Causes of elevated coumestrol in lucerne and mitigation of the subsequent risk to ewe reproductive performance

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Rachel Lilian Fields

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Coumestrol in lucerne can reduce the ovulation rate of ewes, which lowers lambing rates. This thesis isolated the factors with potential to increase the coumestrol levels of lucerne. The management of ewes during the mating season to avoid suppressed ovulation was also investigated. This enabled management strategies to be developed to mitigate the risk of depressed ovulation rate in ewes grazing lucerne during the mating season.

Agronomic factors investigated were lucerne cultivar, cutting frequency, development stage, aphid herbivory, fungal infection, water stress, and fungicide application. Fungal pathogens explained most of the differences in coumestrol with a moderate relationship between fungal damage and coumestrol content in field and glasshouse experiments. Lucerne inoculated with *Stemphylium* sp. had high coumestrol levels, with 169 ± 25.1 mg/kg DM measured in entire shoots, compared with 3.4 ± 0.84 mg/kg in control plants. Pea aphids were a minor cause of a coumestrol response, with an increase from 2.4 ± 0.39 mg/kg DM to 5.3 ± 0.65 mg/kg DM measured in a glasshouse experiment. Flowering did not produce a coumestrol response, with simultaneous increases observed in vegetative and flowering plants. Wilted lucerne had a coumestrol content of 1.3 ± 0.43 mg/kg DM which was not different to well-watered lucerne (2.2 ± 0.59 mg/kg DM) and only increased to 3.0 ± 0.57 mg/kg DM with further stress.

Despite fungal pathogens causing the main coumestrol response, carbendazim fungicidal treatments predicted to decrease coumestrol accumulation were ineffective. In leaves inoculated with stemphylium a newer cultivar ‘Stamina 5’ had higher coumestrol (396 ± 82.4 mg/kg DM) than ‘Wairau’ (143 ± 35.6 mg/kg DM), a 40 year old industry standard. In the field, five cultivars currently on the market did not differ in coumestrol content throughout a growing season and all reached at-risk levels (> 25 mg/kg DM) with an average content of 56.2 ± 3.24 mg/kg DM by late-May 2015. Removal of herbage was an effective method to reduce coumestrol as regrowth material typically had lower levels than the prior herbage.

Based on the field data, a model which used relative humidity and rainfall terms was created to predict when lucerne was likely to have heightened coumestrol. From this model a risk assessment analysis was created for a range of New Zealand locations. The assessment showed that four week old lucerne regrowth was at lower risk of elevated coumestrol than the standard six week regrowth which ewes are typically grazed on. Blenheim was least likely of the four locations to have at-risk levels of coumestrol and in all locations except Napier, the risk of high coumestrol increased as the autumn progressed. This would have greatest effect in Otago/Southland, where mating is concentrated late in the season. Indirect prediction of coumestrol content was also identified through ewe lambs that showed increased growth of teats and udder protrusion as an indicator of the crop oestrogenicity.

Ideally, high quality forage provided by lucerne is beneficial for live weight and thus reproductive performance, but grazing must avoid coumestrol-induced impairment of ovulation rates. A grazing experiment removed ewes at different intervals prior to a CIDR-induced ovulation. This experiment demonstrated that the risk of impaired lambing performance due to moderately oestrogenic lucerne consumption decreased with time on grass prior to ovulation. Removal of ewes two weeks prior to ovulation sufficed to mitigate the risk of decreased lambing performance in this situation.

The main results of this thesis indicate coumestrol levels are elevated by fungal infection of lucerne, but grazing management can be utilised to mitigate the effects on ewe reproductive performance.

Keywords: alfalfa, carbendazim, coumestan, development, drought, dryland, flower, flushing, grazing management, *Medicago sativa* L., nutrition, ovulation, phyto-oestrogen, sheep, *Stemphylium,* water stress.

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Declaration

A substantial amount of the data from the on-farm study described in Chapter 7 has been published in *Journal of New Zealand Grasslands** and therefore includes contributions from co-authors. I was involved in all aspects of the study including sample collection and animal measurements, assay of coumestrol levels and statistical analyses, plus the drafting and final edits of the manuscript. Technical assistance was provided for animal handling and the farm owners were responsible for bringing this event to my attention.

Publications

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Chapter 1

General Introduction

Lucerne (*Medicago sativa* L.) is a high quality, high yield forage legume promoted for use in New Zealand dryland pasture systems. In New Zealand, lucerne was widely used for the majority of the 20th century, with a peak of 220,000 ha during the mid-1970s. Lucerne use then declined, mainly due to susceptibility of the available cultivars to pests and diseases (Douglas, 1986). With the advent of new cultivars, bred specifically for resistance to these factors (Purves and Wynn-Williams, 1989), and renewed interest in productive forages for dryland systems, demand for lucerne has steadily increased throughout New Zealand since the turn of the 21st century, with an estimated 150,000 ha now in lucerne (Monk *et al.*, 2016).

A potential impediment to the further adoption of lucerne as the main pasture source in dryland sheep systems is a perception that it can reduce ewe reproductive performance. Lucerne has been shown to produce a phyto-oestrogen called coumestrol which can reduce the fecundity of grazing ewes through decreased ovulation rate, and therefore lambing rate (Coop, 1977; Scales *et al.*, 1977; Smith *et al.*, 1979). The effect of coumestrol on ovulation rate is temporary and animals fully recover upon removal from the lucerne (Coop, 1977; Adams, 1995). Thus, farmers are generally advised not to feed ewes on lucerne in the weeks prior to and during mating. However, as lucerne is a high quality forage it can also be used to increase live weight gains of grazing stock relative to other non-oestrogenic but potentially lower quality forages, such as grasses. Heightened ewe live weight increases ovulation (Rattray *et al.*, 1980; Thompson *et al.*, 1990) and for this reason, prior to mating, ewes are often put on high quality pastures such as lucerne. This is particularly relevant in dry summer/autumn periods when lucerne may be the only feed available (Brown *et al.*, 2005; King *et al.*, 2010).

Coumestrol levels within lucerne herbage are highly variable. When coumestrol levels are low, mating ewes on lucerne benefits lambing rate (Smith *et al.*, 1979). However, when coumestrol levels are high, lucerne can be detrimental (Coop, 1977; Scales *et al.*, 1977; Smith *et al.*, 1979). Coumestrol levels are elevated in response to fungal infection (Hanson *et al.*, 1965; Bickoff *et al.*, 1967; Sherwood *et al.*, 1970; Saba *et al.*, 1972), and research indicates other factors including aphids and developmental stage may also cause an increase (Bickoff *et al.*, 1960a; Hanson *et al.*, 1965; Loper, 1968). The ability to identify whether a lucerne crop on offer has high or low coumestrol, prior to and during mating, plays a large role in decisions of whether or not to graze lucerne with a reproductive stock class.

1.1 Research aims and objectives

The main aim of this thesis was to identify lucerne crops that may be at risk of high coumestrol content. From this, a second aim was to develop management strategies to mitigate the risk of depressed ovulation from ewes grazing lucerne before and during mating.

This thesis is presented in nine chapters. Chapter 2 is a review of the literature about phyto-oestrogens and the responses of ruminants upon ingestion, with particular emphasis on coumestrol in lucerne and its effect on ewe reproduction. Chapter 4 is a description of the field sites and methodology. In addition, five results chapters (Chapter 3 & Chapters 5-8) are presented. These chapters relate to the following specific objectives developed from the aims:

Objective 1: To refine methods used for coumestrol measurement (Chapter 3):

- Experiment 1 Cellulase versus methanol-only extraction
- Experiment 2 Freeze-dried versus oven-dried material
- Experiment 3 Sonication length
- Experiment 4 Pre-HPLC processing
- Experiment 5 Bioassay and HPLC validation

Objective 2: To isolate factors which increase the risk of high coumestrol in lucerne and identify strategies to minimise coumestrol accumulation (Chapter 5):

- Experiment 6 Cutting frequency and development stage
- Experiment 7 Cultivar
- Experiment 8 Fungicide and insecticide treatment
- Experiment 9 Lucerne infected with fungal pathogens
- Experiment 10 Water stress and recovery from stress
- Experiment 11 Aphids

Objective 3: To develop a predictive tool to estimate coumestrol content of lucerne:

- Chapter 6: Modelling coumestrol in lucerne

Objective 4: To quantify animal responses to elevated coumestrol:

- Chapter 7: Experiment 12 Morphological response of ewes to coumestrol
- Chapter 8: Experiment 13 Ewe fecundity after removal from lucerne

Finally, Chapter 9 provides a general discussion of how the results of this study could be applied in sheep farming systems and opportunities for further research.

Chapter 2

Review of the Literature

This chapter reviews the literature on factors that affect phytoestrogen content in plants, and the effects of those phyto-oestrogens on the ruminants that consume them. The review focuses on lucerne (*Medicago sativa* L.) predominantly, but also considers research with other forages that contain phyto-oestrogens. Finally, this review discusses the research required to identify high coumestrol lucerne crops and to reduce the negative effects of coumestrol on reproductive performance, which forms the basis of the experimental chapters in this thesis.

2.1 Agronomy of common oestrogenic forage legumes

Legumes are grown in pasture systems because they can fix nitrogen through symbiosis with rhizobia. Nitrogen fixation makes legumes high quality, high protein feed sources (Evans and Barber, 1977), and increases the nitrogen status of the soil. This improves the production and quality of other pasture species, such as perennial ryegrass (*Lolilum perenne* L.).

Many forage legumes produce phyto-oestrogenic compounds. Lucerne, red clover (*Trifolium pratense* L.) and subterranean clover (*T. subterraneum* L.) are the main forage legumes that may affect ruminant reproductive performance in New Zealand and Australian dryland pasture systems. This section discusses the growth forms and agronomy of these species.

2.1.1 Lucerne

Lucerne, also known as alfalfa, is a high nutritional quality perennial legume species with a deep taproot which allows it to reach soil moisture that species with shallower rooting depths cannot. Lucerne has comparatively high yield with annual yields of 20 to 28 t DM/ha recorded in Canterbury, New Zealand under irrigation (Brown *et al.*, 2000).

Lucerne is typically grown as a pure sward, although in grazing systems it is sometimes used in mixtures with a grass species (Dunbier, 1983). Direct grazing of lucerne is uncommon in Europe and the United States where most lucerne is cut for hay or silage. However, in New Zealand, Australia and Argentina lucerne is an important forage crop grazed by sheep, beef cattle and dairy cattle (Dunbier, 1983).

Lucerne produces erect stems from a crown at the base of the plant. This means that it is not recommended for set stocking as a rotational grazing system is required to allow the plant to regenerate from new basal shoots (Moot *et al.*, 2003b). In addition, lucerne mobilises its root reserves for spring production and in autumn will replenish these reserves with increased partitioning of photosynthates below ground (Brown *et al.*, 2006). To facilitate this, management advice is to spell the crop in late summer to early autumn until flowering to enable the plant to replenish its root reserves, which improves stand persistence for regrowth in subsequent years (Moot *et al.*, 2003b).

Lucerne produces the phyto-oestrogen coumestrol, a potent phyto-oestrogen that can affect ewe reproductive performance. A background on the chemical structure of coumestrol is provided in Section 2.2.1. The agronomic factors that are implicated to cause and control coumestrol accumulation in lucerne are described in Section 2.6. The literature regarding the affect that coumestrol has on sheep that consume it are described in Section 2.7. Based on the literature, research undertaken in this thesis (Chapters 3 to 8) to improve lucerne grazing management is outlined in Section 2.8.

2.1.2 Red clover

Red clover is a short-lived perennial species (two to four years) that grows from a crown. Its growth form ranges from prostrate to erect. Red clover provides high quality feed and is often used for finishing stock or for dairy cattle.

Red clover yield is typically lower than lucerne with annual irrigated red clover yields of 15 and 20 t DM/ha recorded in Canterbury (Brown *et al.*, 2000). As with lucerne, red clover should be rotationally grazed to increase stem production from basal buds.

Red clover and subterranean clover (Section 2.1.3) produce the compound formononetin which is metabolised in ruminants to the phyto-oestrogen equol. Equol can cause reproductive organ disorders and infertility in ewes that consume it. A background on the chemical structures of formononetin and equol is provided in Section 2.2.1. The agronomic factors that are implicated to cause formononetin accumulation in subterranean and red clover are described in Section 2.4. The literature regarding the affect that formononetin has on sheep that consume it are described in Section 2.5. Due to awareness of the problems formononetin causes and extensive plant breeding to decrease formononetin, subterranean clover and red clover are no longer considered major issues to ewe reproductive performance, and are not the subjects of the research contained in this thesis. A discussion of possible future directions of research is however provided in Section 2.8.

2.1.3 Subterranean clover

In New Zealand, subterranean clover is the recommended annual clover species sown for dryland east coast hill and high country pastures where summer dry can be 3-5 months long (Chapman *et al.*, 1986; Lucas *et al.*, 2015). Subterranean clover is a winter annual species with a prostrate growth form. The plant germinates following autumn rainfall and grows through winter, with the main production period in spring. Subterranean clover sets seed from late spring and produces a burr that is pushed underground as it matures to bury its seeds**.** After seed-set the plants die to avoid the summer drought.

Management of subterranean clover, particularly in the first season after sowing, requires lax grazing in the spring when the plants are flowering and producing seed (Smetham and Dear, 2003). This allows the build-up of a large seed reserve in the soil which can last for many years. Adequate areas of open ground are necessary in the autumn for the plants to successfully re-establish after germination.

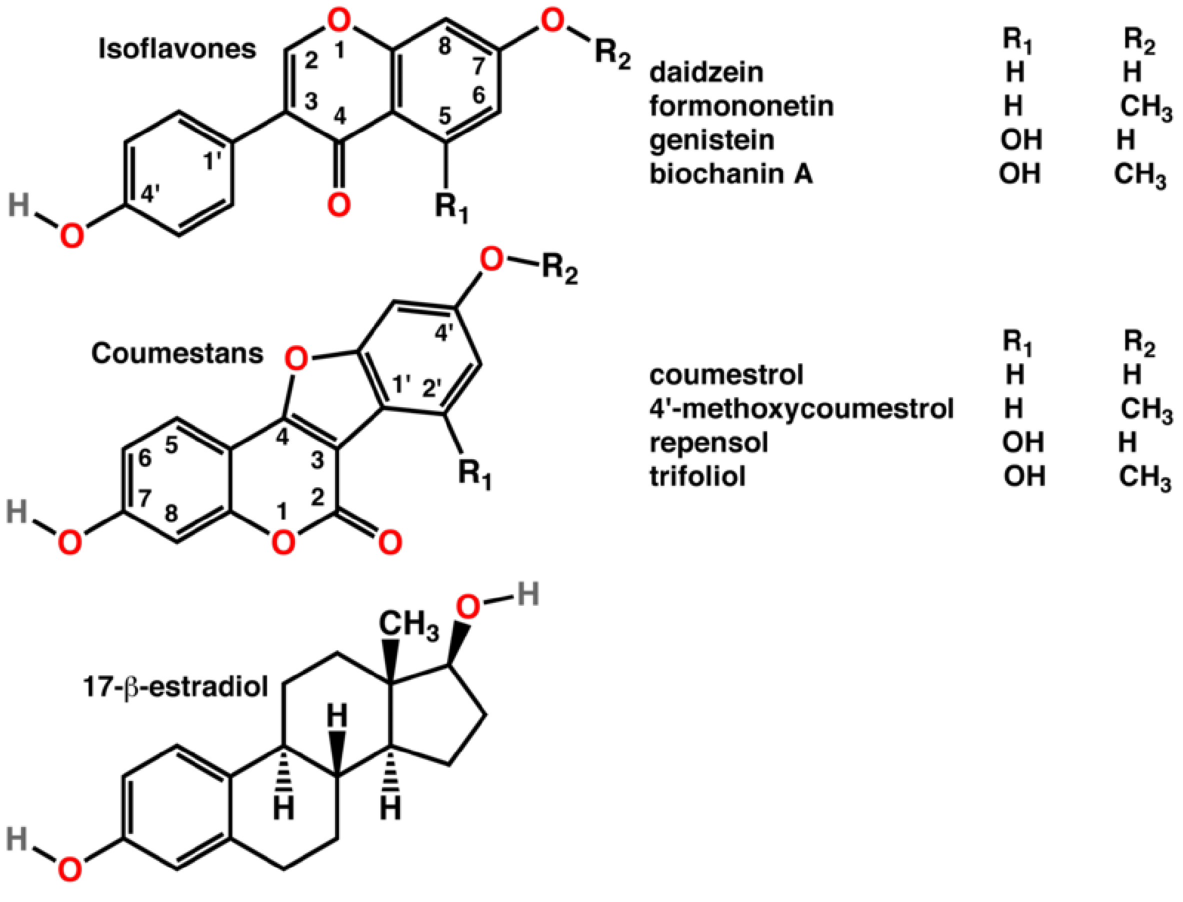
In New Zealand, pure sward yields of 1.8 t/ha in May-germinated subterranean clover and 7 t DM/ha in March-germinated clover were reported (Moot *et al.*, 2003a). In mixed pastures with cocksfoot (*Dactylis glomerata* L.), subterranean clover yields from 2.4 to 3.7 t DM/ha have been measured (Mills *et al.*, 2014).

2.2 Phyto-oestrogens

2.2.1 Phyto-oestrogen structures

The legumes described in Section 2.1 can all produce phyto-oestrogens. Phyto-oestrogens are plant produced, di-phenolic compounds, with structure and molecular weights similar to that of endogenous oestrogens, particularly 17β-oestradiol (E2), and the ability to bind to the oestrogen receptor (Adams, 1995; Turner *et al.*, 2007)**.** A flavonoid structure able to assume a planar configuration, with two hydroxyl groups at a similar distance to that in oestradiol, increases oestrogenicity, while methylation of the R2 group (Figure 2.1) decreases the oestrogenic effect (Miksicek, 1994; Kuiper *et al.*, 1998). Phytoestrogens can also be metabolised in the digestive system, which can either increase or decrease their oestrogenicity (Batterham *et al.*, 1965; Cox and Braden, 1974).

Chemical classes known to contain compounds with oestrogenic properties include coumestans, isoflavones and lignans. The phyto-oestrogenic compounds in lucerne are predominantly coumestans. In subterranean clover and red clover, phyto-oestrogens are mainly isoflavones.



	R_1	R_2
daidzein	H	H
formononetin	H	CH_3
genistein	OH	H
biochanin A	OH	CH_3

	R_1	R_2
coumestrol	H	H
4'-methoxycoumestrol	H	CH_3
repensol	OH	H
trifoliol	OH	CH_3

Figure 2.1 The chemical structures of the phyto-oestrogen containing chemical classes, isoflavones and coumestans, compared with 17β-oestradiol found in animals. Image from Wikimedia Commons.

2.2.1.1 Phyto-oestrogenic coumestans

Coumestrol is a potent phyto-oestrogenic coumestan and the main phyto-oestrogen in lucerne. It is also present in other perennial and annual *Medicago* species, soybean (*Glycine max* L. Merr.) and white clover (*Trifolium repens* L.) (Shutt *et al.*, 1969; Wong *et al.*, 1971; Thompson *et al.*, 2006).

Coumestrol is present in lucerne both in its active form and as 4’-methoxycoumestrol, which is de-methylated in ruminants to coumestrol (Adams, 1995). Coumestrol orients its two hydroxyl groups in the same position as the two hydroxyl groups in 17β-oestradiol. Coumestrol can range between 0-600 mg/kg DM, with highest concentrations primarily measured in diseased plant material (Hanson *et al.*, 1965; Saba *et al.*, 1972). Biological effects on ewe reproduction and reproductive organ development have been suggested to occur in lucerne at coumestrol levels greater than 25 mg/kg DM (Smith *et al.*, 1979).

2.2.1.2 Phyto-oestrogenic isoflavones

Isoflavones have two benzene rings linked through a pyran ring and are the main phyto-oestrogens in species which includes subterranean clover, red clover and soybean. Phyto-oestrogenic isoflavones include biochanin A, genistein, formononetin, and daidzein (Stochmal *et al.*, 2001). These compounds can be metabolised by ruminants to other oestrogenically active or inactive compounds.

Formononetin is an isoflavone that is detrimental to reproduction in ruminants. It is present in older cultivars of subterranean and red clover at levels of 1-2% of dry weight (Cox and Braden, 1974). Formononetin is the 4-methyl ether of daidzein, and has low phyto-oestrogenicity. However, in the rumen, formononetin is demethylated to daidzein and further metabolised to equol (Nilsson *et al.*, 1967; Dickinson *et al.*, 1988). Equol is oestrogenic and rapidly absorbed through the ruminal wall and gastrointestinal tract (Shutt and Braden, 1968; Adams, 1995). It can cause reduced lambing rates, permanent infertility, and prolapse of the uterus (Bennetts *et al.*, 1946; Lundh *et al.*, 1990; Adams, 1995). The proportion of formononetin converted to equol changes little with time and so the oestrogenicity remains high (Lindsay and Francis, 1969). With the release of low-formononetin cultivars of subterranean clover these severe clinical symptoms are now uncommon (Adams, 1995).

Biochanin A and genistein are present in subterranean clover, red clover and lucerne. Genistein along with daidzein are the main phyto-oestrogens in soybean (Thompson *et al.*, 2006). In the digestive tract, biochanin A is metabolised to genistein and then to the non-oestrogenic compound 4-ethylphenol (Batterham *et al.*, 1965). Biochanin A and genistein may produce oestrogenic effects in sheep for a few days following introduction to the pasture. However, once rumen microflora adjust, genistein and biochanin A are metabolised to 4-ethylphenol and are no longer oestrogenic (Lindsay and Francis, 1969; Cox and Braden, 1974). This means that their levels in forage are relatively unimportant compared to formononetin and coumestrol.

2.2.1.3 Phyto-oestrogenic lignans

Lignans are important dietary phyto-oestrogens for humans (Thompson *et al.*, 2006). Plant lignans of interest include secoisolariciresinol and matairesinol. These lignans can be metabolised by gut bacteria to the mammalian lignans enterolactone and enterodiol (Figure 2.2), which both have weak oestrogenic and anti-oestrogenic activity (Axelson and Setchell, 1981; Mousavi and Adlercreutz, 1992). The anti-oestrogenic activity of enterolactone and enterodiol may protect against breast and prostate cancers (Mousavi and Adlercreutz, 1992; Wang, 2002). Plant lignans that can act as phyto-oestrogen pre-cursors are found in many plants, with the richest sources in human food found in flax and sesame seeds (Thompson *et al.*, 2006). Other sources include cereals, brassicas, and soybeans. Mazur *et al.* (1998) suggested that secoisolariciresinol is present in lucerne seeds.

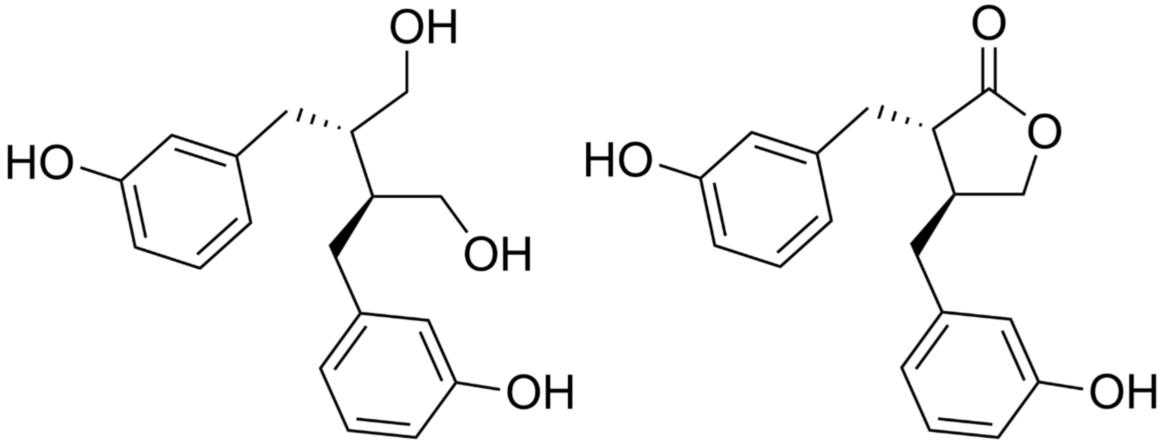


Figure 2.2 The chemical structures of enterodiol (left) and enterolactone (right), which are mammalian lignans with both oestrogenic and anti-oestrogenic activity. Images from Wikimedia Commons.

2.2.2 Oestrogen receptors

Phyto-oestrogens interact with oestrogen receptors. There are two classes of oestrogen receptor, ER-α and ER-β which differ in the C-terminal ligand binding domain and in the N-terminal transactivation domain. In humans, ER-α is located on chromosome 6 (Menasce *et al.*, 1993) and ER-β on chromosome 14 (Enmark *et al.*, 1997) and in sheep ER- α is on chromosome 8 (National Center for Biotechnology Information, 2017b) and ER- β is on chromosome 7 (National Center for Biotechnology Information, 2017a). The ligand, 17β-oestradiol interacts with both receptors while oestrone (E1) binds preferentially to ER-α and oestriol (E3) to ER-β (Zhu *et al.*, 2006).

Oestrogen receptors are present in both males and females and are in many different tissues, including non-reproductive tissues such as brain, liver, bone marrow, heart and lungs. The patterns of expression between the two forms are differential in some, but not all, tissues. For example, in the ovary of rats, ER-β mRNA expression was approximately five times greater than ER-α mRNA expression (Couse *et al.*, 1997), with ER-β expressed in ovarian granulosa cells and ER-α expressed in ovarian theca cells (Sar and Welsch, 1999). In the uterus, ER-α expression was predominant while ER-β was approximately 5% of that expressed in the ovaries (Couse *et al.*, 1997). In the male reproductive tract, ER-α and ER-β was detected in the prostate and epididymis, while only ER- α was detected in the testes (Couse *et al.*, 1997).

Oestrogen receptors in their inactive states are monomers with a heat shock protein (hsp90) that inhibits their action. Hsp90 stabilises proteins and inhibits degradation of unbound oestrogen receptors (Beliakoff and Whitesell, 2004; Sanchez, 2012). When a ligand such as oestradiol-17β binds to the oestrogen receptor, hsp90 is displaced and the receptor becomes a dimer with another oestrogen receptor. This can be a homodimer, or in tissues with co-expression of ER- α and ER-β, a heterodimer comprised of both receptor forms (Cowley *et al.*, 1997; Pettersson *et al.*, 1997). Once activated the oestrogen receptor complex can bind to oestrogen response elements (EREs) in DNA and regulate the activity of different genes (Kushner *et al.*, 2000).

2.2.3 Activation of oestrogen receptors by phyto-oestrogens

Phyto-oestrogens differ in both their binding affinity for oestrogen receptors and their ability to induce transcription through the binding of an oestrogen receptor to the oestrogen response element.

Many phyto-oestrogens have greater binding affinity for ER-β than ER-α. Kuiper *et al.* (1998) reported that compared with a normalised binding affinity of 17β-oestradiol for ER-α of 100, the binding affinity of coumestrol was 20, genistein was 4 and daidzein was 0.1. When compared with a normalised binding affinity of 17β-oestradiol for ER-β of 100, the relative binding affinity of coumestrol was 140, genistein was 87, and daidzein was 0.5. Formononetin and biochanin A had binding affinities less than 0.01 for both oestrogen receptor forms. Another study found that (*S*)-equol preferentially bound ER-β, with an affinity comparable to genistein (Muthyala *et al.*, 2004). The difference in binding affinity between the isoflavones was due to the presence or absence of the two hydroxyl groups. Compounds with one less hydroxyl group than genistein (daidzein, biochanin A) or two less hydroxyl groups (formononetin) had less binding affinity for both oestrogen receptor forms (Kuiper *et al.*, 1998).

Although some phyto-oestrogens have similar binding affinities to 17β-oestradiol they are not as potent. In the Ishikawa cell alkaline phosphatase assay (Markiewicz *et al.*, 1993), compared to oestradiol, coumestrol was 500 times less oestrogenic. Genistein, equol and daidzein were 1250, 1700 and 7700 times less oestrogenic, respectively.

Kostelac *et al.* (2003) reported the concentrations of the phyto-oestrogens that were required relative to 17β-oestradiol to activate the binding of the oestrogen receptor to the ERE. The concentrations of various ligands required to induce an increase in the binding of the oestrogen receptor to the ERE by 50%, relative to oestrogen receptors without a ligand, were:

- For ER-α: 17β-oestradiol (0.03 µM) > coumestrol (0.2 µM) > equol (3.5 µM) > genistein (15 µM) > daidzein (>300 µM)
- For ER-β: 17β-oestradiol (0.01 µM) > coumestrol (0.025 µM) > genistein (0.03 µM) > daidzein (0.35 µM) > equol (0.4 µM).

Lower concentrations of the phyto-oestrogens were required to activate the binding of ER-β to the ERE compared to ER-α Kostelac *et al.* (2003). In particular, 500 times more genistein or 800 times more daidzein was required to activate ER-α than ER-β. Of note however, is that daidzein is metabolised in the rumen to equol, which was only 8.8 times more effective at activating ER-β than ER-α. In addition, coumestrol at concentrations higher than 50 µM inhibited binding of ER-α to EREs. For ER-β coumestrol did not inhibit binding, although it was lowered.

As well as interacting with oestrogen receptors, phyto-oestrogens can regulate endogenous oestradiol concentration by binding or inactivating enzymes and modulate the bio-availability of hormones by increasing sex hormone-binding globulin (SHBG) synthesis, which binds to oestradiol and testosterone, inhibiting their function (Pino *et al.*, 2000; Low *et al.*, 2007).

2.2.4 Oestrogenic and anti-oestrogenic effects

Phyto-oestrogens can act in an oestrogenic or anti-oestrogenic manner. Anti-oestrogenic effects occur when phyto-oestrogens compete with 17β-oestradiol for oestrogen receptors because phyto-oestrogen bound receptors are less able to activate EREs than receptors bound with oestradiol (Tang and Adams, 1980). In contrast, oestrogenic effects occur when the 17β-oestradiol concentration is relatively low or absent. There is less competition for the receptor sites, and a net increase in activation of EREs occurs.

This may explain why oestrogenic effects tend to occur in sheep, which have relatively low levels of endogenous oestradiol, while anti-oestrogenic effects are usually reported in humans, who have relatively high circulating oestrogen concentrations (Adams, 1995). Studies have also suggested a biphasic effect of phyto-oestrogens (Mousavi and Adlercreutz, 1992; Hsu *et al.*, 1999; Miodini *et al.*, 1999). At low concentrations some phytoestrogens have estrogenic activity which stimulates cell growth, while at higher doses these phyto-oestrogens are anti-oestrogenic and inhibit cell growth.

