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**An investigation of the diuretic  
activity of plantain following  
its ingestion by sheep**

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A Dissertation  
submitted in partial fulfilment  
of the requirements for the Degree of  
Bachelor of Science with Honours

at  
Lincoln University  
by  
Catherine Ann O'Connell

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Lincoln University  
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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science with Honours.

An investigation of the diuretic activity of plantain following its ingestion by sheep

by

Catherine Ann O'Connell

Plantain was investigated for its potential to act as a diuretic in sheep. Two groups of sheep ( $n = 8$ ) were provided for 7 days with either ryegrass or plantain diets that had identical water content. Dry matter in feed, fresh faecal weight, and faecal water content were measured daily, as well as volume, pH, specific gravity and osmolality of urine. Urine volumes on Day 1 of the trial were significantly different to those of Days 2-6 of the trial ( $P < 0.05$ ) so the results were analysed separately. Plantain sheep had a higher volume of urine (by 1.7 L,  $P < 0.05$ ) on Day 1 than Ryegrass sheep and continued to produce about 0.5 L more urine each day than Ryegrass sheep for the remainder of the study period. The pH of the urine, its osmolality, faecal water content and faecal weight were all significantly lower ( $P < 0.05$ ) in Plantain sheep than Ryegrass sheep over those days. These results show for the first time that plantain causes a diuresis when it is ingested by sheep, possibly by reducing the reabsorption of water in the kidneys.

**Keywords:** Plantain, *Plantago lanceolata*, diuretic, urine, fluid balance

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# Chapter 1

## Introduction

### 1.1 Plantain – A Potential Diuretic

New Zealand is a country where the economy is largely based on agriculture. However, this agriculture has had a huge effect on the environment of the country (Quinn *et al.*, 1997). In recent times it has become evident that many farming practices may be causing a decrease in water quality, due to factors such as the loss of nitrogen from pastures into fresh water. One of the potential avenues of this loss is through the oversaturation of nitrogen in urine patches. Nitrogen within the urine patch of a dairy cow can reach up to  $1000 \text{ kg/N/ha}^{-1}$ , which is too much to be absorbed by the soil and the pasture (Di *et al.*, 2007). Excess nitrogen leaches out of the soil where it may collect in nearby water systems. This contributes to the growth of algae and a loss of water quality. Research into potential novel methods of minimising the effects of stock on the environment is taking place in an attempt to reduce the impact of farming on the environment. One measure is to investigate the potential of herbaceous plants to act as diuretics and to determine if they can dilute urine.

*Plantago* is a genus of perennial herbaceous plants belonging to the family Plantaginaceae. They are commonly referred to as plantains and there are over 200 different species. Plantains grow low to the ground with only a few species growing into small shrubs. Leaves are long and oval in shape with veins arranged vertically from the base of the leaf to the top (Felter *et al.*, 1898). Plantains have white, many flowered, seed heads which are compact and cylindrical on the end of a stem. Plantain species have been recognised to have some medicinal properties and have been used as natural medicine for centuries (Chiang *et al.*, 2002). Plantains originated in Europe and have eventually spread around the world.

A review by Samuelsen (2000) investigated the various medicinal properties of plantain, specifically *Plantago major*. It was found that plantain acted as preventative treatment stomach ulcers, bacterial infections, and fungal infections. Chiang *et al.* (2002) found that extracts of *Plantago major* could be used to fight viral infections. *Plantago major* could also be used in the treatment of pain and inflammation, and fresh leaves had antioxidant properties (Samuelsen, 2000). *Plantago major* was also found act as a diuretic in laboratory rats by increasing urine output by 108% (Caceres, 1987). However, this result could not be replicated in human trials. Gálvez *et al.* (2003) found that plants from the *Plantago* genus could be used effectively to break down cancer cells. Plantain species were able to be used to reduce the spread of cancer through cells and could selectively disable specified cells but leave healthy cells. Active plant compounds are thought to be responsible for the medicinal properties of

plantains (Gálvez *et al.*, 2003). Experiments into the effects of plantain were influenced by historical and traditional use of plantain species around the world for the treatment of disease. Many of these properties are shared between different species within the Plantaginaceae family.

It is thought that the perennial herb used in agriculture, *Plantago lanceolata*, also exhibits many of these medicinal properties (Stewart, 1996). One of these properties was the ability of plantain to act as a diuretic. Like *Plantago major*, *Plantago lanceolata* (narrow-leaved plantain, also sometimes called ribwort) has been thought to have diuretic properties (Rumball *et al.*, 1997; Tamura *et al.*, 2002). However, only two reports indicate the potential of narrow-leaved plantain to act as a diuretic (Deaker *et al.*, 1994; Wilman *et al.*, 1994).

If plantain was a diuretic it may be able to reduce the concentration of nitrogen in urine. As animals produce more urine then it becomes more dilute (Maloiy *et al.*, 1969). Therefore it has the potential to be used to lower urine nitrogen levels to prevent leaching. This makes it of interest to many plant breeding and seed selling organisations. Farmers may wish to use a plant that will decrease their nitrogen leaching problems as tighter regulations are drafted in response to public outrage at decreasing water quality in rivers and streams.

This project will be an investigation into the diuretic potential of narrow-leaved plantain.

## Chapter 2

### Literature Review

#### 2.1 Plantain (*Plantago lanceolata*)

Plantain (*Plantago lanceolata*), known as narrow-leaf plantain or ribwort, is a common agricultural herb most often used in conjunction with other pasture species (Stewart, 1996). However, until recently plantain was thought to be of little use and was regarded as a pasture weed. Levy (1970) stated that “their presence denotes incompetence and a disregard of the vital need of the world for food” as the presence of plantain in pastures was thought to reduce productivity and efficiency. However, this has changed with the recognition of some health benefits to pastures containing herb species (Stewart, 1996; Rumball *et al.*, 1997; Jurišić *et al.*, 2004). Plantain is now incorporated into pastures and crops as a valuable herb with some proponents claiming it to have beneficial health properties for stock.

Plantain used in agricultural settings has been bred to stand taller in pasture and have increased productivity when compared to uncultivated species from the *Plantago* genus (Stewart, 1996). Common weed species of plantain grow close and flat to the ground, have small to medium sized leaves, medium to high numbers of tillers, very low winter growth, and low summer growth (Stewart, 1996). In contrast, the cultivated versions of plantain have semi to very erect and medium to large sized leaves, high tiller number, and high summer growth. This makes them an ideal plant to use as a forage crop for grazing animals.

In New Zealand there are two main cultivars of plantain used in pastures, Ceres Tonic and Grasslands Lancelot. Grasslands Lancelot is the result of four generations of selective breeding where the best plants were taken from 109 seed populations based in the North Island (Stewart, 1996; Rumball *et al.*, 1997). Grasslands Lancelot has semi-erect leaves (Charlton *et al.*, 2006) but only if grazing is managed to reduce feeding too close to the ground. Leaves are medium to large in size, with a high tiller count. Grasslands Lancelot is winter dormant (Charlton *et al.*, 2006) but has high growth in summer (Stewart, 1996). Ceres Tonic plantain was selected from seeds originating in Portugal (Stewart, 1996) and was chosen for its characteristic of maintaining vertical leaves even under hard grazing, which is a problem in Grasslands Lancelot. Ceres Tonic is also displays more winter growth than Grasslands Lancelot, though has a lower number of tillers. Ceres Tonic, when sown with ryegrass, increases dry matter yields (Moorhead *et al.*, 2009) which means it has the potential to maintain and improve production. This literature review will focus on the properties of Ceres Tonic.

## 2.1.1 Properties

### Mineral content

Plantain has increased levels of many minerals when compared to perennial ryegrass plants (Stewart, 1996). However, estimates of the mineral content of the plantain differs between conditions and experiments. In macrominerals, Ca and Mg levels appear to be consistently higher in plantain when compared to ryegrass (Table 2.1) (Wilman *et al.*, 1993; Wilman *et al.*, 1994; Pirhofer-Walzl *et al.*, 2011). The microminerals Co, Cu, Se, Zn, and B are also higher in plantain than in ryegrass (Table 2.2) (Hoskin *et al.*, 2006; Pirhofer-Walzl *et al.*, 2011).

**Table 2.1:** The macromineral content of plant material from ryegrass and plantain (data from 3 studies).

Plant	Macrominerals (g/kg DM)						Adapted from
	N	P	K	Ca	Mg	Na	
Ryegrass	25.10	3.80	29.90	5.80	2.10	2.20	Wilman <i>et al.</i> (1994)
Plantain	25.00	3.30	29.10	14.00	2.40	1.70	
Ryegrass	39.10	5.42	42.40	6.40	2.56	3.30	Wilman <i>et al.</i> (1993)
Plantain	36.50	4.54	43.80	20.10	2.39	7.20	
Ryegrass	20.00	3.95	33.95	4.65	1.75	1.20	Pirhofer-Walzl <i>et al.</i> (2011)
Plantain	15.95	3.8	29.95	15.65	2.75	0.55	

**Table 2.2:** The micromineral content of plant material from ryegrass and plantain (data from 2 studies).

Plant	Microminerals (mg/kg DM)									Adapted from
	Co	Cu	Fe	Mo	Se	Mn	Zn	Cr	B	
Ryegrass	0.35	9.3	1271	0.31	0.04	-	-	-	-	Hoskin <i>et al.</i> (2006)
Plantain	0.36	14.0	795	0.34	0.06	-	-	-	-	
Ryegrass	-	6.1	66.6	1.25	-	73.2	23.2	0.3	3.9	Pirhofer-Walzl <i>et al.</i> (2011)
Plantain	-	8.2	63.5	0.40	-	33.3	30.8	0.2	20.3	

Ryegrass has higher concentrations of many macrominerals than plantain. However, Wilman *et al.* (1994) found that the apparent availability for many of these constituents was higher in plantain. For instance, K, Ca, Mg, and Na were all more available in plantain crops than in ryegrass crops meaning that animals had a greater access to these nutrients by consuming plantain.

### Aucubin/catapol

Aucubin is an iridoid glucoside which is contained within the leaves plantain (Jurišis *et al.*, 2003). Catapol is an altered form of aucubin produced in lower concentrations within the plant (Bowers *et al.*, 1992; Quintero *et al.*, 2012). It is thought that aucubin and catapol are deposited within leaves to

deter herbivore predation (Fajer *et al.*, 1992; Quintero *et al.*, 2012). Levels of aucubin within plants varies depending on the genetics of the plant and the conditions that it is grown in (Szucs *et al.*, 2013).

Aucubin has been used in traditional medicine for many functions. These include antimicrobial (Davini *et al.*, 1986), liver and pancreas protective, and anti-oxidative properties (Jin *et al.*, 2008). It also has the potential to be used to treat patients with diabetes due to a combination of its ability to increase the activity of enzymes with anti-oxidant properties, regenerate liver tissues, and to reduce hyperglycaemia in rats (Jin *et al.*, 2008). Aucubin can inhibit cytokine production and therefore has some anti-inflammatory properties which make it useful to apply for inflammation caused by allergic reactions (Jeong *et al.*, 2002). Aucubin has also been shown to stimulate the release of uric acid from the tissues and the kidneys (Kato, 1946). Aucubin is thought to induce, or contribute to, a diuretic effect.

Aucubin has been detected in plantain at differing levels. The variation can range between 0.003 and 8.86% of DM (Rumball *et al.*, 1997; Jurišis *et al.*, 2003; Quintero *et al.*, 2012). Levels of aucubin are higher if the plant is under nutrient stress and vary depending on season, with the highest levels present in mature plants (Quintero *et al.*, 2012). An experiment based in Japan by Tamura *et al.* (2002) found aucubin in Ceres Tonic plantain was present in levels of 1.0% DM in Spring which increased to 2.7% DM in Autumn.

