.4 \pm 1.00	91.0* \pm 1.22	92.7 \pm 1.09	93.7 \pm 1.32

CV- Coefficient of variation. SD- Standard deviation. FD Fibre diameter * $P\leq 0.05$, Significantly different than control

Plasma sodium

Concentration of sodium and chloride in plasma was similar between high-salt and control offspring, before and after grazing saltbush for eight weeks. Similarly, there were no differences in plasma sodium and chloride concentrations between saltbush and pasture offspring (Table 13).

Table 13: Plasma sodium and chloride concentration (mmol/l) of offspring before grazing saltbush (week 0) and after 8 weeks of grazing saltbush (\pm s.e.). Sheep are offspring from ewes that were given a control (2%) or high-salt diet (14%) in an animal house or grazed pasture or saltbush in the field during mid pregnancy to early lactation.

	Week	Maternal diet		Maternal diet	
		Animal house offspring		Field offspring	
		Control	High-salt	Pasture	Saltbush
Plasma Na	0	146 \pm 0.43	146 \pm 0.45	147 \pm 0.46	147.3 \pm 0.3
	8	145 \pm 0.52	145 \pm 0.51	145 \pm 0.42	145 \pm 0.29
Plasma Cl	0	110 \pm 0.66	111 \pm 0.65	111 \pm 0.52	111 \pm 0.47
	8	110 \pm 0.58	110 \pm 0.48	111 \pm 0.46	111 \pm 0.37

Saltbush intake

The amount of saltbush consumed by offspring was similar between saltbush and pasture treatments. Saltbush offspring ate an average of 80 % saltbush (S.E. = 1.2) in their diet after 6 and 8 weeks of grazing saltbush. Control pasture offspring ate 79% saltbush (S.E. = 1.6) in their diet during week 6 and 8 of grazing saltbush.

Discussion

My data show that animals whose mother consumed saltbush during pregnancy and early lactation gained weight after grazing saltbush, whereas offspring born to ewes consuming pasture lost weight. However, this difference in weight gain was only apparent when animals returned to grazing a low-salt pasture. A reason for this could be that the weight gain of the saltbush offspring was caused by an increase in body tissue, whereas the weight gain of pasture offspring was caused by an increase in body water. Normally when sheep graze saltbush, they increase their water intake and increase the proportion of water in their weight gain so that most of the weight gain observed in

these animals is water (Casson et al., 1996). When they return to low-salt pastures they lose this water and, accordingly, lose weight, as seen in the pasture offspring. However, when offspring from ewes that consumed saltbush during pregnancy graze saltbush, they may not increase their water intake to the same extent as control offspring. This is because I have shown that water intake following an oral salt dose is attenuated in offspring born to ewes fed saltbush during pregnancy (Chapter 4). Consequently, any weight gain is likely to contain less water and more body tissue. Therefore, I propose that offspring born to ewes consuming saltbush during pregnancy and early lactation are likely to make better use of saltbush as adults and gain more tissue weight than sheep that have not had similar exposure to saltbush early in life. However, confirmation of my conclusion through direct measurement of body water in future studies is warranted.

Reduced activity of plasma renin is probably the mechanism that underlies the reason why saltbush offspring gained more weight grazing saltbush than control pasture offspring. Decreased activity of renin allows more sodium to be excreted because it slows the formation of the salt retaining hormones angiotensin II and aldosterone (Lumbers, 1999, Morgan, 2001). Since the saltbush offspring had a lower renin activity, they may have been able excrete salt more rapidly, a result supported by my previous experiment (Chapter 4). A more efficient excretion of salt, in turn, would allow them to gain more weight when grazing saltbush because it may allow a higher intake and/or reduced expenditure in renal salt excretion.

Feed intake was not measured in this study but the proportion of saltbush in the diet was, and this did not differ between groups. Perhaps the young age that the offspring were exposed to saltbush before weaning explains why no difference was found. Squibb et al. (1990) showed that lambs consumed more of a shrub as adults if they were

exposed to it at 4-8 weeks or 8-12 weeks of age, but not if they were exposed when they were younger (1-5 weeks of age). The reason I did not see a difference in intake of saltbush could be because at 3 weeks of age, saltbush offspring were too young to consume saltbush themselves and gain a preference for it. Although the proportion of saltbush the offspring consumed in their diet did not differ, the total food consumption of the saltbush offspring could have been higher so they were eating more saltbush and also more grain and understorey. This may explain how the saltbush offspring gained more tissue weight when grazing saltbush, but the percentage of saltbush in their diet did not increase.