The protective effects of the mammalian lignans enterodiol and enterolactone in humans against breast and prostate cancer are thought to be by competing with 17β-oestradiol for ER-β, by inducing

SHBG, and by acting as antioxidants. Breast cancer cells in culture with either 17β-oestradiol or a low concentration (0.5-2 µM) of enterolactone had proliferative growth, while both compounds together, or a high concentration (10 µM) of enterolactone, had no stimulatory effect (Mousavi and Adlercreutz, 1992). In postmenopausal women the oestrogenic lignans enterodiol and enterolactone were associated with increased SHBG and decreased plasma testosterone, while equol was associated with decreased oestradiol levels (Low *et al.*, 2007).

2.3 Determination of phytoestrogen concentration

Isoflavones can comprise up to 5% of the dry weight of pasture plants and are not difficult to measure, while coumestrol occurs in 10-fold lower concentrations and may also be more difficult to extract (Adams, 1995).

2.3.1 Material preparation

2.3.1.1 Storage of fresh samples

Hanson *et al.* (1965) compared effects of aerobic and anaerobic storage of freshly cut lucerne on coumestrol content. Heavily diseased lucerne was stored at 3°C and 29°C, in humid conditions. The aerobic storage of lucerne was comparable to unfavourable field-curing conditions which would normally result in mould and decomposition, while anaerobic conditions were compared to silage production conditions. Aerobic samples were placed in perforated cellophane bags, and anaerobic samples were placed in jars and air was replaced with nitrogen.

Coumestrol content of freshly cut lucerne ranged from 70 to 83 mg/kg DM. Under aerobic conditions, coumestrol content increased during the first two days of storage. Material stored at 3°C then levelled off slightly above the original amount while material stored at 29°C coumestrol content declined rapidly, reaching 26 mg/kg DM on day six. After eight days the 29°C samples had begun to decompose. Under anaerobic conditions coumestrol content of the material at 29°C remained elevated but was variable, while at 3°C coumestrol remained constant between days 4 and 12.

2.3.1.2 Sample Drying

The preparation of material can alter the phytoestrogen concentration of plant samples. Bickoff *et al.* (1960a) assayed lucerne as fresh forage and after oven drying. Although some of the samples lost no oestrogenic activity on drying, others lost as much as three quarters or more of their original oestrogenic activity. Drying time ranged from 45 minutes to 24 hours at 70°C, which depended on the maturity and original moisture content of the sample. Little additional loss after 24 hours as compared to 1 hour was found. Livingston *et al.* (1961) found that drying at 80°C in a forced-air oven degraded 25% of a coumestrol sample.

Bickoff *et al.* (1961) compared freshly harvested and oven dried subterranean and red clover samples. Samples were extracted with acetone for 24 hours. Drying the clover prior to bioassay did not reduce oestrogenic activity, and in some cases dried samples contained greater estrogenic activity than the fresh material. The greater retention of the activity of red and subterranean clover during prolonged storage or drying of the plant samples may reflect a greater stability of the isoflavones over coumestrol.

Based on these studies, plant material where a measurement of coumestrol is required should be stored in a cooler and not oven dried or stored fresh, while plant material for isoflavone measurements can be oven dried. However, this area is worth further research. Cayley and Bird (1996) recommend drying herbage samples at 60°C for chemical analyses of nutritive components, which is cooler than the temperatures used in the above experiments. The coumestrol yields from oven drying at 60°C versus freeze drying are tested in Chapter 3. Should a lower temperature of 60°C be successful then samples routinely measured from field experiments could also be assayed for coumestrol content.

2.3.2 Chemical assays

Chemical assays quantify the concentration of oestrogenic compounds. They do not quantify the oestrogenic potency of these compounds in organisms. Early research used semi-quantitative assays with paper or thin layer chromatography (Beck, 1964; Drane *et al.*, 1980; Adams, 1995). Detectable limits of 10 mg/L for genistein, daidzein and formononetin, and of 1 mg/L for coumestrol were reported for thin layer chromatography (Drane *et al.*, 1980).

High performance liquid chromatography (HPLC) is more accurate and now commonly used to measure concentrations of coumestrol and isoflavones in forage (Lookhart, 1980; Patroni *et al.*, 1982; Seguin and Zheng, 2006) and tissues (Lundh *et al.*, 1990). Most recently ultra-performance liquid chromatography (UPLC) methodology has been developed which has a shorter run time per sample and high sensitivity (Kiss *et al.*, 2010). This method provides a greater throughput than HPLC.

2.3.3 Bioassay

Biological assays are used to measure the concentration or potency of a substance by its effect on living tissues or cells. Bioassays can be used to give a measure of the oestrogenicity of pastures, taking into account the oestrogenic compounds, diet of the animal, metabolism of the animal, and relative potency of the oestrogenic compounds (Adams, 1995).

2.3.3.1 Animal assays

Uterine weight of mice was commonly used as an indicator of feed or compound oestrogenicity during the 1950s and 1960s (Bickoff *et al.*, 1959; Bickoff *et al.*, 1960a). However, ruminant metabolism differs from mice. Therefore the potency of oestrogen to sheep from consuming forage is likely to differ from that indicated by mouse uterine weights (Cox and Braden, 1974). Ruminant microflora can de-methylate methylated coumestans which increases biological activity and therefore pasture oestrogenicity (Cox and Braden, 1974). Breakdown of oestrogenic compounds also occurs, which can decrease pasture oestrogenicity (Cox and Braden, 1974).

Adams (1995) reviewed a range of bioassays that can be performed in sheep. Uterine weight of ovariectomised ewes gives an accurate indication of oestrogenic effects, however this is expensive. RNA/DNA ratios in uterine biopsy has been proposed as a way to reduce this cost. Weight of cervical mucus can give a rapid result, but is inaccurate after a couple of days due to cervical refractoriness. Increase in the teat length of wether lambs can give a measure of plant oestrogenicity. This is a sensitive and simple test, but considered imprecise (Adams, 1995).

2.3.3.2 Mammalian cells

Oestrogen-dependent mammalian cells grown in culture, such as breast cancer cells can be used to determine the concentrations at which oestrogenic compounds have oestrogenic and anti-oestrogenic effects, and in oestradiol and phyto-oestrogen competition assays to determine whether cell proliferation is inhibited by their combined action (Mousavi and Adlercreutz, 1992; Hsu *et al.*, 1999; Miodini *et al.*, 1999).

2.3.3.3 Yeast assay

A laboratory assay that uses a recombinant yeast (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen) was developed by Routledge and Sumpter (1996) to determine oestrogenic activity of surfactants. The assay has also been used for determining oestrogenic activity of plants and phyto-oestrogens (Breithofer *et al.*, 1998).

The mechanism of the oestrogen-induced system is shown in Figure 2.3. The yeast genome was modified with the human oestrogen receptor (hER) DNA sequence and with expression plasmids carrying an ERE sequence (Routledge and Sumpter, 1996). The ERE controls the expression of the *lac-Z* gene which encodes the enzyme β-galactosidase. Upon activation by an oestrogenic compound, hER binds to the ERE sequence on the plasmid. This initiates expression of lac-Z, which results in production of the enzyme β-galactosidase. This β-galactosidase is secreted out of the yeast cell and into the growth medium. In the growth medium β-galactosidase metabolises the yellow ‘chlorophenol red-β-D-galactopyranoside’ (CPRG) into a red product that can be measured by absorbance at 570 nm within 72 hours of yeast incubation. The assay was reported to be able to detect 17β-oestradiol concentrations as low as 2 ng/L (Routledge and Sumpter, 1996).

Compared to mammalian cells, yeast is more resistant to environmental factors, such as heavy metals, and to bacterial toxins (Zysk *et al.*, 1995). This makes it easier to assay field grown plants. As with mouse bioassays it does not take into account the metabolism of phyto-oestrogens in ruminants. The yeast assay can have results available within seven days of collection from the field, is not costly and can be used in labs without easy access to an HPLC machine. However due to its genetically modified status, in New Zealand, the yeast assay required an approved containment facility, biosafety training and compliance records.

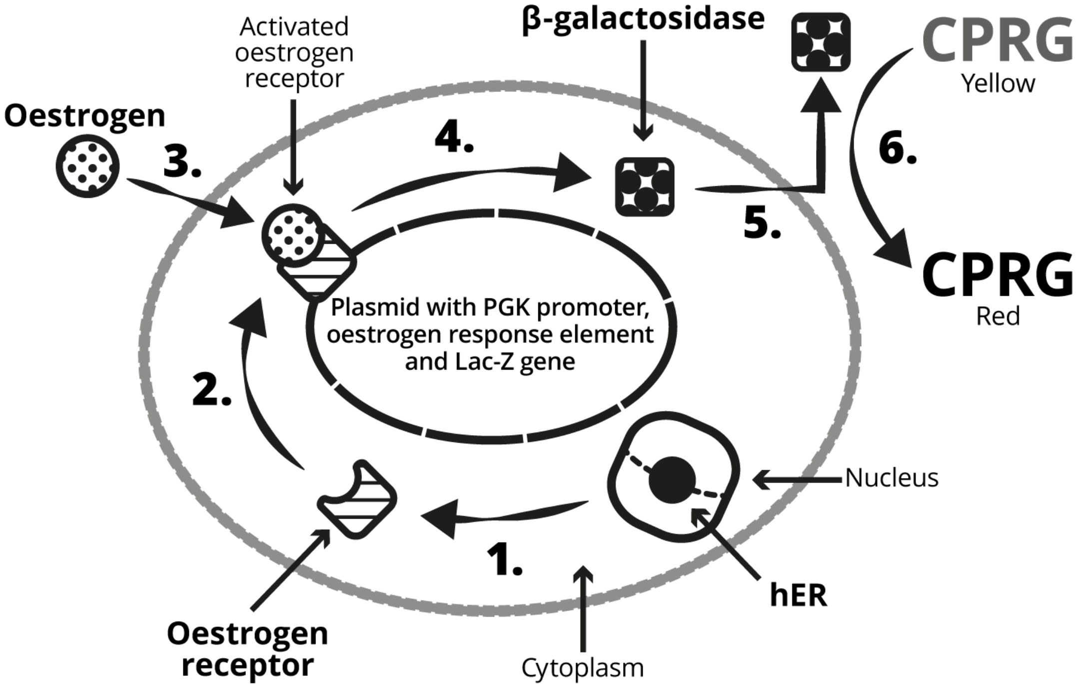


Figure 2.3 The oestrogen-induced expression system of the recombinant yeast bioassay, which causes chlorophenol red- β-D-galactopyranoside to change from yellow to red in the presence of oestrogenic compounds. Diagram commissioned from Adam Fields Illustration & Design for this thesis, 2017.

2.4 Factors which affect formononetin content of red and subterranean clovers.

Equol, a metabolite of formononetin, is the compound responsible for the oestrogenic effects of subterranean and red clovers. The most important factor that influences the formononetin content of these species is the cultivar. In the 1960s, analytical techniques to determine the levels of plant phyto-oestrogens were developed and it was recognised that there was a wide variation in the levels of formononetin among different cultivars of subterranean clover (Beck, 1964; Francis and Millington, 1965). By the 1970s, three low-formononetin subterranean clover cultivars had been commercialised in Australia and formononetin level was considered one of the easier criteria to satisfy in the selection of new cultivars (Lightfoot, 1974). Low-formononetin content is also a breeding objective in red clover, which has successfully led to lower levels in the current cultivars (McDonald *et al.*, 1994; Ford and Barrett, 2011). For both red and subterranean clovers, a formononetin concentration below 1.0 g/kg DM is preferred, to limit the likelihood of impaired fertility (Collings and Gladstones, 1984).

Agronomically, replacement of old oestrogenic subterranean clover pastures was more difficult than breeding the cultivars. This was because established clovers were well adapted to their environments and had built up large reserves of hard seed. The methods used to reduce the seedbank of undesirable cultivars included successive years of cropping, herbicidal sprays, and heavy spring grazing to prevent seed-set (Lightfoot, 1974).

Other factors that can affect formononetin content are nutrient deficiency and development stage, however these are unlikely to cause problematic levels in low-formononetin cultivars (Sivesind and Seguin, 2005). In both red and subterranean clovers, phosphate deficiency increased formononetin content (Rossiter and Beck, 1966; McMurray *et al.*, 1986). Sulphur and nitrogen deficiencies were also reported to increase formononetin content of subterranean clover (Rossiter, 1969; Rossiter and Barrow, 1972).

Maturity of red clover has been reported to affect formononetin content. Dedio and Clark (1968) found highest formononetin contents in red clover leaves during vegetative growth, which declined at flowering. McMurray *et al.* (1986) also reported that total formononetin content declined over time from 10.8 g/kg DM to 4.8 g/kg DM between a late vegetative and dying inflorescence stage, although increased to 7.9 g/kg DM at the final harvest a week later. In subterranean clover, the formononetin content decreased during plant growth for the cultivar ‘Dwalganup’ but increased for the cultivar ‘Yarloop’ (Rossiter and Beck, 1967).

2.5 The effect of isoflavones on ewes

2.5.1 Lambing performance

2.5.1.1 Permanent infertility

Oestrogenic subterranean and red clovers can cause two distinct forms of infertility: temporary infertility and permanent infertility (Lightfoot and Wroth, 1974).

Prolonged grazing of high formononetin clover, particularly subterranean clover, can lead to a condition termed 'clover disease'. Clover disease symptoms include permanent infertility, uterine prolapse, and dystocia (Bennetts *et al.*, 1946). Clover disease is progressive and permanent, with infertility which worsens with time spent on subterranean clover and symptoms that persist even after the ewes are removed from the oestrogenic pasture.

The main cause of infertility from clover disease is the failure of ovum fertilisation due to impaired sperm transport through the cervix (Turnbull *et al.*, 1966; Lightfoot *et al.*, 1967; Kaltenback and Davies, 1970). Turnbull *et al.* (1966) suggested that higher embryo mortality in the first 30-60 days may also contribute to low ewe fertility. However, Kaltenback and Davies (1970) did not observe a difference in embryo survival. The failure to come into oestrus and ovarian activity, as assessed by follicle development and ovulation rate, were not considered to be associated with permanent infertility (Lightfoot *et al.*, 1967).

Bennetts *et al.* (1946) reported lambing rates at tailing of a mob of ewes with long term exposure to subterranean clover decreased from 80% in 1941 to 57% in 1942 down to 8% in 1944. Similarly, Davenport (1967) reported that after three years on subterranean clover 'Dwalganup' 73% of ewes lambed, compared with 93% of ewes on oats. After five years the proportions of ewes that lambed were 46% and 87%, respectively. For red clover, Barrett *et al.* (1965) reported that over six years the proportion of ewes lambing fell progressively over time, from 87 to 25% in ewes grazing red clover for eight months per year, compared with a decline from 88 to 66% in control animals on non-oestrogenic pasture.

The proportion of clover in the sward also affected ewe fertility. Davies and Maller (1970) reported that after four years of grazing subterranean cultivar 'Yarloop' in grass mixes of various proportions, the percent of ewes pregnant on 100% clover was 54%, compared with 77% pregnant on 30% clover and 88% pregnant on the grass control. Shackell *et al.* (1993) reported that ewes grazing pasture containing 60% 'Pawera' red clover for four years had a higher proportion of ewes return to service at least once compared with ewes grazing pasture with 30% red clover and ewes in the control group. In the fourth year 49% of ewes grazing 60% red clover returned to service at least once, compared with 11% of the control and 16% of the 30% red clover treatment. Higher return to service rates continued in the ewes from the 60% red clover treatment in the following two years, despite no access to red clover. The proportion of ewes that did not lamb was also higher, with 34-62% of ewes in the 60% red clover group barren, compared with 7-25% and 5-29% in the 30% red clover and control treatments, respectively.

2.5.1.2 Temporary infertility

Temporary infertility occurs when animals are grazed on oestrogenic pasture during the mating period. It is characterised by an increase in returns to service, reduced incidence of oestrus and lower ovulation rate (Lightfoot and Wroth, 1974). However, some studies have reported no incidence of decreased ovulation rate due to formononetin. Holst and Braden (1972) reported that ewes grazing oestrogenic clover during the mating period instead had abnormal ovum transport, which may contribute to a decreased fertilisation rate. As with permanent infertility, sperm transport is impaired but fertility recovers after removal from the oestrogenic pasture (Lightfoot and Wroth, 1974).

Lightfoot and Wroth (1974) reported that ewes grazing subterranean clover ‘Dinninup’ with an average formononetin content of 9.1 g/kg DM had reduced incidence of oestrus compared with control animals (66% vs. 79%). Ovulation rates for ewes on the subterranean clover treatment were lower than those of the control animals (1.15 vs. 1.45).

Kelly *et al.* (1980) reported that 80% of ewes grazed on pure ‘Pawera’ red clover with 8 - 12 g formononetin/kg DM, from eight days before mating to 17 days after mating, were mated in the first cycle compared with 100% of ewes on a white clover/grass pasture. Over three cycles of mating 66% of ewes on ‘Pawera’ returned to service compared with 22% of control ewes. Mean ovulation rates in the first cycle were also lower for ewes grazing ‘Pawera’ than for control ewes (0.79 vs. 1.55). Only 25% of ‘Pawera’ ewes lambed in the first cycle compared with 75% of control ewes.

2.5.1.3 Performance of ewes on low formononetin cultivars of clover

With the advent of low formononetin cultivars, permanent infertility is no longer considered a major problem for grazing with subterranean and red clovers. Davies *et al.* (1970) compared high formononetin (8.6 - 13.6 g/kg DM) and lower formononetin (0.6 - 1.7 g/kg DM) subterranean clover cultivars, and reported that the proportion of ewes that conceived after four years grazing on the high formononetin clovers declined to 61%, compared with 85% on the low formononetin cultivars. The differences in conception rates between the two groups were significant from the second year on.

Low formononetin cultivars of red and subterranean clovers have also been shown to decrease the severity of temporary infertility. Clark (1965) compared the reproductive performance of ewes grazing ‘Dwalganup’ or ‘Bacchus Marsh’ subterranean clover for 17 days prior to mating and during the mating period. The lambing percentage of 'Dwalganup' was 74%, with 47% barren, compared with a lambing percentage of 90% on 'Bacchus Marsh', with 30% barren. The rate of return to service was also greater in 'Dwalganup' subterranean clover with 1.6 matings per lambing ewe on 'Dwalganup' compared with 1.42 on 'Bacchus Marsh'. The differences between the two cultivars of subterranean clover were attributed to a higher oestrogenicity in 'Dwalganup' than 'Bacchus Marsh'. Later measurements of the formononetin contents of 'Dwalganup' and 'Bacchus Marsh' were 12 and 1 g/kg DM, respectively (Nichols *et al.*, 2013).

Anwar *et al.* (1993) compared ovulation rates of low formononetin (3.4-5.9 g/kg DM) red clover cultivar 'G27', high formononetin (10.9-13.1 g/kg DM) cultivar 'Pawera' and ryegrass/white clover pastures. Ovulation rates were 1.63 for 'G27', 0.63 for 'Pawera', and 1.93 for ryegrass/white clover, in one trial; and 1.17, 0.31, and 1.53 in another. There was no significant difference between G27 and ryegrass/white clover.

McDonald *et al.* (1994) grazed ewes for six weeks prior to mating on 'Pawera', 'G27' or ryegrass white clover. There was no difference in ovulation rates between grazing treatments, but there was a higher incidence of returns to service in ewes that had previously been on 'Pawera' (73%) than G27 (33%). Lowest rates of return were observed in ryegrass/white clover (9.5%) which indicated some impairment was still occurring in the low formononetin 'G27' cultivar.

Today's red clover and subterranean cultivars have formononetin levels below those reported in these experiments. In red clover, levels of 1.0 g/kg DM were reported for 'Grasslands Relish' and 0.6 g/kg DM in 'Crossway' (Ford and Barrett, 2011). In subterranean clover, levels are below 0.5 g/kg DM in many subterranean cultivars including 'Denmark', 'Leura' and 'Narrikup' (Nichols *et al.*, 2013). This would be expected to further reduce the risk of grazing with these species. Therefore, subterranean and red clovers are not the focus of this thesis.

2.6 Factors which affect coumestrol content of lucerne

In lucerne, factors such as cultivar, development stage, insect herbivory, and fungal disease have all been reported to affect coumestrol concentrations. The previous research undertaken to assess these factors is described in this section.

2.6.1 Fungal pathogens

Fungal pathogens are considered the main factor that influences coumestrol content in lucerne (Hanson *et al.*, 1965; Sherwood *et al.*, 1970; Saba *et al.*, 1972). Environmental factors that make lucerne more susceptible to disease such as humidity, temperature and plant age can therefore also increase coumestrol. On the other hand, factors such as genetic resistance to fungal pathogens can reduce it.

2.6.1.1 Common fungal pathogens of lucerne in New Zealand

The following species are common foliar and stem diseases of lucerne in New Zealand. Root and crown pathogens, and species not commonly encountered in New Zealand are not described.

Common leaf spot

Common or Pseudopeziza leaf spot (*Pseudopeziza medicaginis* (Lib.) Sacc.) produces a dark brown 'cushion' or apothecium in the middle of the 1-3 mm lesion on lucerne leaflets (Stuteville and Erwin, 1990; Harvey and Harvey, 2009) (Figure 2.4). Common leaf spot has optimal growth in moist, cool conditions and although it does not kill lucerne plants, it can cause severe defoliation with yield reductions of 40% reported (Morgan and Parbery, 1977).

Figure 2.4 A leaflet infected with *Pseudopeziza medicaginis.* Photo: R. L. Fields, 2016.

Stemphylium leafspot

Stemphylium leafspot in lucerne is caused by pathogens including *Stemphylium botryosum* Wallr., *S. vesicarium* (Wallr.) Simmons*,* and *S. globuliferum* (Vestergr.) Simmons*.* It was originally thought that there were two biotypes of *S. botryosum* - cool temperature (Californian) and warm temperature (eastern North America) (Cowling *et al.*, 1981). Morphological and pathogenicity experiments comparing Australian and North American isolates suggested that the cool biotype was *S. vesicarium* (Irwin *et al.*, 1986; Irwin and Bray, 1991); however the biotype is a useful division of the two distinct stemphylium leafspot symptoms.

Warm temperature biotype stemphylium pathogens cause expanding concentrically ringed lesions surrounded by a light yellow halo on leaflets. In contrast, cool biotype stemphylium has lesions that are tan in the middle with a sharply defined brown margin (Figure 2.5). Cool biotype lesions do not expand once the border is formed and are approximately 3-5 mm in diameter (Cowling *et al.*, 1981; Stuteville and Erwin, 1990; Harvey and Harvey, 2009). Cool biotype stemphylium infection is common in New Zealand and affects mainly young leaves (Harvey and Harvey, 2009).

Figure 2.5 Cool biotype stemphylium leaf spot symptoms. Photo: R. L. Fields, 2016.

Lepto leaf spot

Lepto or pepper leaf spot is caused by *Leptosphaerulina trifolii* (Rostovzev) Petr., (1959). Leaf symptoms vary with the environment and physiological state of the leaf. Lesions (Figure 2.6) begin as small, black spots and may enlarge to 1-3 mm in diameter, with light brown or tan centres and a dark brown border. Lesions are often surrounded by a chlorotic area. In favourable conditions, the lesions enlarge and join together to produce a bleached area. This results in leaf senescence. Most damage occurs on leaves of young regrowth following harvest under cool, moist conditions (Stuteville and Erwin, 1990).

Figure 2.6 A leaf infected with *Leptosphaerulina trifolii*. Photo: R. L. Fields, 2016.

Anthracnose

Anthracnose (*Colletotrichum trifolii* Bain, (1906); *C. destructivum* O'Gara, (1915)) spreads in warm, humid weather, with an optimum temperature range of 25-28°C (Stuteville and Erwin, 1990; Harvey and Harvey, 2009). Symptoms range from small and irregularly shaped blackened areas to large diamond-shaped straw-coloured lesions. Lesions may join together and kill stems that become encircled. Within the lesions, black fruiting structures called acervuli are produced which are visible to the eye. Rain and dew wash spores from the acervuli onto uninfected stems (Stuteville and Erwin, 1990).

Spring black stem

Spring black stem is caused by *Phoma medicaginis* Malbr. & Roum. var. *medicaginis* Boerema. Spring black stem affects both the stem and leaves of lucerne. Symptoms begin with small, dark coloured spots on the lower leaves and stems (Stuteville and Erwin, 1990; Harvey and Harvey, 2009). Lesions expand along the stem (Figure 2.7) and leaves, and can cause severe defoliation and yield losses of over 40% (Hijano, 1981). Growth of spring black stem occurs in cool, moist conditions, and despite its name it can occur in autumn (Stuteville and Erwin, 1990).

Figure 2.7 Spring black stem lesions on a section of lucerne stem. Photo: R. L. Fields, 2016.

Yellow leaf blotch

Yellow leaf blotch is caused by *Leptotrochila medicaginis* (Fuckel) H. Schüepp and occurs in lucerne stands with high humidity in the canopy (Stuteville and Erwin, 1990; Harvey and Harvey, 2009). The initial symptoms of the disease are small yellow spots on the leaves which expand into yellow to brown streaks between the leaf veins. Within the lesions small apothecia are produced. Ascospores are ejected from the apothecia to land on new host tissue.

Downy mildew

Downy mildew is caused by *Perononspora trifoliorum* (de Bary, (1863)) under cool, wet or humid conditions (Stuteville and Erwin, 1990; Harvey and Harvey, 2009). It seldom causes forage loss, and usually only before the first cut in spring. Symptoms of downy mildew are twisting and curling of upper leaves and grey to light purple downy growth on the undersides of the leaflets (Harvey and Harvey, 2009).

Verticillium wilt

Verticillium wilt (*Verticillium albo-atrum* Reinke & Berthier) can reduce yields and shorten the life span of a lucerne stand. It grows between 15-30°C and is most serious in irrigated lucerne crops (Stuteville and Erwin, 1990). Early symptoms are a V-shaped chlorosis at the tips of leaflets as the disease progresses and leads to total leaflet necrosis. New leaves may grow from the axils of symptomatic leaves, as the infected stem remains green until all leaves are dead (Stuteville and Erwin, 1990).

2.6.1.2 The effect of fungal pathogens on coumestrol content

Loper and Hanson (1964) found that non-infected lucerne grown under a wide range of environmental conditions had low levels of coumestrol. However, coumestrol content increased with infection of common leaf spot. They reported that leaves with two or more lesions per leaflet had a coumestrol content of 184 mg/kg DM, while leaves with no lesions had 1.1 mg/kg DM. They also reported that infection with lepto leafspot increased coumestrol accumulation.

Hanson *et al.* (1965) investigated a range of lucerne pathogens in greenhouse experiments. Lucerne clones were used, with one healthy and one infected. Four fungi (spring black stem, common leafspot, lepto leafspot and stemphylium leafspot), and one virus (alfalfa yellow mosaic virus) were tested. It was not stated whether the stemphylium symptoms were of the warm or cool temperature biotype, as the distinct types had not yet been determined. However the experiment took place on the east coast of the USA where the warm biotype symptoms are most commonly found. The fungi caused leaf spots and senescence; and some produced stem lesions. The virus caused leaf mottling. Fungal infection caused an increase in coumestrol content, while the virus did not. In healthy plants coumestrol was not detected in most cases. Plants infected with spring black stem had the highest coumestrol content with an average of 200 mg/kg DM, compared with 0.7 mg/kg DM for the healthy plants. Coumestrol was detected in only two of 17 healthy samples. All of the fungal pathogens except lepto leafspot consistently caused coumestrol in infected plant material with levels ranging from 9 to 219 mg/kg DM, and an average of 47 mg/kg DM.

Loper *et al.* (1967) reported that although coumestrol increased with rust (*Uromyces striatus* Schröt.) infection, it was sometimes unexpectedly low in leaves with high levels of sporulation. It was suggested that the coumestrol was removed from the plant within spore discharge, as remnant spores collected in containers contained more than 400 mg/kg DM. Bickoff *et al.* (1967) also reported that when inoculated with rust, the coumestrol levels were not high and attributed this to the hypothesis, of Loper *et al.* (1967), that it was lost in spores.

Sherwood *et al.* (1970) found that coumestrol accumulated in lucerne in response to inoculation with all pathogenic fungi tested including spring black stem, Cylindrocladium root and crown rot (*Cylindrocladium scoparium* Morgan), anthracnose, and rust. The concentration of coumestrol was related to the severity of infection. When inoculated with non-pathogenic fungi, or with a pathogenic bacterium (*Xanthomonas alfalfae* (Riker *et al.* 1935) Schaad *et al.* 2007) little coumestrol accumulated (Sherwood *et al.*, 1970). No coumestrol accumulated in response to alfalfa mosaic virus, stem nematode (*Ditylenchus dipsaci* (Kuhn)), or mechanically injured leaves (Hanson *et al.*, 1965;

Sherwood *et al.*, 1970). Coumestrol was not translocated from areas of the plant with foliar disease to other areas (Sherwood *et al.*, 1970).

2.6.1.3 Management of fungal pathogens

Hanson *et al.* (1965) compared coumestrol content in diseased lucerne with fungicide (Dithane M-45) treated lucerne. Fungicide was applied weekly, from 15 cm stem height to nine days post full bloom. At the full bloom stage, senescence of unsprayed lucerne was in the lower 25 cm while the sprayed lucerne senescence was in the lower 13 cm. Ten days after full bloom no further senescence had occurred in the sprayed lucerne, while senescence in the unsprayed had increased to the lower 30 cm. Overall plant height ranged from 45 to 55 cm between early bud and 10 days post-bloom. Coumestrol content of leaves and stems in unsprayed lucerne increased with successive stage of growth, with the greatest increase in the stems. Coumestrol contents averaged over growth stages were 28.9 and 72.9 mg/kg DM for sprayed and unsprayed treatments, respectively.

Bickoff *et al.* (1967) inoculated ‘Atlantic’ and ‘Cayuga’ lucerne with common leafspot. Coumestrol content increased from 2 mg/kg DM to 49 mg/kg DM after 12 days and to 219 mg/kg DM after 18 days in ‘Atlantic’, and from 2 to 50 to 137 mg/kg DM in ‘Cayuga’. The difference between the cultivars was attributed to the higher disease resistance of ‘Cayuga’, which had less severe symptoms. Loper *et al.* (1967) also reported that selection over four recurrent cycles for increased resistance to common leafspot resulted in fewer leafspots and lower coumestrol content of plants from two gene pools.

Purves *et al.* (1981) tested the effects of cultivar, cutting management and fungicide application on the amount of fungal infection and coumestrol content of lucerne between February and April 1980. Cut treatments were mown in March, halfway through the two month trial, and fungicide was applied every 10 days, with aphid control when necessary. The main diseases during the experiment were common leaf spot, stemphylium leaf spot and yellow leaf blotch, and at the end of March following heavy rainfall downy mildew was observed. The authors found a correlation between the severity of foliar infection and coumestrol content, and lower coumestrol levels in the more disease resistant cultivar ‘Saranac’. Cutting and fungicide decreased coumestrol levels, however the fungicide applications used were not considered economic.

2.6.2 Aphids

It has been reported that aphids have increased coumestrol levels and that cultivars resistant to insect attack accumulate less coumestrol in the presence of lucerne aphids.