### **Acteoside (verbascoside)**

Acteoside is a phenylpropanoid glycoside that can be present in high levels within the leaves of plantain (Fajer *et al.*, 1992). Like aucubin, acteoside is thought to have developed in plants as a defence mechanism against predation from insects (Fajer *et al.*, 1992). Also like aucubin, acteoside is known for its antimicrobial (Andary *et al.*, 1982; Chen *et al.*, 2012), and antioxidant effects (Zhou *et al.*, 1991; Chen *et al.*, 2012). Acteoside is also known for its hypertension fighting properties (Andary *et al.*, 1982; Chen *et al.*, 2012). Following oral administration of a dose of acteoside, hypertensive rats had a decline in both systolic and diastolic blood pressure. Acteoside inhibited angiotensin converting enzyme, an important factor in the regulation of blood pressure (Kang *et al.*, 2003; Chen *et al.*, 2012).

Acteoside can be found in differing concentrations depending on the time of year that plantain is harvested. Spring harvested plantain (Ceres Tonic) contained concentrations of acteoside at 1.5% of dry matter and in autumn this increased to 4.1% (Tamura *et al.*, 2002).

### **Sorbitol (D-glucitol) and Mannitol**

Sorbitol is a sugar alcohol found within plantain that acts as an osmotic regulator (Stewart, 1996). Sorbitol levels are usually around 2% in plantain, however this can increase when the plant is under drought stress (Stewart, 1996). Levels of up to 6.2% of dry matter were found by Briens *et al.* (1983)

when plantain was grown in dry conditions. Sorbitol is thought to contribute to the drought tolerance of the plant.

Sorbitol has been shown to act as a diuretic in dogs (Todd *et al.*, 1939) and for humans when used as an irrigation fluid for the kidneys and bladder (Akan *et al.*, 1997). It is not always well absorbed from the diet by the body (ADA, 2004) and attracts water. Therefore, it is excreted with faeces and can act as a laxative (Stacewicz-Sapuntzakis, 2001; ADA, 2004). Sorbitol is stored in the renal medulla and metabolised sorbitol is used by the body as a regulator of cell volume (Burg *et al.*, 1988; Yancey *et al.*, 1989).

Sorbitol is used to regulate the concentration of solutes within cells. Instead of cells absorbing large amounts of sodium and potassium they absorb sorbitol to maintain osmotic balance (Burg *et al.*, 1988). Similarly, when concentrations of within cells become too high the production of sorbitol can be stopped. Sorbitol is produced by aldose reductase and removed by sorbitol dehydrogenase (Burg *et al.*, 1988; Sands *et al.*, 1990). Sands *et al.* (1990) found that when water surplus occurred more sorbitol is released into the terminal inner medullary collecting ducts and when in deficit sorbitol release is limited. Some sorbitol is passed in the urine, approximately 7% of the dose, and has the potential to act as an osmotic diuretic (Todd *et al.*, 1939; Akan *et al.*, 1997).

Mannitol is an isomer of sorbitol which is found at much lower levels than sorbitol in plantain (Stewart, 1996). It is not as readily absorbed by the body as sorbitol (Todd *et al.*, 1939). It is known as a strong osmotic diuretic (Atherton *et al.*, 1968; Akan *et al.*, 1997; Mattila *et al.*, 1996). Mattila *et al.* (1996) found that mannitol acted as a strong diuretic in rats, almost doubling urine output. Mathisen *et al.* (1981) found that mannitol can cause between 30 and 70% increase in sodium excretion in urine. They found that mannitol reduces the ability of the proximal tubule to reabsorb both sodium and water, thus causing a diuretic effect. Seely, *et al.* (1969) found that the largest effect of mannitol was the inhibition of sodium and water reabsorption in the loop of Henle. They found that mannitol also inhibited reabsorption in the proximal tubule, but its effect in the loop of Henle created a greater diuretic effect. Mannitol is used as a diuretic in human medicine.

### **Plantain as a Diuretic**

Plantain and members of the same genus have been known as a diuretics in traditional medicines (Rumball *et al.*, 1997). Closely related species have also been known for their diuretic properties but under experimental conditions have varied results in regards to diuretic activities. Caceres (1987) found that *Plantago major* could act as a diuretic in lab rats and increase urine output by 108%. However, a double blind experiment conducted on humans in Vietnam found that there was no diuretic effect of the species *Plantago major* over a 12-24 hr period (Doan *et al.*, 1992). Plantain is thought to have similar effects to *Plantago major* due to the presence of similar plant compounds.

Like *Plantago major*, there has been some evidence of a diuretic property in plantain (*Plantago lanceolata*). An experiment by Deaker *et al.* (1994) conducted a trial to determine the effects of plantain diets on the growth of lambs. Lambs fed plantain had heavier kidneys (145 g) compared with lambs fed ryegrass (109 g) when weights were adjusted to a lamb weight of 37.4 kg. Lambs fed plantain were not the heaviest animals, nor were they the largest. Therefore, changes in kidney weight cannot be explained by differences in growth rate between trial groups (Deaker *et al.*, 1994). Kidneys were larger in plantain fed animals, but creatinine clearance and serum urea levels were within normal ranges. This indicates that the kidneys were functioning normally. Deaker *et al.* (1994) concluded that plantain diets enhanced kidney function.

Another study by Wilman *et al.* (1994) found that animals fed a diet of dried plantain produced more urine than animals fed ryegrass. Animals fed on a plantain diet produced 1.32 kg of urine per day compared to animal fed ryegrass, who produced between 0.73 and 0.86 kg. This provides further evidence of a diuretic effect resulting from the consumption of plantain.

These two studies indicate that there may be a diuretic property in plantain that causes increased urine output and which requires the kidneys of sheep to work harder. However, there is very little other information on the potential for plantain to act as a diuretic, or the exact mechanism of diuresis.

## **2.2 Water Balance and the Effects of Diuretics**

### **2.2.1 Fluid Balance**

Mammals are made up of approximately 60% water (Akers *et al.*, 2008). This water is contained within two 'compartments' within the body known as the intracellular (ICF) and extracellular (ECF) fluids (Verbalis, 2003). The ICF accounts for between 55 and 65% of total body water, and the ECF is between 35 and 45% of total body water. The ECF is further broken up into two compartments, the interstitial fluid (ISF) and the intravascular fluid (IVF) – also called plasma. The IVF is the water contained within the blood portion of the body (plasma) in contrast to the ISF which is the fluid that bathes the cells. Water flows between these compartments and carries ions and nutrients with it (Verbalis, 2003). ICF contains large amounts of potassium ions and ECF contains large amounts of sodium ions. This gradient is maintained by a sodium/potassium ion pump.

The water within these compartments must be maintained in order to preserve normal body functions. This is because many of the chemical reactions that occur within the body require constituents to be dissolved in the fluid of the ICF and ECF. The balance of water is critical to maintaining the levels of solutes present and allowing reactions such as energy synthesis and nerve impulses to occur. The monitoring of the excretion and intake of water, and is known as water balance. Water balance is controlled through a feedback mechanism in the kidneys and the hypothalamus of the brain.

## **Body Water Level Maintenance**

The body produces urine and faeces to remove waste produced from cells and left over waste from food digestion. Evaporative losses play a huge part in the maintenance of body temperature. Animals eat and drink to gain nutrients and fluids. However, the gains and losses of body water must be balanced. Water loss is required to rid the body of unwanted material, however losses that are too great for the body to handle will result in illness or death for the individual. Therefore, there must be a regulation system to prevent animals becoming dehydrated.

Body water levels are maintained by a feedback mechanisms located in the hypothalamus of the brain and the juxtaglomerular apparatus (JGA) of the kidney (Campbell *et al.*, 2005). These two areas monitor blood solute concentration to ensure the osmotic levels within the body remain constant and to ensure blood pressure stays at the correct levels (Verbalis, 2003). The production of anti-diuretic hormone (ADH), also known as vasopressin, from the pituitary gland in the hypothalamus of the brain is increased at times when the blood osmolarity is above the set point of the individual (Campbell *et al.*, 2005). This stimulates the distal tubules and collecting ducts in the kidney to become more permeable to water and reabsorb more from the kidney filtrate. Similarly, stimulation of the JGA also causes an increase in the amount of water reabsorbed from the kidney filtrate. When blood pressure drops below the set point it activates the renin-angiotensin-aldosterone system (RAAS) which causes the proximal tubules within the kidney to reabsorb sodium and water.

If there is too much water in the animal's system, negative feedback is relayed to the brain. The osmoreceptors within the hypothalamus cease the production of ADH and thus the distal tubules and collecting ducts become less permeable to water. This means more is excreted by the animal as urine. The heart also plays a role in osmoregulation (Campbell *et al.*, 2005). If blood pressure or volume is too high the atria of the heart produces atrial natriuretic factor (ANF) which prevents the release of renin from the JGA, reduces the release of aldosterone, and prevents the reabsorption of sodium from the collecting ducts, thus also preventing water reabsorption.

## **Water Intake**

Water can be gained by animals by three routes. Water can be gained by drinking free water, eating food, and water gained through metabolism, (Sileshi *et al.*, 2003).

Animals consume water as free water. This is consumed as the animal requires it. Different animals require varying amounts of water depending on their metabolism and the environment they are in. Free water intake is controlled by the thirst mechanism (Verbalis, 2003). When an animal does not gain enough through other sources the release of ADH makes them feel thirsty. Sheep are estimated to drink between 3.79 and 15.14 L/water/day (Sileshi *et al.*, 2003). This variation accounts for the differing water requirements according to their conditions.

Different feeds have different compositions that yield varying levels of water depending on that composition. Therefore, different feeds contribute different amounts of water to the total water balance of the animal. Ryegrass and plantain can have varying levels of dry matter depending on the time of year, stage of maturity in the plant, and the conditions the plant is grown in (Schils *et al.*, 1999). The dry matter represents the solid plant material that remains once all free water has been removed. Dry matter for ryegrass/white clover pastures can vary from between 12 and 30% (Dairy NZ, 2012). Therefore, 70 to 88% of an animal's diet may be made up of the water contained within their feed. Sheep consume very low amounts of free water if the dry matter content of their feed is less than 30% (Sirohi *et al.*, 1997; Sileshi *et al.*, 2003). This indicates that sheep can receive enough water in their diet without needing to drink free water.

As well as water contained within feed, animals can gain water from the metabolism of the dry constituents of their diet through metabolism. Every 100 g of fat, protein, and carbohydrate yields 107, 40, and 57 mL of water respectively (Edney, 1977). This water is gained due to the oxidation of the compounds during digestion. Therefore, animals with little or no access to free water are able to maintain some of their water balance through the metabolism of, and the water contained within, their feed. Sirohi *et al.* (1997) found that metabolisable water can contribute between 7 and 15% of daily water intake depending on diet. When the animal is experiencing water deficits, and there is no free water available, metabolisable water can become the sole source of water for the body (Sileshi *et al.*, 2003). This is because fat and protein stores can be mobilised to gain water and energy.

### **Water Losses**

Water is lost from the body through three main routes. These are the losses within urine, faeces, and evaporative losses, such as insensible evaporation and sweat (Sileshi *et al.*, 2003).

Sheep urine losses can contribute up to 30% of total water losses (Sileshi, 2003). Sheep have been shown to lose between 0.41 and 3.14 L of water per day in urine depending on feed intake and environmental factors (Degen *et al.*, 1981). As feed intake and the temperature increases water lost as urine also increases. Sirohi *et al.* (1997) had similar results with sheep losing between 0.2 and 2.1 L of water per day depending on feed type. Sheep in Nigeria have been shown to have average daily urine outputs of 0.5 L (Aganga, 1992). The variation of water losses indicates the adaptation of the animal to environmental pressures such as temperature and varying levels of water present in their diet. As dietary water increases the more that is surplus to the animal and is excreted as urine.