Offspring from ewes that consumed a high-salt diet during pregnancy and early lactation did not gain weight when grazing saltbush, a result that supported my hypothesis. This may be because they do not possess the same beneficial adaptations as the saltbush offspring, a conclusion supported by my earlier study (Chapter 4). Both high-salt and control offspring lost weight when grazing saltbush, but high-salt offspring did not show the usual increase, and then decrease, of weight gain associated with higher water turnover on saltbush (Casson et al., 1996). In previous experiments I found that high-salt offspring consumed less water per unit food intake than control sheep when consuming both a normal salt (0.5%) and high-salt diet (7%) (Chapter 4). The same may have occurred in the current field study where the high-salt offspring may have been drinking less water which may account for their lack of bodyweight change. The concentration of sodium and chloride in the plasma of high-salt offspring was not altered by grazing saltbush, which suggests that despite possibly having a low water intake, as found in the previous study (Chapter 4), they were still able to maintain salt balance.

There could be several reasons for the different adaptations seen in saltbush and high-salt offspring, despite both offspring being born to ewes that consumed high amounts of NaCl from mid-pregnancy to early lactation. First, saltbush not only contains high amounts of NaCl, it also contains high levels of other minerals such as K, Mg and S as well as secondary compounds such as oxalates and tannins (Norman et al., 2004). Consumption of these other minerals and compounds by the ewe could influence the environment of the fetus *in-utero*, or influence the milk of the ewe in the early post-natal period. Another difference is that ewes consuming the high-salt diet had a constant 14% salt in their diet whereas the saltbush ewes had to be fed more grain supplement as pregnancy progressed so the percentage of salt in their diet decreased towards the end of pregnancy. The salt content of the ewe's diet in particular stages of fetal or early postnatal development could determine what adaptations the offspring possess (Emanuelli and Madeddu, 1999).

Offspring from ewes that consumed a high-salt diet during pregnancy had an increased wool growth and fleece weight, as anticipated, but the results for saltbush offspring were less clear-cut since there was only an increase in fleece weight at 22 months, not at 14 months. However, contrary to my hypothesis, the renin-angiotensin system (RAS) does not seem to be directly involved in the increased fleece weight observed since it did not correlate to plasma renin activity of the pregnant ewe or her offspring in early life. There was, however, a significant correlation between the plasma sodium of the ewe at day 130 of gestation and the fleece weight of the offspring ($P < 0.02$, $R^2 = 0.17$), which suggests that sodium itself may be involved more directly with wool growth. How salt (sodium) controls fleece weight is uncertain but one possibility is a direct influence on the density of wool follicles formed in mid to late gestation.

An increase in the density of wool follicles is the most likely explanation for the increased fleece weight in high-salt and saltbush offspring because the other components that influence fleece weight, fibre diameter and fibre length, remained unchanged (Adams and Cronje, 2003). Although I did not measure follicle number or density, the decrease in fibre curvature observed in the high-salt animals fits this theory as curvature is inversely correlated to density of follicles per unit area of skin as well as fleece weight (Jackson et al., 1975). Adelson et al. (2002) suggest that, during development, follicle density (primary and secondary follicle) is determined independently from follicle diameter. Therefore, it may be that the high-salt diet of the mother influenced the mechanisms or genes that determine follicle density, but did not alter follicle diameter.

Sheep from mothers that grazed saltbush during pregnancy and early lactation grew more fleece (8%) than control sheep at 22 months but strangely, not at 15 months. Like the high-salt offspring, the saltbush offspring were also exposed to a high-salt diet *in-utero* so I would expect them to also have a higher fleece weight than the pasture offspring when they gain similar amounts of weight. However, the saltbush offspring gained less weight (Chapter 4) and consequently grew less wool in the period before they grazed saltbush ($P=0.1$), which would have lowered their fleece weight at 15 months. But, in the 8 months following the saltbush grazing experiment, the weight gains of saltbush offspring and pasture offspring were comparable and, during this time, the higher fleece weight of the saltbush offspring was expressed.