Loper (1968) investigated coumestrol accumulation due to aphids in lucerne cultivars with different aphid resistance. The tissues damaged by pea aphid (*Acyrthosiphon pisum* (Harris)) or spotted alfalfa aphid (*Therioaphis maculata* (Buckton)) had coumestrol concentrations between 41 and 119 mg/kg DM, while tissues with no visible aphid damage had less than 2 mg/kg DM coumestrol. Coumestrol contents of the total above ground growth of the aphid susceptible cultivar 'Vernal' was 67 mg/kg DM with pea aphid infestation and 51 mg/kg DM with spotted alfalfa aphid, but in aphid resistant 'Nevada Syn T-P' coumestrol was < 1 mg/kg DM for pea aphid and 6 mg/kg DM for spotted alfalfa aphid. This means that selection for aphid resistance could reduce coumestrol accumulation (Loper, 1968).

Kain and Biggs (1980) reported that infestations of pea aphid and blue green aphid in both field and glasshouse experiments increased the coumestrol content of 'Wairau' lucerne. In glasshouse experiments blue green aphids caused higher coumestrol levels than similar populations of pea aphids. They also found no difference in the coumestrol levels between leaf and stem tissues. In their field experiments, aphidicide treatments were effective at controlling the aphid populations and preventing coumestrol accumulation. The earlier sprays, when approximately 5-10 aphids per stem were present, were more effective than spraying when 30 aphids per stem were present.

2.6.3 Stage of development

Stage of development has been reported to affect lucerne coumestrol content. The flowering stages were typically reported to have highest coumestrol content, while early vegetative stages may also have had relatively higher contents than mid to late vegetative stages.

Bickoff *et al.* (1960a) compared the estrogenic activity of lucerne at different stages of maturity in six consecutive crops. Crops one to four took place between spring and autumn during 1958; crop five over winter; and crop six over the first spring growth of the 1959 season. A bioassay measuring the uterine weight of mice was used to determine apparent coumestrol content.

Apparent coumestrol content had a similar pattern among crops. The apparent coumestrol content was low in the vegetative stages and increased as the plant matured. Coumestrol content was usually greatest at the full bloom or seed head stages. The third crop had an increase in coumestrol content from 6 mg/kg DM in the vegetative stage to 108 mg/kg DM in the early bud stage. Coumestrol then remained between 89 and 142 mg/kg DM through the remainder of the growth cycle. Crops one, two, four and six also had low coumestrol (1-13 mg/kg DM) during vegetative growth and the levels did not increase until at least ¼ or ½ bloom. The winter growth attained high levels of coumestrol without flowering (80 mg/kg DM at the dormant pre-bud stage). The 1959 crop did not attain more than one-third of the peak coumestrol of the comparable 1958 crop, with a peak of 44 mg/kg DM.

Hanson *et al.* (1965) also reported an increase in coumestrol content with successive development stages. Peak coumestrol was observed 25 days after full bloom in Nebraska and Pennsylvania. They suggested that this could be due to greater degrees of fungal disease with crop age, and noted that at their California and Utah sites there was lower severity of disease and lower coumestrol.

Seguin *et al.* (2004) reported that coumestrol had a quadratic response to increasing stage of maturity in field grown lucerne. They collected material between the late vegetative (>30 cm tall) and late seed pod developmental stages. Coumestrol concentrations ranged between 50 and 135 mg/kg DM. Coumestrol was high at late vegetative stage and late seed pod and low at early flowering.

In contrast, Loper and Hanson (1964), using lucerne grown in a controlled environment, did not find a relationship between development stage and coumestrol content, though noted a tendency for lower coumestrol in the vegetative stage of growth.

2.6.4 Plant part

Loper and Hanson (1964) reported that in uninfected lucerne plants there was no difference in coumestrol content between leaves and stems, and that no coumestrol was found in the flowering racemes. In contrast, lucerne infected with leafspot diseases had lower levels of coumestrol in the stems with no or only a few lesions present (5.7-7.2 mg coumestrol/kg DM), than in the lightly to heavily infected leaves (28.5-183.7 mg coumestrol/kg DM).

Seguin *et al.* (2004) reported no difference in coumestrol concentration between flowers, stems, and leaves. Highest coumestrol content was observed in the top segment (61 mg/kg DM), intermediate levels in the 40-60 cm segment (41 mg/kg DM) and lowest below this (average 25 mg/kg DM). This was in contrast to the expectation of highest fungal infection and therefore coumestrol, in older leaves and material exposed to high humidity below the canopy.

2.6.5 Water stress

Coumestrol has been shown to respond to water stress in the roots of hydroponically grown soy bean (Tripathi *et al.*, 2015; Tripathi *et al.*, 2016). For example, Tripathi *et al.* (2015) noted an increase in coumestrol content of soybean roots after three hours dehydration from 0.15 to 0.55 mg/kg FW, as estimated from a graph. Soybean plant leaves did not have increased coumestrol in response to dehydration. Coumestrol content of lucerne under water stress has not been examined. Despite a lack of evidence for or against coumestrol accumulation in water stressed lucerne, some sheep farmers in New Zealand conservatively avoid mating stock on water stressed lucerne.

2.6.6 Mechanism of coumestrol accumulation

Coumestrol has no demonstrated fungicidal or insecticidal activity (Loper, 1968; Sherwood *et al.*, 1970). He *et al.* (1998) reported that stresses which increase coumestrol affect the transcription of enzymes in coumestan synthesis pathways (e.g. isoflavone 7-O-methyltransferase) causing increased medicarpin, a phytoalexin (antimicrobial compound) and the precursor to coumestans. This suggests that an increase in coumestrol is part of a general increase in flavonoid production in affected leaves and stems. Lucerne cultivars least resistant to a given stress develop the most coumestrol when exposed to that stress (Loper, 1968; Purves *et al.*, 1981) and breeding to reduce coumestrol accumulation should not have a negative impact on the defence system of lucerne.

Coumestrol is not translocated around the plant. This means variation in coumestrol content represented variation in synthesis among areas of the plant. This is why studies have found that only infected material has high coumestrol levels and that after cutting new regrowth does not contain high levels of coumestrol (Loper and Hanson, 1964; Sherwood *et al.*, 1970).

2.7 The effect of coumestrol on ewes

2.7.1 Lambing performance

Unlike formononetin (Section 2.5), coumestrol in lucerne does not cause drastic or permanent reproductive disorders. However, coumestrol has been shown to reduce ovulation rate leading to fewer multiple births on lucerne relative to grass pastures (Coop, 1977; Scales *et al.*, 1977; Smith *et al.*, 1979). Ovulation rate of ewes is the number of eggs released by the ovary during an oestrous cycle and can be measured by the number of corpora lutea on the ovaries. It is dependent on the stage of breeding season, the genetics and live weight of the ewes, the nutritional value of the pasture and the amount of phytoestrogen present (Adams, 1995).

In the early 1960s, shortly after the identification of coumestrol, a 10-15% reduction in conception rate of ewes on lucerne and approximately 10% reduction in lambing rate was reported (Coop and Clark, 1960). A later experiment (Coop, 1977), in which ewes were put on grass pasture during four weeks pre-mating and six weeks of mating, or on lucerne with 80-120 mg/kg DM coumestrol throughout this period, reported ewes on lucerne had reduced lambing performance. The mean lambing rate per ewe was 1.8 on grass and 1.6 on lucerne, and 2.6% of ewes on lucerne were barren compared with 0.3% barren on grass. Coop (1977) showed that having animals on lucerne prior to mating did not decrease lambing rate but that mating on lucerne did decrease lambing rate. Ewes flushed on lucerne but grazed on grass produced 1.75 lambs per ewe, compared with 1.7 lambs per ewe when grazed on grass during both the pre-mating and mating periods. Mating on lucerne reduced lambing rate to 1.5-1.6 lambs/ewe regardless of whether the animals were on grass or lucerne pre-mating. Coop (1977) did not report whether lucerne had an effect due to a change in coumestrol content during mating relative to the pre-mating period, or an increased effect on ovulation rate closer to ovulation.

Scales *et al.* (1977) compared the reproductive performance of sheep on lucerne versus ryegrass-clover at two New Zealand stations- Mt Somers and Winchmore. Coumestrol levels in lucerne ranged between 82 and 157 mg/kg DM at Winchmore and 26-104 mg/kg DM at Mt Somers, before and during mating. Ewes flushed and mated on lucerne produced fewer lambs than ewes on ryegrass-clover pastures. Reduced twinning accounted for most of this difference. At Mt Somers in 1976, 61.3% of ewes flushed and mated on grass had multiple births compared with 29% on lucerne. This led to a lambing rate of 165% on grass and 123% on lucerne. Winchmore station trials also showed similar decreases in multiple births and lambing rate. The decreased twinning rates were attributed to lower ovulation rates which were 1.77 per ewe on grass and 1.53 per ewe on lucerne at Mt Somers in 1976. Partial embryonic mortality (cases where ewes had ovulated two eggs but there was only one embryo or lamb), barrenness, mean lambing date and live weights were not different between lucerne and grass treatments. Most of the ewes were mated in the first 17 days (cycle 1) of mating, with no significant difference in conception rate between treatments. Scales *et al.* (1977) concluded that lucerne containing 100 mg/kg DM of coumestans was sufficiently potent to reduce reproductive performance in ewes.

Ramòn *et al.* (1993) reported reduced ovulation rate in Rasa Aragonesa ewes which consumed a diet of lucerne that provided approximately 27 mg coumestrol/ewe/d before and during mating. Ewes fed on Italian ryegrass (*Lolium multiflorum* L.) had an ovulation rate of 170% compared with 150% in ewes fed on lucerne. Live weight (47 kg), conception rate (86%), barrenness and embryonic losses were unaffected by lucerne. In contrast to Scales *et al.* (1977), lambing rate was not affected with 140% for lucerne and 144% for Italian ryegrass, suggested to be due to higher than usual barrenness and embryonic loss in the grass-fed ewes.

Smith *et al.* (1979) reported decreased ovulation rate relative to grass controls from 1.38 to 1.04 in ewes fed diseased lucerne, with high (> 100 mg/kg DM) coumestrol for most weeks before and during mating, which led to fewer ewes bearing twins. There was no effect of diet on oestrus behaviour or conception rate. In contrast, a diet of substantially higher coumestan levels (>1000 mg/kg DM) did affect the incidence of oestrus (Kelly *et al.*, 1976) with most ewes on this diet not displaying oestrus.

In ewes fed different coumestrol contents, ewes fed a coumestrol-free diet had an ovulation rate of 1.43, while ewes fed a diet with a coumestrol content of 100 mg/kg DM had an ovulation rate of 0.98. A diet of 25 mg coumestrol/kg DM was sufficient to reduce ovulation rate to 1.23 (Smith *et al.*, 1979). This is lower than the 100 mg/kg DM level suggested to decrease reproductive performance by Scales *et al.* (1977).

When ewes were fed lucerne with no detectable foliar disease or coumestrol, ewes had increased live weight, while ewes on the grass control had decreased live weight (Smith *et al.*, 1979). The lucerne-fed ewes had higher ovulation rates relative to grass-fed ewes with an increase of 0.06 for each kilogram of live-weight gain. However, there was no difference in subsequent lambing due to higher barrenness and estimated embryonic loss in the lucerne-fed ewes.

More recently, King *et al.* (2010) observed a higher ovulation rate on lucerne pasture than on senesced phalaris (*Phalaris aquatica* L.) or phalaris plus 500 g lupin grain per day. On lucerne, 36% of ewes had multiple ovulations while 27% on phalaris and 33% on phalaris plus lupin had multiple ovulations. The proportion of ewes with multiple ovulations increased with the quantity of live herbage. The authors concluded that short-term grazing of lucerne could increase ovulation rates compared with dead or low quality pastures in late summer.

On the other hand, Cranston *et al.* (2017; in press) reported that in ewes with low or high body condition score (BCS), high coumestrol (ca. 100 mg/kg DM) lucerne caused ewes to have a greater return to service and fewer fetuses per ewe joined than grass fed ewes. No effect of BCS on the pattern of conception was found between diets, but the high BCS sheep had an overall greater number of fetuses per ewe. This meant that the use of lucerne as a nutritional feed was not beneficial when coumestrol was elevated, even in ewes with poor initial body condition score.

2.7.2 Morphological changes in organs

Grazing of high coumestrol feed can alter the development of ewe and ewe lamb reproductive organs. Ewes and ewe lambs fed high coumestrol lucerne tend to have increased uterine size as oestrogens stimulate uterine blood flow which causes cell proliferation and uterine growth (Reynolds *et al.*, 1998) and decreased ovarian weight (Newsome and Kitts, 1980; Sexson, 2002).

Newsome and Kitts (1980) orally administered coumestrol to pre-pubertal lambs for 12 days at a rate of 132 mg/lamb/d. The rate of coumestrol was calculated from the relative affinity of coumestrol and oestradiol for the uterine cytosol oestrogen receptor (1:53). The 132 mg rate was equivalent to 2.5

mg of oestradiol. The authors suggested that the dose was within the range of coumestrol that could be consumed each day in lucerne hay containing 100 to 140 mg/kg DM coumestrol. Coumestrol increased uterine weight, and decreased ovarian weight to a similar extent as oral administration of 2.5 mg oestradiol.

The consequences these developmental changes have on the subsequent reproductive performance of ewe lambs are unclear.

2.7.3 Live weight

Oldfield *et al.* (1966) observed an increase in the live weight gains of wether lambs and a decrease in weight gains of ewe lambs with high coumestrol (99 mg/kg DM) lucerne meal, relative to lambs fed moderate levels of coumestrol (35 mg/kg DM) lucerne meal. In contrast, Trenkle and Burroughs (1978) reported that isoflavones and coumestans found in clovers and lucerne did not increase live weight gain in sheep.

2.7.4 Mechanism of reproductive disruption

Coumestrol affects reproductive performance through reduction of ovulation rate. The mechanism behind decreased ovulation rate in lucerne grazing trials is not well understood but may be due to an alteration in the function of the hypothalamic–pituitary–gonadal (HPG) axis. The normal function of the HPG axis is described by Meethal and Atwood (2005).

Studies have shown coumestrol to alter luteinizing hormone (LH) either through decreasing the amplitude of LH released during pulses (Montgomery *et al.*, 1985), or increasing the amplitude or delaying the timing of the LH surge that precedes ovulation (Hettle and Kitts, 1983).

Montgomery *et al.* (1985) reported that ovariectomised Ile-de-France ewes fed lucerne diets with high coumestrol concentrations during the breeding season had altered LH release. Ewes (3-4 per group) were fed lucerne supplying 4, 18 or 40 mg coumestrol/ewe/day. The concentration of LH was lower with the high coumestrol diets. The mean amplitude of pulses decreased from 10.3 ng/mL with the low coumestrol diet to 4.2 ng/mL with the high coumestrol diet. The interval between LH pulses did not differ between diets. This was in contrast to oestradiol which increased LH pulse frequency. This suggests that coumestrol does not have an effect on the hypothalamus, but is active at the pituitary gland to reduce LH pulse amplitude. This differs from the view of Mathieson and Kitts (1980) who reported that coumestrol competes with 17β-oestradiol for binding sites in both hypothalamic and pituitary tissue of sheep.

Hettle and Kitts (1983) measured plasma LH concentrations in five Dorset ewes fed cocksfoot hay and five ewes fed lucerne hay and found that the LH peak during the surge was higher in ewes fed lucerne hay (66 ng/mL) than for control ewes (40 ng/mL). The interval between onset of behavioural oestrus and the LH peak was also lengthened by lucerne and it was suggested that this could affect reproductive performance.

2.8 Implications for future research

Phyto-oestrogens in legumes can affect ewe reproductive performance. This is particularly the case with formononetin in traditional subterranean clover cultivars, which cause permanent infertility and severe symptoms in the reproductive organs of ewes following prolonged consumption (Bennetts *et al.*, 1946; Davenport, 1967; Davies *et al.*, 1970). However, due to the severity of the symptoms following ingestion of subterranean clover, a large amount of effort has successfully been invested into producing low formononetin cultivars of both subterranean and red clovers (Lightfoot, 1974; McDonald *et al.*, 1994) and formononetin concentration is no longer considered to be a major issue in forage consumption. It is possible however that some non-obvious effects on reproductive performance are still occurring, as studies from the 1990s with red clover showed that the low formononetin cultivar of that time still appeared to cause some suppression of reproductive performance (Anwar *et al.*, 1993; McDonald *et al.*, 1994). However, since then red clover cultivars with even lower levels have been released. Selecting for low formononetin cultivars in breeding programmes continues to have merit and contemporary research with both subterranean clover and red clover could be worthwhile, but is outside of the scope of this thesis.

In contrast to formononetin in subterranean and red clovers, coumestrol in lucerne does not cause obvious nor permanent effects on ewe reproductive performance. Ovulation rates can be depressed leading to fewer multiple births, but conception rates and embryo mortality do not appear to be affected (Coop, 1977; Scales *et al.*, 1977; Smith *et al.*, 1979). Ewes do not display clinical symptoms such as prolapse and dystocia, and removal of the animals from lucerne allows full recovery (Coop, 1977). The low severity of effects of coumestrol is possibly the reason attempts to specifically breed low coumestrol lucerne, as has been done with formononetin, appear uncommon. Instead cultivars that accumulate less coumestrol are likely to be a side effect of breeding for resistance to aphids and major fungal pathogens. For this reason it is important to investigate the status of modern cultivars currently used in New Zealand to determine if they still produce high levels of coumestrol, or if the breeding programmes have been sufficient to control disease infection and thus prevent formation of high levels of coumestrol (Sections 5.2, 5.4, 5.5, 5.6). Research from the 1960s to 1980s indicated that resistant cultivars ‘Saranac’ and ‘Nevada Syn T-P’ did not accumulate as much coumestrol as susceptible cultivars in response to fungal infection or aphid infestation (Loper, 1968; Purves *et al.*, 1981). It could be expected that in the time since then, modern cultivars would have greater resistance and be even less likely to accumulate coumestrol.

Fungal disease is considered the main factor affecting coumestrol content of lucerne, and so far all pathogenic fungi tested have caused an increase in coumestrol. In terms of limiting coumestrol by controlling fungal pathogens, studies have shown fungicidal treatments to be effective (Hanson *et al.*, 1965; Purves *et al.*, 1981), however these experiments have used a weekly application rate which is unlikely to be economic. The effect of one-off applications is not known and is therefore tested in Section 5.3. Researching the effect of the cool biotype of stemphylium is of interest as the lesions do not expand once the border has formed. Only one study (Hanson *et al.*, 1965), which, due to its location on the East Coast of the USA, likely used the warm biotype with lesions that continue to expand, has looked at the effect of stemphylium on coumestrol accumulation. As the lesions of the cool biotype do not expand it is possible that coumestrol accumulation will be restricted to within the borders of the lesions, as it is not translocated (Sherwood *et al.*, 1970), leaving the majority of the plant with a low coumestrol content. Research on the cool biotype is therefore reported in Section 5.4.

Aphids have also been shown to cause increased coumestrol content in lucerne (Loper, 1968; Kain and Biggs, 1980). Whether the accumulation of coumestrol in the presence of aphids is a response to the aphids themselves, or that the wounding of the plant tissues and secretion of honeydew by the aphid increases susceptibility to fungal pathogens, has not been explored. Section 5.6 tests whether lucerne leaves that have accumulated coumestrol in the presence of aphids have fungal damage around the sites of damage.

The effect of water stress on coumestrol content in lucerne has not been tested, but no relationship was found in the leaves of soybean (Tripathi *et al.*, 2015; Tripathi *et al.*, 2016). As some farmers in New Zealand avoid grazing ewes on wilted lucerne, this factor is investigated in Section 5.5.

There is no consensus on whether developmental stage of the lucerne plant causes increased coumestrol. The field studies (Bickoff *et al.*, 1960a; Hanson *et al.*, 1965; Seguin *et al.*, 2004) that reported an effect of developmental stage did not compare different stages of maturity simultaneously nor quantify all of the non-developmental properties of the stand that prevailed during plant development, such as fungal infection, pest pressure, and climatic conditions. Separation of growth stage from these factors is important to determine whether growth stage in isolation has an effect on coumestrol content and this is investigated in Section 5.1. Despite the tentative link between developmental stage and coumestrol content, farmers in New Zealand have largely been advised to avoid grazing flowering lucerne.

A model that accounts for the various factors that control coumestrol accumulation would be a useful tool to assist farmers to estimate whether the coumestrol content of a lucerne crop is likely to be high. Development of such a model is described in Chapter 6.

In addition to estimating the coumestrol content based on the environmental factors, it would be useful to have a method that could be used to quickly and cheaply quantify the levels of coumestrol or phyto-oestrogen in the plant. The yeast bioassay produced by Routledge and Sumpter (1996) could be a cheap and efficient way to test plant samples, as the only analytical machine that is required is a microplate spectrophotometer. Drawbacks in New Zealand are the requirement for physical containment due to its genetically modified status and that the plant extraction and assay incubation takes approximately one week. This thesis will test the accuracy and precision of the yeast assay for measuring oestrogenicity of lucerne, which may have greater application outside of New Zealand (Chapter 3).

Further research into grazing management during the mating season would also be of merit. To decrease the risk of depressed lambing rates the current recommendation is for farmers to remove ewes from lucerne well in advance of mating if it is diseased, has aphids or is flowering. Even more conservative recommendations advise removing ewes from lucerne prior to mating regardless of the crop's condition. The time frame following coumestrol consumption during which reproductive performance is affected is investigated (Chapter 8) along with its implications for the management of lucerne grazing. Removal of animals results in the inability to utilise high quality feed to increase the condition of ewes prior to mating. This is particularly of issue in dryland systems, where lucerne, due to its long taproot, and therefore greater drought tolerance, may be the only pasture species available. Research into this area could allow a longer grazing period on high-risk lucerne prior to mating without impacting on ovulation rates.

Biological effects on ewe reproduction have been suggested to occur in lucerne at coumestrol levels greater than 25 mg/kg DM (Smith *et al.*, 1979). This is the level that will be used as a threshold in the experiments of this thesis. However, this level should be regarded with caution. It is likely that the true safe level is below this value.

2.9 Further research

This section summarises the further research requirements, identified from this literature review, which would be useful for improving the management of lucerne. These were used to design the experiments required to address the aims of the thesis (Section 1.1).

- Optimise and test the yeast bioassay for suitability as a coumestrol screening method.
- Coumestrol accumulation under different cutting frequencies and development stages
- Coumestrol accumulation in modern lucerne cultivars
- Control of coumestrol with one-off fungicidal sprays
- Effect of the cool biotype of stemphylium
- Testing whether aphids cause coumestrol accumulation by increasing disease incidence
- The effect of water stress on coumestrol content
- Determine the duration that ewes are affected by coumestrol.

Chapter 3

Method Development

3.1 Introduction

The objective of the research reported in Chapter 3 (Objective 1) was to refine the methods used for coumestrol measurement. Identification of whether lucerne has a high or low content of coumestrol prior to and during mating of sheep is crucial to enable decisions on grazing to be made. A method allowing rapid screening of lucerne was desirable for use in further experiments and also for monitoring lucerne stands prior to and during the mating period. A bioassay which used yeast modified with a human oestrogen receptor was readily available at the onset of this research project and was more cost-effective per sample than using high performance liquid chromatography (HPLC).

This section investigated the suitability of the yeast bioassay for measuring oestrogenicity in lucerne. The yeast bioassay was subsequently compared with HPLC. HPLC is the method used by the majority of researchers for measuring compounds in plant material. It directly quantifies the concentration of a molecule, such as coumestrol, in solution.

The methodology for extracting coumestrol from lucerne was also tested. The aim was to produce a simple, cost-effective extraction method. Freeze drying, the addition of a cellulase digestion step, and sonication were used by Davis (2013) to prepare plant samples for measurement by the yeast bioassay. Each of these steps is time consuming, which reduces the applicability for screening pasture coumestrol during the mating season, and also adds to the expense of extracting coumestrol from lucerne. Testing the necessity of these steps could simplify the methodology and decrease the time required to determine coumestrol levels. In addition, different methods for removing plant debris from samples prior to HPLC were compared.

To meet the objective of this chapter, the accuracy and precision of HPLC and the yeast assay were compared, and extraction methodology was optimised for further experimental work. This could ultimately provide a screening method to assist farmers with lucerne grazing decisions during the mating period. The following null hypotheses were tested:

- There is no correlation in coumestrol yield between cellulase extracted and methanol-only extracted lucerne samples.

- There is no correlation in coumestrol yield between freeze-dried and oven-dried lucerne samples.

- There is no effect of duration of sonication on coumestrol extracted from lucerne samples.
- The use of filtration prior to HPLC to ensure the column lasts longer does not affect the coumestrol concentration of a solution.
- The yeast assay measurement of oestrogenicity is not correlated with the HPLC method of coumestrol measurement.

3.2 Materials and Methods

3.2.1 Lucerne sampling

Lucerne samples for the experiments in this chapter were collected from crops at Lincoln University (site described in Section 4.1.1) and Ashley Dene (Section 4.1.2), between January 2014 and May 2016, using 0.2 m^2 quadrats. Lucerne was cut to approximately 40 mm above the ground. The lucerne represented a range of high and low coumestrol levels. Latex gloves were worn while handling lucerne material.

3.2.2 Lucerne processing and extraction experiments

3.2.2.1 Experiment 1: Cellulase versus methanol-only extraction

In Experiment 1, the use of a cellulase digestion as part of the extraction process was tested. Davis (2013) found that digestion of soya bean with cellulase following the methanol extraction increased the oestrogenicity slightly, as measured by the yeast bioassay. However, the cellulase digestion adds an additional 24 hours to the extraction length and purchasing of the cellulase enzyme increases cost.

Samples (n = 12) were oven dried, and ground sequentially with two machines, one that coarsely ground the material to cut the lucerne stems and an Ultra Centrifugal Mill ZM 200 (Retsch, Germany) which ground the lucerne through a 1.0 mm sieve. From this material, 0.5 g was weighed into 5 mL of >99.9% methanol (Sigma-Aldrich, Missouri, USA) in a 15 mL conical bottom tubes (Axygen Scientific, California, USA). Solutions were vortex mixed for 20 seconds, sonicated for 10 minutes with tubes sitting in an ice bath, and then put on an end over end mixer for 18 hours at room temperature. Samples were centrifuged at 4,700 x *g* for 5 minutes and 2.5 mL supernatant transferred by pipette to a new 15 mL tube.

Cellulase-treated samples had the methanol removed by ‘CentriVap Centrifugal Vacuum Concentrator’ (Labconco, Missouri, USA) at 30°C while untreated samples were stored in a refrigerator. Treated-samples were re-suspended in 2.5 mL of sodium acetate buffer solution (0.1 M, pH 5) with 100 units of cellulase from *Aspergillus niger* (Sigma-Aldrich, Missouri, USA) and mixed with a vortex mixer. The samples were incubated overnight at 37°C, whilst shaken at 200 rpm. Digestion was terminated by incubating samples at 65°C for 5 minutes before cooling on ice. Samples were centrifuged at 4,700 x *g* for 5 minutes and 1.5 mL transferred to a microcentrifuge tube (Axygen Scientific, California, USA).

3.2.2.2 Experiment 2: Freeze dried versus oven dried lucerne

In Experiment 2, the coumestrol levels extracted from freeze dried and oven dried lucerne were compared. Oven drying has previously been shown to reduce coumestrol levels in samples, however, these samples were dried at 70°C and 80°C (Bickoff *et al.*, 1960b; Livingston *et al.*, 1961) which is above the 60°C generally used to preserve phyto-chemicals in plants (Cayley and Bird, 1996). Being able to oven dry lucerne without affecting the coumestrol content would simplify the process of lucerne extraction, as it does not require access to freeze drying equipment. It would also mean that farmers could dry and send lucerne samples to a laboratory for analysis.

Fresh lucerne samples (n = 9) were mixed by hand and separated into halves. Samples were either oven dried for 48 hours at 60°C or freeze dried. Samples were ground and extracted as described for the non-cellulase samples in Experiment 1.

3.2.2.3 Experiment 3: Sonication duration

In Experiment 3, the duration of sonication was tested. Davis (2013) used sonication in phyto-oestrogen extraction methodology to break apart plant tissue and potentially increase the yield of phyto-oestrogen. However, sonication of samples increases the labour input of the coumestrol extraction. Therefore reducing the sonication length reduces the cost of the methodology.

Lucerne samples (n = 14) were ground and extracted as described for non-cellulase samples in Experiment 1, with the exception that samples were sonicated with a ‘W-225’ probe-type sonicator (Heat Systems-UltraSonics Inc, New York, USA) immersed in the sample, for either 0, 2.5, 5 or 10 minutes.

3.2.2.4 Experiment 4: Pre-HPLC processing

For HPLC, the pre-assay processing to remove plant debris is important to extend the life-span of the column. The most reliable way to ensure all plant material is removed is via a syringe filter. However, some filters can remove the compound of interest from the solution. If it is not possible to retain the compound in the filtrate, centrifugation can be used as a less effective alternative but is not recommended as the column lifespan will be decreased.

For this research, three different syringe filters were compared with centrifugation at 13,500 x *g* for their ability to retain coumestrol in solution. The three types of syringe filters used were Minisart®

SRP 0.45 µm polytetrafluorethylene (PTFE) Membrane Filters, 0.45 µm Nylon Membrane Filters (Sigma-Aldrich, Missouri, USA) and MicroScience 0.7 µm Glass Fibre Filters (MicroAnalytix, New Zealand). The filters were all contained in polypropylene housing.

3.2.3 Measurement of coumestrol by HPLC

Following filtration or centrifugation, samples of approximately 300 µL volume were transferred into 2 mL glass auto-sampler vials (Thermo Scientific, Massachusetts, USA). Glass fibre syringe filters were used prior to analysis of samples from Experiments 1 to 3.

Extracted samples were analysed for coumestrol content using HPLC with methodology adapted from Wang *et al.* (1990). HPLC analyses were performed with an Agilent 1100 series instrument (Agilent Technologies, San Francisco, CA, USA) equipped with binary pumps, and a fluorescence detector set at 365 nm for excitation and 418 nm for emission. The injection volume was 20 µL. Separation was carried out on an ACE (Aberdeen, Scotland) reverse phase column (C18, 3 µm, 150 mm x 2.1 mm) at 25°C, with the flow rate set at 0.5 mL/min. Solvent A was deionized water and solvent B was 100% methanol. Elution of coumestrol was performed by linearly increasing the percentage of solvent B from 40% to 100% over 14 minutes. Solvent B was maintained at 100% for 2 minutes. The column was re-equilibrated for 9 minutes between samples. Coumestrol (Sigma-Aldrich, Missouri, USA) was used to make a calibration curve from 0.5 to 20 mg/L.