Faecal water losses are generally less than urine losses. Up to 18% of water losses occur through faecal water content (Sileshi *et al.*, 2003). Degen *et al.* (1981) found Suffolk cross sheep lost between 0.11 and 0.57 L of water in faeces depending on their feed intake. Greater losses were found by Sirohi *et al.* (1997). They found that faecal water losses in crossbred Ethiopian sheep amounted to between 0.8

and 1.1 L per day. This may be attributed to the different conditions and feed that the animals were exposed to. Aganga (1992) found that Yankasa sheep produced a faecal water volume of 16.08 mL/kg<sup>0.73</sup>/day. In all experiments, faecal losses represented the smallest water volume loss.

Evaporative losses make up the final route of water loss from the body of the animal. This pathway accounts for around 52% of water losses (Sileshi, 2003). Evaporative losses include losses through respiration, evaporation of moisture through the skin, and perspiration. Aganga (1992) found that evaporative water losses accounted for 162 mL/kg<sup>0.73</sup>/day for sheep in Nigeria (temperature between 21-39°C) and made up 72% of all water losses. Evaporative losses are linked to the thermoregulation of the animal and thus as temperature increases so do these losses. At an environmental temperature of 0°C, evaporative losses can be as low as 0.49 L/day (Degen *et al.*, 1981). When the environmental temperature is increased to 30°C losses of up to 3.01 L/day can occur as long as there is sufficient water consumed to support excretion.

### **2.2.2 Diuretics**

Diuretics are substances that interfere with the body's normal maintenance of water balance by altering the environment of the kidneys or interfering with signalling molecules. This is achieved by altering the ability of the kidney to reabsorb certain ions and molecules which causes them to be excreted as urine and leads to increased urine volume (Rose, 1991). This is often used in medicine as a way to counteract diseases in which water is retained by the body.

#### **Water Diuresis**

Water diuresis is caused by the excessive consumption of water, and therefore the inhibition of anti-diuretic hormone (ADH), or its receptors (Atherton *et al.*, 1968). This results in the production of an increase volume of dilute urine.

#### **Carbonic anhydrase Inhibitors (CBI)**

The carbonic anhydrase enzyme is found in the proximal convoluted tubule of the nephron. The greatest percentage (up to 55%) of water and filtered sodium is reabsorbed within this section of the nephron (Rose, 1991). The carbonic anhydrase enzyme increases the amount of sodium that can be reabsorbed by exchanging sodium ions for hydrogen ions. The exchanger plays a direct role in the reabsorption of sodium bicarbonate. However, CBIs prevent the activity of this enzyme and result in the reduced reabsorption of sodium and an increase in sodium bicarbonate in lumen (Rose, 1991). This in turn prevents water reabsorption and thus leads to a diuretic effect. Urine from diuresis caused by CBIs has high levels of bicarbonate and can be detected by a urine pH increase. Urine pH levels rise above 7 when humans have used a CBI. The most commonly used CBI in humans is acetazolamide (Rose, 1991).

CBI's are not strong diuretics because they effect the proximal tubule (Rose, 1991). The proximal tubule is responsible for the majority of water reabsorption, some of the extra water left in the lumen can be removed by other areas of the nephron.

### **Loop Diuretics**

Loop diuretics act by preventing sodium from being reabsorbed from within the ascending section of the loop of Henle. This is caused by an inhibition of the  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  cotransporter leading to less calcium, potassium, and sodium reabsorption from filtrate as the diuretics bind to this transporter (Wittner *et al.*, 1991). This pulls water into the tubule causing diuresis.

Loop diuretics can remove calcium from the body by decreasing voltage in the lumen. (Frick *et al.*, 2003). This also prevents water from being reabsorbed and thus increases water excretion (Rose, 1991). Decreasing the voltage within the lumen may also result in increased hydrogen excretion in the later sections of the nephron (Kavocikova *et al.*, 2006). Therefore, loop diuretics can increase urine pH. Loop diuretics can sometimes be detected in urine due to the increased levels of calcium, potassium, and sodium present from this disruption of reabsorption.

### **Thiazide Diuretics**

Thiazide diuretics act by preventing sodium reabsorption from the distal convoluted tubule in the nephron (Rose, 1991). The sodium-chloride symporters found in the distal tubule are inhibited by the presence of thiazides such as hydrochlorothiazide. This causes water to be retained within the tubule and therefore increases the production of urine. Potassium excretion into urine is increased as an effect of the use of these diuretics (Rose, 1991). These diuretics also alter the excretion of calcium in urine by promoting increased calcium reabsorption. Therefore, they are referred to as being calcium sparing diuretics.

### **Potassium-Sparing diuretics**

Potassium-Sparing diuretics act in the collection duct of the nephron and reduce the amount of potassium that is excreted (Rose, 1991). They also act within the distal tubule (Horisberger *et al.*, 1987). Medicines interact with the sodium/potassium exchangers. Potassium-sparing diuretics can be broken into two groups, those which act as an aldosterone antagonist to prevent sodium reabsorption and those which block the sodium channels (Horisberger *et al.*, 1987). Much like thiazides, potassium-sparing diuretics are also calcium sparing.

### **Osmotic Diuretics**

Osmotic diuretics are substances contained within the filtrate that attract water to them by increasing the osmotic pressure of filtrate within the nephron tubules (Atherton *et al.*, 1968). Their presence prevents water reabsorption by reducing the osmotic driving force between the lumen and

surrounding cells (Mathisen *et al.*, 1981). Tubular secretion of chemicals into the proximal tubule is a common way to eliminate drugs from the body (Rose, 1991). These drugs can act as osmotic diuretics and their effect on the filtrate environment can be large. Glucose (in diabetics) and mannitol are both substances that can act as osmotic diuretics (Atherton *et al.*, 1968; Mathisen *et al.*, 1981).

## 2.3 Conclusions

- Plantain (*Plantago lanceolata*) has many properties that make it of interest when investigating the potential for a diuretic effect. Plantain contains higher levels of Ca, Mg, Co, Cu, Se, Zn, and B than ryegrass which may have an effect on how it interacts within the body. Many of the minerals within plantain are more available for digestion than those in ryegrass. Plantain also contains many compounds that, on their own, can have a diuretic effect.
- Water levels within the body are monitored by feedback mechanisms within the hypothalamus and the JGA which prevent the concentration of solutes in the blood and blood pressure becoming too high or too low.
- Water can be gained by animals in three ways: drinking water, water contained in feed, and metabolised water. Sheep can gain most of the water to meet their daily requirements through their feed.
- Water is lost from the animal in three ways: urine, faeces, and evaporative losses. The largest loss is through evaporative losses, followed by urine losses, and then faecal losses.
- Diuretics alter the environment of the kidney leading to altered excretion of water and solutes. There are many different sections of the kidney that diuretics can act on. Each type of diuretic works in a different way which alters the characteristics of the urine.

## 2.4 Objectives

The objective of this study is to determine whether plantain can act as a diuretic when fed to sheep.

## Chapter 3

### Methods

#### 3.1 Trial Design

##### 3.1.1 Sheep

Animals used in this study were 6-month-old Romney X Southdown breed ewes owned by a commercial seed company (PGG Wrightson Seeds, Hornby 8042, New Zealand). They were grazed on predominantly ryegrass/white clover pasture near Lincoln, Canterbury, New Zealand (Kimihi Research Centre) then transported on 2 June 2015 to the nearby (5.2 km) Johnstone Memorial Laboratory of Lincoln University (153 Ellesmere Junction Rd, Lincoln, -43.644175 E, 172.451685 S) where they were held indoors for 7 days in individual metabolism crates without access to drinking water. The sheep were allocated to two dietary treatment groups (Plantain, Ryegrass,  $n = 8$ ) that were balanced for live weight (average 38.4 kg for both groups) which was measured the day before the experiment and urine production ( $4.3 \pm 0.10$  L/day for Ryegrass and  $4.2 \pm 0.09$  L/day for Plantain) which was based on average daily urine production from a previous study conducted by PGG Wrightson Seeds. They were held in two separate rooms at the Johnstone Memorial Laboratory, with 4 animals from each group per room. Either plantain (*Plantago lanceolata*, cultivar Ceres Tonic) or perennial ryegrass (*Lolium perenne*, cultivars Base and Expo) provided by PGG Wrightson Seeds from the Kimihi Research Centre was supplied so that each sheep received a daily feed water allocation of 4 litres. Each day's individual sheep feed allowance was computed from the dry matter content of the previous day's feed to maintain the constant daily water allocation. Any refused feed was collected the next day and weighed, then discarded. Refusals were subtracted off the daily feed allocation to give true feed input for that day. All procedures involving these animals were approved by the Lincoln University Animal Ethics Committee.

##### 3.1.2 Feed Harvesting

Plantain and ryegrass were grown as monocultures in separate paddocks at the Kimihi Research Centre. Each crop was harvested at about 0830 h every day using a motorised forage harvester (Wintersteiger Cibus F, Wintersteiger AG, 4910 Ried, Austria) that weighed out the required amount of feed and transferred it to a large, synthetic wool pack (wool fadge). The plantain crop was two years old and the perennial ryegrass crop was one year old. Plantain and ryegrass crops were harvested at 2000 kg/DM/ha. This was chosen to maximise the amount of leaf material and minimise the occurrence of stem or dead matter. The feed was immediately transported to the Johnstone Memorial Laboratory where it was weighed out into individual sheep aliquots and placed into each sheep's feed

bin within half an hour of being harvested. Two samples of each day's harvest were collected in perforated bags, weighed, and placed in a 90°C oven for 24 h or until dry. These were removed and weighed again. The dry matter percentage for each day's feed was determined by the equation: average dry weight/average fresh weight X 100.

### **3.1.3 Urine and Faeces**

Urine and faecal matter were collected separately into collection trays and buckets situated under the grating of each metabolism crate. To prevent cross-contamination net separators (shade cloth stretched over a metal frame and fixed underneath the metabolism crates) were used to trap faeces and allow urine to pass through the mesh.

Urine was acidified to prevent nitrogen losses by adding 250 mL of 5% sulphuric acid to the collection trays. Daily urine volume for each sheep was measured in a volumetric flask and an approximately 70 mL sample was placed into a polypropylene container and stored in a freezer. The collection trays were rinsed with tap water, returned to each metabolism crate and left until a non-acidified (fresh) sample (approximately 70 mL) of urine could be collected. Acid was then placed in the tray for urine collected during the remainder of the day and overnight. The pH of fresh urine was measured with a portable pH reader (Pocket ISFET pH meter S2K712, ISFETCOM Co Ltd, Hidaka, Saitama-Prefecture, Japan) and its specific gravity was determined with a portable refractometer (Uricon, Atago USA Inc, Kirkland, WA, USA). Measurements of pH and specific gravity were taken within an hour of the animal urinating. Fresh and acidified urine samples in their polypropylene containers were bagged separately in plastic bags and stored frozen for subsequent analyses. The frozen, fresh urine samples were later thawed for measurement of their osmolality (mmol/kg) with a vapour pressure osmometer (VAPRO 5520, Wescor Inc., Logan, Utah, USA) and multiplying this value by the daily urine volume provided a measure of the total osmols excreted per day.

The metabolism crates were cleaned daily to collect all faecal matter and each sheep's daily faecal production was weighed. A small sample was set aside to be freeze dried for subsequent analysis. The rest was divided between two perforated plastic bags which were weighed and placed in 90°C ovens for 48 h to dry. Drying time depended on the size of the sample, therefore if a sample did not dry in the allocated time it was left for a further 48 h. Once dry, the bags were re-weighed and the dry matter percentage calculated (i.e. dry weight/wet weight X 100). This gave two values for faecal dry matter percentage (DM %) for each sheep's daily output. The mean of these two DM% values was applied to the total faecal weight to determine faecal water content.