In summary, grazing pregnant ewes on saltbush from mid pregnancy to early lactation induces salt handling adaptations in their offspring that allows them to gain more tissue weight when grazing saltbush as adults. Consuming high amounts of salt or saltbush

during pregnancy also increases the fleece weight of the offspring. These results can help increase farm productivity in three ways. First, grazing pregnant ewes on saltbush not only reduces supplementary feeding costs to the farmer, but also makes use of otherwise unproductive saltland (Masters et al., 2006). Second, the offspring from these ewes can gain weight when grazing saltbush, rather than losing weight, at least over a 2 month period of grazing. Third, the increase in fleece weight of the offspring can make a significant contribution to farm profits.

CHAPTER 6

General Discussion

Fetal programming can be used to increase sheep production on saltbush. Grazing pregnant ewes on saltbush programs adaptations in the salt balance of their offspring that allow them to gain more weight when grazing saltbush. In contrast, different salt balance mechanisms were programmed in offspring born to ewes fed a high-salt diet during pregnancy, which was not conducive to them performing well on saltbush in later life. The main reason for these differences in performance was that saltbush offspring were more efficient at excreting salt, whereas high-salt offspring tended to retain salt. However, salt balance mechanisms were not the only changes in physiology that were programmed in these offspring; they also showed alterations in food and water intake and an increased fleece weight. Using fetal programming to develop beneficial adaptations in animals could be a powerful tool when trying to increase animal production from particular forages or environments.

Feeding saltbush to the ewe did not have the same effects on the offspring as feeding a high-salt diet. Saltbush offspring could excrete a salt load faster, whereas high-salt offspring tended to retain more salt than normal animals. In the literature review, I discussed some possible alterations in physiology that could occur in the offspring; these are shown in Figure 16 below. The RAS of the saltbush offspring was more responsive to salt, whereas the RAS of high-salt animals was less responsive to salt. The responsiveness of the RAS to salt dictates the rate at which salt is excreted, which ultimately influences the performance of livestock on saltbush. Therefore, offspring

born to ewes that consume saltbush, but not a high-salt diet, from mid pregnancy to early lactation possess beneficial adaptations in salt excretion that allows them to gain tissue weight when grazing saltbush in later life.