3.2.4 Experiment 5- HPLC and Yeast Assay Validation

In this experiment the HPLC analysis and yeast bioassay were evaluated and compared.
The extraction method used for validation of the HPLC and yeast assays was based on the results of Experiments 1 to 3 (Section 3.2.2). In summary, oven-dried, finely-ground lucerne samples of 0.5 g were weighed into 5 mL HPLC-grade 99.9% methanol. Solutions were vortexed for 20 seconds and put on an end over end mixer for 16 hours at room temperature. Solutions were centrifuged at 4,700 x *g* for 5 minutes and 1.5 mL of supernatant was transferred to a microcentrifuge tube and stored in the freezer.

3.2.4.1 HPLC Validation

HPLC analyses for evaluating the HPLC method were performed as described in Section 3.2.3 using glass fibre filters for sampling preparation.

To test the method validity the linearity of the calibration curve was assessed over four separate assays.

To test the precision between and within HPLC runs, the inter- and intra-assay coefficients of variation (CV) were determined. To measure the intra-assay variability of the HPLC method a high coumestrol sample was injected 10 times in a run. To measure the inter-assay variability of the HPLC method a high coumestrol sample and a low coumestrol sample were injected across three runs.

3.2.4.2 Yeast Assay Validation

Yeast assay methodology

For the bioassay, the recombinant yeast strain and methodology were supplied by Victoria University, Wellington, New Zealand (Davis, 2013)**.** Media solution components for the assays were stored in separate parts and a high Cu^{2+} concentration was used to help avoid contamination as the recombinant yeast had copper tolerance. The relative concentrations and preparation of the assay media are given in Table B.1 (Appendix B)**.** For each assay 25 mL of media solution with 0.125 mL of yeast was grown for 24 hours at 28°C (until OD_{640} was ca. 1.0). A fresh media solution was then made with 25 mL required per 96 well plate. To the fresh media, 1 mL of yeast and 0.25 mL of 10 mg/mL chlorophenol red-β-D-galactopyranoside (CPRG; Sigma-Aldrich, Missouri, USA) was added per 25 mL of media solution.

For each assay samples and standards were repeated in duplicate. To an empty plate 10 µL of $2x10^{-7}$ mol L^{-1} oestradiol was added to the first well and serial diluted with 10 µL ethanol across the plate with the final well an ethanol blank. Samples were also added in 10 µL aliquots and serial diluted with ethanol across four or six wells depending on the expected concentration. Ethanol was evaporated and 200 µL of assay media was added to each well. The initial absorbance at 570 and 620 nm was measured and the plate was incubated at 32°C for 2 or 3 days, until colour in the standard curve had developed.

The absorbance at 570 and 620 nm was again measured and the difference between the final and initial readings for both wavelengths was calculated. The wavelength of 570 nm measured the ‘redness’ of the assay wells, while the wavelength of 620 nm measured the optical density of the yeast cells.

These values were used to calculate the ratio between 570 and 620 nm. The sample activity equivalent to oestradiol was then determined from the oestradiol standard curve.

Yeast Assay tests

To determine linearity of the yeast assay the oestradiol standard curve was produced in duplicate across 15 assays. The average 570:620 ratio between the duplicates was plotted against the concentration of β-oestradiol.

To determine the intra-assay variability of the yeast assay a total of 40 samples were assayed in duplicate using the yeast assay. To measure the inter-assay variability a sample was measured over nine separate assays.

3.2.4.3 Yeast assay and HPLC Comparison

To compare the yeast assay to HPLC, 157 samples collected between November 2014 and January 2016 were used. These samples were measured by both the yeast assay and HPLC.

3.2.5 Statistical analysis

Statistical analyses were performed using Genstat 16. Pearson correlation coefficient was used to analyse whether the resultant coumestrol levels of different methodologies were correlated. Paired *t*-tests were used to compare the effect of method on the coumestrol concentration. Bland-Altman plots were used to visualise the differences between methods with upper and lower limits of agreement calculated as the mean ± 1.96 x the standard deviation.

Linearity of HPLC and yeast inter-assay standard curves was determined by linear regression with groups, where groups were the separate assays.

Coefficients of variation (CV) were calculated to assess inter- and intra-assay precision of HPLC and the yeast assay.

The standard error of the mean (SEM) is presented where mean data are reported in the format (mean ± SEM).

3.3 Results

3.3.1 Experiment 1 results: Cellulase versus methanol-only extraction

Cellulase extracted coumestrol was related ($r = 0.906$) to methanol-only extracted coumestrol (Figure 3.1). Coumestrol content was greater ($P < 0.001$) in methanol-only extracts than cellulase extracts. The mean of the difference between the two methods was 55.7 mg/kg DM with standard deviation of 42.07 mg/kg DM. There was a relationship ($P < 0.001$; $R^2 = 0.921$) between the difference and the average coumestrol content of the two methods (Figure 3.2). As average coumestrol content increased the difference between the methodologies also increased.

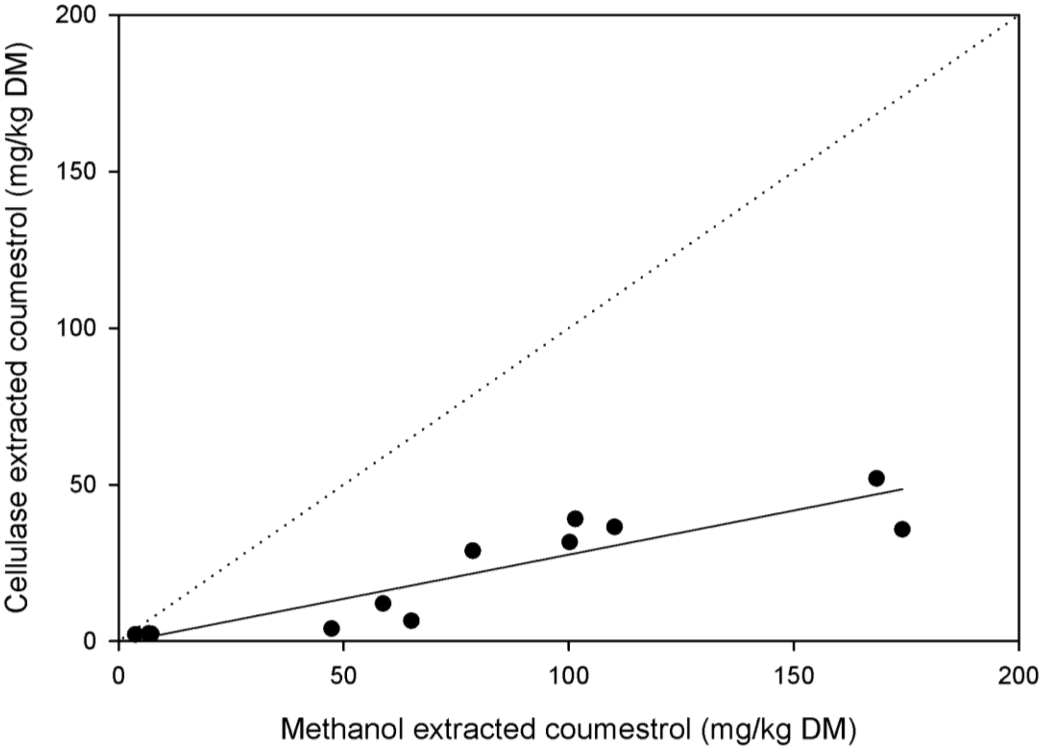


Figure 3.1 **Coumestrol content (mg/kg DM) of cellulase-extracted lucerne samples correlated (r = 0.906) with methanol-only extracted lucerne samples. Solid line is the line of best fit and the dotted line is the line of equality.**

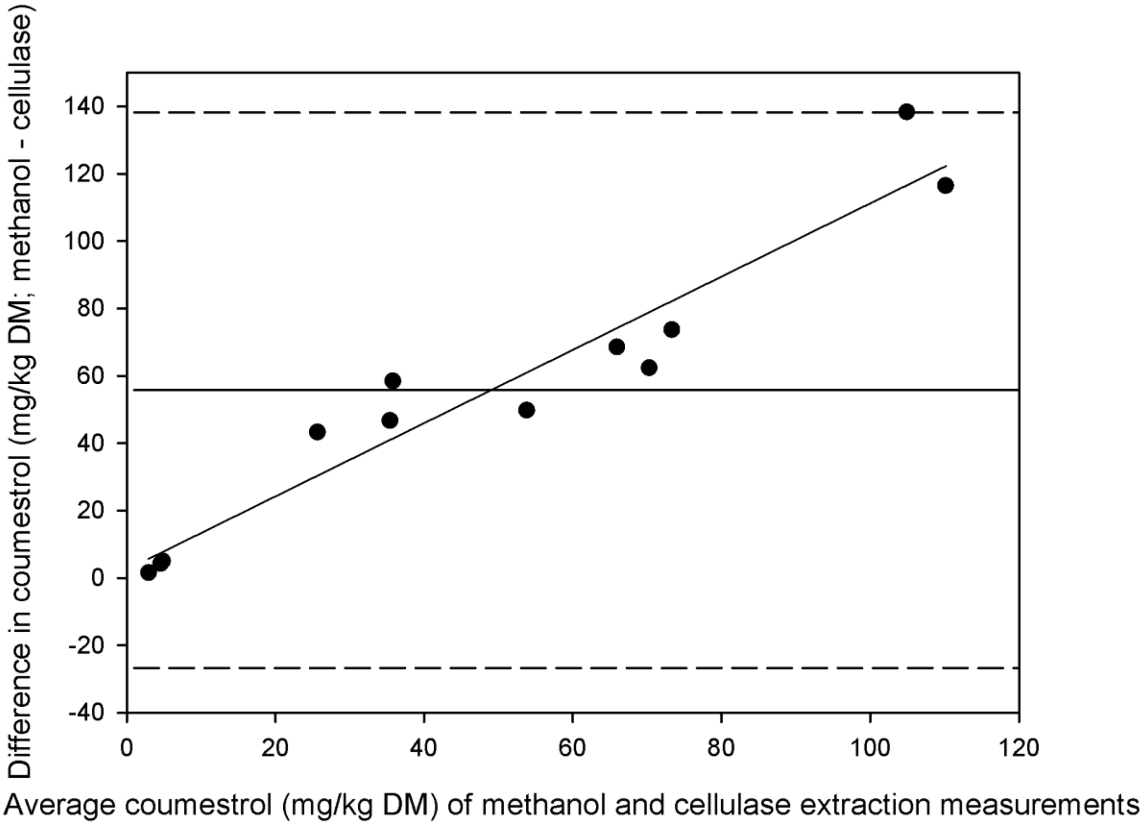


Figure 3.2 **Difference in average coumestrol content (mg/kg DM) measurements of methanol and cellulase extracted samples increased (R^2 = 0.921) with the average coumestrol measured with both methods. Solid horizontal line is the average difference (55.7 mg/kg DM); dashed lines are 95% limits of agreement.**

3.3.2 Experiment 2 results: Freeze-dried versus oven-dried lucerne

Coumestrol extracted from freeze-dried lucerne was related to coumestrol extracted from oven-dried lucerne (Figure 3.3; r = 0.991). The mean of the difference between freeze dried and oven dried measurements was 2.89 mg/kg DM with a standard deviation of the difference of 8.96 mg/kg DM (Figure 3.4). There was no relationship (P = 0.277; R^2 = 0.166) between the difference and the average coumestrol content of the two methods.

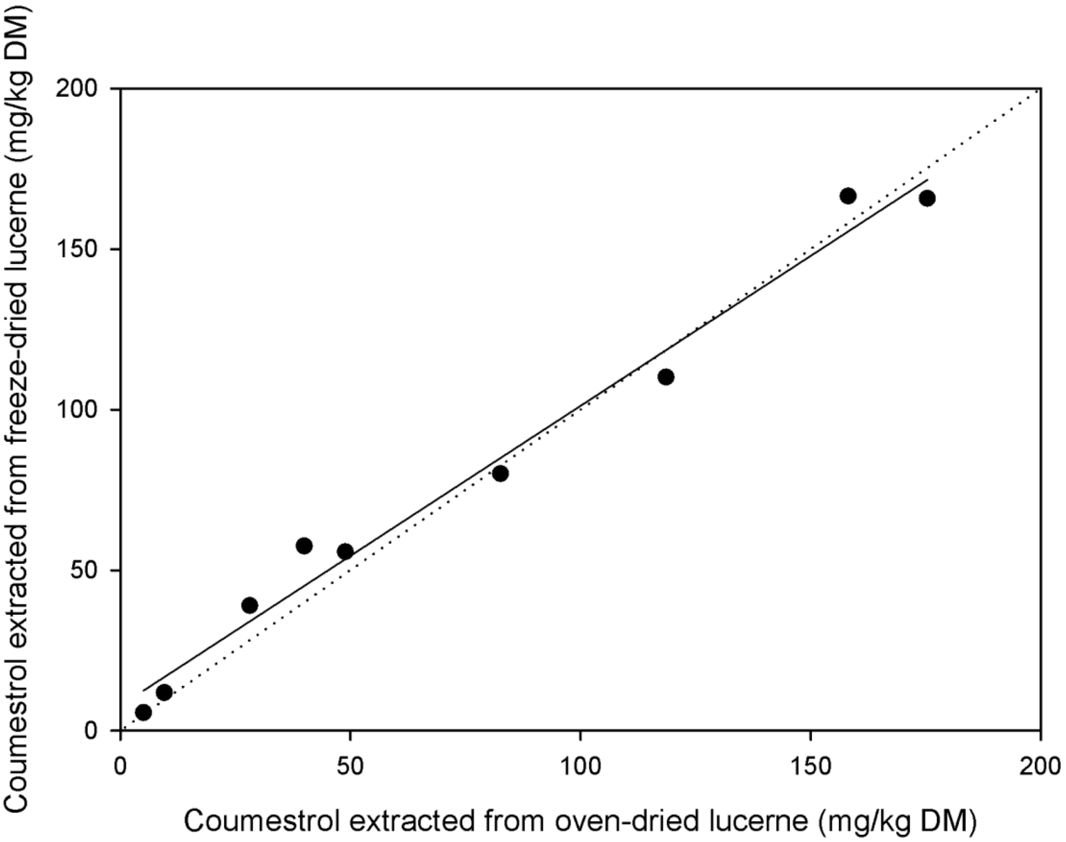


Figure 3.3 Coumestrol content (mg/kg DM) of freeze dried lucerne correlated (r = 0.991) with oven dried lucerne. Solid line is the line of best fit and the dotted line is the line of equality.

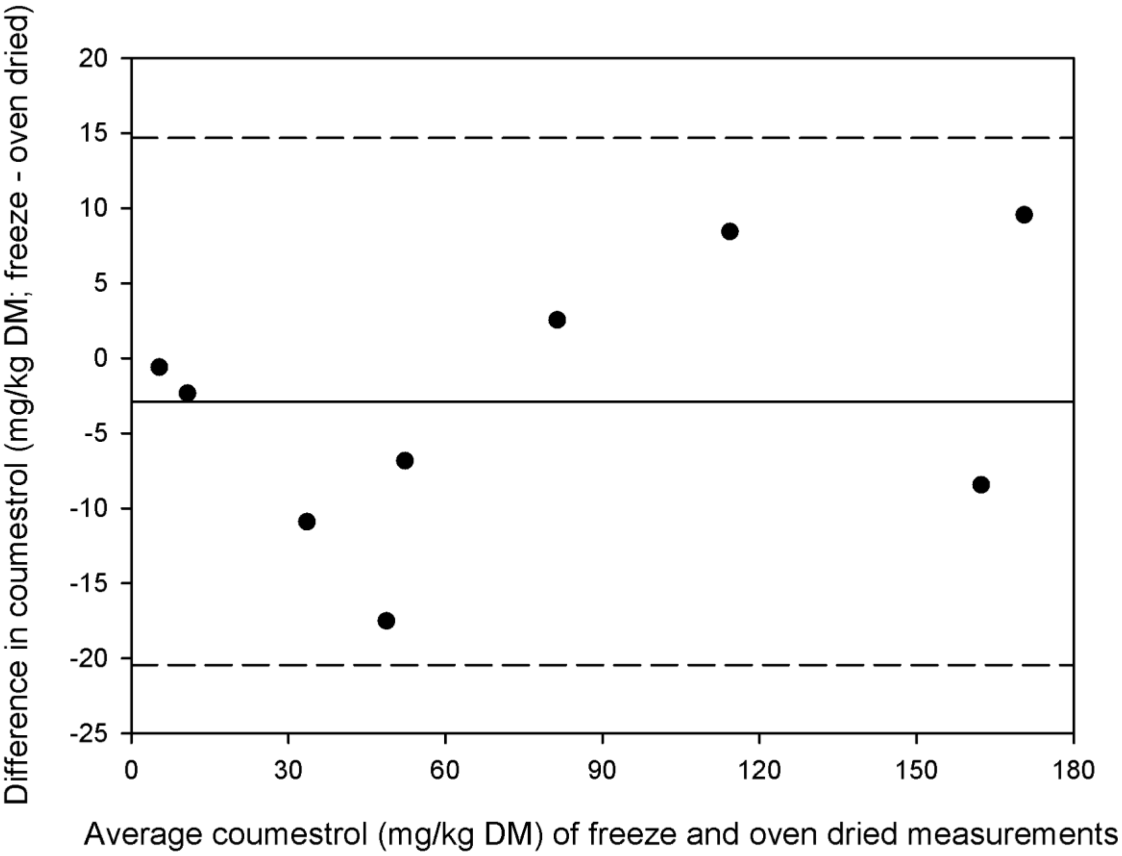


Figure 3.4 Difference in average coumestrol content (mg/kg DM) measurements of freeze dried and oven dried samples versus the average coumestrol content measured by both methods. Solid line is the average difference (2.89 mg/kg DM); dashed lines are 95% limits of agreement.

3.3.3 Experiment 3 results: Duration of sonication

Regression analysis with separate lines for each group of duplicate or triplicate samples collected on the same date, showed no effect (P > 0.173) of duration of sonication on the coumestrol content measured. The only difference in coumestrol content was between groups; with each sampling date different (P < 0.001) from the others (Figure 3.5).

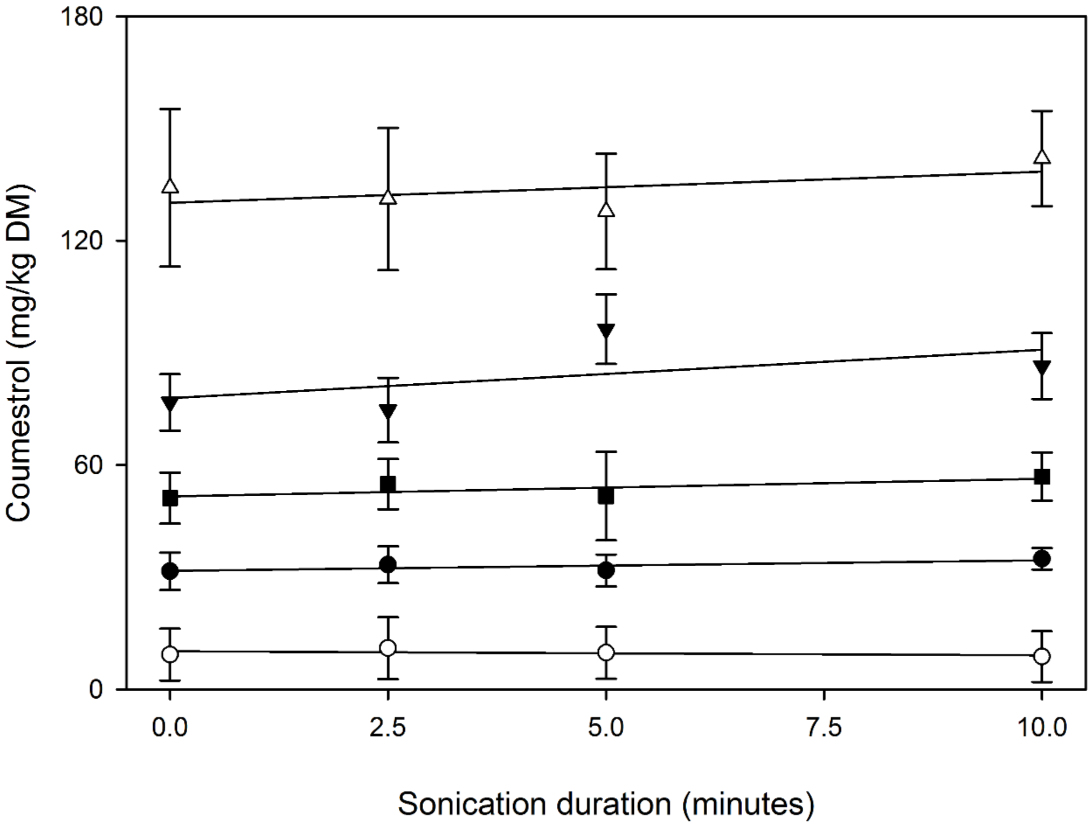


Figure 3.5 Coumestrol extracted after sonication for 0, 2.5, 5 or 10 minutes of lucerne samples collected from Iversen 12 on 21 April 2014 and 25 May 2014 in duplicate (△; ■) and on 25 March 2015, 15 April 2015 and 17 May 2015 in triplicate (●;○;▼). Error bars are standard error of the mean of the duplicate or triplicate samples.

3.3.4 Experiment 4 Pre-HPLC processing

Compared to centrifugation, nylon membrane and PTFE membrane filter treatments were not effective at leaving the coumestrol in solution (Figure 3.6). The nylon membrane and PTFE membrane filters reduced coumestrol in solution by 22.2 and 27.5% respectively relative to centrifugation (n = 1). This was not replicated to determine statistical significance as a > 20% decrease even on one event, was sufficient to determine the unsuitability of the preparation method. Especially when, in contrast, the use of glass fibres had a strong (r = 0.999), almost 1:1 correlation (n = 5) with centrifugation, encompassing both low (4-5 mg/kg DM) and high (144 mg/kg DM) coumestrol extremes (Figure 3.7).

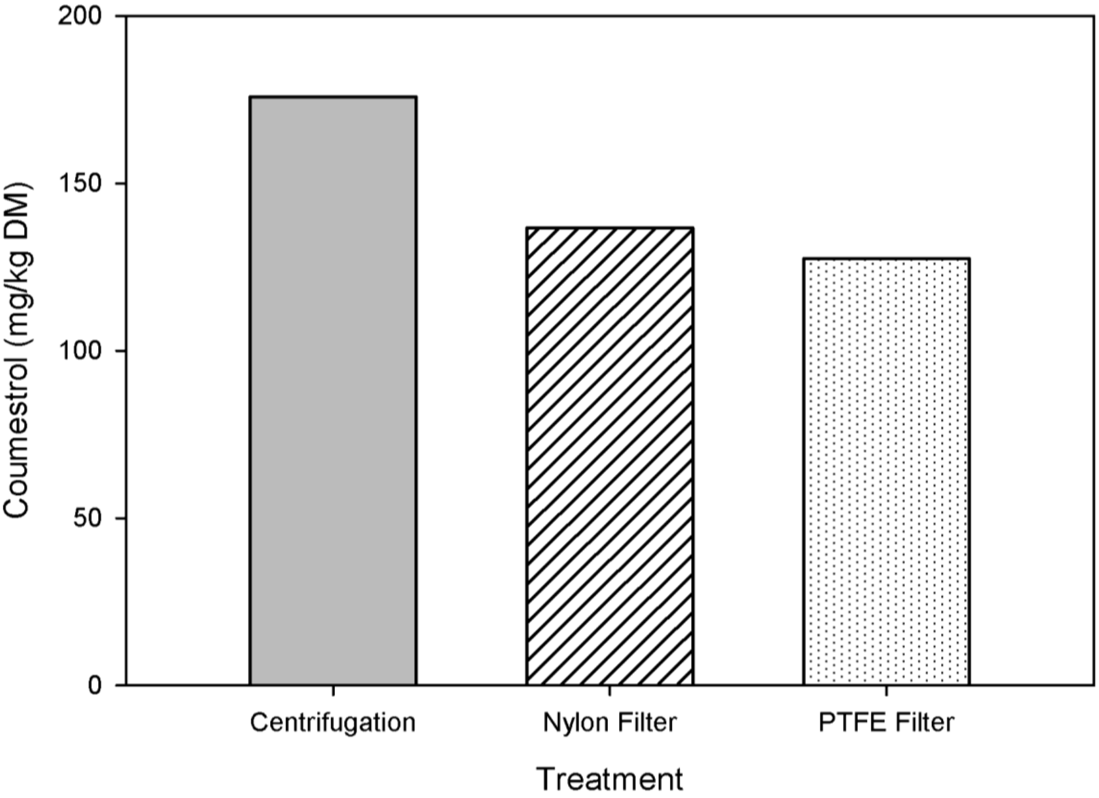


Figure 3.6 Peak area measured by HPLC of a lucerne sample (n = 1) following plant debris removal by centrifugation, nylon membrane filter or PTFE membrane filter.

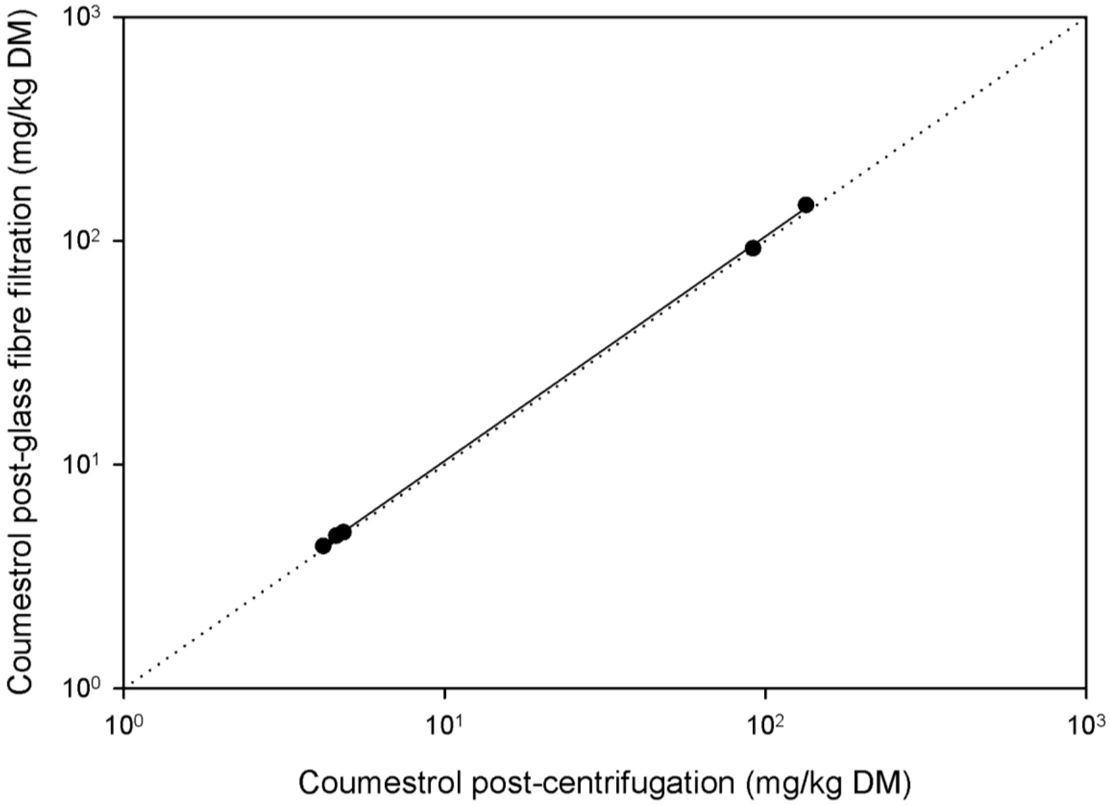


Figure 3.7 The correlation (r = 0.999) in peak area measured by HPLC between lucerne samples (n = 5) prepared with glass fibre filters and samples prepared with centrifugation. Solid line is the line of best fit, dotted line is the line of equality.

3.3.5 Experiment 5 HPLC and yeast assay validation

3.3.5.1 Validity of the HPLC assay

HPLC was able to consistently produce linear calibration curves. For each individual calibration curve, there was a linear relationship ($R^2 \geq 0.999$) between the peak area measured by HPLC and the known concentration of the coumestrol standard dissolved in the mobile phase (Figure 3.8). There was some spread among calibration curves among runs, with an overall R^2 of 0.981 for all peak areas versus the concentration of the coumestrol standard.

The intra-assay CV of HPLC, as measured by 10 injections of a high coumestrol sample, was 1.05%. The inter-assay coefficients of variation (n = 3) were 0.24% in the low coumestrol sample with average coumestrol of 6.37 ± 0.009 mg/kg DM and 3.6% in the high coumestrol sample with average coumestrol of 79.5 ± 1.66 mg/kg DM. The average inter-assay CV was 1.4%.

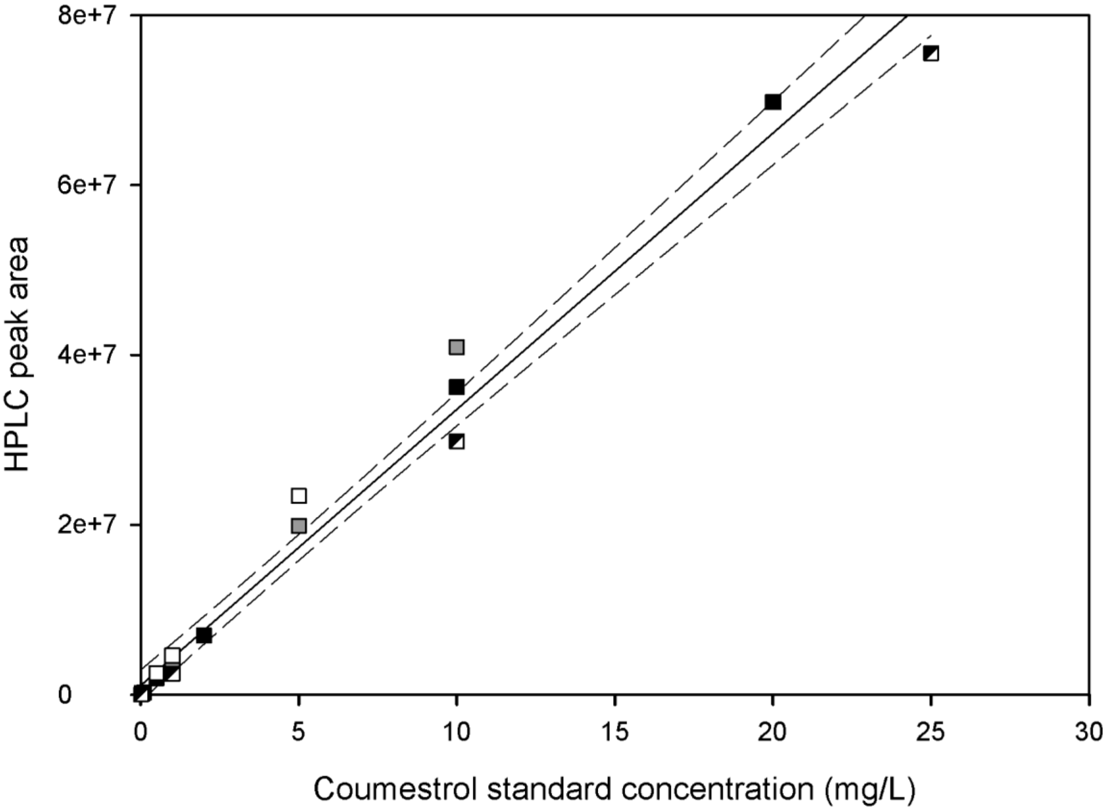


Figure 3.8 Calibration curves of the HPLC measured peak area against the coumestrol standard solution concentration (mg/L) for four separate HPLC runs. Regression line is the overall relationship ($R^2 = 0.981$) over the four runs described by the equation: $y = 3.3 \times 10^6 \cdot x + 1.1 \times 10^6$ ($\pm 0.11 \times 10^6$; $\pm 0.88 \times 10^6$), with 95% confidence interval (- - -). The individual R^2 values of runs one (■; n = 6), three (□; n = 4) and four (◩; n = 5) were 0.9999, the R^2 of run two (■; n = 5) was 0.9987.