### 3.1.4 Blood

Venous blood samples were collected from all 16 sheep on two occasions (at approximately 1200 h on 2 and 10 June 2015). The blood samples (10 mL) were obtained by venepuncture from an external jugular vein whilst the sheep were manually restrained using evacuated plastic tubes containing potassium ethylene diamine tetra acetic acid (K2E, B D Vacutainer®, Becton Dickinson and Company, Franklin Lakes, NJ, USA) as anticoagulant and a 0.9 x 25 mm needle (PrecisionGlide™, Becton Dickinson and Company). Immediately on withdrawal of the sample, each tube was gently inverted a few times to ensure dispersal of the anticoagulant.

Packed cell volume of each blood sample was determined in duplicate using glass microhaematocrit tubes that were filled with blood and plugged with vinyl plastic putty (Leica Critoseal®, Wetzlar, Germany). The tubes were spun in a microhaematocrit centrifuge (Heraeus Sepatech Haemofuge, New York, USA) for 5 minutes. A micro-haematocrit reader (Hawksley Micro-Haematocrit Reader, London, England) was used to determine the packed cell volume of the sample in each tube.

The number of red blood cells in each blood sample was determined in duplicate using a haemocytometer (Levy Double Neubauer Chamber, Clay Adams, Parsippany, NJ, USA) viewed under a light microscope (40X stage magnification). A 1/1000 dilution of blood in isotonic (0.9 %) saline solution was prepared by serial dilution of 0.1 mL of blood in 0.9 mL of the saline solution and diluting 0.1 mL of this solution in another 0.9 mL of saline solution. After application of the diluted solution to both chambers of the haemocytometer the total number of red blood cells in 80 squares in the central region of the microscope field (5 blocks of 16 squares – shown in blue in Fig. 3.1) was counted. The number of red blood cells per  $\mu\text{L}$  of blood was calculated (sum of cells counted in each square  $\times 50 \times 1000$ ).

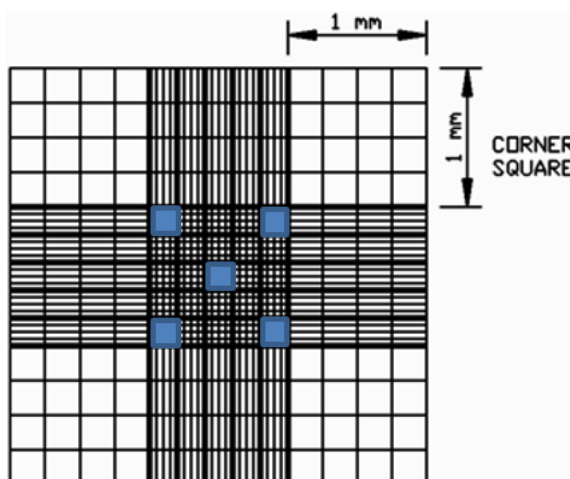


Figure 3.1: The squares shown in blue on the haemocytometer were counted. This was repeated in on an identical grid located on other side of the same haemocytometer to give a duplicate for comparison. (Image source: Nexcelom Bioscience, 2015)

### **3.1.5 Water balance**

Daily water balance was determined for each animal by subtracting the sum of urine volume plus faecal water volume from the total water content of the daily water in feed.

### **3.1.6 Statistical Analysis**

Data were averaged for each day. Results for Days 2-6 were computed as an average of all the data for each group (Plantain and Ryegrass) from those days, as determined by using a pivot table in Microsoft Excel 2013. The data were analysed using Student's *t*-test. This was completed using a One-Way ANOVA on the computing software Minitab® 17. Results were considered significant at  $P < 0.05$ . To test for the interaction between variables, a General Linear Model stepwise regression was used, interaction terms were added if the  $\alpha$  was greater than 0.15. Again this was completed on Minitab® 17 software and the results were considered significant if  $P < 0.05$ . Data was further analysed by a fit regression model with the probability of the slope of the line considered significant if  $P < 0.05$ .

## Chapter 4

### Results

#### 4.1 Trial Results

A sheep in the Plantain group was removed from the trial due to failure to eat the allocated food ration and general poor health. The blood data (packed cell volume, number of red blood cells) recorded on the first day of the trial were deemed to be unreliable and have been excluded from analysis. There was a loss of dry matter data for the feeds and faeces on the seventh day of the trial. Therefore, full analysis of the results was confined to the first six days of the study. On the first day of introduction of sheep to the study (Day 1) animals had very high osmolality readings (Table 4.2) unique to that day, and the urine volumes of Plantain sheep exceeded those of Ryegrass sheep by more than 1.5 L. This was significantly different ( $P < 0.05$ ) from all subsequent measurements, therefore results from the 1<sup>st</sup> day were analysed separately from those of the other 5 days (Days 2-6), which were analysed as a single data set. Data from Days 2-6 were computed as the mean of the average output for each group over the five days. Data from Day 1 and Days 2-6 are summarised in Table 4.2. There was no evidence of dehydration from the haematological data (red blood cell counts and packed cell volumes) or plasma sodium concentrations in blood obtained on Day 7 (see Table 4.1). Sheep in both groups lost weight during the experiment with Plantain sheep losing more ( $P = < 0.001$ ) than Ryegrass sheep (mean  $\pm$  s.e.m. loss for seven days,  $3.7 \pm 0.2$  and  $1.0 \pm 0.6$  kg, respectively). The Plantain sheep had a very much lower ( $P < 0.001$ ) daily water balance than the Ryegrass sheep (mean  $\pm$  s.e.m. daily average for Days 1-6,  $27 \pm 50$  and  $533 \pm 81$  mL, respectively).

Table 4.1: Haematological data (mean  $\pm$  SEM) for sheep fed ryegrass (Ryegrass) or plantain (Plantain) recorded from blood collected on Day 7 of the study. Number of animals per group are given in parentheses. Statistical significance (determined by Student's *t*-test) is indicated as ns (not significant,  $P > 0.05$ )

	Ryegrass (n = 8)	Plantain (n = 7)	Significance
Red blood cells ( $\times 10^7$ per $\mu\text{L}$ of blood)	$1.63 \pm 0.09$	$1.69 \pm 0.11$	ns
Packed cell volume (%)	$37 \pm 1.2$	$35 \pm 0.95$	ns
Plasma sodium (mmol/L)	$147 \pm 0.5$	$147 \pm 0.4$	ns

Table 4.2: Measurements of inputs and outputs (mean  $\pm$  SEM) for sheep fed ryegrass (Ryegrass) or plantain (Plantain) for seven days. Number of animals per group are given in parentheses. Statistical significance (determined by Student's *t*-test) is indicated as ns (not significant,  $P > 0.05$ ), \* ( $P < 0.05$ ), \*\*\* ( $P < 0.001$ ).

	Ryegrass (n = 8)	Plantain (n = 7)	Significance
<b>Day 1</b>			
Water in feed (L)	4.0 $\pm$ 0.04	3.7 $\pm$ 0.1	*
Dry matter in feed (g)	839 $\pm$ 9	634 $\pm$ 17	***
Urine volume (L)	2.9 $\pm$ 0.2	4.6 $\pm$ 0.3	***
Fresh faecal weight (g)	612 $\pm$ 63	545 $\pm$ 59	ns
Faecal water (mL)	450 $\pm$ 55	399 $\pm$ 52	ns
Urine pH	7.0 $\pm$ 0.4	6.1 $\pm$ 0.3	ns
Urine specific gravity	1.035 $\pm$ 0.001	1.037 $\pm$ 0.002	ns
Urine osmolality (mmol/kg)	1147 $\pm$ 31	1106 $\pm$ 61	ns
Osmols excreted per day (mmol)	3290 $\pm$ 272	5171 $\pm$ 519	*
<b>Days 2-6</b>			
Water in feed (L)	3.8 $\pm$ 0.07	4.0 $\pm$ 0.02	ns
Dry matter in feed (g)	686 $\pm$ 14	463 $\pm$ 2	***
Urine volume (L)	3.0 $\pm$ 0.1	3.5 $\pm$ 0.05	*
Fresh faecal weight (g)	494 $\pm$ 24	284 $\pm$ 17	***
Faecal water (mL)	360 $\pm$ 23	198 $\pm$ 16	***
Urine pH	7.4 $\pm$ 0.3	5.5 $\pm$ 0.3	***
Urine specific gravity	1.014 $\pm$ 0.001	1.009 $\pm$ 0.001	*
Urine osmolality (mmol/kg)	499 $\pm$ 52	327 $\pm$ 19	*
Osmols excreted per day (mmol)	1490 $\pm$ 169	1092 $\pm$ 82	ns

## Chapter 5

### Discussion

#### 5.1 Discussion of the Potential to Use Plantain as a Diuretic

The experiment was intended to determine whether the perennial herb, plantain (*Plantago lanceolata*), has diuretic activity following its ingestion by grazing animals. Results of the study indicate a considerable diuresis plus a substantial lowering of urinary pH in sheep that have consumed plantain. This is the first substantive evidence to support the anecdotal (and folklore) reports of diuretic activity in this plant and the effect on lowering urinary pH appears to be a new discovery.

##### 5.1.1 Urine Volume and Water Balance

###### Water In

The daily water input for both groups of sheep varied due to alterations in dry matter content of feed. The plantain diet had an average dry matter percentage of  $11 \pm 0.8\%$  whereas average dry matter percentage of the ryegrass tended to be higher, at  $15 \pm 1.2\%$  (Fig. 5.1). The dry matter of crops varies with seasonal and daily effects (Penston, 1938; Ackley, 1954; Schenk, 1996). Therefore, dry matter content of plants varied throughout the experiment due to the effect changes in the day to day environment had on the crops. Dry matter accumulation within plants is shown to increase with increased periods of photosynthesis (Penston, 1938). Factors such as temperature, light intensity, and soil moisture alter the dry matter content of leaves. Hourly fluctuations in dry matter also occur, with the highest dry matter content occurring at 1100 h, and can be seen in a diurnal pattern (Penston, 1938). The trial minimised the effect of this diurnal pattern by harvesting crops at the same time every day. However, alterations to dry matter caused by changing climatic conditions could not be avoided. Therefore diets were adjusted daily based on the dry matter content of the previous days feed. The variation in grass minimum temperature and rainfall over the course of the experiment can be seen in Figure 5.2. The effect these changes had on the composition of the plants is not immediately obvious, but may explain some of the dry matter variation seen in this experiment. Although the dry matter content of plantain did not vary as much as ryegrass it did increase and decrease in a similar pattern. However, the increases were not as extreme. In spite of the dry matter variation over the course of the experiment, the difference between water inputs was not significant between groups.

Although the mean water input on Day 1 differed between the two treatment groups, the lack of difference and its reversal over the ensuing 5 days suggests that overall the effort to provide constant and equal water intakes for each sheep was achieved very well.

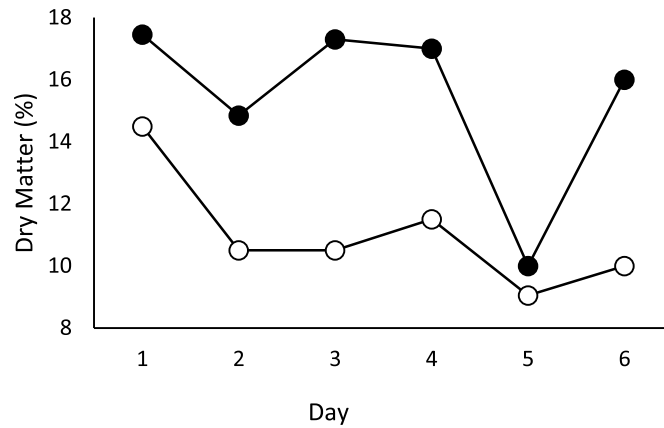


Figure 5.1: The dry matter percentage of the Ryegrass (●) and Plantain (○) diets over the course of the experiment.