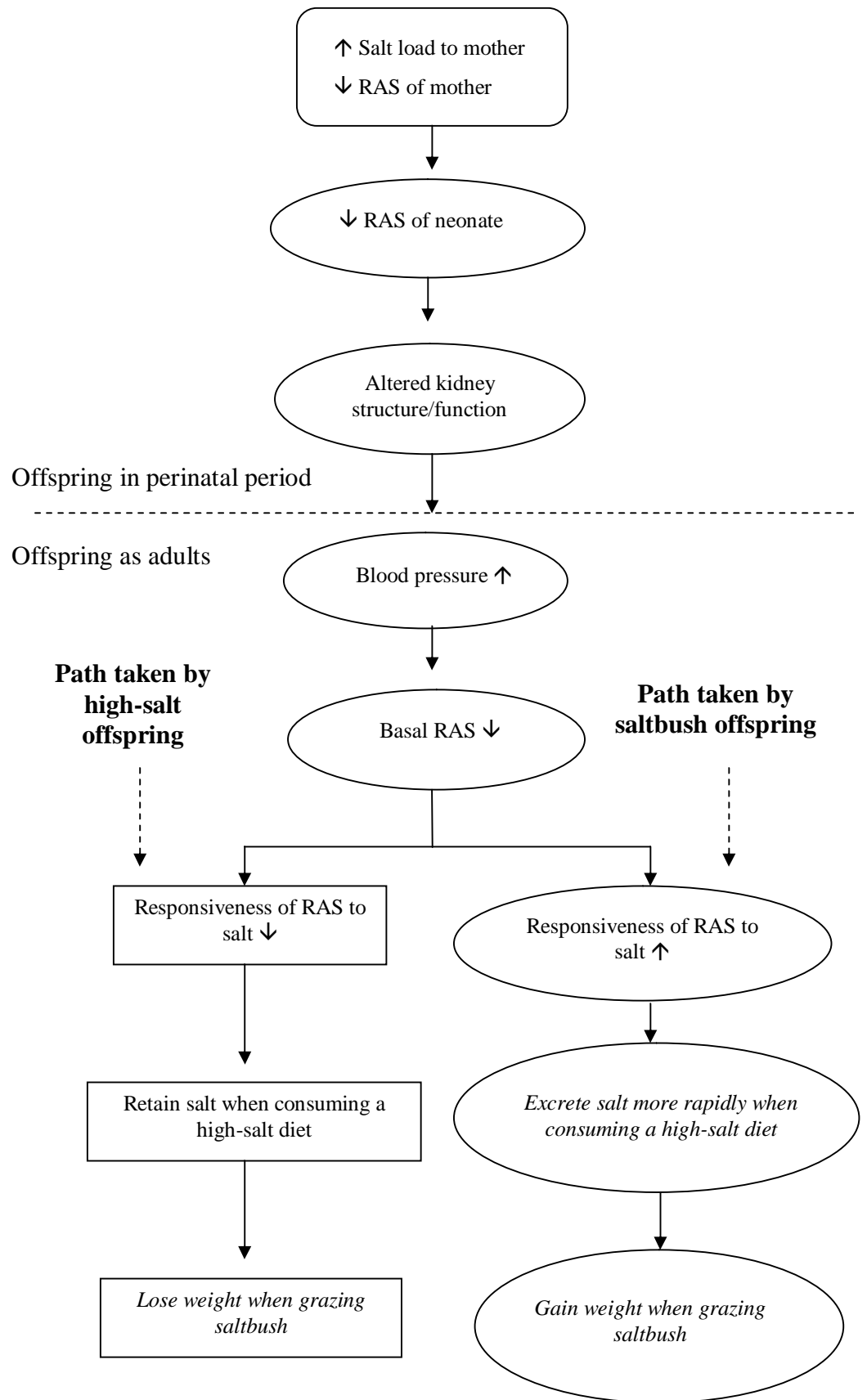


Figure 16: Potential alterations in physiology of offspring from mothers consuming a high-salt diet or a saltbush diet, showing divergent responses depending on changes to the RAS, as discussed in section 6 of the Literature Review. Responses in *italics* are results that my experiments are the first to report.

An explanation for the different physiological adaptations between the high-salt and saltbush offspring could be the different mineral and secondary compound components of their diets, and also differences in the amount of salt they consumed. Saltbush contains not only NaCl, but also other minerals such as K, Mg and S. The excretion of these minerals, especially potassium, may interact with the excretion of sodium. Consuming high amounts of both sodium and potassium decreases feed intake and digestibility more than consuming sodium alone (Masters et al., 2005). Therefore, the added pressure on the physiology of the saltbush ewes to excrete high amounts of both sodium and potassium could have affected the physiological adaptations of their offspring, as opposed to the high-salt offspring whose mothers' only consumed high amounts of sodium. In addition to increased minerals, saltbush ewes also consumed high amounts of secondary compounds such as oxalates, which can affect kidney function (Abu-Zanat et al., 2003). Nothing is known about the fetal programming effects of the secondary compounds in saltbush, but it is possible that they may have influenced the adaptations of the saltbush offspring. Another reason for the differences in physiology between the high-salt and saltbush offspring are the changes in salt content of the ewe's diet at different stages of pregnancy. Saltbush ewes consumed about 150 g of NaCl per day throughout pregnancy and lactation. In contrast, the diet of the high-salt ewes started at 115 g of NaCl at day 60 of pregnancy and as their nutrient requirement increased they were fed more pellets, so during lactation they were consuming about 300 g of NaCl per day. Consuming different quantities of salt at different stages of pregnancy may have influenced the adaptations of the high-salt and saltbush offspring. However, more work needs to be conducted on which stage of pregnancy and which level of salt intake is most critical for the high-salt diet of the mother to produce beneficial adaptations in the offspring.