3.3.5.2 Validity of the yeast assay

Yeast assay calibration curve

The oestradiol standard curve of the yeast assay was logarithmic. At high concentrations of oestradiol, or when left to incubate for extended periods of time, the standard curve plateaued due to saturation of red pigment in the wells. At lower levels of oestradiol, a lack of accumulated pigmentation in the wells limited detection. At moderate oestradiol concentrations there was a linear relationship between the oestradiol concentration and the absorbance ratio. The relationship between oestradiol concentration and absorbance, and the linear range of the calibration curve across 15 runs are given in Table 3.1.

The average lower limit of linearity following 72 hours incubation was 21.3 ± 2.70 ng oestradiol/L media solution in a well. The lower limit ranged from 5.3 ng/L to 42.6 ng/L (CV = 49.1%). The average absorbance ratio (570:620) at the lower limit was 1.14 ± 0.014 and ranged from 1.06 to 1.26 (CV = 4.70%). The average upper limit of linearity following 72 hours incubation was 210 ± 21.8 ng/L and ranged from 85.1 to 341 ng/L (CV = 40.2%). The average absorbance ratio (570:620) at the upper limit was 1.81 ± 0.091 and ranged from 1.40 to 2.37 (CV = 19.3%).

Among assays the slope of the calibration curve in the linear range was variable, with slopes ranging from 0.0015 to 0.0075 (CV = 53.2%). The average slope was 0.0039 ± 0.00054. The average intercept of the calibration curve was 1.085 ± 0.01120. There was low variation in the intercept value among assays, with the intercept ranging from 1.02 to 1.17 (CV = 4.27%). Although the slopes differed, the standard curve had linearity. The average R^2 of the assays (n = 15) was 0.974 ± 0.0069, ranging from 0.902 to 0.998 (CV = 2.74%).

Table 3.1 The relationships between oestradiol (E2) concentration (ng/L) and 570:620 absorbance in media solution across 16 separate assays.

Assay Date	Slope of equation	Intercept of equation	R^2	Linear range (ng E2/ L media solution)
6 May 2014	0.0031	1.025	0.9940	21.3 – 170.2
29 May 2014	0.0022	1.010	0.9451	42.6 – 340.5
5 July 2014	0.0025	1.046	0.9972	21.3 – 340.5
17 July 2014	0.0015	1.053	0.9930	42.6 – 340.5
9 Sept 2014	0.0024	1.074	0.9968	21.3 – 170.2
8 April 2015	0.0029	1.124	0.9871	5.3 – 170.2
17 April 2015	0.0073	1.144	0.9527	10.64 – 170.2
7 July 2015	0.0035	1.038	0.9701	21.3 – 170.2
16 July 2015	0.0027	1.050	0.9694	21.3 – 170.2
13 Aug 2015	0.0042	1.048	0.9955	5.3 – 85.1
21 Aug 2015	0.0031	1.095	0.9825	21.3 – 340.5
6 Oct 2015	0.0060	1.054	0.9627	21.3 – 170.2
13 Oct 2015	0.0075	1.158	0.9592	21.3 – 170.2
20 Oct 2015	0.0023	1.169	0.9021	21.3 – 170.2
13 Nov 2015	0.0074	1.102	0.9982	21.3 – 170.2

Yeast assay sample analysis

The yeast assay was not precise. The intra-assay co-efficient of variation between 40 duplicate samples was 26.1%. The inter-assay co-efficient for nine runs of a sample was 55.5%. In comparison, the average intra-assay CV for HPLC was 1.05% and the average inter-assay CV was 1.4% (Section 3.3.5.1).

There was a correlation (r = 0.873) between the measurement of oestrogenicity by the yeast assay and the measurement of coumestrol by HPLC (Figure 3.9). The root mean square deviation (RMSD) was 43.8 µg oestradiol equivalent activity/kg DM.

The ratio between coumestrol (mg/kg DM) and oestradiol (mg/kg DM) was 427:1. This mean that 100 mg/kg DM was required to produce the response of 0.234 mg E2 equivalent. In mole terms, 381 µmol coumestrol was required to produce the response of 0.859 µmol E2, a ratio of 443:1.

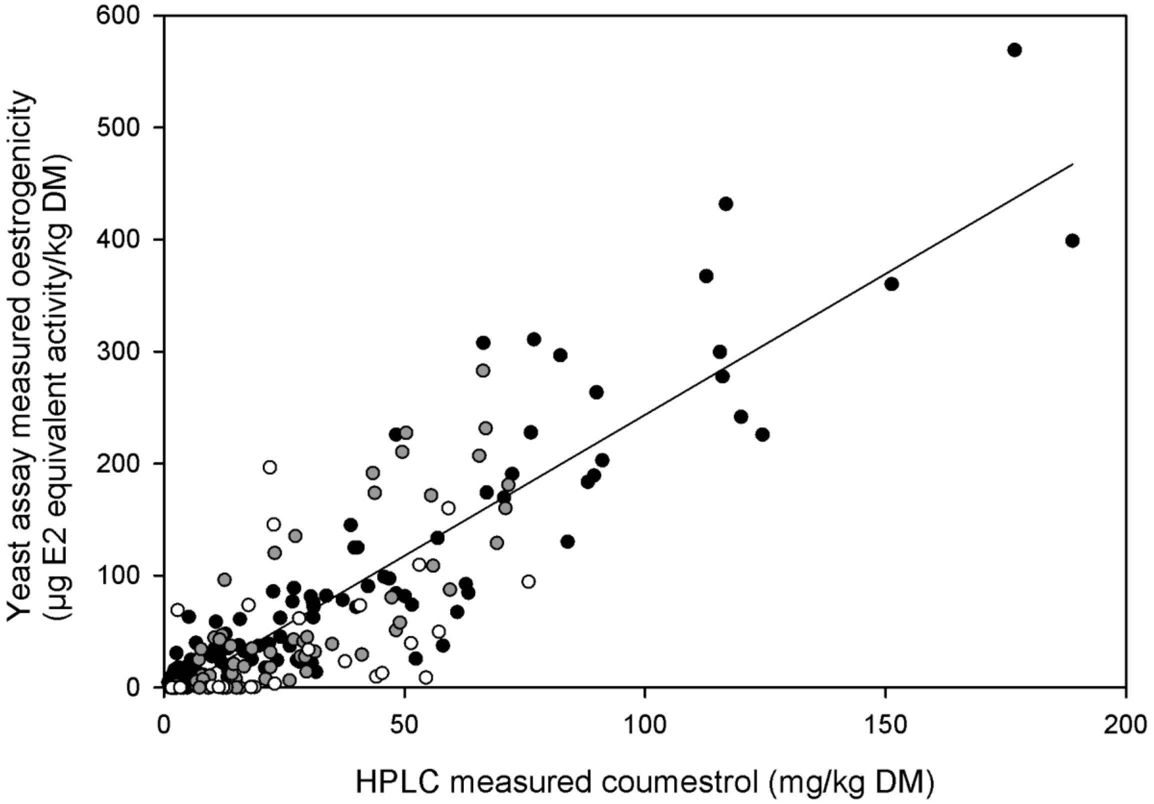


Figure 3.9 Lucerne oestrogenicity measured by the yeast assay (µg oestradiol (E2) activity/kg DM) against (r = 0.873) coumestrol content measured by HPLC (mg/kg DM). Samples were from Iversen in autumn 2014 (●) and spring 2015 (○) and from Ashley Dene between spring 2014 and autumn 2015 (●).

3.4 Discussion

The objective of the research reported in this chapter was to refine the methods used for coumestrol measurement. This was achieved by comparing different methods of sample extraction, and evaluating the yeast bioassay as a way of measuring sample oestrogenicity, a cost-effective method that did not require HPLC access.

3.4.1.1 Lucerne processing

Lucerne could be processed by either freeze drying or oven drying at 60°C. There was no adverse effect of either process relative to the other on the extracted coumestrol content. This result was in contrast to Livingston *et al.* (1961) who found that drying at 80°C in a forced-air oven destroyed 25% of a coumestrol sample, and Bickoff *et al.* (1960a) who found decreases in lucerne oestrogenic activity ranging from no loss to up to 75% loss when oven dried at 70°C. Oven drying is advantageous to researchers as it allows samples to be easily processed into a stable state. A lower temperature of 60°C as recommended by Cayley and Bird (1996) appears to have successfully prevented coumestrol loss.

3.4.1.2 Lucerne extraction

Cellulase extraction resulted in less coumestrol yielded than methanol-only extraction. Based on this the cellulase extraction technique should not be used. The low coumestrol yield may have been because coumestrol has low solubility in water (Bickoff and Emerson, 1959). It also may have been due to plant compounds adhering to tube walls following removal of the methanol by 'CentriVap'. This was in contrast to the results of Davis (2013) who found cellulase digestion to have the same or greater capability of extracting oestrogenic compounds from soy compared with methanol extraction. The exact compounds were not determined, however were likely to be genistein and daidzein, as these are the major oestrogenic compounds of soy (Thompson *et al.*, 2006). These compounds, like coumestrol, are solvent-soluble and only sparingly water soluble, based on this, the results of Davis (2013) were unexpected.

Removal of the cellulase step reduced the length of time the extraction took as digestion added an additional 24 hours. Overall the extraction process was decreased from three days to overnight.

Sonication

Sonication did not increase the amount of coumestrol extracted in lucerne. Removal of this step would reduce the labour required to extract samples, as only a single sample could be sonicated with the probe-type sonicator at a time.

3.4.1.3 Pre-HPLC processing

Plant extracts can be prepared for HPLC with either centrifugation at 13,500 x *g* or through filtration with glass fibre filters. Nylon and PTFE filters were unsuitable for solution filtration, the use of these filters reduced filtrate coumestrol by 22.5 and 27.5% respectively relative to centrifugation. While, the use of glass filters did not decrease coumestrol.

3.4.1.4 HPLC validity

HPLC was a reliable method for measuring coumestrol extracted from dried lucerne. Although the slope of the calibration curve varied slightly across runs, there was high linearity between the concentration of the coumestrol standard solution and the peak area for each assay. This lead to a low inter-assay CV for plant sample controls (1.4%). The CV for the intra-assay was also low (1.05%).

3.4.1.5 Yeast assay validity

The yeast assay produced a logarithmic standard curve. This means that there was a linear range within which oestrogenicity could be measured, but at higher levels the wells were saturated with pigment and differentiation could not occur. This highlighted the importance of having a dilution series for plant samples to increase the likelihood of oestrogenicity falling within the linear range. The standard curve was variable across runs, possibly due to differences in duration of assay and growth rate of yeast. This meant that at the end of some assays, more wells and therefore lower concentrations of oestradiol along the calibration curve had developed colour compared with other assays. This should not be an issue however, as the samples will also be relatively more developed too.

Linear regressions run through the linear sections of the calibration curves had a high goodness of fit with an average R^2 of 0.974, but were not as precise as the HPLC standard curve ($R^2 > 0.9999$). The detection limit was variable among assays, the lowest level that fell within the linear range was approximately 5 ng/L of oestradiol, and this was not dissimilar to the quoted detection limit of 2 ng/L (Routledge and Sumpter, 1996).

Agreement in the analysis of coumestrol and oestrogenicity in lucerne between the yeast assay and HPLC results showed that the yeast assay gave a qualitative, but inaccurate indication of whether the coumestrol level in lucerne was low, medium or high (r = 0.873). This would make it a cost-effective tool to monitor lucerne in situations where HPLC is unavailable. Some variation from the HPLC values could have been due to non-coumestrol phyto-oestrogens such as daidzein and genistein, however the high inter and intra-assay variations in the yeast assay indicate that this is a major source of imprecision. HPLC and yeast bioassay run in parallel would allow both a measurement of present compounds and of oestrogenic activity. The ratio between the yeast assay response to oestradiol and coumestrol was 443:1 on a mole per mole basis. This is similar to the 500:1 reported by Markiewicz *et al.* (1993) with the Ishikawa cell test.

As the yeast assay was too imprecise to give reliable quantitative values it was unsuitable for experimental studies. This imprecision could have been due to other oestrogenic compounds that were not measured by HPLC, but was mostly likely due to the high inter- and intra-assay variations. A further limitation of the yeast assay is its genetically modified status. This means that in New Zealand a laboratory with compliant physical containment is required, and transfer permits are required to move the yeast between laboratories. For these reasons, a field kit cannot be developed for New Zealand farmers to use and so samples would have to be sent to laboratories.

3.5 Conclusions

Based on the results of this chapter the following conclusions can be made:

- The use of freeze drying, cellulase digestion and sonication were unnecessary for extracting coumestrol.
- The imprecision of the yeast assay limited it to providing preliminary and rough approximations of oestrogenicity.
- HPLC was a precise and accurate method for measuring coumestrol content.

Therefore, for the experiments of this thesis lucerne will be oven dried to process it to a stable state. No sonication will be used and only methanol will be used for the extraction. Coumestrol will be measured with HPLC following removal of plant debris from solution with glass-fibre syringe filters.

Chapter 4

Materials and Methods

This chapter describes the experimental field sites and general methodology used for harvesting lucerne in the field, the measurements taken and the subsequent analysis of coumestrol. Further methodology specific to each experiment is reported in individual chapters.

4.1 Main Experimental Sites

4.1.1 I12

I12 is located at the Lincoln University Field Research Centre, Canterbury, New Zealand (43° 38' 53.29" S, 172° 27' 54.92" E; 11 m above sea level) on flat land in Iversen Field. The soil is a Wakanui silt loam classified as a Mottled Immature Pallic soil in the New Zealand Soil Classification (Hewitt, 2010). Wakanui silt loams typically have 1.8-3.5 m of fine textured, greywacke-derived material overlying gravels and are imperfectly drained (Cox, 1978), making them susceptible to water logging in winter. The paddock had a history of lucerne from 2004-2007, forage turnip (*Brassica rapa* L.) in 2008 and annual ryegrass (*Lolium multiflorum* Lam.) from 2009-2010.

To prepare the site for lucerne the site was ploughed on 1 September 2010, harrowed and Cambridge rolled. After ploughing, 'Sulphur Super 20' (0, 8, 0, 20; 250 kg/ha) and pre-emergence herbicide Treflan (trifluralin; 0.8 kg a.i./ha) were applied and incorporated with secondary cultivation. Coated lucerne seed (cv. 'Stamina 5') was sown in November 2010 at a rate of 16 kg/ha (10.5 kg/ha bare seed) to 20 mm depth using an Øyjord cone seeder. Herbage was cut for hay or grazed by sheep as needed, and grass and broadleaf weeds were controlled with herbicide each winter with Gallant (haloxyfop; 0.025 kg a.i./ha) and Preside (flumetsulum; 0.052 kg a.i./ha).

Several experiments were undertaken using this stand. They were Experiment 6 (Section 5.1- Cutting frequency), Experiment 8 (Section 5.3- Fungicide and insecticide) and Experiment 13 (Chapter 8- Ewe fecundity after removal from lucerne). In addition, samples for Experiments 1-5 (Chapter 3- Method development) were collected from I12. Lucerne was removed from experimental plots by mowing to 65 mm with a 'Walker MTGHS 23HP' mower (Ft. Collins, Colorado, USA).

4.1.2 H7

Experiment 7 (Section 5.2- Cultivar) was on flat land in H7 of the Homestead Block, Ashley Dene Farm, Springston, Canterbury, New Zealand (43° 39' 24" S, 172° 19' 32" E) between October 2014 and May 2015. In addition, samples for Experiments 1-5 (Chapter 3- Method development) were collected from H7.

The soils are Lowcliffe moderately deep and Lowcliffe stony silt loam (Webb and Bennett, 1986) classified as Mottled Argillic Pallic soils (Hewitt, 2010). The stony Lowcliffe soils are imperfectly drained and shallow with <20 cm of moderately stony silt loam overlying stony silt loam. From 50-80 cm there are dense gravel pans which perch water. Rooting depth is typically less than 70 cm. The moderately deep profiles have 45-90 cm of imperfectly drained silt loam overlying gravels. The subsoil horizons are dense with slow permeability but allow root penetration.

The paddock was divided into six blocks and each block sown with seven cultivars of inoculated lucerne in November 2008. The cultivars were ‘Grasslands Kaituna’, ‘Stamina 5’, ‘Stamina 6GT’, ‘Rhino’, ‘Runner’ and two non-commercial genotypes not examined in this thesis. During the experiment, the lucerne was grazed under a 35 - 40 day six block rotation from late September to mid-November 2014. The entire paddock was again grazed for approximately two weeks from 17 March 2015. Further details of the grazing management were reported by Moot *et al.* (2016).

4.1.3 HRA14

Lucerne grazing for Experiment 13 (Chapter 8) occurred in I12 in April 2016 and the HRA14 in May 2016. HRA14 is located on flat land at the Lincoln University Research Area, Canterbury, New Zealand (43° 38' 54" S, 172° 27' 30" E). The soil is a Templeton silt loam classified as a Typic Immature Pallic Soil with a depth ranging from 55 to over 100 cm. The HRA14 was sown in autumn 2012 with ‘Force 4’ lucerne.

4.1.4 I9

Ryegrass/white clover grazing for Experiment 13 (Chapter 8) was performed in paddock 9 of Iversen Field. The species present were ‘Arrow’ AR37 perennial ryegrass and ‘Tribute’ white clover. Species were drilled in August 2014.

4.1.5 Glasshouse experiments

Glasshouses were used for Experiments 10 and 11 (Section 5.5- Water stress and Section 5.6- Aphids, respectively). The glasshouse used for Experiment 10 was the ‘Aluminex Glasshouse’. Experiment 11 was located in an ‘Eden 8 Single Door Glasshouse’.

4.1.5.1 Potting Mix

Potting mix used for the glasshouse experiments consisted of 80% composted bark and 20% pumice grade 1-7 mm. The potting mix was mixed with 3 g per litre of Everris Osmocote® Exact 3-4 month patterned release fertiliser (NPK 16-3.9-10), 1 g per litre horticultural lime (Southern Horticultural Products Ltd, Christchurch, New Zealand) and 1 g per litre of Hydraflo® Wetting Agent.

Seeds were applied at a rate of 10 per pot and following establishment, lucerne was thinned to three plants per pot.

4.1.5.2 Inoculation

Lucerne planted in the glasshouse experiments were inoculated with rhizobia (*Sinorhizobium meliloti* (Dangeard 1926) De Lajudie *et al*. 1994), using methodology adapted from Vincent (1970). A single colony of *S. meliloti,* isolated on yeast mannitol agar (YMA) from the nodules of lucerne grown with Group AL peat inoculant, was transferred to 500 mL sterile yeast mannitol broth (YMB). The YMB contained 1.0 g/L yeast extract, 10 g/L mannitol, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4$ and 0.1 g/L NaCl. The YMA also contained these ingredients, plus 1.0 g $CaCO_3$ and 15 g agar. The inoculated YMB was incubated at 27°C at 220 rpm on a shaking incubator to log phase, ca. 1 x 10^8 colony forming units (CFU)/mL. A control tube of YMB, not inoculated with a bacterial isolate, was included to ensure against contamination. At the time of sowing, each pot received 10 mL of inoculant.

4.2 Meteorological conditions

Experiments 6-8 and 12a were performed in rain-fed systems without irrigation. Irrigation was provided in Experiments 12b and 13.

4.2.1 Measurements

Rainfall (mm), air temperature (°C), relative humidity (%) and Penman potential evapotranspiration (mm) were recorded at Broadfield Meteorological Station, Lincoln, Canterbury (43° 37' 34.392'' S, 172° 28' 13.44" E; NIWA, National Institute of Water and Atmosphere Research, New Zealand), located 2 km north of Iversen Field and 12 km northeast of Ashley Dene. Rainfall at Ashley Dene was recorded between 1 August 2014 and 25 May 2015 at Burnham Sewage Plant Meteorological Station, Canterbury (4 km northwest of Ashley Dene; 43° 37' 20.1'' S, 172° 18' 33.3'' E). Data were sourced from CliFlo database (NIWA). Meteorological data are provided in Appendix A.

4.2.2 Long-term meteorological conditions

The average long-term meteorological conditions at Broadfield Meteorological Station between 1960 and 2015 are provided in Table 4.1. Average temperature ranged from 6.1°C in July to 16.7 °C in January. The mean annual rainfall during this period was 631 mm compared with an average annual potential evapotranspiration of 1090 mm.

Table 4.1 Monthly long-term means from 1960 to 2015 for air temperature (°C), rainfall (mm), and Penman potential evapotranspiration (mm). Data from the CliFlo database (NIWA), recorded at Broadfield Meteorological Station, Lincoln, Canterbury, New Zealand.

Month	Air temperature (°C)	Rainfall (mm)	PET (mm)
Jan	16.7	48.2	157
Feb	16.5	42.4	126
Mar	14.8	52.9	102
Apr	12.0	55.5	64.7
May	9.2	56.8	45.1
Jun	6.6	61.8	33.1
Jul	6.1	61.7	36.3
Aug	7.3	62.1	51.9
Sep	9.4	39.8	74.9
Oct	11.4	47.2	110
Nov	13.2	50.2	133.0
Dec	15.2	52.6	152
Annual	11.5	631	1090

4.3 Soil water budget

Plant available water capacity (AWC) is the difference between the field capacity (FC) and the lower limit of water extraction by a mature crop (McLaren and Cameron, 1996). The potential soil moisture deficit (SMD) is the difference between the AWC and the available soil water present in the soil. Daily SMD was calculated, beginning at a time when the soil was known to be fully recharged, by adding daily water use (WU) and subtracting daily rainfall (R) from the previous day's SMD. When SMD was less than 0 the amount was attributed to runoff or drainage. When the SMD was less than half of the AWC, daily WU was equal to the daily potential evapotranspiration (PET) values measured at the climate station, Broadfield EWS. When SMD was greater than half of the AWC the daily WU was calculated as: (AWC-SMD) / (0.5 x AWC) x PET (Moot *et al.*, 2012).

4.3.1 SMD in Iversen 12 for the entire experimental period

Figure 4.1 shows the rainfall events and changes in modelled SMD over time between 23 May 2013 and 1 June 2016 at Iversen 12, Lincoln, with notation for each individual sampling date. During this period three experiments were performed. The first and second cutting frequency experiments (Experiments 6a & 6b; Section 5.1) took place from March 2014 to June 2014 and from November 2014 to the end of May 2015, respectively. The fungicide and insecticide experiment (Experiment 8; Section 5.3), took place from November 2015 to the end of May 2016. The SMDs for these periods are described in further detail in Sections 4.3.2 to 4.3.4, while this section provides an overview of the entire duration.

The FC of Iversen 12, in a fallowed state, five days after complete soil recharge, was calculated to be 760 mm for the soil profile to 2.3 m depth with an AWC of 360 mm (Sim, 2014). The threshold for water stressed status was 180 mm. The soil water deficit model for Iversen 12 began on 23 May 2013. On this date, the SMD in the NIWA database (Broadfield EWS), which assumes an AWC of 150 mm, was 0.0 mm for the first time since summer. Therefore in Iversen 12, assuming an AWC of 360 mm, the SMD was considered to be 210 mm.

The soil profile was fully recharged during the winter months of 2013. Between 15 June and 23 June 2013, the SMD decreased by 21 mm per day from 194 mm to 16.0 mm. The SMD decreased to lows of 2 mm by 16 July 2013 and 0 mm by 19 August 2013. The SMD increased to a peak of 300 mm during the 2013/14 summer. In winter 2014, the soil profile was recharged to a minimum SMD of 14 mm. During the 2014/15 summer the SMD peaked at 330 mm. Soil moisture was not substantially recharged in the winter of 2015, reaching a minimum SMD of 174 mm. During the 2015/16 summer the SMD peaked at 312 mm.

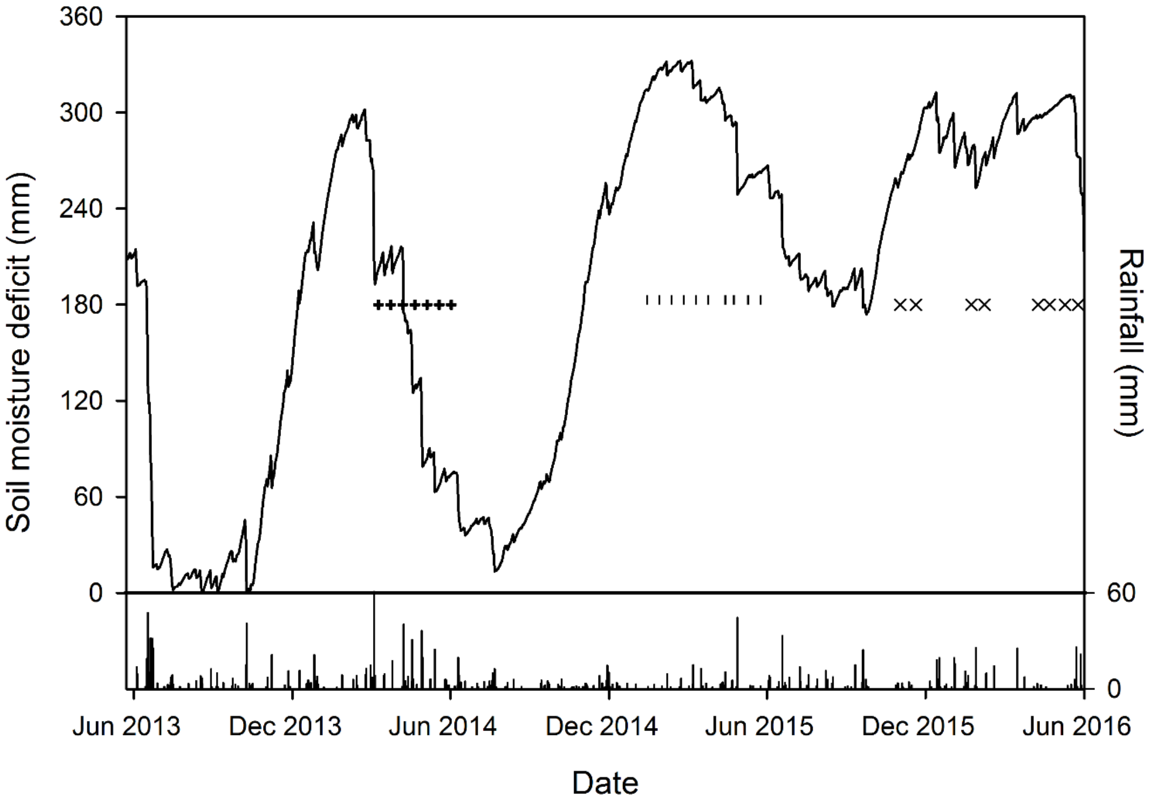


Figure 4.1 **The modelled soil moisture deficit (mm) and actual rainfall events (mm) in Iversen 12 between 23 May 2013 and 1 June 2016, before and during the experimental periods of Experiment 6a (+), Experiment 6b (|) and Experiment 8 (x). The water stress threshold was 180 mm. Sampling dates with a symbol below the SMD line are considered to be water stressed with reduced rate of transpiration, while sampling dates with a symbol above the SMD line have access to readily available water.**

4.3.2 SMD in Iversen 12 for cutting frequency experiment: first season

By the start of Experiment 6a (Section 5.1) on 10 March 2014, the modelled SMD (Section 4.3.1) was 200 mm (Figure 4.2). Following a rainfall event of 40 mm on 8 April 2014, the SMD decreased from 215 to 175 mm. A second large rainfall event on 18 April of 31 mm decreased the SMD from 160 to 130 mm and a third rainfall event on 29 April and 30 April decreased the SMD from 134 to 79 mm. A fourth event on 14 May decreased the SMD from 88 to 63 mm.

Across the entire experiment the SMD decreased at a rate of 2.2 mm/d. The SMD was greater than half AWC between 10 March and 7 April 2014 and less than half between 21 April and 2 June 2014. Sampling date symbols (+) below the SMD line are considered water stressed, while symbols above the SMD line have access to readily available water.

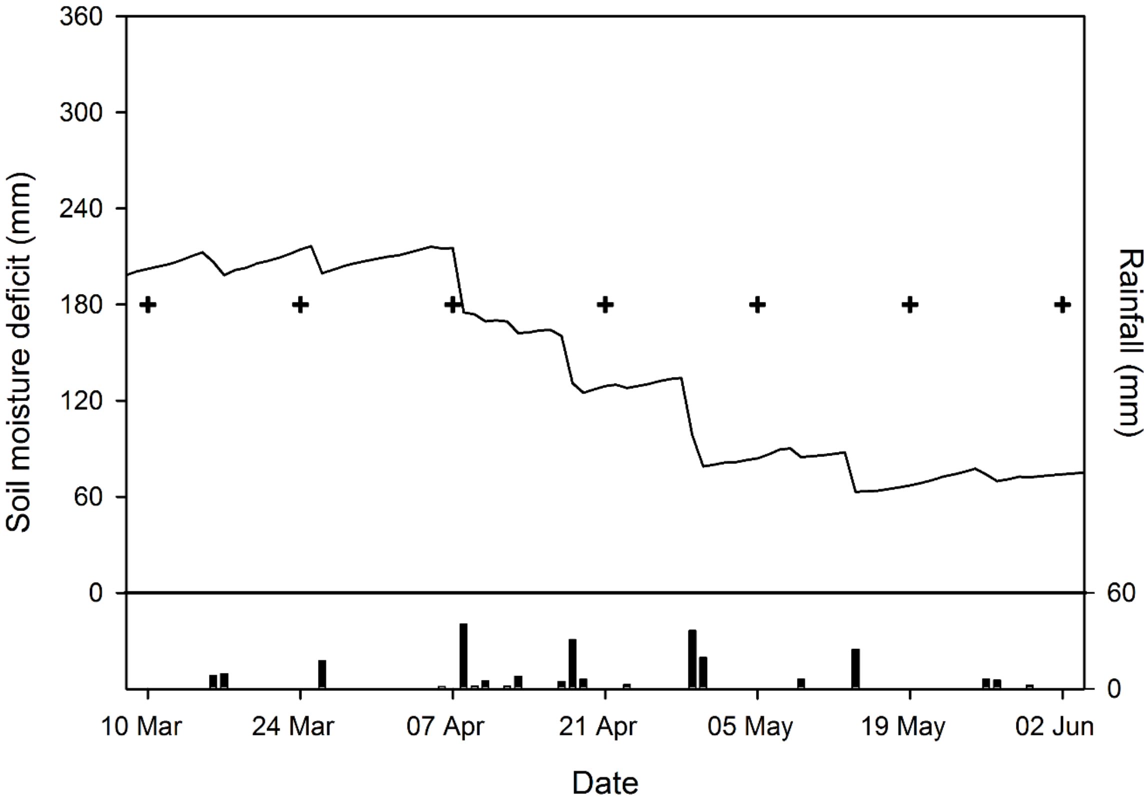


Figure 4.2 The modelled soil moisture deficit (mm) and actual rainfall events (mm) in Iversen 12 for Experiment 6a between 10 March 2014 and 2 June 2014 with relation to lucerne sampling dates (+). The water stress threshold was 180 mm. Sampling dates with a symbol below the SMD line are considered water stressed, while sampling dates with a symbol above the SMD line have access to readily available water.