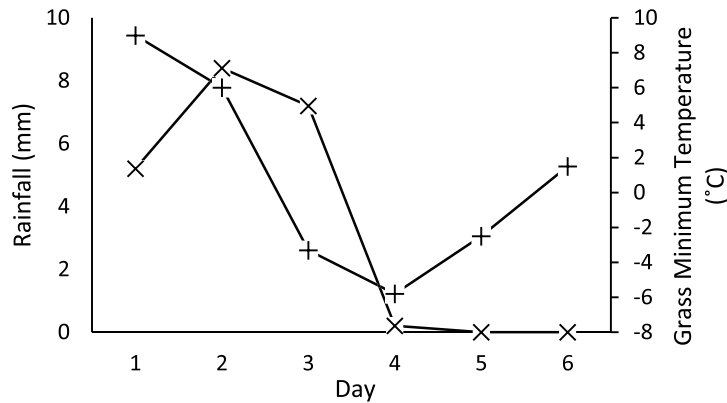


Figure 5.2: The change in temperature (+) and rainfall (x) over the course of the experiment (Source: NIWA Weather Station, Lincoln (Broadfield Ews 17603))

### Urine Production

The volume of urine produced each day by animals consuming plantain was greater than that produced by the animals on a ryegrass diet throughout the experiment. This was most notable on Day 1 of the trial where sheep fed plantain produced an average of 1.7 L more urine than those fed ryegrass. Day 1 was the only day in the trial where the water content of diets was different. On that day, Plantain sheep received  $3.7 \pm 0.1$  L of water in their feed as opposed to the  $4.0 \pm 0.04$  L given to ryegrass animals. So, in spite of receiving less water with their feed Plantain animals produced greater volumes of urine than Ryegrass animals. This diet-related difference in urinary volume continued, but at a reduced magnitude, for the rest of the trial with the average urine production of sheep on Days 2-6 being 17% higher by volume for Plantain than for Ryegrass sheep. This is evidence for a form of diuresis in sheep caused by consumption of plantain.

Throughout the experiment there was an apparent decline in the average daily volume of urine produced in the Plantain animals (Fig. 5.4). This is in spite of little change or possibly a slight increase

in daily water input in their feed. The initial water losses may have been higher because excess water output could have been supplied by the rumen. The rumen can account for 10-30% of the total water content of the body (Hecker *et al.*, 1964). This volume can decrease rapidly in response to dehydration. Camels have been shown to lose as much as 52% of their body weight by losing gut fluid during times of increased water stress (MacFarlane *et al.*, 1963). Sheep can lose up 45% of their body weight in cellular and ruminal fluid losses. Hecker *et al.* (1964) found that the greatest decrease in rumen volume occurs during the first two days of increased water stress. This fits with the results of the trial where urine output for Plantain animals was the greatest on Days 1 and 2 (Fig. 5.4).

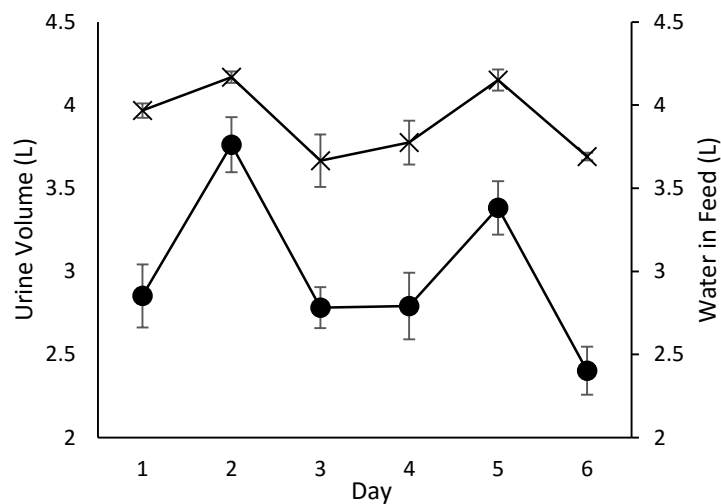


Figure 5.3: The average urine volume over six days of sheep (n = 8) feed on a ryegrass diet (●) and the water inputs from feed (x) over the course of the experiment.

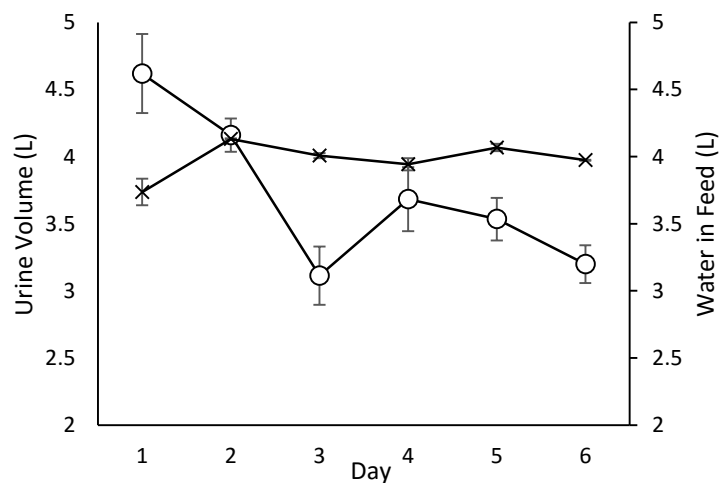


Figure 5.4: The average urine volume over six days of sheep (n = 8) feed a diet of plantain (o) and the water inputs from feed (x) over the course of the experiment.

Hecker *et al.* (1964) also noted that the rumen prevents much of the loss of water from other areas of the body, such as within the body cells. This prevents the animal becoming dehydrated even in times of water stress. Their results may help to explain why, under the same dietary water inputs and in spite

of Plantain sheep having higher urine volumes than the Ryegrass animals over the trial, the blood results show no evidence of dehydration in either group.

### **Urine solute content**

At the beginning of the experiment both the Ryegrass and Plantain groups had urine specific gravity within the normal range (Swenson *et al.*, 1993; Radostits *et al.*, 2000). Urine specific gravity for stock normally ranges between 1.015 and 1.045 (Swenson *et al.*, 1993). Radostits *et al.* (2000) suggest that specific gravity readings that are between 1.008-1.012 in animals displaying increased urine production may indicate renal disease. Urine specific gravity of both the Ryegrass and the Plantain groups decreased after the first day of the trial. However, only the Plantain group had urine specific gravities that indicate kidney dysfunction as outlined by Radostits *et al.* (2000). These authors stated that decrease in specific gravity can be a result of failure for the kidney to reabsorb water or when the osmotic gradient within the kidney has been altered, thus preventing animals from concentrating their urine. This supports the hypothesis that plantain is acting as a diuretic, as diuretics alter the ability for the kidney to reabsorb water, and also that plantain has the potential to dilute urine. However, it does not explain the drop in the specific gravity of the Ryegrass group.

Not surprisingly, the values obtained for urine osmolality echoed the specific gravity readings. There is a high correlation between urine osmolality and its specific gravity (Fig. 5.5) which suggests that specific gravity can be measured as a proxy for osmolality. However, the correlation between the two readings is not always so strong. In the measurement of pathological urine samples the correlation between the two measurements is reduced (Imran *et al.*, 2010). The present results showed some loss of linearity in this correlation, indicating that specific gravity increased more quickly than osmolality. This may have been due to the presence of compounds that altered the refractive index of urine but did not contribute equally to the molar increase in concentration. Compounds such as haemoglobin, ketones, and bilirubin may be present in the urine of ill individuals, or those with irregular urine outputs, and alter the readings made by the refractometer (Imran *et al.*, 2010). Measuring osmolality is recommended as very reliable measure of solute content of urine.

The results for Day 1 were very different to those of Days 2-6. The total osmols excreted per day was higher for the Plantain sheep than in the Ryegrass group. However, this can be attributed to the urine volumes of the Plantain sheep. Increase in solute excretion is a characteristic of an osmotic diuresis (Atherton *et al.*, 1968). Day 1 may be different due to the presence of higher molecular weight solutes within urine samples. They may have been present in animals from both Plantain and Ryegrass groups on Day 1, having just come off pasture, and causing the increased readings in both SG and osmolality. These readings could have been a result of compounds left over from the diet of the animals before

they began the experiment. However, further investigation would be required to determine just what was happening to urine production on the first day of the study.

On the following days the total daily osmols excreted was not different between groups. However, the concentration (specific gravity and osmolality) of the urine in the samples was reduced. This indicates that although animals within the Plantain group were producing more urine their total daily excretion of solutes was similar to Ryegrass sheep. Therefore, the kidneys were excreting more water without removing excess solutes. This suggests that the water reabsorption mechanism within the kidney has been altered, possibly due to an interaction with antidiuretic hormone (ADH) or its receptors (Atherton *et al.*, 1968). This type of diuresis is most commonly seen in animals that have consumed too much water and is known as water diuresis.

The low osmolality and specific gravity readings indicate that plantain will be able to be used as a way of decreasing solute load in urine patches. Data from Days 2-6 shows that urine has become diluted but still maintains similar total daily solute excretion as the Ryegrass group. If the assumption that nitrogen has also been reduced in concentration by this dilution is correct, then plantain can be used as a measure to reduce the nitrogen load of a urine patch.

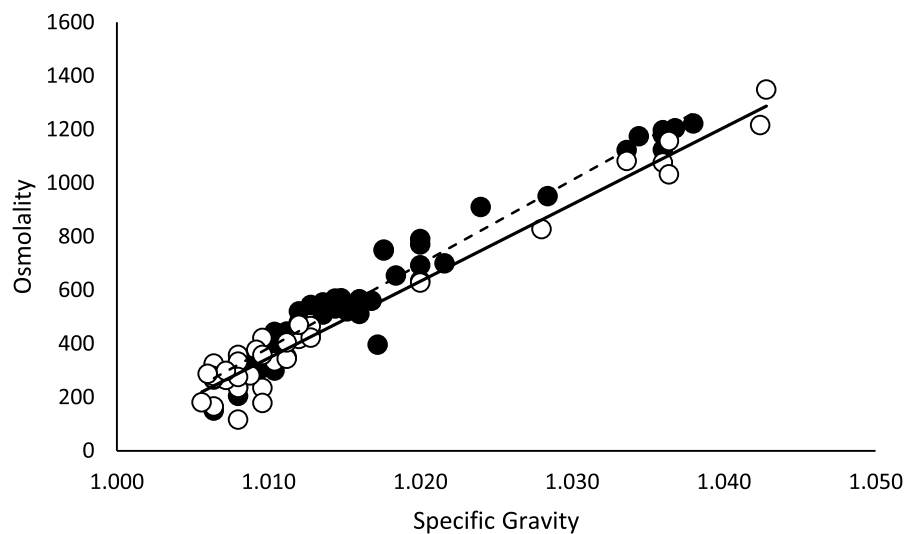


Figure 5.5: Regression analysis of osmolality and specific gravity readings for animals on different diets over the course of six days. Plantain diets (○) are displayed with the solid trendline ( $y = 28695x - 28636$ ,  $R^2 = 0.97$ ) and Ryegrass diets (●) are displayed with the dashed trendline ( $y = 31334x - 31264$ ,  $R^2 = 0.9504$ ).

### Faecal Water

The water content and weight of the faeces was similar for both groups of sheep on the first day of the trial. However, on Days 2-6 there was a difference between groups with Plantain animals having lighter, drier faeces. The water content within the faeces of all animals decreased over the course of the experiment (Fig. 5.6). This may be explained by a physiological response to loss of water in the urine

of the animal. Other literature (Maloiy *et al.*, 1970; Skadhauge *et al.*, 1980) found that as water is restricted in the diets of sheep, deer (*Cervus elaphus*), and the dik-dik antelope (*Rhynchotragus kirkii*) the faecal water content decreases. The amount of water lost per gram of faecal matter could be decreased by as much as 35% (Skadhauge *et al.*, 1980) in times of water stress. Sheep in the Plantain group had faecal water contents reduced by as much as 45% when compared to those in the Ryegrass group. This is most likely due to the reabsorption of ruminal and gut fluids (MacFarlane *et al.*, 1963; Hecker *et al.*, 1964).