The experiments reported here show beneficial fetal programming effects of feeding a particular forage to a pregnant animal. In many studies where the effects of a particular forage plant have been investigated, the main nutritive or anti-nutritive quality of the plant is tested on animals in isolation. For example, the high-salt content of saltbush is tested by adding NaCl to the diet or, in other forages, secondary compounds such as tannins or terpenes may be the major limitation to intake so they are studied in isolation. My results emphasize that focusing on a single major compound in the plant (such as high salt content) may be misleading when tested in isolation compared to when it is part of the mixture of compounds in the plant (such as feeding saltbush). My experiments also highlight the benefits of using the natural adaptation mechanisms of animals to allow them to be prepared for the environments they will encounter in later life. Increasing the performance of animals when consuming low-quality forage, such as saltbush, effectively increases the feeding value of that forage. Therefore, it may no longer be such low quality in terms of its impact on animal performance. The feeding value of saltbush may remain higher than normally considered if beneficial adaptations in offspring accumulate in subsequent generations that are exposed to the forage in early life. It may also be possible that the physiological adaptations seen in the saltbush offspring from my experiment may be passed to the next generation without further exposure to saltbush. My results support the view that fetal programming can be used to develop beneficial adaptations in animals that allow them to perform better and, in turn, increase the feeding value of low quality forages such as saltbush.

Alterations in epigenetics could underlie the physiological changes seen in the offspring born to ewes fed high amounts of salt or saltbush during pregnancy. A mechanism of fetal programming is the methylation of DNA which takes place *in-utero* and early in postnatal development. During this time, it can be influenced by environmental factors

such as maternal diet (Bogdarina et al., 2007). DNA methylation influences the expression of genes (Bogdarina et al., 2007). Maternal high-salt diet in rats has been shown to alter the expression of RAS genes in the offspring specifically, angiotensin II (da Silva et al., 2003, Balbi et al., 2004) and angiotensin receptor one (AT1) (Balbi et al., 2004). It is possible that the high-salt diet of the ewe altered the DNA methylation of the offspring which led to changes in gene expression, particularly of the RAS, which caused the alterations in plasma renin activity observed in the offspring.

Many of the results I found in sheep were similar to those reported in rat offspring born to dams consuming a high-salt diet. For example, similar to studies in rats (Balbi et al., 2004, Ingelfinger et al., 1998), I also found that when mothers are fed a high-salt diet it suppresses their offspring's RAS in early life. Some of the changes in physiology that this causes in sheep are also similar to rats; for example, the tendency of the high-salt offspring to retain salt (Dichtchekian et al., 1989, Rasch et al., 2004, Simchon et al., 1999). However, some of my results differ to what has been shown in rats (Contreras et al., 2000, Curtis et al., 2004), such as the reduced feed intake and reduced ratio of water to food intake of the high-salt offspring. The faster salt excretion of the saltbush offspring has not been shown in offspring from rats fed a high-salt diet. However, rather than being a species difference, this is likely to be due to the differences between consuming salt and saltbush since the high-salt offspring in my experiment did not show this adaptation either. The amount of salt consumed by the mother is probably the main reason that results from the high-salt offspring in my experiment were different from those in rat offspring from dams fed a high-salt diet. In most studies with rats, the dams are fed a diet containing 3% NaCl, whereas the sheep in my experiment were fed nearly five times that much salt (14% NaCl) to mimic the salt content of the diet of ewes grazing a saltbush-based pasture. The higher consumption of salt by the ewes in

my experiment may have caused more profound changes in RAS activity than in the rat offspring. As a consequence of this, physiological changes such as reduced food and water intake in offspring were shown in my study, and not in the rat studies (Contreras et al., 2000, Curtis et al., 2004).

The offspring's salt balance mechanisms, but not their preference for salt in their food, were altered from feeding their mothers a high-salt diet during mid-late gestation and early lactation. Other authors have suggested this "programming window" of mid-late gestation and early lactation is the most important period of neonatal development for altering salt balance mechanisms (Hilgers et al., 1997, Konje et al., 1996, Manning and Vehaskari, 2005). In these studies, RAS inhibitors were used on fetal rats at different stages of development, but my study is the first to show changes in salt balance mechanisms in offspring from mothers fed a high-salt diet only during the programming window of mid-late gestation and early lactation. Most other studies have involved exposing the mother to a high-salt diet either during the entire pregnancy (Digby et al., 2008, Mohamed and Phillips, 2003) or, more commonly in rats, from conception to weaning (Contreras and Ryan, 1990, Curtis et al., 2004). However, the programming window for altering the salt preference of the offspring may be different to that of salt handling mechanisms. I did not show any difference in salt preference in my offspring, but a difference in salt preference was shown in rat offspring whose mothers consumed the high-salt diet until their offspring were weaned (Contreras and Kosten, 1983, Contreras and Ryan, 1990, Curtis et al., 2004). Presumably the rat offspring would have tasted some of their mother's diet prior to weaning so they may have developed a feeding behaviour that sought salt. Short exposure to the taste of salt in the early postnatal period in rats has been shown to increase their preference for salt in later life