4.3.3 SMD in Iversen 12 for cutting frequency experiment: second season

Figure 4.3 shows the SMD modelled between spring 2014 and autumn 2015, during Experiment 6b (Section 5.1) in Iversen 12. The soil moisture did not fully recover in winter 2014 with a SMD of 14 mm on 22 July 2014 (Section 4.3.1). The SMD increased through the spring reaching 104 mm Sat the beginning of the experiment (10 October 2014). The SMD reached 180 mm (half of the max AWC) by 2 November 2014. The SMD increased to 244 mm by 20 November and remained above 300 mm between 4 January and 13 April 2015. Following a rainfall event on 28 April 2015, the SMD decreased from 293 to 249 mm. The SMD was 262 mm by 25 May 2015. Based on the SMD model, lucerne was water stressed on all sampling dates, however after the rainfall event on 28 April, it is likely that as water was in the top section of the soil profile, rather than distributed throughout, it would have been readily available for the period before the subsequent sampling date on 10 May 2015.

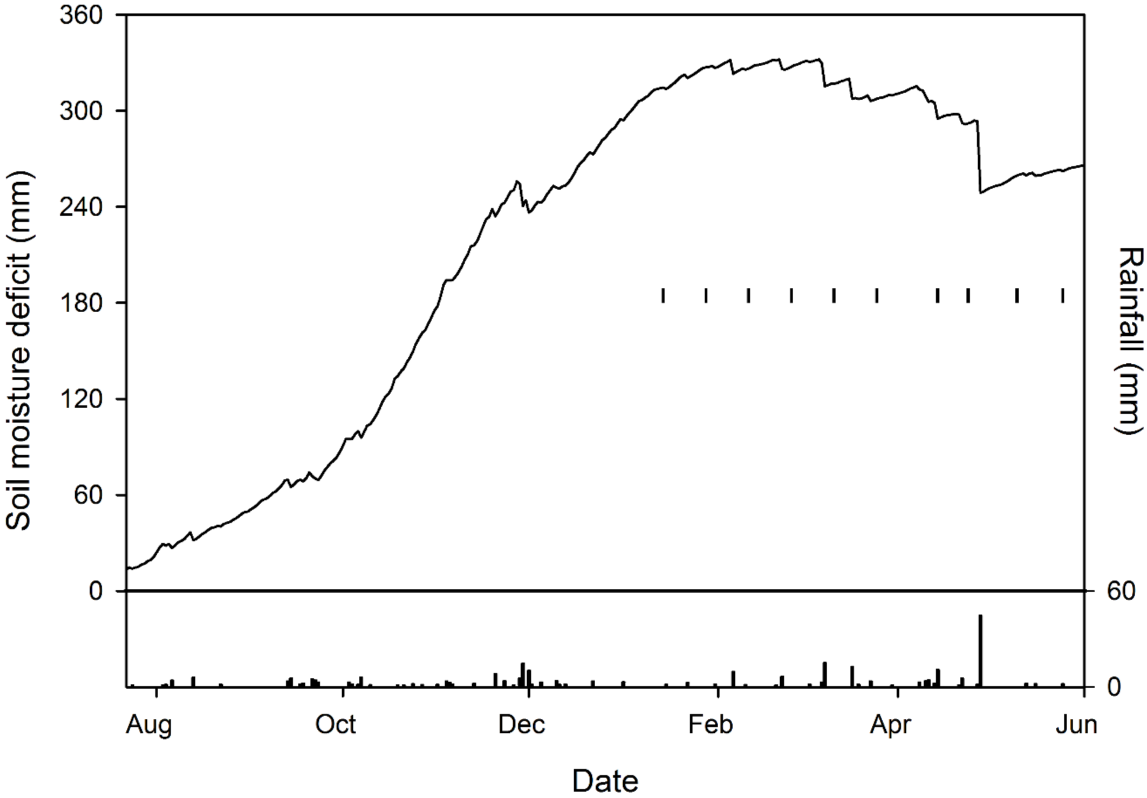


Figure 4.3 The modelled soil moisture deficit (mm) and actual rainfall events in Iversen 12 for Experiment 6b between 22 July 2014 and 1 June 2015 with relation to lucerne sampling dates (|).The water stress threshold was 180 mm meaning lucerne was considered to be water stressed with reduced rate of transpiration on each sampling date.

4.3.4 SMD in Iversen 12 for fungicide and insecticide experiment

Directly following on from, but in a different part of Iversen 12 than Experiments 6a and 6b (Section 5.1), was Experiment 8 (Section 5.3). The SMD modelled during the experimental period between 1 October 2015 and 25 May 2016 is given in Figure 4.4. The SMD at the beginning of the experimental period was 187 mm. This was similar to half of the max AWC (180 mm). The SMD increased to 312 mm by 13 December 2015 and remained above 250 mm for the remainder of the experimental period. Based on the SMD model lucerne was under water stress throughout the experimental period.

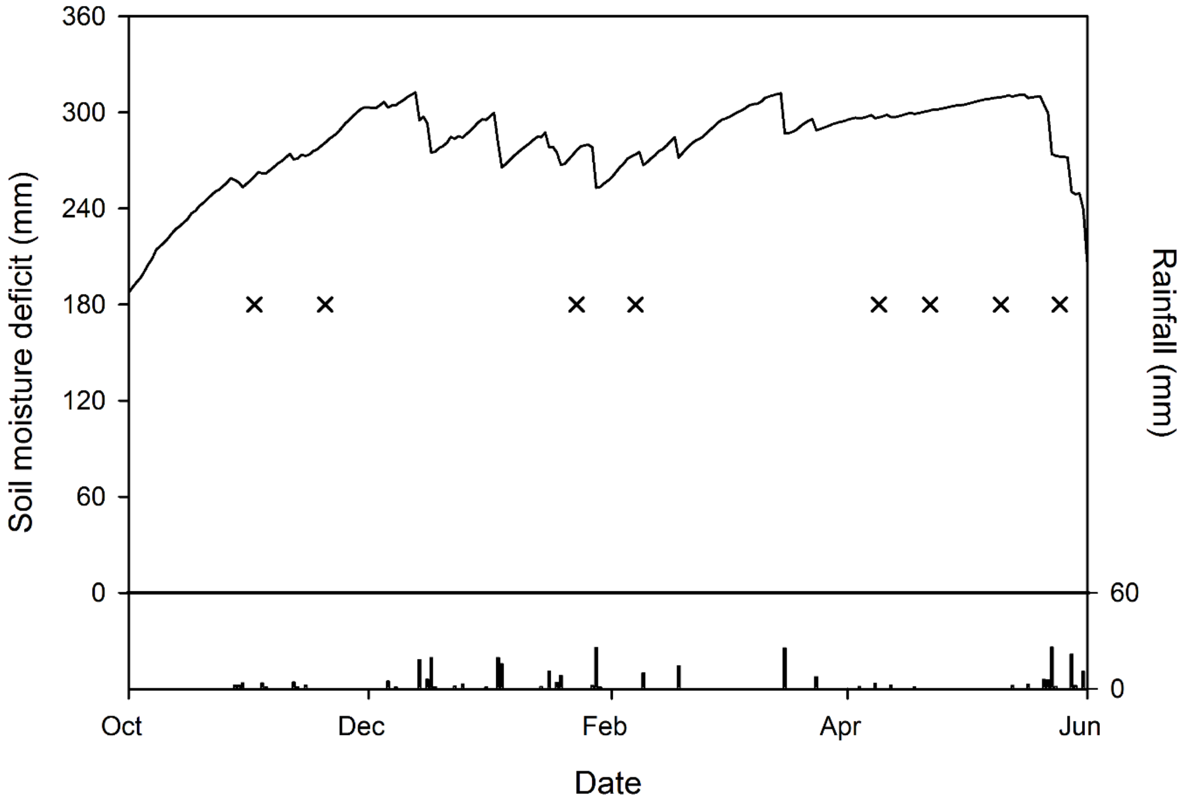


Figure 4.4 The modelled soil moisture deficit (mm) and actual rainfall events in Iversen 12 for Experiment 8 between 25 May 2015 and 25 May 2016 with relation to lucerne sampling dates (X). The water stress threshold was 180 mm meaning lucerne was considered to be water stressed with reduced rate of transpiration on each sampling date.

4.3.5 SMD at Ashley Dene for cultivar experiment

The Lowcliffe soils in paddock H7 at Ashley Dene have an AWC of between 70-120 mm AWC (McLenaghen and Webb, 2012). For this model an estimated AWC of 100 mm was used. Rainfall data was from Burnham Sewage Plant while PET was from Broadfield EWS Station. The model began on 21 July 2014, when soil moisture deficit was 0.0 mm in NIWA's model of a soil of 150 mm AWC.

Prior to the cultivar comparison experiment (Experiment 7; Section 5.2) the SMD (Figure 4.5) increased from 0 to 90 mm between late July and late October 2014 with 50% of max AWC reached by 9 September 2014. SMD remained above 80 mm from late October to early March. The SMD was above 88 mm at the start of Experiment 7 in January and through February 2015. SMD between 8 March and 27 April 2015 ranged between 53 and 85 mm. A rainfall event on 28 April decreased the SMD from 60 mm to 2 mm, bringing the SMD below 50% of max AWC. The SMD then increased over the subsequent month to 34 mm on 2 June 2015. Based on the SMD model, lucerne was water stressed on the sampling dates between 15 January and 5 March 2015 and not water stressed on 2 or 17 May 2015.

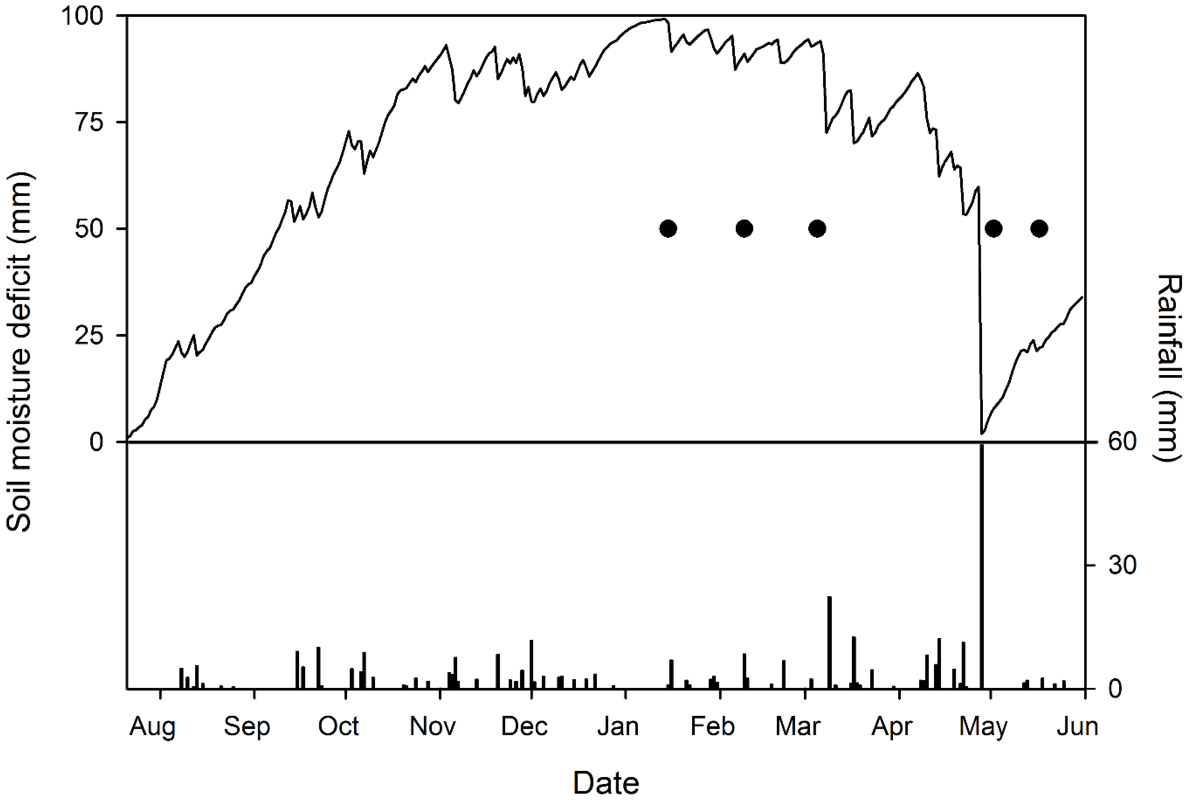


Figure 4.5 The modelled soil moisture deficit (mm) and actual rainfall events in H7 of Ashley Dene between 21 July 2014 and 1 June 2015 for Experiment 7 with relation to lucerne sampling dates (●).The water stress threshold was 50 mm. Sampling dates with a symbol below the SMD line are considered water stressed, while sampling dates with a symbol above the SMD line have access to readily available water.

4.4 Field measurements and lucerne collection

Lucerne was sampled fortnightly using one 0.2 m^2 quadrat per plot for Experiments 6- 8 (Sections 5.1 to 5.3), or using three quadrats pre- and post-grazing for each break in Experiment 13 (Chapter 8). Random placements of the quadrats were pre-determined for each plot to avoid re-cutting a previously sampled area. For each quadrat, the average height of the lucerne was determined using a 1 m ruler. Lucerne was cut above crown height (approximately 40 mm above the ground) to avoid damaging the plants. After samples were cut, they were stored in a cooler at 4°C until they were processed.

4.5 Lucerne processing

From the lucerne samples, the fungal pathogen species were identified and fungal damage was scored using the criteria based on Harvey and Martin (1980) and plant assessment keys from James (1971). The categories of the scoring system are outlined in Table 4.2. Insect damage was also scored using the damage area thresholds.

Plant material was scored for mean stage of development using the criteria and calculations given in Fick and Mueller (1989). A random sample of 25 stems was obtained. Each stem was individually scored using the criteria in Table 4.3 and the mean stage determined using the following formula:

$$Mean\ Development\ Stage = \frac{\Sigma(Stage\ number, x *\ number\ of\ stems\ at\ stage, n)}{\Sigma n}$$

The lucerne was oven dried for 48 hours at 60°C and weighed to calculate tonnes of dry matter (DM) yield per hectare (t DM/ha).

Table 4.2 Damage severity score categories for fungal and insect damage

Damage Score	Score description
1	No symptoms
2	Trace: 1-4% of area affected
3	Light to moderate: 5-20% of area affected
4	Heavy: 21-50% of area affected
5	Severe; over 50% of area affected

Table 4.3 Morphological stages of development of lucerne shoots.

Stage number	Stage name	Stage definition
1	Early vegetative	Stem length less than 15 cm, no buds flowers or seed pods.
2	Mid vegetative	Stem length 15-30 cm, no buds flowers or seed pods.
3	Late vegetative	Stem length greater than 30 cm, no buds flowers or seed pods.
4	Early flower bud	1-2 nodes with flower buds, no flowers or seed pods.
5	Late flower bud	3 or more nodes with flower buds, no flowers or seed pods.
6	Early flower	1 node with one open flower, no seed pods.
7	Late flower	2 or more nodes with open flowers, no seed pods.
8	Early seed pod	1-3 nodes with green seed pods.
9	Late seed pod	4 or more nodes with green seed pods.
10	Ripe seed pod	Nodes with mostly brown, mature seed pods.

4.6 Coumestrol extraction

The extraction method used was based on the results provided in Chapter 3. In summary, material was ground with two machines, one that coarsely ground the material and could cut the lucerne stems, followed by a second machine (Ultra Centrifugal Mill ZM 200, Retsch, Germany) that ground the lucerne finely through a 1 mm sieve. Material was then stored in a freezer for long term (weeks) or refrigerator for short term (days). Lucerne samples of 0.5 g were weighed into 5 mL HPLC-grade 99.9% methanol (Sigma-Aldrich, Missouri, USA) in 15 mL conical bottom tubes (Axygen Scientific, California, USA). Solutions were vortex mixed for 20 seconds and put on an end over end mixer for 16 hours at room temperature. Solutions were centrifuged at 4,700 x *g* for 5 minutes and 1.5 mL of supernatant was transferred to a 1.5 mL microcentrifuge tube (Axygen Scientific, California, USA) and stored in the freezer.

4.7 HPLC measurement of coumestrol in lucerne

Glass fibre syringe filters were used to filter samples of approximately 500 µL volume into 2 mL glass auto-sampler vials (Thermo Scientific, Massachusetts, USA). Extracted samples were analyzed for coumestrol content using HPLC with methodology adapted from Wang *et al.* (1990). HPLC analyses were performed with an Agilent 1100 series instrument (Agilent Technologies, Walbronn, Germany) equipped with binary pumps, and a fluorescence detector set at 365 nm for excitation and 418 nm for emission. The injection volume was 10 µL. Separation was carried out on an ACE reverse phase column (C18, 3 µm, 150 mm x 4.6 mm, Winlab, Scotland) at 25°C, with the flow rate set at 0.5 mL/min. Solvent A was deionized water and solvent B was 100% methanol. Elution of coumestrol was performed by linearly increasing the percentage of solvent B from 40% to 100% over 14 minutes. Solvent B was maintained at 100% for 2 minutes. The column was re-equilibrated for 9 minutes between samples. Coumestrol (Sigma-Aldrich, Missouri, USA) was used to make a calibration curve from 0.5 to 20 mg/L.

4.8 Coumestrol rating scale

For the purposes of this thesis the criteria in Table 4.4, on a scale from negligible to extreme, are used to describe the coumestrol level in lucerne. A coumestrol level of 25 mg/kg DM is used throughout this thesis as a threshold above which coumestrol has been shown to reduce ewe reproductive performance (Smith *et al.*, 1979). However, this level should be regarded with caution. It is likely that the true safe level is below this value.

Table 4.4 Coumestrol content (mg/kg DM) rating scale for lucerne samples.

Coumestrol content (mg/kg DM)	Rating
<1	Negligible
1-10	Low
10-25	Moderately low
25	**Risk threshold**
25-50	Moderate
50-100	Moderately high
100-150	High
>150	Extreme

4.9 Plasma collection

Blood samples (10 mL) were obtained by venepuncture from an external jugular vein whilst the sheep were manually restrained using evacuated plastic tubes containing sodium ethylene diamine tetra acetic acid (BD 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. Blood samples were centrifuged for 10 minutes at 1300 x *g* to obtain plasma which was transferred to glass vials and stored frozen (at -20 °C).These samples were collected from four ewes per treatment at the beginning of the experiment, at the time points when a group was transferred from lucerne to grass and three and seven days after transfer.

4.10 Plasma extraction

To measure the concentration of free coumestrol, 1.5 mL plasma was extracted with 3 x 3 mL ethyl acetate (Sigma-Aldrich, Missouri, USA) by vortex mixing for one minute, followed by centrifugation (4000 x *g* for 10 minutes). The ethyl acetate layers were collected and evaporated to 1.5 mL by CentriVap Centrifugal Vacuum Concentrator (Labconco, Missouri, USA) at 40°C. The solution was transferred to a glass microcentrifuge tube and centrifuged (13,400 x *g* for 15 minutes). The supernatant was transferred to a 15 mL conical bottom tube (Axygen Scientific, California, USA) and evaporated to dryness by the CentriVap Centrifugal Vacuum Concentrator. The residue was dissolved in 300 µL 99% methanol, centrifuged (13,400 x *g* for 15 minutes) and transferred to a 2 mL glass auto-sampler vial (Thermo Scientific, Massachusetts, USA).

To measure concentration of conjugated coumestrol, plasma (1.5 mL) was incubated with 50 units of H-1 sulphatase plus 1,000 units of β-glucuronidase (Sigma-Aldrich, Missouri, USA) in 3 mL 0.2 M sodium acetate buffer solution (pH 5.5) in a shaking bath at 37 °C for 16 hours prior to extraction, as described above.

4.11 HPLC Measurement of Coumestrol in Plasma

Prior to HPLC injection, the solution was centrifuged (15 minutes, 13,000 x *g*) and due to the small sample volume, not filtered. HPLC analyses were performed as described in Section 4.7, but with a 50 µL injection volume and elution of coumestrol was performed by linearly increasing the percentage of solvent B from 40% to 100% over 20 minutes. Solvent B was kept at 100% for 2 minutes. The column was re-equilibrated for 9minutes between samples. The coumestrol calibration curve was 0, 0.05, 0.5, 1 and 5 mg/L coumestrol.

4.12 Statistical Analyses

The standard error of the mean (SEM) is presented where mean data are reported in the format: mean ± SEM. The standard errors of the coefficients are presented where regression equations are reported in the format: $y = mx + c$ ($\pm SE_m$; $\pm SE_c$).

4.12.1 Agronomic screening chapter

In Chapter 5, statistical analyses were performed using Genstat 16.1. Analysis of variance (ANOVA) was used to analyse variables in experiments with a randomised complete block design (Sections 5.1 to 5.6). When samples were collected over time the experiment was analysed as a split-plot ANOVA with each block containing plots that were randomly allocated a treatment. Fisher’s protected least significant difference (LSD) post hoc test was used to separate means when the ANOVA was significant ($\alpha = 0.05$).

In field experiments (Sections 5.1 to 5.3), linear regression analysis was used to analyse rates of dry matter production between and within growing seasons. Pearson correlation coefficient (r) was used to assess the relations between coumestrol levels and dry matter yield, plant height, average number of leaves per stem and fungal damage score.

4.12.2 Chapters 6-8

Statistical analyses for these sections are described within the relevant chapters.

Chapter 5

Agronomic Screening

This chapter includes the experiments undertaken to meet Objective 2. This objective was to isolate factors which increase the risk of high coumestrol in lucerne and identify strategies to minimise coumestrol accumulation. Specifically:

- Experiment 6 isolated the effect of lucerne stand cutting frequency and development stage.
- Experiment 7 isolated the effect of lucerne cultivar.
- Experiment 8 isolated the effect of fungicide and insecticide treatments.
- Experiment 9 isolated the effect of cool temperature biotype stemphylium infection.
- Experiment 10 isolated the effect of water stress and recovery from water stress.
- Experiment 11 isolated the effect of aphid infestation.

Each experiment is presented individually with relevant material and methods, results and discussion sections.

5.1 Experiment 6 Cutting frequency and development stage

5.1.1 Introduction

Previous studies (Section 2.4) reported that coumestrol content of lucerne was related to developmental stage. The flowering and seed set stages typically had higher coumestrol content than vegetative stages (Bickoff *et al.*, 1960a; Hanson *et al.*, 1965; Seguin *et al.*, 2004). This could be a problem in autumn when farmers are recommended to spell lucerne in late summer or early autumn until it reaches 50% flowering to give the crop time to recharge its root reserves (Moot *et al.*, 2003b). The start of the ewe mating season coincides with this recommended flowering period. This could create contradictory management between plant and animal priorities.

However, the studies which showed a relationship between developmental stage and coumestrol content did not simultaneously compare stands at different stages of maturity. They also did not quantify the non-developmental properties of the stand that prevailed during plant development, such as fungal infection and meteorological conditions. Separation of growth stage from these factors is important to determine whether developmental stage in isolation has an effect on coumestrol or if an external factor caused an apparent effect in these studies.

The objective of Experiment 6a and 6b was to determine any variation in coumestrol content of field grown lucerne in summer and autumn under different cutting frequencies and isolate the effect of development stage on coumestrol content in lucerne. Therefore, the null hypotheses are that coumestrol content of lucerne is not affected by development stages nor is it affected by cutting frequency.

5.1.2 Methodology

5.1.2.1 Experimental Site

This experiment was located in I12, Lincoln University during autumn 2014 (first season; Experiment 6a) and from late spring 2014 to the end of autumn 2015 (second season; Experiment 6b). Plots were 2 x 4 m plots of 'Stamina 5' lucerne. A description of the site and mowing method is given in Section 4.1.

5.1.2.2 Experimental Design

Experiment 6a took place in autumn 2014, between 24 March and 2 June. The experiment was a split plot design with three blocks, five cutting frequency treatments (2, 4, 6, 8, or 12 week intervals) and six sampling dates (5 x 3 x 6, N = 90). Each block contained five plots which were randomly allocated one of the five cutting frequency treatments and samples were taken every two weeks beginning 24 March. Lucerne regrowth was two weeks old at the onset of the experiment.

Experiment 6b took place between 22 October 2014 and 25 May 2015 in a different section of I12 to that studied in Experiment 6a. The experiment was a split plot design with three blocks which each contained three plots. These plots were each randomly allocated one of the three cutting frequency treatments (approximately 4, 6 and 12 week intervals). Lucerne yield was sampled at two week intervals on 14 dates beginning 22 October 2014 (3 x 3 x 14, N = 126) while coumestrol content was measured at two week intervals on 10 dates beginning 14 January 2015 (3 x 3 x 10, N = 90). Lucerne regrowth was four weeks old at the onset of the cutting treatments and yield sampling.

The lucerne samples were processed and coumestrol was extracted as described in Sections 4.4 to 4.6 (Chapter 4). Coumestrol was measured by HPLC with the methodology described in Section 4.7. The coumestrol rating scale (Table 4.4) was used to rate coumestrol content from negligible to extreme. Statistical analyses are described in Section 4.12.1. A square root transformation was used to normalise coumestrol data for Experiment 6a and a $\log_{10}$ transformation was used for Experiment 6b. Graphs were drawn with the dependent variable back transformed.

5.1.2.3 Meteorological Data

Rainfall and temperature data were recorded at Broadfield Meteorological Station, Lincoln, 2.5 km from the field site, for the duration of the experiment. The daily rainfall and mean daily temperature during the autumn 2014 are shown in Figures A.1 and A.2 (Appendix A). The total rainfall during the first experimental period between 10 March 2014 and 2 June 2014 was 242 mm, with 161 mm of this falling in April 2014.

The daily rainfall and mean daily temperature during the second experimental period are shown in Figures A.3 and A.4.The total rainfall was 257 mm between 15 September 2014 and 25 May 2015. Total monthly rainfall ranged from 6.4 mm in May 2015 to 77.6 mm in April 2015. A rainfall event of 44.6 mm occurred on 28 April 2015.

5.1.3 Experiment 6a results

5.1.3.1 Lucerne yield

Lucerne remained in a vegetative state (stage 1-3; Table 4.3) throughout Experiment 6a. Figure 5.1 shows the interaction ($P < 0.001$) of cutting frequency and date on lucerne yield. At the onset of the experiment on 24 March 2014, the two week old lucerne regrowth ($n = 15$) had a yield of 0.59 t DM/ha and a height of 15 ± 1.0 cm (Stage 1-2). Between 24 March and 21 April 2014, unmown lucerne grew at a rate of 33 kg DM/ha/d ($R^2 = 0.739$). Lucerne ($n = 9$) reached a pasture mass of 1.5 ± 0.09 t DM/ha and height of 37 ± 0.9 cm (Stage 3) in uncut, six week old regrowth on 21 April 2014. This was higher ($P < 0.001$) than two week old lucerne regrowth ($n = 6$) with yield of 0.15 ± 0.019 t DM/ha and height 3.5 ± 0.6 cm (Stage 1). From 21 April 2014, pasture mass did not change ($P = 0.142$) over time in the uncut plots (Stage 3). Once plots were cut ($n = 42$), there was no effect ($P > 0.1$) of harvest date or cutting treatment with an average dry matter yield of 0.17 ± 0.012 t DM/ha (Stage 1).

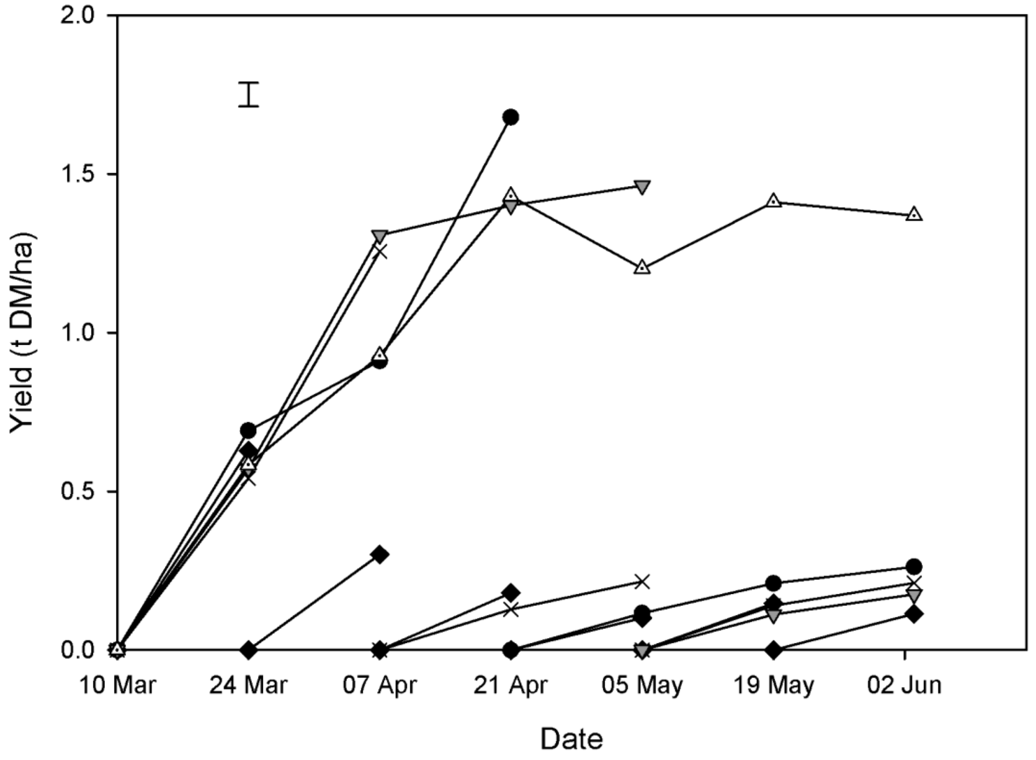


Figure 5.1 Dry matter (DM) yield (t/ha) of lucerne measured fortnightly in autumn 2014 under cutting intervals of 2 (◆), 4 (X), 6 (●), 8 (▲) or 12 (△) weeks. Line breaks indicate when lucerne was mown. Error bar is the standard error of the mean for the interaction between cutting frequency and date.