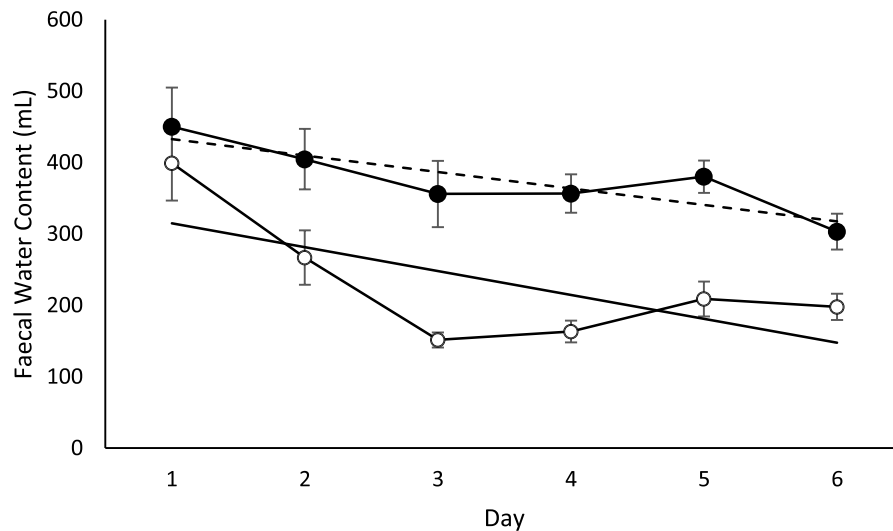


Figure 5.6: The average faecal water content in of sheep feed a diet of ryegrass (●) ( $y = 455.8 - 23.04 x$ ,  $P$  of the slope = 0.014) or plantain (○) ( $y = 348.2 - 33.40 x$ ,  $P$  of the slope = 0.001) over the six days of the experiment. Water content of the faeces decreased over the experiment.

### Blood Characteristics

The red blood cell counts were slightly higher than the normal range for sheep as indicated by Radostits *et al.* (2000). The normal red blood cell concentration for sheep should be between  $0.9$  and  $1.5 \times 10^7/\mu\text{L}$  blood. However, the present sheep had average counts of  $1.6 \times 10^7/\mu\text{L}$  blood for ryegrass diets and  $1.7 \times 10^7/\mu\text{L}$  blood for plantain diets. This indicates that the sheep may have been slightly dehydrated. However, the blood samples were within normal range for packed cell volume (normal range is between 27-45%) which suggests that although there were more red blood cells they might have been smaller, which could be evidence for dehydration. However, plasma sodium levels were within the normal range of 145-152 mmol/L which tends to dispel any suggestion of dehydration in these sheep.

### Fluid Balance

The water balance at the end of Day 6 was a surplus for both groups. However, the Plantain animals had a lower water surplus than the Ryegrass animals, providing further evidence that this group of sheep was exporting more of its water reserves into urine and faeces than the Ryegrass sheep.

For this experiment it was assumed that evaporative losses in each group of animals were similar. Evaporative loss, through respiration and insensible loss can account for up to 52% of water lost from the body of sheep (Sileshi, 2003). However, this changes with the temperature of the environment and the amount of feed in the diet (Degen *et al.*, 1981). The sheep here were exposed to the same environmental temperature, because they were housed together. However, the two groups were fed differing levels of dry matter. This is a consequence of balancing the two diets for total water input. Because ryegrass usually has a higher dry matter percentage than plantain, the Ryegrass sheep were consistently fed higher levels of dry matter. Degen *et al.* (1981) found that as feed allocation is increased there is an increased evaporative water losses. This is due to the effect of the extra metabolic activity required to assimilate the additional nutrients, which causes a rise in body temperature (Degen *et al.*, 1981) and therefore greater evaporative water losses. This may have implications for the water balance of the animals in this study. Ryegrass sheep may have lost more water from evaporative losses due to the increased metabolism of higher levels of dry matter.

However, water can also be gained by metabolism of the protein, carbohydrate, and fat components of the dry matter. Every 100 g of protein, carbohydrate, and fat can contribute 40, 57, and 107 mL of water respectively (Edney, 1977). Plantain and ryegrass diets contribute differing amounts of these constituents. In plantain, 16-18% of dry matter is made up of protein, 11-20% in soluble carbohydrates (Dairy NZ, 2013), and 2.1% crude fat (Pastoral Improvements Ltd., 2014). For every kg of dry matter consumed, the sheep will receive 148-208 mL of water through metabolism. For ryegrass, 12-28% of dry matter is protein, 8-21% carbohydrate (Dairy NZ, 2013), and 2.7% crude fat (Elgersma *et al.*, 2003). This means that the metabolism of a kg of dry matter would yield between 122-260 mL of water. Therefore, in this experiment sheep fed ryegrass were gaining an estimated 87-185 mL of water daily through the metabolism of their diet and Plantain sheep gained 73-102 mL. At both high and low levels of metabolisable water the difference gained through metabolism of the diet is significantly different ( $P < 0.001$ ).

### **5.1.2 Other effects of a plantain diet on kidney function**

#### **pH**

On the first day of the trial urinary pH was similar for both groups of sheep. However for the remainder of the study period, the average pH of Plantain sheep urine was lower than that of the Ryegrass sheep (by almost 2 pH units). In both groups the pH of the urine appeared to decline over the course of the experiment except for on the final day in which Ryegrass urine pH rose by 1.5 and Plantain by 1. The decline was more pronounced within the Ryegrass group than the Plantain group. There are several mechanisms through which a decrease in urine pH can be attributed to. The drop in urine pH may be in response to gradual dehydration. In an experiment described by MacFarlane *et al.* (1961), as sheep

were dehydrated their urine pH dropped. The urine pH of the Plantain group remained consistently lower than that of the Ryegrass group. The decline in pH was not as dramatic in this group. The assumption can be made that this was due to factors already interacting with hydrogen excretion within the kidney. Both groups had a pH rise on Day 6 of the trial. It is possible that some aspect of their feed intake on Day 6 changed their hydration status.

The dry matter contents for both Ryegrass and Plantain diets were at their lowest on Day 5 of the experiment. Dry matter percentages of 10 and 9.05 were recorded for Ryegrass and Plantain respectively. This means that they received higher water inputs on that day. The apparent increase in pH on Day 6 (Fig. 5.7) may indicate slight rehydration within both sheep groups and thus less acid excretion in urine.

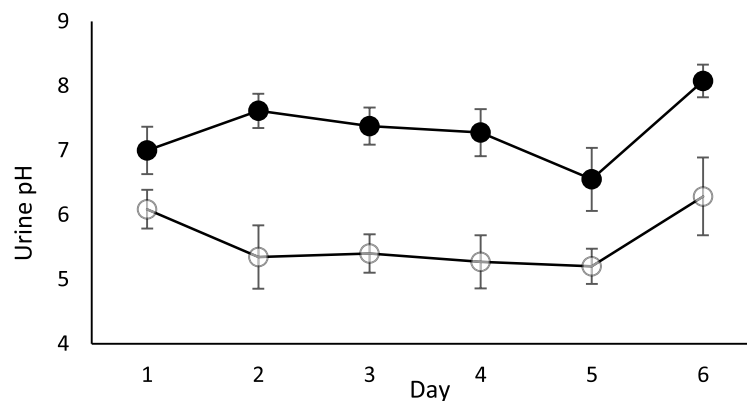


Figure 5.7: The pH of urine from sheep feed a diet of plantain (o) or ryegrass (●) over the course of the experiment. There appears to be a downwards trend for urine pH except on Day 1 for Ryegrass sheep and Day 6 for both groups.

Hydrogen ions are released in the connecting tubule or collecting duct via electrogenic vacuolar H<sup>+</sup>-ATPases and the K-H exchanger (Kovacikova *et al.*, 2006). Factors that alter the environment of these areas alter the excretion of H<sup>+</sup> ions and thus can decrease the pH of the urine. Plantain animals had urine pH that was consistently lower than the Ryegrass group. The mechanism for this pH reduction is not clear. However, it may indicate that a molecule or metabolite within plantain has either inhibited the mechanism of hydrogen excretion or altered the electron gradient within the tubule leading to greater hydrogen excretion (Kovacikova *et al.*, 2006). Furosemide, a loop diuretic, has been shown to cause urine acidity by this same mechanism (Kovacikova *et al.*, 2006).

Alterations in urine pH may have implications for how the constituents within the urine behave within the soil. Different soil pH has an effect on the microbes which will work optimally in that area. When the pH of the soil is around 7, the microbes which work to convert nitrate into nitrous oxide and nitrogen gases work at their optimal level (Hall *et al.*, 1998). However, when pH levels drop this microbes become less active and the reaction to remove nitrogen from the soil into its gas forms is less

favoured (Cameron *et al.*, 2012). The temporary reduction in soil pH which is caused by the plantain urine patch may work to increase the amount of nitrate in soil by inhibiting denitrification. This would potentially increase the risk of this nitrate leaching from the soil and into ground water reserves. Urine does not remain at a low pH for long periods of time. Standing urine will revert to a neutral or basic pH when left in the air (Cook *et al.*, 2007). This may minimise any long lasting effects of the lowered urine pH on the soil biota. However, soil is a multifaceted system, and without the correct research the implications of decreased urine pH, even for a short time before, and urine patches is unknown.

It is possible that plantain has acted as some form of vasopressin receptor antagonist (vaptan). Vaptans are a class of drug which have been used in the treatment of water retention within the body (Aditya *et al.*, 2012). They are favoured due to their ability to cause increased water loss but retaining solutes. This helps to prevent some of the ill effects that can be seen in other diuretics. Vaptans work by interacting with ADH receptors throughout the body.

### **Precipitate in Urine**

During the experiment, it was noted that sheep fed on a ryegrass diet produced a precipitate within their acidified urine samples after overnight collection. It is unclear whether the addition of acid to the urine aided in the formation of a precipitate or not. The formation of crystals in the urine of herbivores is not uncommon and is usually due to the presence of calcium carbonate or triple phosphate (Radostits *et al.*, 2000). The precipitate was present throughout the experiment in the urine of animals fed ryegrass. Future experiments may wish to analyse this precipitate to find out what it is and whether it is present in fresh urine. If the precipitate was present, in a dissolved form, in fresh urine it would have altered the specific gravity and osmolality readings. This may explain the higher readings obtained for Ryegrass sheep.

### **Weight Change**

Between the beginning and the end of the experiment the Plantain group lost an average of 4 kg of weight. The Ryegrass group lost less weight as they only lost an average of 1 kg. It can be assumed that much of this loss can be accounted for in lost water, as this can account to up to 45% of body weight losses in sheep (MacFarlane *et al.*, 1963). However further investigation is required to confirm this possibility. This can be done by using radiolabelled chemicals such as sodium thiosulphate (Macfarlane, 1975; Ross *et al.*, 1992). Sodium thiosulphate can be used to detect extracellular water. This would make it useful to detect the changes in water content of the extracellular compartments in response to a diet of plantain. Sodium thiosulphate concentration alterations over the course of the experiment would allow the determination of whether water was lost from the rumen and gut or from extracellular areas.

Weight loss may also be due to the mobilisation of body stores within the sheep. If this project is repeated it would be useful to slaughter animals at the end of the study to detect changes in fat and muscle deposition in animals. Alternatively subcutaneous fat samples could be analysed from live sheep for adipocyte size and the depth of adipose tissue (Reid *et al.*, 1986). This could be measured at the beginning and the end of the trial to determine if some fat stores were mobilised. Blood tests to detect constituents, such as non-esterified fatty acids (NEFA), released during the metabolism of fats can also be used to determine if animals have mobilised body stores (Reid *et al.*, 1986; Rodrigues *et al.*, 2010). If animals had been using more of their body stores then the levels of these compounds would be increased when compared to the beginning of the experiment. If the mobilisation of body stores explains the weight loss of these sheep it will have an effect on the water balance. Therefore it is an important consideration for future experiments.