(Smriga et al., 2002). Results from these studies suggest that altering the salt preference of the offspring depends on offspring ingesting the salt themselves during the postnatal period, rather than just their mothers consuming the high-salt diet, as in my experiment. Overall, my experiments show that mid-late pregnancy and early lactation is the programming window for altering salt balance mechanisms in the offspring, but the programming window for altering salt preference appears to be later in postnatal development, possibly from 4 weeks after birth until weaning for sheep.

One of the more intriguing findings of my study was the lower food intake of the high-salt and saltbush offspring when they were consuming a pelleted diet, regardless of salt content. This effect has not been found in rat offspring whose dams consumed a high-salt diet during pregnancy (Contreras et al., 2000, Curtis et al., 2004) and conflicts with results from transgenic rats which, like the high-salt and saltbush offspring in my study, show chronic suppression of their RAS (Jayasooriya et al., 2008). My results may differ due to species differences or, more likely, the extent to which the RAS is suppressed. The pregnant rat dams were only fed 3% salt in their diet compared to the 14% salt in the diet of the pregnant ewes in my study so I would expect the RAS of the offspring in my work to be suppressed more than the rat offspring. At the other extreme, transgenic rats can show up to 90% reduction in circulating RAS (Jayasooriya et al., 2008), which is well above the 20% reduction I found in the offspring in this study. Interestingly, the lower feed intake I found in the high-salt and saltbush offspring was only apparent when they were fed a pelleted diet. Although feed intake was not measured in the field while grazing pasture, all offspring showed similar weight gains, suggesting that they were consuming similar amounts of feed. While the high-salt and saltbush offspring may gain as much weight as their respective controls while grazing, they may not gain

as much weight in a feedlot situation where they may be given a concentrate or pelleted ration.

Another interesting result was the increased wool production in the high-salt and saltbush offspring. This increased fleece weight is most likely due to an increase in follicle density since the other components of fleece weight, fibre diameter and fibre length, were similar between groups. I expected fleece weight to increase because the suppressed RAS of the fetus may increase blood flow and nutrient delivery to the skin, enabling more follicles to develop. However, regression analysis showed that the increased fleece weight in the high-salt and saltbush offspring was not associated with changes in RAS, but was more closely correlated to the plasma sodium of the ewe at 130 days gestation. There is no evidence linking plasma mineral composition of the ewe to the follicle development of the fetus. However, it is possible that there is an indirect effect involving hormones such as epidermal growth factor. Epidermal growth factor (EGF) decreases sodium re-absorption in the distal nephron (Shen and Cotton, 2003, Vehaskari et al., 1989). Therefore, if animals are consuming large amounts of salt I would expect epidermal growth factor to also be high. Epidermal growth factor is also involved in the development of follicles and cell proliferation (Yuspa et al., 1993). So it is possible that the high-salt diet of the mother increased her EGF and that of her fetus, which increased the density of wool follicles in the offspring.

In conclusion, my experiments have important implications for farmers with salt-affected land. Planting saltbush can make use of otherwise unproductive land and can also help to slow the spread of salinity (Barrett-Lennard et al., 2005). Grazing pregnant ewes on saltbush during autumn can help to lower supplementary feeding costs to the farmer. My results indicate that the offspring from these ewes would also be better

adapted to cope with the high-salt content of saltbush and would gain more weight than normal animals when they graze saltbush as adults. These offspring would also produce more wool than normal sheep. The results from this research could increase the profit that can be made from the increasing amount of saline land on farms and improve the sustainability of agriculture in southern Australia.

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