5.1.3.2 Coumestrol concentrations over time

Square root transformed coumestrol content of lucerne was affected ($P < 0.001$) by a time × cutting treatment interaction in Experiment 6a. Figure 5.2 shows the square root transformed coumestrol variable against cutting frequency and time.

Pre-coumestrol spike (24 March and 7 April 2014)

For the first four weeks of the experiment, coumestrol content was low to moderately low in all plots. Coumestrol was higher ($P < 0.001$) on 7 April (23.0 ± 7.30 mg/kg DM) than 24 March 2014 (5.8 ± 3.66 mg/kg DM). There were no differences ($P > 0.05$) in coumestrol content among cutting frequencies on either date. On 24 March 2014 all plots contained two week old regrowth with early vegetative (Stage 1-2) lucerne. The lucerne had a fungal damage score of 4 due predominantly to cool-type *Stemphylium* sp. symptoms. On 7 April 2014, lucerne of the two week cutting frequency plots had two weeks regrowth and lucerne of all other plots had four weeks regrowth. Stemphylium symptoms were still present with a fungal damage score of 2.8.

Coumestrol spike (21 April 2014)

Coumestrol further increased ($P < 0.001$) on 21 April 2014 across all plots. This marked elevations was designated as a 'coumestrol spike'. The six week old regrowth of the six, eight, and 12 week cutting frequency plots had a high mean coumestrol content of 138 ± 17.9 mg kg^{-1} DM compared (P =

0.398) with the two week regrowth of the fortnightly cut alfalfa which had 137 ± 17.8 mg kg^{-1} DM. The two week regrowth of the four week cutting frequency plots had a lower (P = 0.025) coumestrol content with a moderately high 82.3 ± 13.79 mg kg^{-1} DM. There was no relationship between the coumestrol content of lucerne and dry matter yield which ranged from 0.1 t DM/ha to 1.9 t DM/ha (P = 0.276), height which ranged from 2 cm to 40 cm (P = 0.238) or average number of leaves per stem which ranged from 2 per stem to 17 per stem (P = 0.196). Lucerne had an average fungal damage score of 3.5 and spring black stem (*Phoma* sp.) symptoms were present on both leaves and stems of lucerne in addition to stemphylium.

Post-coumestrol spike (5 May to 2 June 2014)

From 21 April to 2 June 2014 coumestrol content remained elevated in the plots that had not yet been mown, with an average extreme coumestrol content of 161 ± 19.3 mg/kg DM (n = 21). These plots had average fungal damage scores of 4 on 5 May and 5 on 2 June 2014. Cutting of the unmown lucerne resulted in lower coumestrol levels. For example, two week regrowth of lucerne cut at six weeks or eight weeks had decreased (P < 0.001) coumestrol contents of 29.4 ± 8.24 mg/kg DM (moderate) and 53.3 ± 11.10 mg/kg DM (moderately high), respectively.

In contrast, coumestrol content of the two week old regrowth of the lucerne cut at four week intervals increased (P = 0.005) to a high 139 ± 17.9 mg/kg DM on 19 May 2014 compared with 73.6 ± 13.04 mg/kg DM on 5 May 2014. It then decreased (P < 0.001) to a moderate 36.5 ± 9.18 mg/kg DM on 2 June 2014. The coumestrol content of the lucerne cut every two weeks remained elevated and did not respond (P > 0.116) to the subsequent cuts.

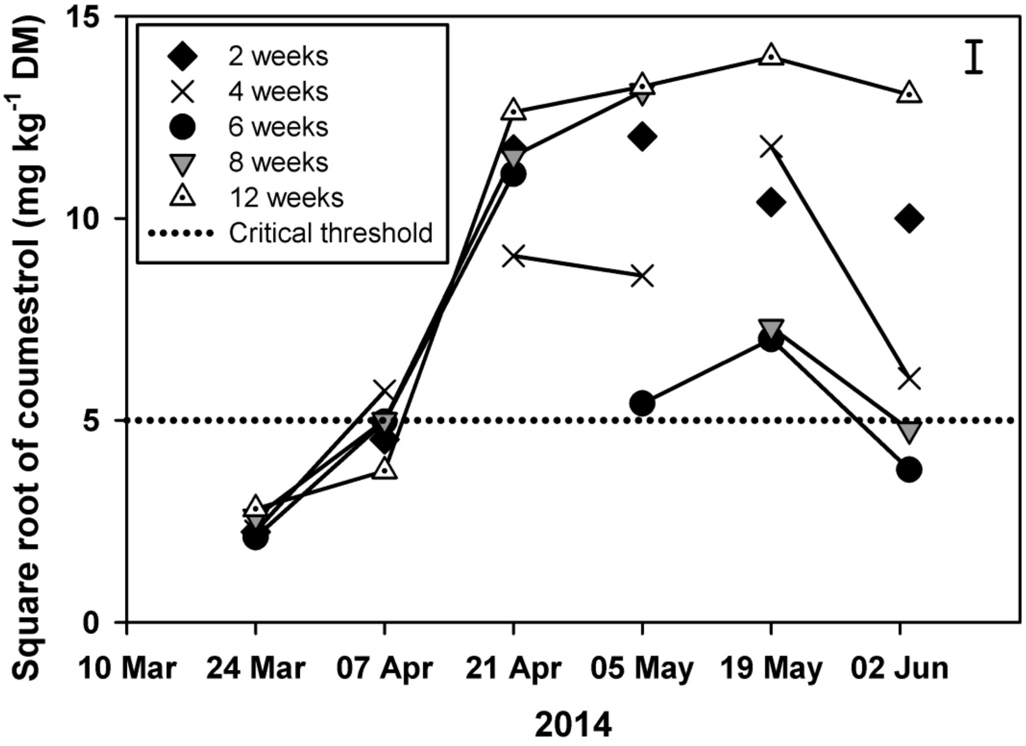


Figure 5.2 Square root transformed coumestrol content of lucerne under cutting intervals of 2 (◆), 4 (×), 6 (●), 8 (▼) and 12 weeks (△) from 24 March to 2 June 2014. Line breaks indicate when lucerne had been mown. Dotted line is the square root of the critical value (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance. Error bar is the standard error of the mean for the interaction between treatment and time.

5.1.3.3 Coumestrol content versus vegetative growth

Figure 5.3 shows the coumestrol content versus dry matter production (t DM/ha), plant height (cm) and average number of leaves per stem on 7 and 21 April 2014.

On 7 April 2014, when coumestrol levels were moderate (25.5 mg/kg DM; Section 5.1.3.2), there was no relationship between coumestrol content and dry matter production (P = 0.739), plant height (P = 0.625) or the average number of leaves per stem (P = 0.989).

On 21 April 2014, coumestrol had increased (P < 0.001) to a high level in both the short (Stage 1) two week (114 mg/kg DM) and tall (Stage 3) six week old (140 mg/kg DM) lucerne regrowth, as detailed in Section 5.1.3.2. Again, there was no relationship between the coumestrol content of lucerne and dry matter production (P = 0.276), height (P = 0.238) or average number of leaves per stem (P = 0.196).

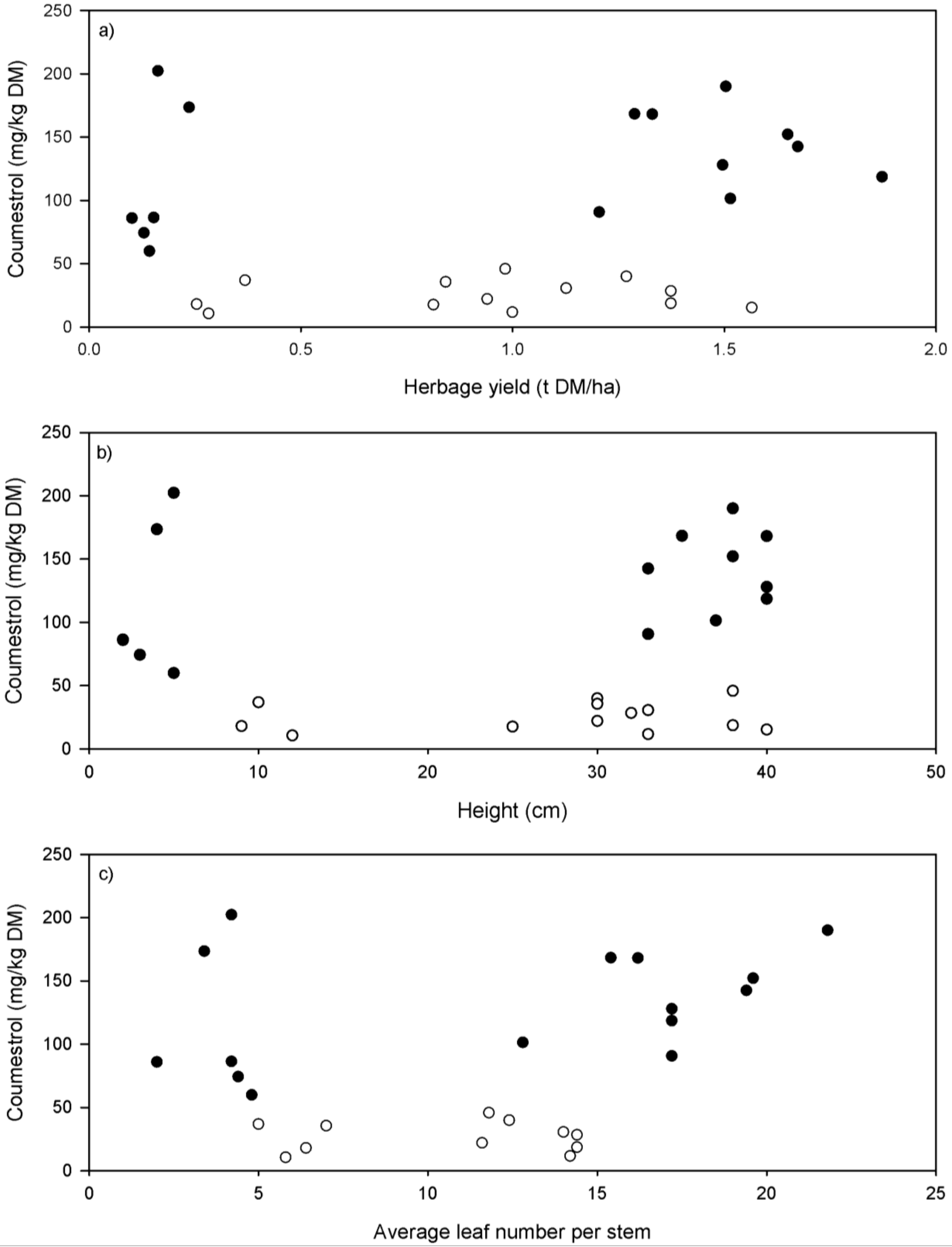


Figure 5.3 Coumestrol content (mg/kg DM) of lucerne against a) dry matter yield (t/ha), b) plant height (cm), and c) number of leaves per stem on 7 April 2014 (○) and 21 April 2014 (●).

5.1.3.4 Fungal damage score over time

Figure 5.4 shows the change in fungal damage score (Table 4.2) over time across the different cutting treatments in Experiment 6a. There was no difference (P = 0.426) in lucerne fungal pathogen damage between the treatment designations on 24 March 2014 (Stage 1-2) and 7 April 2014 (Stage 1 and Stage 3). Lucerne fungal pathogens on these dates were predominantly stemphylium (Figure 5.7). Fungal damage score was higher (P< 0.001) on 24 March 2014 than 7 April 2014, with average scores of 4.0 and 2.8 respectively.

By 21 April 2014, spring black stem was also present with visual symptoms of this infection present on both leaves and stems (Figure 5.8). Fungal damage score, relative to 7 April 2014, had increased (P < 0.05) to 3.7 ± 0.33 in the unmown treatments (Stage 3) and did not change (P > 0.05) in the two week re-growths of the two and four week cutting intervals (Stage 1), with damage scores of 3.3 and 3.0, respectively. There was no difference (P = 0.133) in the fungal damage scores between these mown and unmown treatments.

By 5 May 2014, fungal damage (4.0) in unmown plots (Stage 3) was unchanged (P = 0.131) relative to 21 April 2014 (3.7). The unmown plots had a higher (P < 0.001) damage score than four week old regrowth of the four week cutting treatment (3.3), two week old regrowth of the two week cutting treatment (3.0) and two week old regrowth of the six week cutting treatment (2.7). The mown plots were all Stage 1.

On 19 May 2014, there was no change (P = 0.116) in the fungal damage score of the unmown plots (Stage 3) relative to 5 May 2014 (4.7 vs 4.0). There was a difference (P < 0.001) in some mown (Stage 1) treatments. The fungal damage score of the eight week cutting interval treatment decreased (P < 0.05) from 4.0 to 3.0 in new regrowth, following mowing on 5 May 2014. Although the damage score of the two week cutting interval treatment increased (P < 0.05) from 3.0 to 4.0, the damage scores of the four week and six week cutting frequency treatments did not change (P > 0.05) relative to 21 April 2014.

On 2 June 2014, damage score (5) of the unmown plots (Stage 3) was not different to that of 19 May 2014 (4.7), but was higher (P < 0.05) than on all other harvest dates. The unmown plots had a higher (P = 0.007) damage score than the previously mown plots (Stage 1). The four week old regrowth of the eight week cutting frequency treatment had a lower (P < 0.05) damage score (3) than all other mown treatments, except (P > 0.05) the four week old regrowth of the four week cutting frequency treatment (3.3). There was no difference (P>0.05) in damage score between the two, four and six week cutting interval treatments and these had not changed (P>0.05) relative to 19 May 2014.

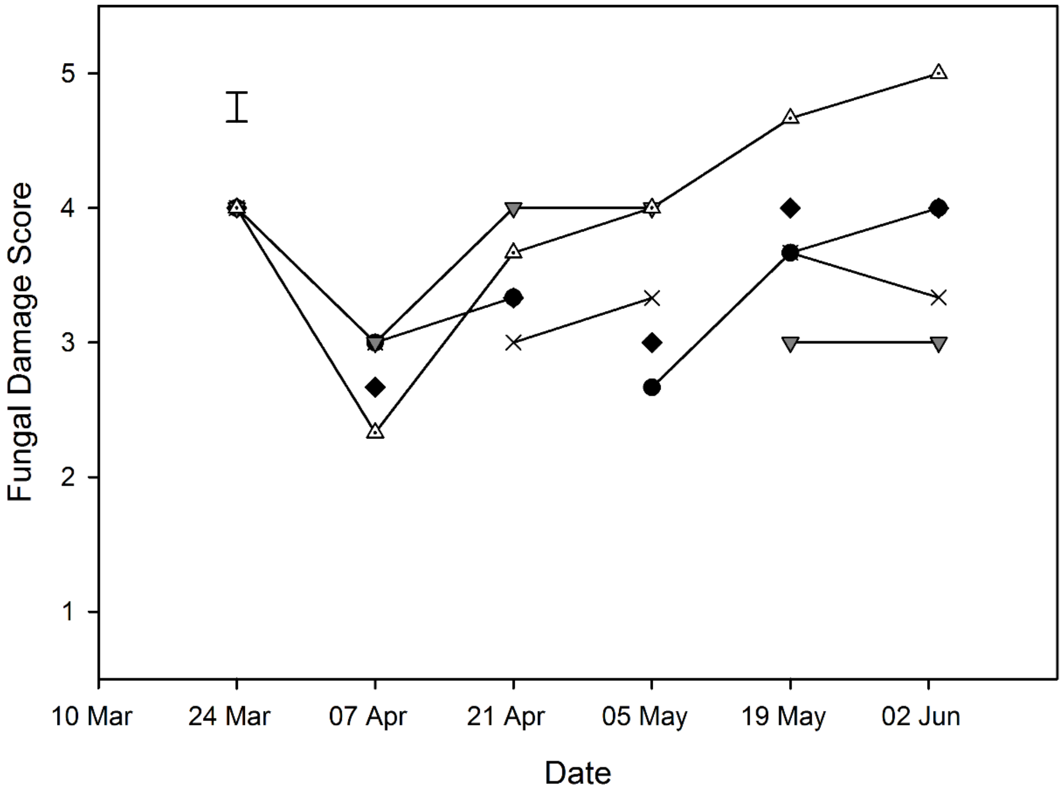


Figure 5.4 Fungal damage score (Table 4.2) of lucerne measured fortnightly in autumn 2014 under cutting intervals of 2 (◆), 4 (X), 6 (●), 8 (▲) weeks and unmown (◬). Line breaks indicate when lucerne has been mown. Error bar is the standard error of the mean for the cutting frequency treatment x date interaction.

5.1.3.5 Coumestrol content versus fungal damage

Figure 5.5 shows a weak relationship (P = 0.001; r = 0.361) between coumestrol content and fungal damage score. On 24 March 2014, the plots (Stage 1-2) had fungal damage scores of four due to stemphylium symptoms (Figure 5.6) but the coumestrol contents were low (5.9 mg/kg DM; Section 5.1.3.2). Samples from these plots are portrayed by the black data points at the bottom of Figure 5.5, at the fungal damage score of '4'. In addition, on 19 May 2014 and 2 June 2014, samples from the six and eight week cutting intervals (Stage 1) were assessed as high damage score (Figure 5.8), again due to stemphylium presence, but had lower coumestrol than expected, with moderate to moderately high average coumestrol contents of 49 and 54 mg/kg DM respectively as detailed in Section 5.1.3.2. These are the light and dark grey squares below 60 mg coumestrol/kg DM at fungal damage score '4'.

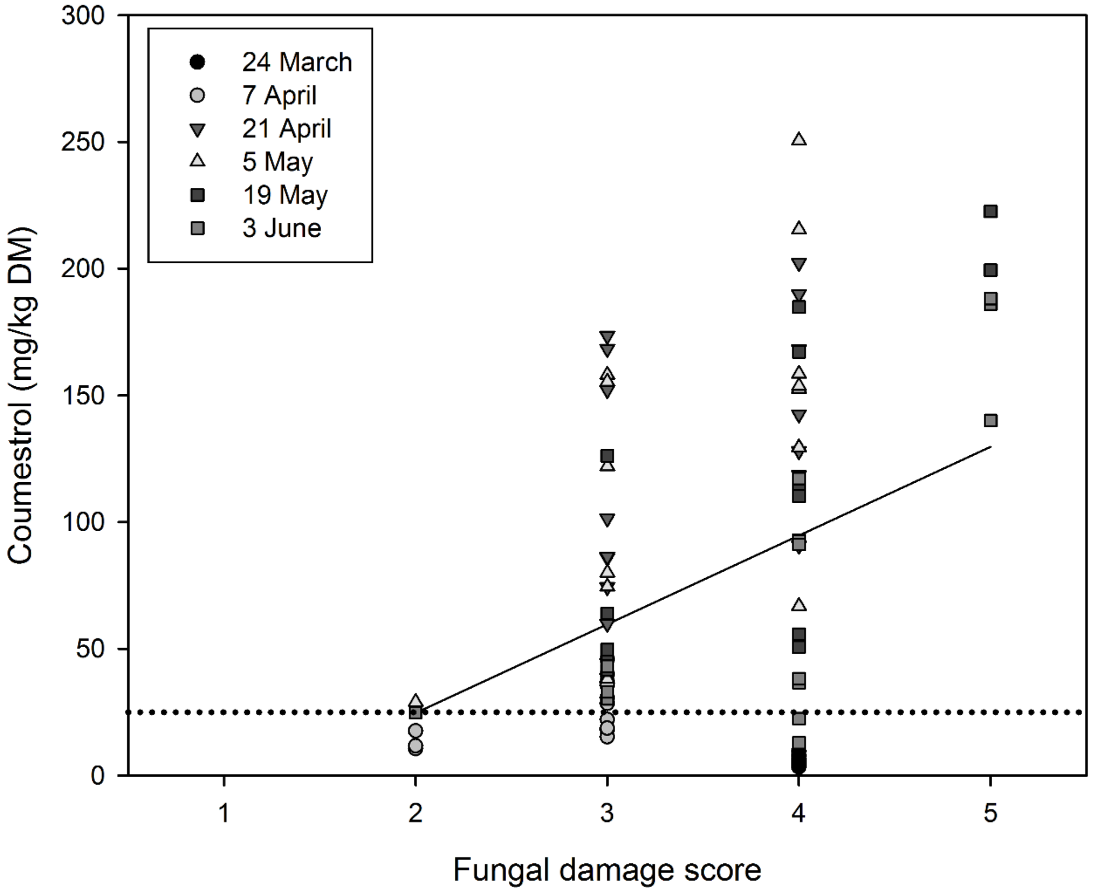


Figure 5.5 Coumestrol content (mg/kg DM) against fungal damage score was weakly correlated (r = 0.361) in lucerne sampled during autumn 2014. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

Figure 5.6 Fungal damage due to stemphylium on two week old lucerne regrowth (Stage 1-2) from 24 March 2014 in Experiment 6a. This lucerne had a low mean coumestrol content of 5.92 ± 0.483 mg/kg DM. Photo: R. L. Fields, 2014.

Figure 5.7 Fungal damage symptoms from stemphylium and spring black stem on six week old lucerne regrowth (Stage 3) from 21 April 2014 in Experiment 6a. This lucerne had a high coumestrol content of 140 ± 11.0 mg/kg DM. Photo: R. L. Fields, 2014.

Figure 5.8 Fungal damage due to stemphylium on four week old lucerne regrowth (Stage 1) from 19 May 2014. This lucerne had a moderate mean coumestrol content of 49.3 ± 4.16 mg/kg. Photo: R. L. Fields, 2014.

5.1.4 Experiment 6b results

5.1.4.1 Lucerne yield

In the 2014 to 2015 growing season (Experiment 6b), dry matter yield was measured fortnightly from 22 October 2014 to 16 December 2014 and from 14 January 2015 to 25 May 2015. At the dates of sampling onset lucerne plots each had four week old regrowth. Dry matter yield was affected ($P < 0.001$) by an interaction of cutting treatment and date (Figure 5.9).

Long cutting interval

There was no difference ($P = 0.273$) in the growth rates between the first and second regrowth periods.

For the first regrowth period, long rotation lucerne grew at a rate of approximately 63 ± 9.3 kg DM/ha/d ($P < 0.001$; $R^2 = 0.866$) between four and nine weeks regrowth to 3.4 ± 0.36 t DM/ha ($n = 3$) before production slowed, reaching 3.8 ± 0.18 t DM/ha (late-bud to early flowering; Stage 5-6; Table 4.3) at 11 weeks regrowth, on 16 December 2014.

In the second regrowth period, lucerne grew ($P < 0.001$; $R^2 = 0.902$) at an average rate of 78 ± 9.7 kg DM/ha/d between four and eight weeks regrowth to 4.0 ± 0.26 t DM/ha on 11 February 2015 (green seed pod; Stage 8-9). Production then plateaued with no change ($P = 0.362$) in dry matter yield between 11 February and 25 February 2015 (green seed pod; Stage 9). Dry matter yield then declined ($P = 0.014$; $R^2 = 0.552$) at a rate of 61 ± 19.4 kg/ha/day from 25 February until cutting at 14 weeks regrowth on 25 March 2015 at ripe seed pod stage (Stage 10), with a dry matter yield of 2.7 ± 0.51 t DM/ha ($n = 3$).

In the third regrowth period, lucerne dry matter production was lower ($P < 0.05$) than in the first and second regrowth periods. Lucerne grew ($P < 0.001$; $R^2 = 0.753$) at a rate of 23 ± 4.0 kg DM/ha/d. After eight weeks regrowth, at the final harvest for the growing season on 25 May 2015, the dry matter yield was 1.6 ± 0.13 t DM/ha ($n = 3$) and lucerne was vegetative (Stage 2).

Medium cutting interval

The average dry matter yield of the medium cutting interval treatment at mowing was 2.1 ± 0.15 t DM/ha. The exception was on 10 May 2015 (Stage 1-2), when the average dry matter yield at cutting was lower ($P < 0.001$) at 1.0 ± 0.10 t DM/ha.

The average dry matter production rates of each regrowth period were 57 ± 12.9 kg DM/ha/d ($P = 0.012$; $R^2 = 0.828$) between 22 October 2014 (Stage 1) and 10 November 2014 (Stage 2), 65 ± 18.1 kg DM/ha/d ($P = 0.023$; $R^2 = 0.762$) between 27 November 2014 (1-2) and 16 December 2014 (late vegetative to early bud; Stage 3-4), 60 ± 15.8 kg DM/ha/d ($P = 0.019$; $R^2 = 0.785$) between 14 January

(Stage 4) and 28 January 2015 (flowering; Stage 6-7), and 64 ± 9.5 kg DM/ha/d ($P = 0.003$; $R^2 = 0.919$) between 11 February (Stage 1) and 25 February (late vegetative to early bud; Stage 3-4).

After 25 February 2015 (Stage 3-4), until cutting on 25 March 2015 (Stage 6), the regrowth plateaued ($P = 0.070$) with an average production rate of 14 ± 6.7 kg DM/ha/day and an average dry matter yield of 1.4 ± 0.09 t DM/ha ($n = 9$) across the three sampling dates. This is the same period during which growth in the long cutting interval plots declined. Between 14 April (Stage 1) and 10 May 2015 (Stage 1-2), lucerne yield increased ($P < 0.05$) at a lower rate of 20.4 kg DM/ha/day relative to the pre-February regrowth periods.

Short cutting interval

The average dry matter yield of the short cutting interval treatment at mowing was 1.2 ± 0.08 t DM/ha for all cutting dates but 16 December 2014 (Stage 1), 24 April 2015 (Stage 1) and 25 May 2015 (Stage 1), when the average yield at cutting was lower ($P < 0.05$), at 0.47 ± 0.059 t DM/ha. The regrowth period which ended on 16 December 2014 was of shorter duration than the other regrowth periods, at just under three weeks.

The average dry matter production rates between 10 November 2014 (Stage 1) and 27 November 2014 (Stage 2), between 28 January 2015 (Stage 1-2) and 11 February 2015 (Stage 3-4), and between 25 February (Stage 1) and 25 March 2015 (Stage 3) were not different ($P = 0.517$) with an average increase of 29 kg/ha/day. The growth between 25 February and 25 March was in contrast to the medium cutting interval treatment which did not grow, and the long cutting interval treatments which had a decrease in dry matter yield, during this period.

Between 14 April 2015 (Stage 1) and 24 April 2015 (Stage 1) and between 10 May 2015 (Stage 1) and 25 May 2015 (Stage 1), growth rate plateaued ($P = 0.195$), with no difference in dry matter yields between the two regrowth periods ($P = 0.723$).

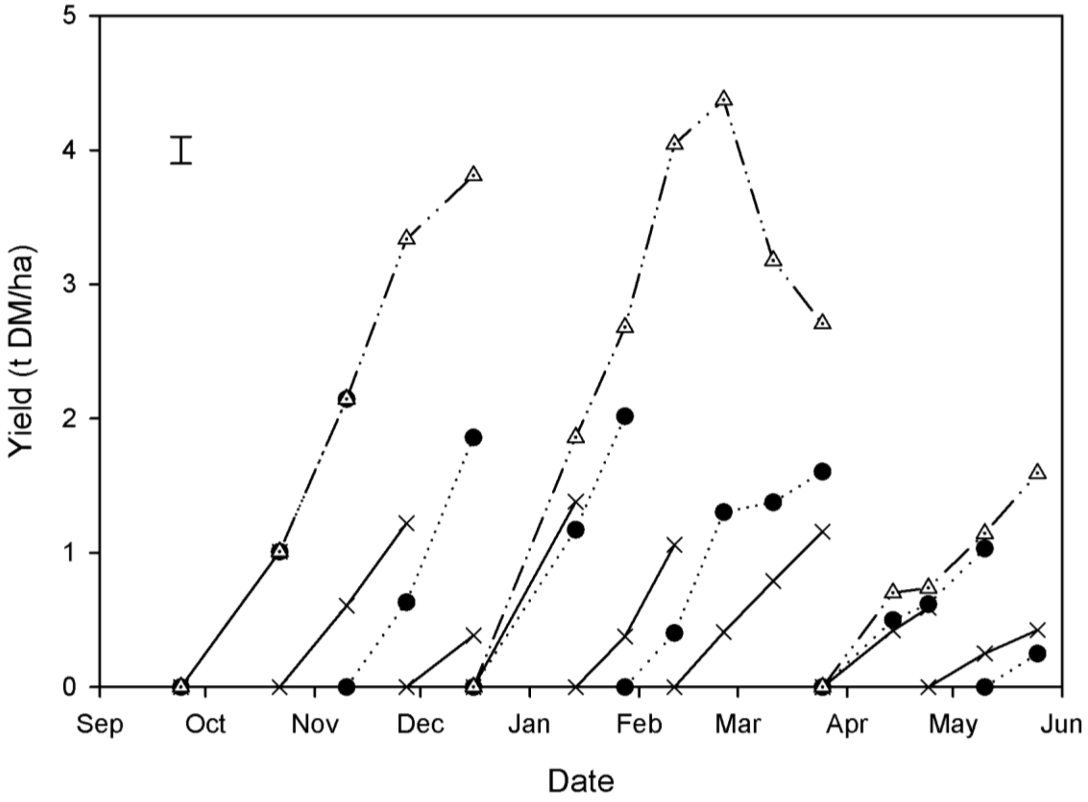


Figure 5.9 Dry matter (DM) yield (t/ha) of lucerne measured between Dec 2014 and May 2015 under approximately four (X), six (●) and 12 (◬) week cutting intervals. Coumestrol content was measured between January 2014 and June 2015 of this period. Error bar is the standard error of the mean for the interaction between date and cutting treatment.

5.1.4.2 Coumestrol content over time

In Experiment 6b, $\log_{10}$ transformed coumestrol content was affected by an interaction (P = 0.001) between time and cutting frequency. Figure 5.10 shows $\log_{10}$ transformed coumestrol variable between January 2015 and May 2015 under short, medium and long cutting intervals.

14 January to 11 February 2015

By 14 January 2015, all plots had received three months under a short, medium or long cutting regime. During this period, the short rotation was mown three times, the medium rotation twice, and the long rotation once. Between 14 January and 11 February coumestrol was low and. On 14 January coumestrol was higher (P = 0.008) in the short rotation plots than the medium rotation (5.3 ± 1.30 vs 2.0 ± 0.49 mg/kg DM, respectively). All plots four weeks old and at the bud stage (Stage 4-5). On 11 February 2015, coumestrol was a low 3.5 ± 0.86 mg/kg DM with no differences (P = 0.061) among cutting frequencies. The short rotation lucerne was four weeks old (Stage 4), the medium rotation was two weeks old (Stage 1), and the long rotation lucerne was eight weeks old (Stage 8-9).