### **The effect of measured variables on urine Volume**

There was a significant difference between the urine outputs of Ryegrass and Plantain animals. A generalised linear model stepwise regression was used in an attempt to determine whether any of the variables measured may have caused the difference in urine volume seen throughout the experiment. Of all the measurements the only variable that had an effect on the volume of urine produced was the water they received in their feed. It is intuitive to expect that the volume of water given to the animals would have an effect on the amount of urine they produced. The analysis also determined whether the feed water inputs explained all of the differences in urine volume between groups. It shows that, water input has a significant effect on urine output, however it could not account for the all the difference in urine volume found between groups in the experiment.

This suggests not only that Plantain diets will increase urine production even when water inputs are accounted for, but also that other variables (such as pH and osmolality) did not cause a statistically significant change in urine production. This reinforces that both of these effects are caused by the plantain diet.

### **5.1.3 Suggestions for future research**

The trial showed that there was a diuretic effect caused by the feeding of plantain but it did not indicate the cause of this diuresis. Further research is required to understand the underlying mechanism of diuresis. There are many putative diuretic components present in plantain including aucubin, acteoside, sorbitol, and mannitol. Possible future investigations could attempt to isolate the various compounds of plantain and test them all for diuretic effects. There were also differences in the mineral content within the plants. Future experiments should attempt to balance the mineral content of both diets in order to see if these mineral changes have an impact on the apparent diuretic effect. It is

possible that plantain itself does not act as a diuretic, but a component of plantain that is produced during metabolism or by gut microflora may be responsible for the diuretic effect.

The study was not designed to determine the dose of plantain required to cause a diuretic effect. Dose-response experiments in which different amounts of plantain are added to the diet of sheep may help to provide this information. If on-farm application of these results was intended it would be important to research at what level plantain needs to be available in swards to gain the benefits of diuresis.

There should be more research done into the mineral content within the urine of animals fed plantain versus those fed ryegrass. The trial was based around determining if plantain acted as a diuretic. This was to investigate the possibility of this diuresis decreasing the levels of nitrogen excreted through urine. However, the nitrogen levels within the urine were not tested within this experiment. Therefore, another experiment which recorded the urine nitrogen levels between the two groups would be advantageous for further application of plantain research.

Measurements of urine sodium, potassium, and calcium would also help to identify the cause of diuresis. This is because many of the diuretic effects act on specific transporters within the kidney. Thus the mechanism of diuresis can be more accurately estimated if a full urine nutrient analysis was completed.

The long term effect of the diuretic action of plantain was not investigated in this study. There may be health and welfare implications due to the effects of plantain. There can be negative health effects associated with the long term use of some types of diuretics. Diuretics that cause an increased loss of calcium or other minerals may have adverse side-effects such as issues with kidney stones or mineral deficiency (Hufnagle *et al.*, 1982; James *et al.*, 1984). There is also the increased function of the kidneys in response to producing more urine. Deaker *et al.* (1994) found that kidney sizes increased in animals fed plantain. This may indicate that kidney function has been amplified which is compensated by the increased size.

#### **5.1.4 Conclusions**

- Plantain acts as a diuretic when fed as the sole diet for sheep. However, the mechanism for the cause of this diuresis is unknown.
- Plantain has dramatic effects on urine volume for the first day of administration but these effects appear to lessen over time.

- Although urine volume is increased in animals fed plantain, they are no more dehydrated than those fed ryegrass. This may indicate that water reserves within the rumen of the animal could have been mobilised to make up for urine losses.

## References

- Ackley, W. B. (1954). *Seasonal and Diurnal Changes in the Water Contents and Water Deficits of Bartlett Pear Leaves*. *Plant Physiology*. 29 (5): 445-448
- ADA (American Dietetic Association). (2004). *Position of the American Dietetic Association: Use of Nutritive and Nonnutritive Sweeteners*. *Journal of the American Dietetic Association*. 104 (2): 255-275
- Aditya, S., and Rattan, A. (2012). *Vaptans: A New Option in the Management of Hyponatremia*. *International Journal of Applied and Basic Medical Research*. 2 (2): 77-83
- Aganga, A. A. (1992). *Water Utilization by Sheep and Goats in Northern Nigeria*. *Revista Mundial de Zootecnia*. 73
- Akan, H., Sargin, S., Dalva, I., Akman, Y., Yazicioglu, A., and Cetin, S. (1997). *Effects of Distilled Water and Mixture of Sorbitol – Mannitol Irrigation Fluids on Fluid – Electrolyte Balance in Patients Undergoing Transurethral Prostatectomy*. *International Urology and Nephrology*. 29 (5): 575-580
- Akers, R. M., and Denbow, D. M. (2008). *Anatomy and Physiology of Domestic Animals*. Iowa, USA. Blackwell Publishing.
- Andary, C., Wylde, R., Laffite, C., Privat, G., and Winternitz, F. (1982). *Structures of Verbascoside and Orobanchoside, Caffeic Acid Sugar Esters from Orobanche rapum-genistae*. *Phytochemistry*. 21 (5): 1123-1127
- Atherton, J. C., Hai, M. A., and Thomas, S. (1968). *Effects of Water Diuresis and Osmotic (Mannitol) Diuresis on Urinary Solute Excretion by the Conscious Rat*. *Journal of Physiology*. 197 (2): 395-410
- Bowers, M. D., and Stamp, N. E. (1992). *Chemical Variation Within and Between Individuals of Plantago lanceolata (Plantaginaceae)*. *Journal of Chemical Ecology*. 18 (7): 985-995
- Briens, M., and Larher, F. (1983). *Sorbitol Accumulation in Plantaginaceae; Further Evidence for a Function in Stress Tolerance*. *Zeitschrift für Pflanzenphysiologie*. 110 (5): 447-458
- Burg, M. B., and Kador, P. F. (1988). *Sorbitol, Osmoregulation, and the Complications of Diabetes*. *The Journal of Clinical Investigation*. 81: 635-640
- Cáeres, A., Girón, L. M., and Martínez, A. M. (1987). *Diuretic Activity of Plants Used for the Treatment of Urinary Ailments in Guatemala*. *Journal of Ethnopharmacology*. 19: 233-245

- Cameron, K. C., Di, H. J., and Moir, J. L. (2013). *Nitrogen Losses from the Soil/Plant System: A Review*. Association of Applied Biologists. 162: 145-173
- Campbell, N. A., and Reece, J. B. (2005). *Biology*. San Francisco, USA. Pearson Education Ltd.
- Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T., and Lin, C. C. (2002). *Antiviral Activity of Plantago major Extracts and Related Compounds In Vitro*. Antiviral Research. 55 (1): 53–62
- Charlton, D., & Stewart, A. (2006). *Pasture and Forage Species for New Zealand*. (3rd Ed.). Christchurch, New Zealand. Grasslands Research and Practice Series.
- Chen, C.-H., Lin, Y.-S., Chien, M.-Y., Hou, W.-C., and Hu, M.-L. (2012). *Antioxidant and Antihypertensive Activities of Acteoside and its Analogs*. Botanical Studies. 53: 421-429
- Cook, J. D., Strauss, K. A., Caplan, Y. H., LoDico, C. P., and Bush, D. M. (2007). *Urine pH: The Effects of Time and Temperature After Collection*. Journal of Analytical Toxicology. 31: 486-496
- Dairy NZ. (2012). *Chicory and Plantain Programme*. Retrieved from <http://www.dairynz.co.nz/what-we-do/research/key-projects/chicory-and-plantain-programme/> on 19.05.15
- Dairy NZ. (2013). *Plantain Management (1-78b)*. Retrieved from [http://www.dairynz.co.nz/media/253818/1-78b\\_Plantain\\_management.pdf](http://www.dairynz.co.nz/media/253818/1-78b_Plantain_management.pdf) on 9.11.15
- Davini, E., Lavarone, C., Trogolo, C., Aureli, P., and Pasolini, B. (1986). *The Quantitative Isolation and Antimicrobial Activity of the Aglycone of Aucubin*. Phytochemistry. 25: 2420–2422
- Deaker, J. M., Young, M. J., Fraser, T. J., and Rowarth, J. S. (1994). *Carcass, Liver and Kidney Characteristics of Lambs Grazing Plantain (Plantago lanceolata), Chicory (Cichorium intybus), White Clover (Trifolium repens), or Perennial Ryegrass (Lolium perenne)*. Proceedings of the New Zealand Society of Animal Production. 54: 197-200
- Degen, A. A., and Young, B. A. (1981). *Effect of Air Temperature and Feed Intake on Live Weight and Water Balance in Sheep*. Journal of Agricultural Science Cambridge. 96: 493-496
- Di, H. J., and Cameron, K. C. (2007). *Nitrate Leaching Losses and Pasture Yields as Affected by Different Rates of Animal Urine Nitrogen Returns and Application of a Nitrification Inhibitor—A Lysimeter Study*. Nutrient Cycling in Agroecosystems. 79 (3): 281-290
- Doan, D. D., Nguyen, N. H., Doan, H. K., Nguyen, T. L., Phan, T. S., van Dau, N., Grabe, M., Johansson, R., Lindgren, G., and Stjernström, N. E. (1992). *Studies on the Individual and Combined Diuretic Effects*

- of Four Vietnamese Traditional Herbal Remedies (Zea mays, Imperata cylindrica, Plantago major and Orthosiphon stamineus)*. Journal of Ethnopharmacology. 36 (3): 225-231.
- Edney, E. B. (1977). *Metabolic Water*. Zoophysiology and Ecology. 9: 189-195
- Elgersma, A., Ellen, G., van der Horst, H., Muuse, B. G., Boer, H., and Tamminga, S. (2003). *Comparison of the Fatty Acid Composition of Fresh and Ensiled Perennial Ryegrass (Lolium perenne L.), Affected by Cultivar and Regrowth Interval*. Animal Feed Science and Technology. 108 (1-4): 191-205
- Fajer, E. D., Bowers, M. D., and Bazzaz, F. A. (1992). *The Effect of Nutrients and Enriched CO<sub>2</sub> Environments on Production of Carbon Based Allelochemicals in Plantago: A Test of the Carbon/Nutrient Balance Hypothesis*. The American Naturalist. 140 (4): 707-723
- Felter, H. W., & Lloyd, J. U. (1898). *Practical Herbs 2*. (18th Ed.). Cincinnati, USA. Ohio Valley Co. <http://www.henriettes-herb.com/eclectic/kings/plantago.html>
- Frick, K. K., and Bushinsky, D. A. (2003). *Molecular Mechanisms of Primary Hypercalciuria*. Journal of the American Society of Nephrology. 14 (4): 1082-1095
- Gálvez, M., Martín-Cordero, C., López-Lázaro, M., Cortés, F., and Ayuso, M. J. (2003). *Cytotoxic Effect of Plantago spp. on Cancer Cell Lines*. Journal of Ethnopharmacology. 88 (2-3): 125-30
- Hall, J. M., Paterson, E., and Killham, K. (1998). *The Effect of Elevated CO<sub>2</sub> Concentration and Soil pH on the Relationship Between Plant Growth and Rhizosphere Denitrification Potential*. Global Change Biology. 4: 209-216
- Hecker, J. F., Budtz-Olsen, O. E., and Ostwald, M. (1964). *The Rumen as a Water Store in Sheep*. Australian Journal of Agricultural Research. 15: 961-968
- Horisberger, J. D., and Giebisch, G. (1987). *Potassium-Sparing Diuretics*. Renal Physiology. 10 (3-4): 198-220
- Hoskin, S. O., Wilson, P. R., Ondris, M., and Bunod, A. H. (2006). *The Feeding Value of Forage Herbs: Studies with Red Deer*. Proceedings of the New Zealand Grasslands Association. 68: 199-204
- Hufnagle, K. G., Khan, S. N., Penn, D., Cacciarelli, A., and Williams, P. (1982). *Renal Calcifications: A Complication of Long-Term Furosemide Therapy in Preterm Infants*. Pediatrics. 70 (3): 360-363
- Imran, S., Eva, G., Christopher, S., Flynn, E., and Henner, D. (2010). *Is Specific Gravity a Good Estimate of Urine Osmolality?* Journal of Clinical Laboratory Analysis. 24 (6): 426-430

- James, M. D., and Knochel, P. (1984). *Diuretic-Induced Hypokalemia*. The American Journal of Medicine. 77 (5): 18-27
- Jeong, H. J., Koo, H. N., Na, H. J., Kim, M. S., Hong, S. H., Eom, J. W., Kim, K. S., Shin, T. Y., and Kim, H. M. (2002). *Inhibition of TNF-Alpha and IL-6 Production by Aucubin Through Blockade of NF-KappaB Activation RBL-2H3 Mast Cells*. Cytokine. 18 (5): 252-259
- Jin, L., Xue, H.-Y., Jin, L.-J., Li, S.-Y., and Xu, Y.-P. (2008). *Antioxidant and Pancreas-Protective Effect of Aucubin on Rats with Streptozotocin-Induced Diabetes*. European Journal of Pharmacology. 582: 162-167
- Jurišić, R., Debeljak, Ž., Vladimir-Knežević, S., and Vukovic, J. (2004). *Determination of Aucubin and Catapol in Plantago Species by Micellar Electrokinetic Chromatography*. Zeitschrift für Naturforschung. 59: 27-31
- Kang, D. G., Lee, Y. S., Kim, H. J., Lee, Y. M., and Lee, H. S. (2003). *Angiotensin Converting Enzyme Inhibitory Phenylpropanoid Glycosides from Clerodendron trichotomum*. Journal of Ethnopharmacology. 89 (1): 151-154
- Kato, Y. (1946). *Mechanism of Uric Acid Excretion Stimulation by Aucubin*. Folia Pharmacology Japan. 42: 37-40. [Ref. Deaker *et al.*, 1994]
- Kovacikova, J., Winter, C., Loffing-Cueni, D., Loffing, J., Finberg, K. E., Lifton, R. P., Hummler, E., Rossier, B., and Wagner, C. A. (2006). *The Connecting Tubule is the Main Site of the Furosemide-Induced Urinary Acidification by the Vacuolar H<sup>+</sup>-ATPase*. Kidney International. 70: 1706-1716
- Levy, E. B. (1970). *Grasslands of New Zealand*. (3<sup>rd</sup> ed.). Wellington, New Zealand. A. R. Shearer Govt, Print.
- MacFarlane, W. V. (1975). *Distribution and Dynamics of Body Fluids in Sheep*. The Blood of Sheep: Composition and Function. New York USA. Springer-Verlag.
- MacFarlane, W. V., Morris, R. J. H., and Howard, B. (1963). *Turn-Over and Distribution of Water in Desert Camels, Sheep, Cattle and Kangaroos*. Nature. 197: 270-271
- Macfarlane, W. V., Morris, R. J. H., Howard, B., McDonald, J., and Budtz-Olsen, O. E. (1961). *Water and Electrolyte Changes in Tropical Merino Sheep Exposed to Dehydration During Summer*. Australian Journal of Agricultural Research. 12 (5): 889-912

- Maloiy, G. M. O., Kay, R. N. B., and Goodall, E. D. (1970). *Digestion and Nitrogen Metabolism in Sheep and Red Deer Given Large or Small Amounts of Water and Protein*. *British Journal of Nutrition*. 24: 843-855
- Maloiy, G. M. O., and Scott, D. (1969). *Renal Excretion of Urea and Electrolytes in Sheep and Red Deer*. *The Journal of Physiology*. 205 (1): 91-101
- Mathisen, O., Raeder, M., and Kiil, F. (1981). *Mechanism of Osmotic Diuresis*. *Kidney International*. 19: 431-437
- Mattila, P. T., Svanberg, M. J., Makinen, K. K., and Knuutila, M. L. E. (1996). *Dietary Xylitol, Sorbitol, and D-Mannitol but Not Erythritol Retard Bone Resorption in Rats*. *The Journal of Nutrition*. 126 (7): 1865-1870
- Moorhead, A. J. E., and Piggot, G. J. (2009). *The Performance of Pasture Mixes Containing 'Ceres Tonic' Plantain (Plantago lanceolata) in Northland*. *Proceedings of the New Zealand Grassland Association*. 71: 195-199
- Nexcelom Bioscience. (2015). *Manual Cell Counting and Viability Using a Hemacytometer*. Retrieved from <http://www.nexcelom.com/Applications/counting-cells-with-a-hemacytometer.php> on 9.11.15
- Pastoral Improvements Ltd. (2014). *Hercules Plantain – Technical Sheet*. Retrieved from <http://herculesplantain.co.nz/wp-content/uploads/2014/05/Hercules-Plantain-technical-sheet-1.pdf> on 9.11.15
- Penston, N. L. (1938). *Studies of the Physiological Importance of the Mineral Elements in Plants: The Variation in Potassium Content of Maize Leaves During the Day*. *New Phytologist*. 37 (1): 1-14
- Pirhofer-Walzl, K., Sjøgaard, K., Høgh-Jensen, H., Eriksen, J., Sanderson, M. A., Rasmussen, J., and Rasmussen, J. (2011). *Forage Herbs Improve Mineral Composition of Grassland Herbage*. *Grass and Forage Science*. 66: 415-423
- Quinn, J. M., Cooper, A. B., Davies-Colley, R. J., Rutherford, R. J., Williamson, R. B. (1997). *Land Use Effects on Habitat, Water Quality, Periphyton, and Benthic Invertebrates in Waikato, New Zealand, Hill-Country Streams*. *New Zealand Journal of Marine and Freshwater Research*. 31: 579-597
- Quintero, C., and Bowers, M. D. (2012). *Changes in Plant Chemical Defences and Nutritional Quality as a Function of Ontogeny in Plantago lanceolata (Plantaginaceae)*. *Oecologia*. 168: 471-481

- Radostits, O. M., Gay, C. C., Blood, D. C., and Hinchcliff, K. W. (2000). *Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses*. (9 ed.). London, England. W. B. Saunders. pp. 479-492, 1819-1822
- Swenson, M. J., and Reece, W. O. (1993). *Dukes' Physiology of Domestic Animals*. (11<sup>th</sup> ed.). New York, USA. Cornell University Press.
- Reid, I. M., Roberts, C. J., Treacher, R. J., and Williams, L. A. (1986). *Effect of Body Condition at Calving on Tissue Mobilisation, Development of Fatty Liver and Blood Chemistry of Dairy Cows*. *Animal Production*. 43 (1): 7-15
- Rodrigues, R. O., Trevisanuto, C., Cooke, R. F., and Vasconcelos, J. L. M. (2010). *Effects of Body Weight Loss on Serum Progesterone Concentrations of Non-Lactating Dairy Cows*. *Theriogenology*. 75 (1): 131-137
- Rose, B. D. (1991). *Diuretics*. *Kidney International*. 39: 336-352
- Ross, J. G., Preston, R. L., and Bartle, S. J. (1992). *Evaluation of Sodium Thiosulfate as an Extracellular Water Marker in Cattle*. *Journal of Animal Science*. 70: 434-438
- Rumball, W., Keogh, R. G., Lane, G. E., Miller, J. E., and Claydon, R. B. (1997). *'Grasslands Lancelat' Plantain (Plantago lanceolata L.)*. *New Zealand Journal of Agricultural Research*. 40: 373-377
- Samuelsen, A. B. (2000). *The Traditional Uses, Chemical Constituents and Biological Activities of Plantago major L.: A Review*. *Journal of Ethnopharmacology*. 71: 1–21
- Sands, J. M., and Schrader, D. C. (1990). *Coordinated Response of Renal Medullary Enzymes Regulating Net Sorbitol Production in Diuresis and Antidiuresis*. *Journal of the American Society of Nephrology*. 1 (1): 58-65
- Schenk, M. K. (1996). *Regulation of Nitrogen Uptake on the Whole Plant Level*. *Plant and Soil*. 181: 131-137
- Schils, R. L. M., Vellinga, T. V., and Kraak, T. (1999). *Dry-Matter Yield and Herbage Quality of a Perennial Ryegrass/White Clover Sward in a Rotational Grazing and Cutting System*. *Grass and Forage Science*. 54: 19-29.
- Seely, J. F., and Dirks, J. H. (1969). *Micropuncture Study of Hypertonic Mannitol Diuresis in the Proximal and Distal Tubule of the Dog Kidney*. *The Journal of Clinical Investigation*. 48 (12): 2330-2340

- Sileshi, Z., Tegegne, A., and Tsadik, G. T. (2003). Water Resources for Livestock in Ethiopia: Implications for Research and Development. Conference Papers from International Water Management Institute. 66-79
- Sirohi, S. K., Karim, S. A., and Misra, A. K. (1997). Nutrient Intake and Utilisation in Sheep Fed with Prickly Pear Cactus. *Journal of Arid Environments*. 36: 161–166
- Skadhauge, E., Clemens, E., and Maloiy, G. M. O. (1980). *The Effects of Dehydration on Electrolyte Concentrations and Water Content Along the Large Intestine of a Small Ruminant: The Dik-dik Antelope*. *Journal of Comparative Physiology*. 135 (2): 165-173
- Stacewicz-Sapuntzakis, M., Bowden, P. E., Hussain, E. A., Damayanti-Wood, B. I., and Farnsworth, N. R. (2001). Chemical Composition and Potential Health Effects of Prunes: A Functional Food? *Critical Reviews in Food Science and Nutrition*. 41 (4): 251-286
- Stewart, A. V. (1996). Plantain (*Plantago lanceolata*) – A Potential Pasture Species. *Proceedings of the New Zealand Grassland Association*. 58: 77-86
- Szucs, I., Escobar, M., Leonardos, D., Crain, S., and Grodzinski, B. (2013). Photosynthetic Labeling and Partitioning of Major Sugars and Monoterpenes in Leaves of *Plantago lanceolata* L. *Photosynthesis Research for Food, Fuel and Future*. 15: 304-308
- Tamura, Y., and Nishibe, S. (2002). *Changes in the Concentrations of Bioactive Compounds in Plantain Leaves*. *Journal of Agricultural and Food Chemistry*. 50: 2514-2518
- Todd, W. R., Myers, J., and West, E. S. (1939). On the Metabolism of Sorbitol and Mannitol. *Journal of Biological Chemistry*. 127: 275-284
- Verbalis, J. G. (2003). Disorders of Body Water Homeostasis. *Best Practice and Research Clinical Endocrinology and Metabolism*. 17 (4): 471-503
- Wilman, D., and Derrick, R. W. (1994). Concentration and Availability to Sheep of N, P, K, Ca, Mg, and Na in Chickweed, Dandelion, Dock, Ribwort, and Spurrey, Compared with Perennial Ryegrass. *Journal of Agricultural Science*. 122: 43-49
- Wilman, D., and Riley, J. A. (1993). *Potential Nutritive Value of a Wide Range of Grassland Species*. *Journal of Agricultural Science*. 120: 43-49
- Wittner, M., Di Stefano, A., Wangemann, P., and Greger, R. (1991). *How Do Loop Diuretics Act?* *Drugs*. 41 (3): 1-13

Yancey, P. H., and Burg, M. B. (1989). *Distribution of Major Organic Osmolytes in Rabbit Kidneys in Diuresis and Antidiuresis*. The American Journal of Physiology. 257 (4): 602-607.

Zhou, Y. C., and Zheng, R. L. (1991). *Phenolic Compounds and an Analog as Superoxide Anion Scavengers and Antioxidants*. Biochemical Pharmacology. 42 (6): 1177-1179