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First coumestrol spike (25 February to 25 March 2015)

On 25 February 2015, mean coumestrol had increased (P = 0.003) for all treatments relative to their values on 11 February. Coumestrol was higher (P = 0.032) in the short rotation than medium and long rotation lucerne plots with 25.6 ± 6.3 and 10.6 ± 2.6 mg/kg DM, respectively. Coumestrol continued to increase and reached a moderately high peak of 75.5 ± 18.6 mg/kg DM on 25 March 2015 with no differences (P = 0.168) among cutting frequencies. On this date there were regrowth ages of six (Stage 3; 27.1 ± 2.1 cm tall), eight (Stage 6-7; 45 ± 3.7 cm tall) and 14 (Stage 10; 64 ± 3.0 cm tall) weeks.

Second coumestrol spike

All plots were cut after sampling on 25 March 2015. On 14 April 2015, the coumestrol content in the three week old regrowth (Stage 1) of these plots was lower (P < 0.001) than on 25 March 2015, at a low 6.55 ± 2.64 mg/kg DM. The short rotation plots had higher coumestrol than the medium and long rotations with 7.9 ± 1.94 vs. 3.3 ± 0.80 mg/kg DM, respectively.

Coumestrol content increased (P < 0.001) in all plots on 10 May 2015 relative to the 14 and 24 April. On this date all lucerne was vegetative (Stage 1-2). The short rotation lucerne had two weeks regrowth (3.7 ± 0.67 cm tall), and the medium and long rotation lucerne had six weeks regrowth (22 ± 1.1 cm). Short rotation plots had lower coumestrol than the long rotation plots with 58.2 ± 14.3 and 120 ± 29.6 mg/kg DM, respectively. Medium rotation plots had 100 ± 24.6 mg/kg DM, which was not significantly different from the other cutting frequencies. On 25 May 2015, there was no change (P = 0.105) in coumestrol relative to 10 May 2015, including in the regrowth of the lucerne cut at six weeks regrowth on 10 May 2015. All lucerne was vegetative (Stage 0-1) and regrowth ages of four (short rotation), two (medium rotation) and eight weeks (long rotation) were present.

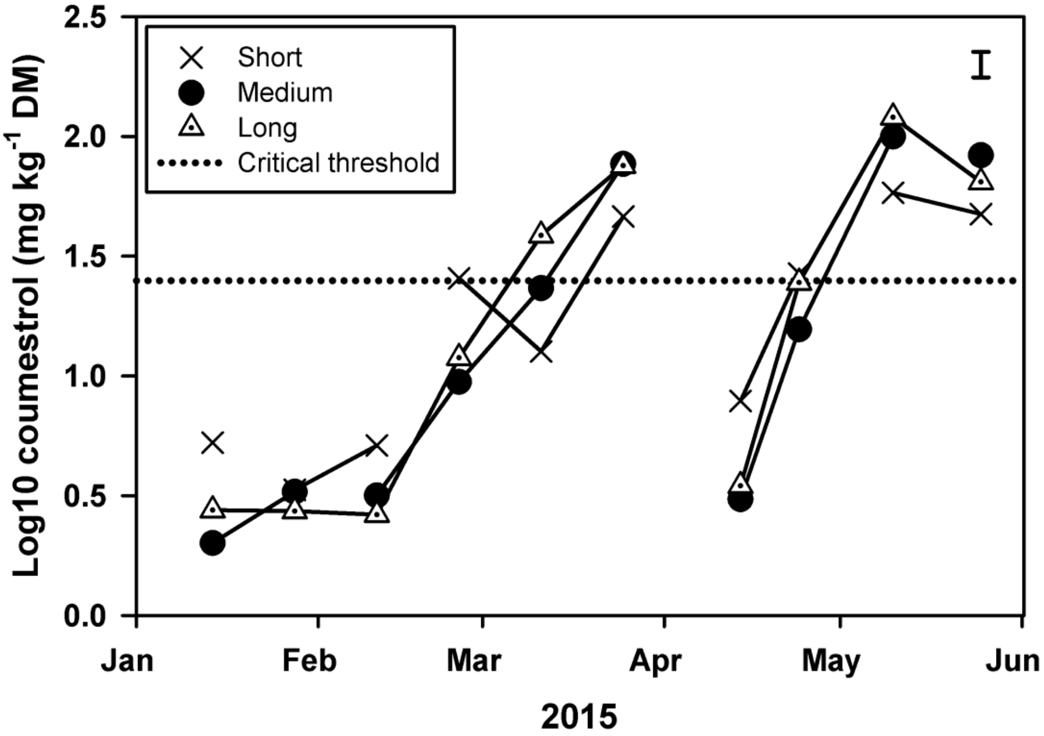

Figure 5.10 Log$_{10}$ transformed coumestrol content of lucerne under short (×), medium (●) and long (△) cutting intervals between January 2015 and May 2015. Line breaks indicate when lucerne had been mown. Dotted horizontal line is the log of the critical value (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance. Error bar is the standard error of the mean for the interaction between treatment and time.

5.1.4.3 Coumestrol content vs. developmental stage

Log$_{10}$ coumestrol content was not correlated (r = -0.134; P = 0.218) with development stage (Figure 5.11). In the late developmental stages, i.e. from flowering (Stage 7) to mature seed pods (Stage 10), both low (1-10 mg/kg DM) and high (100-150 mg/kg DM) coumestrol contents were measured. Likewise at the early vegetative developmental stages (Stage 1-3), a range of low to extreme (>150 mg/kg DM) coumestrol levels were measured. At the bud stages (Stage 4-5) only low coumestrol contents were measured, however no samples were at the bud stages on the dates that coumestrol was high (25 March, 10 May and 25 May 2015).

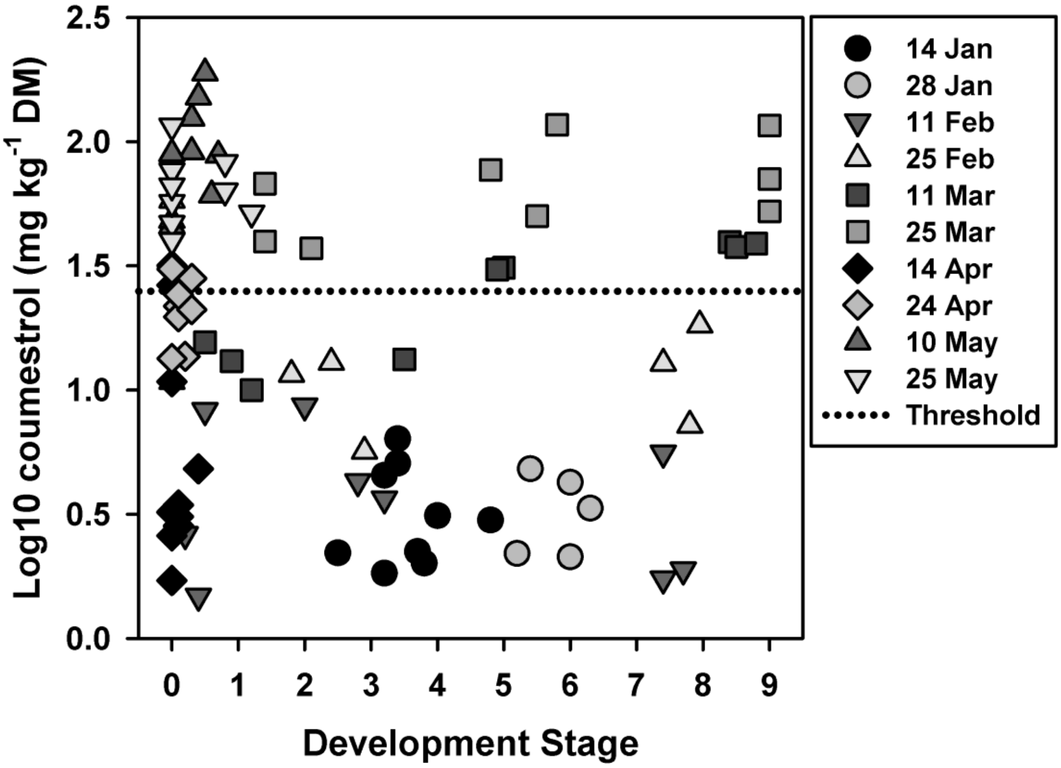

Figure 5.11 Log$_{10}$ coumestrol content (mg/kg DM) between 14 January and 25 May 2015 against (r = -0.134; P = 0.218) with development stage, where Stage 1-3 is vegetative, Stage 4-5 is bud, Stage 6-7 is flowering and Stage 8-10 is seed pod (Table 4.3). Dotted horizontal line is the log of the critical value (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

5.1.4.4 Coumestrol content vs. fungal score

There was an interaction ($P < 0.001$) between date and cutting frequency on fungal damage score. The fungal damage score of the long rotation lucerne crops tended to increase over time, while the short and medium rotation lucerne had more variable levels of damage over time. This was because cutting of lucerne stands removed the damaged material resulting in lower damage scores in subsequent regrowth periods. When stands were not cut, fungal damage tended to increase over time. Fungal scores were highest ($P < 0.05$) on 11 March, 25 March, 10 May and 25 May 2015. The dominant pathogens on these dates were stemphylium and common leaf spot. Coumestrol content was correlated ($r = 0.707$; $P > 0.001$) with fungal damage score (Figure 5.13).

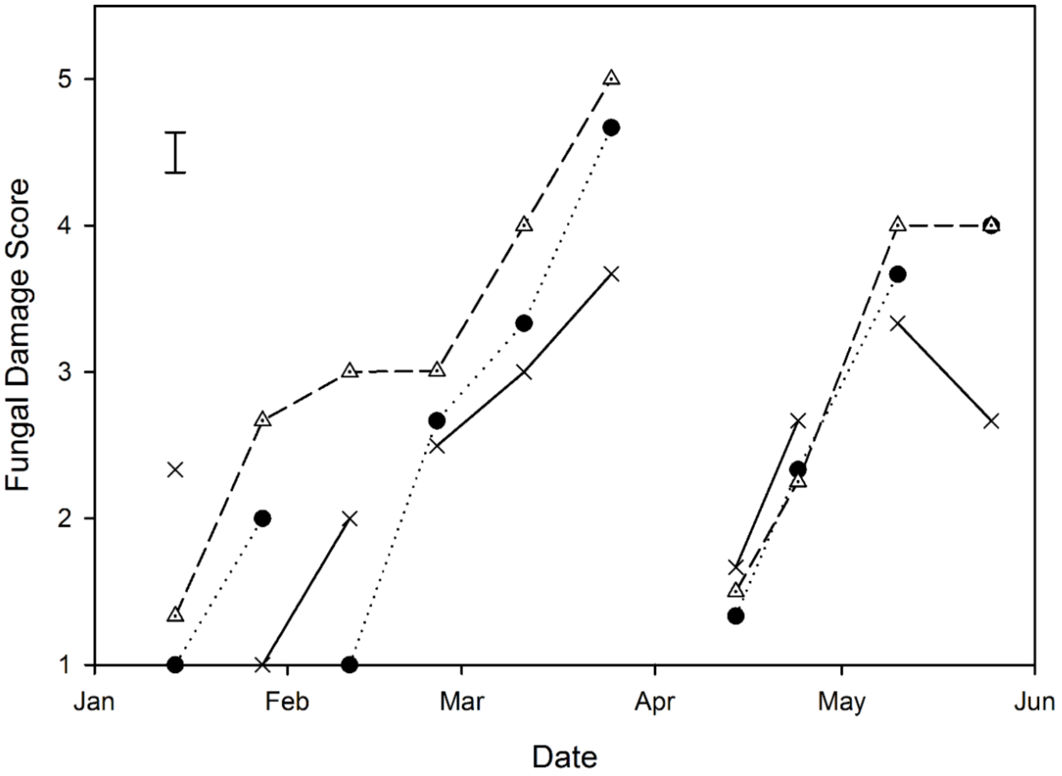

Figure 5.12 Fungal Damage Score of lucerne measured between 14 January and 25 May 2015 under short (X), medium (●) and long (◬) cutting intervals. Error bar is the standard error of the mean for the interaction between date and cutting frequency.

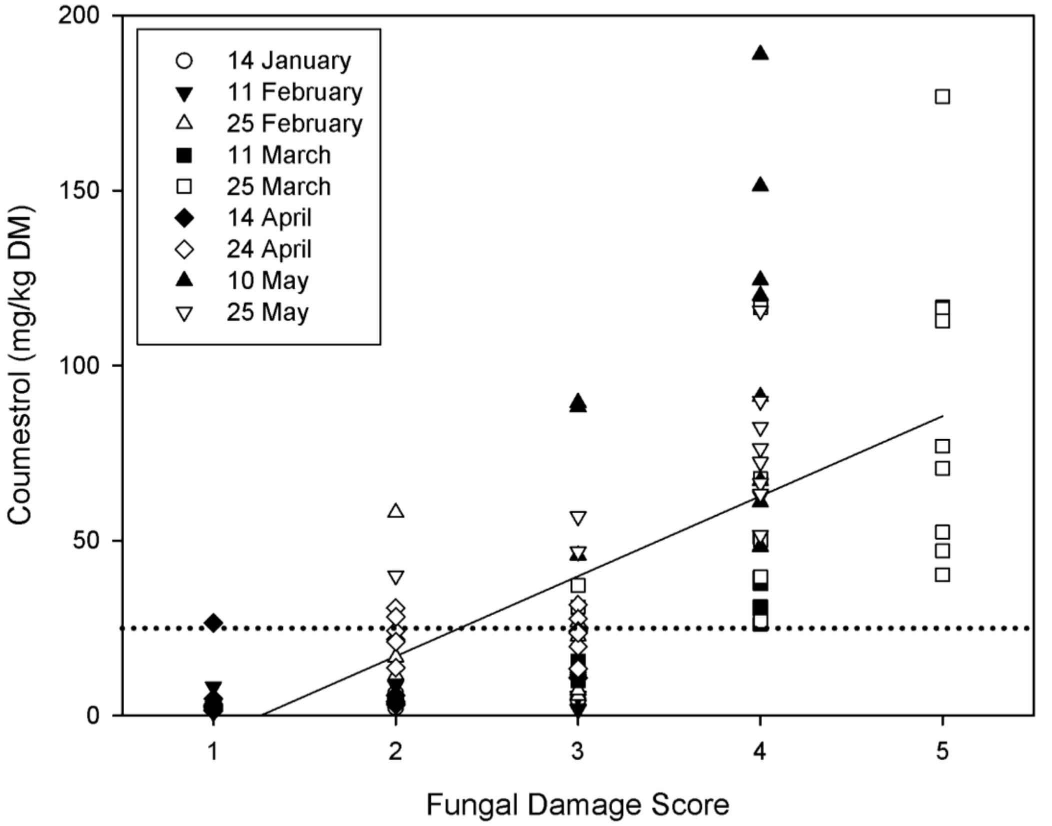

Figure 5.13 Coumestrol content of lucerne was correlated (r = 0.707; P < 0.001) with fungal damage score in summer and autumn 2015. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

5.1.5 Discussion

In Experiment 6a coumestrol content increased in the fortnight to 21 April 2014 in all plots that each had two or six weeks of regrowth (Figure 5.2) relative to the earlier harvests. In Experiment 6b coumestrol increased during two regrowth periods, reaching maximum coumestrol contents on 25 March and 25 May 2015.

Based on the results of the developmental stage comparison, the morphological status of the stand did not have an effect on coumestrol content. In the Experiment 6a spike, coumestrol content increased simultaneously to ~140 mg/kg DM in two weeks old (3.5 cm height) and six weeks old (37 cm height) vegetative lucerne (Figure 5.2). In Experiment 6b (Figures 5.10 & 5.11), coumestrol content increased simultaneously to 70 mg/kg DM, in six (vegetative; 27 cm), eight (early flowering; 45 cm height) and 14 (mature seed set; 64 cm) week old lucerne in the first spike, and to ~100 mg/kg DM in two (vegetative; 3.7 cm) and six (vegetative; 22 cm) week old in the second spike. In addition, lucerne had low coumestrol levels on 11 February 2015, in two (4 mg coumestrol/kg DM; vegetative; 13 cm), four (5.5 mg/kg DM; early bud; 27 cm), and eight (3 mg/kg DM; early seed pod; 64 cm) week old lucerne regrowth.

These results are in contrast to previous studies which showed flowering and seed set stages to have higher coumestrol. These studies followed a stand through its growing season, but did not isolate developmental stages from harvest date (Bickoff *et al.*, 1960a; Hanson *et al.*, 1965; Seguin *et al.*, 2004). This gave an appearance of elevated coumestrol levels during flowering which was inconsistent with our results. When crops at different developmental stages were simultaneously compared over time in Experiment 6b, the vegetative stands increased in coumestrol during the same chronological periods as the reproductive stands (Figures 5.10 & 5.11). These results suggest that coumestrol content was not dependent on the morphological age of the lucerne crop.

Foliar pathogens, in particular a cool temperature biotype of stemphylium leafspot and spring black stem, were present (fungal damage score ≥ 3) on the plants when coumestrol spikes occurred. In contrast, when foliar pathogens were not present (damage score of 1) or present only at low levels (damage score of 2) the coumestrol levels were low. In some cases, particularly in Experiment 6a, coumestrol content was low to moderate, despite the presence of fungal pathogens. The fungal pathogen present in each of these cases was stemphylium and the plant material was young regrowth (Figure 5.6). Additionally a fortnight following the 24 March 2014 sampling, which determined stemphylium infection to be high with a damage score of 4 but coumestrol to be low (5.9 mg/kg DM), the fungal damage score decreased to 2.8. This may have been due to production of new, uninfected foliage, and a lack of spread of the stemphylium on the older leaves. A spike in coumestrol occurred a fortnight later (21 April 2014), and by this date an additional fungal pathogen (spring black stem) was present. This suggests that stemphylium did not cause elevated coumestrol in the lucerne. This is in contrast to research by Hanson *et al.* (1965) who showed that all pathogenic fungi tested, including stemphylium, caused elevated coumestrol. However, methodology was sparse and, as discussed in Section 2.6.1.2, the stemphylium biotype that was used was likely to be the warm biotype, in contrast to the cool biotype present in this experiment. Further research to investigate the effect of the cool biotype of stemphylium is performed in Experiment 9 (Section 5.4). However, in that experiment stemphylium did increase coumestrol content.

The increased presence and diversity of fungal pathogens at the Experiment 6a coumestrol spike on 21 April 2014 could be attributed to the weather conditions during March and April 2014 (Figure A.1). There was 161 mm of rainfall in April, with rainfall on 12 of the 14 days prior to 21 April 2014, including two large rainfall events of 40.4 mm on 8 April and 30.8 mm on 18 April 2014. Based on the SMD model (Figure 4.2), plants were not water stressed (SMD < 50% AWC) from 7 April to 2 June 2014.

The fungal damage and lucerne coumestrol levels recorded during Experiment 6b were not as high as in Experiment 6a, but coumestrol spikes were still observed. Rainfall during the autumn season of Experiment 6b was lower (Figure A.3). In March 2015 there was 40.5 mm rainfall and in April there was 77.6 mm, with 44.6 mm of this falling on 28 April, prior to the 10 May peak. Based on the SMD model (Figure 4.3), plants were water stressed throughout the experimental period, with a high SMD well above 50% AWC.

After high coumestrol plots were mown, coumestrol was, in most cases, lower in the regrowth a fortnight later. The four week cutting interval in Experiment 6a did not show this response after cutting of lucerne with a moderately high (74 mg/kg DM) coumestrol content on 5 May 2014; these plants had increased coumestrol content to a high 140 mg/kg DM a fortnight later on 19 May 2014. The fortnightly cutting regime also did not show this response to cutting following a similar, albeit lower, coumestrol pattern to the unmown lucerne. These inconsistencies in the more frequently cut plots may have been due to the low quantity and height of the regrowth between cuts (i.e. less than the mower height of 6.5 cm). This would have resulted in the mower leaving behind leafy residual which was then followed by further low growth. This may have resulted in a large proportion of leaf material from previous re-growths remaining simultaneously with the younger regrowth material which could not be differentiated. Future research should use ewes to graze to ensure all leafy material is removed.

Based on Experiment 6b, it is not necessary to avoid grazing flowering lucerne however the longer the duration of lucerne regrowth, the more likely it is to experience environmental conditions suitable for fungal infection (Chapter 6).

5.2 Experiment 7 Cultivar

5.2.1 Introduction

Experiment 7 was part of Objective 2. Specifically, this experiment examined the variation in coumestrol content among cultivars of field grown lucerne. Previous studies from New Zealand (Purves *et al.*, 1981) and the United States (Hanson *et al.*, 1965) showed that cultivars more resistant to disease produced less coumestrol than susceptible cultivars (Section 2.6.1).

Since these studies, new cultivars have been bred and it is expected that there could be variation in the coumestrol content produced by each cultivar. Experiment 7a compared five cultivars in an established lucerne crop between mid-summer and late autumn 2015. These dates encompass the mating period of ewes and therefore is the period that coumestrol in lucerne is most likely to affect ewe reproductive performance. Experiment 7b compared 10 cultivars of a young lucerne crop in autumn 2016. The null hypothesis was that no difference in coumestrol content between cultivars would exist.

5.2.2 Methods

5.2.2.1 Experimental Design

For Experiment 7a, five cultivars (‘Grasslands Kaituna’, ‘Stamina 5’, ‘Stamina 6GT’, ‘Rhino’ and ‘Runner II’) at Ashley Dene (paddock H7), which were established in November 2008 in 6.3 x 24.5 m plots were used. A description of the site is provided in Section 4.1. The experiment was a split plot design with three blocks which contained randomly allocated plots of each of the five cultivars. In total, samples were collected on five dates (5 x 3 x 5, N = 75). Samples were taken three times between 15 January and 5 March 2015. The paddock was grazed for approximately two weeks from 17 March 2015 and the re-growth was then sampled twice, on 2 and 17 May 2015.

The established cultivars tested at Ashley Dene during summer and autumn 2015 were:

- ‘Grasslands Kaituna’ which has a fall dormancy (FD) rating of 5 and was bred in New Zealand for its quality and resistance to pests and diseases.
- ‘Rhino’, a winter dormant (FD 3) cultivar also considered to have pest and disease resistance
- ‘Runner II’, a winter dormant (FD 2) cultivar of lucerne that contains *Medicago falcata* L. parentage, which enables it to ‘creep’ producing new plants from buds on lateral roots.
- ‘Stamina 5’ (FD 5), a cultivar selected for grazing persistence under set-stocking and close grazing.

- ‘Stamina GT6’ (FD 6), a cultivar selected from Australian lucerne trials for grazing tolerance, has higher winter growth than Stamina 5, persistent stand density, and is resistant to pests and diseases.

For Experiment 7b, Paddock 9 of the Lincoln University Horticultural Research Area (HRA9) had 10 cultivars (‘Grasslands Kaituna’ (FD 5), ‘Takahe’ (FD 5), ‘Force 4’ (FD 4), ‘Force 7’ (FD 7), ‘Force 11’ (FD 11), ‘P54V09m (FD 4)’, ‘P55Q27 (FD 5)’ ‘Torlesse’ (FD 4), ‘SFR27-018’(FD N/A), and ‘Venus’ (FD 5) which were compared five to six months after sowing, in 42 day regrowth on 7 March, and in 44 day old lucerne on 20 April 2016 from two blocks (10 x 2 x 2, N = 40). These dates were chosen because it was during a time of year when lucerne stands had previously shown elevated coumestrol levels (Section 5.1) and also coincides with the mating season of sheep.

The resistances of each of these cultivars to pests and diseases are shown in Table 5.1. The lucerne samples were processed and extracted as described in Sections 4.4 to 4.6. Coumestrol was measured by HPLC with the methodology described in Section 4.7. The coumestrol rating scale (Table 4.4) was used to rate coumestrol content from negligible to extreme. Statistical analyses performed are described in Section 4.12.1.

Table 5.1 Stated resistance ratings of the 5 established lucerne cultivars at Ashley Dene H7 and 10 lucerne cultivars sown at Lincoln University HRA9[1].

	Pea Aphid	Spotted Alfalfa Aphid	Blue-green Aphid	Verticilium Wilt	Phytophthora Root Rot	Stem Nematode	Anthracnose	Bacterial Wilt	Fusarium Wilt	Leaf Diseases (*Stemphylium* & *Leptosphaerulina*)
‘Grasslands Kaituna’	R	R	R	MR	R	R	-	R	HR	MR
‘Rhino’	HR	-	-	R	R	R	R	HR	R	-
‘Runner II’	S	S	-	S	S/R	LR	S	R	R	-
‘Stamina 5’	-	HR	R	-	R	HR	HR	-	-	-
‘Stamina GT6’	-	HR	R	-	R	HR	HR	-	-	HR
Torlesse	R	R	R	MR	R	R	R	R	-	MR
Force 4	-	-	MR	HR	HR	R	-	R	-	-
Force 7	HR	R	-	-	-	HR	MR	R	-	-
Force 11	HR	HR	-	-	HR	HR	-	-	-	-
P54V09	HR	R	-	HR	-	HR	-	HR	-	-
P55Q27	R	R	-	HR	HR	HR	-	HR	-	-
Venus	-	HR	MR	-	MR	-	LR	-	-	-
Takahe	-	-	-	-	-	-	-	-	-	-
SFR27-018	-	-	-	-	-	-	-	-	-	-

[1] Table adapted from (NAAIC, 1993; Lattimore, 2013; Pioneer, 2015; S&W Seed Company, n.d; Agricom, n.d.). Highly resistant (HR) stands have over 51% of plants with resistance, resistant (R): 31-50%, moderately resistant (MR): 15-30%, low resistant (LR): 6-14% and susceptible (S): 0-5%. Dashes indicate information was unavailable.

5.2.2.2 Meteorological Data

Rainfall data were recorded between 1 August 2014 and 17 May 2015 at Burnham Sewage Plant Meteorological Station, Burnham; 4 km from the field site. Temperature data were recorded during this period at Broadfield Meteorological Station, Lincoln; 12 km from the field site. The rainfall and mean daily temperature are shown in Figures A.5 and A.6. The total rainfall between 1 January and 17 May 2015 was 195 mm, a rainfall event of 57.3 mm occurred on 28 April 2015.

5.2.3 Results

5.2.3.1 Height and yield

For Experiment 7a, dry matter yield was not affected (P = 0.145) by cultivar but there was an effect (P < 0.001) of date (Figure 5.14). Crop yields were low across sampling dates, which reflects the shallow soil and lack of soil moisture present in H7 (Figure 4.5). The highest (P < 0.001) yields were during the second regrowth period, with 0.70 and 0.83 t DM/ha on 2 May 2015 (Stage 1) and 17 May 2015 (Stage 2), respectively. This contrasts with the earlier regrowth period from 15 January 2015 (Stage 1) to 5 March 2015 (Stage 7-8) with yields from 0.16 to 0.34 t DM/ha respectively. In total, the average biomass accumulated during the two regrowth periods was 1.2 t DM/ha.

Lucerne height was affected by cultivar (P < 0.001) and by date (P < 0.001). Between 15 January and 17 May 2015, Stamina 5 (15.5 ± 1.55 cm, n = 15) and Grasslands Kaituna (13.5 ± 1.72 cm) were taller (P < 0.05) than Rhino (9.7 ± 0.99 cm) and Runner (7.9 ± 0.71 cm). Runner was also shorter (P < 0.05) than Stamina 6GT (13.2 ± 1.56 cm). On average lucerne was tallest (P < 0.001) during May (16 ± 1.6 cm) and shortest from 15 January to 5 May (10 ± 1.3 cm).

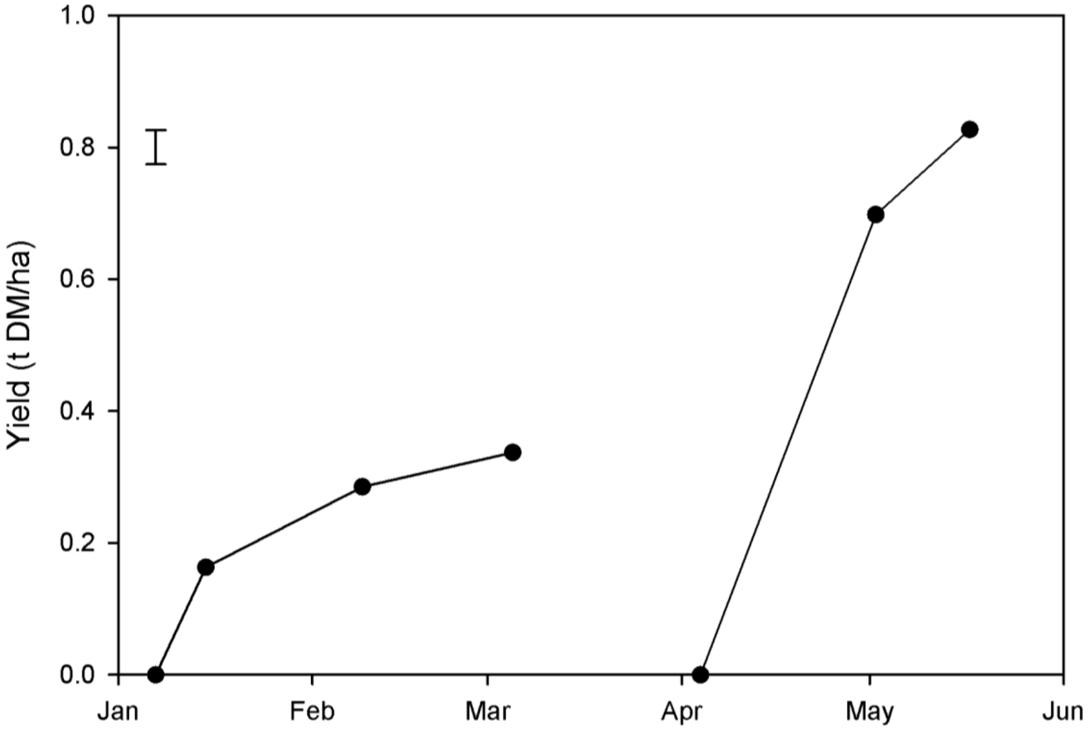

Figure 5.14 Mean dry matter (DM) yield (t/ha) of five lucerne cultivars sampled at Ashley Dene, over two regrowth periods between 15 January and 25 May 2015. Error bar is the standard error of the mean for the effect of date.

5.2.3.2 Coumestrol content

Cultivar did not affect the coumestrol content (P = 0.692) in Experiment 7a. It was affected (P < 0.001) by the date of sampling, with samples from the subsequent regrowth in May containing higher coumestrol than samples from January to March (Figure 5.15). In the first regrowth period average coumestrol (n = 15) increased (P < 0.001) from a low 9.7 ± 1.51 mg/kg DM on 15 January (Stage 1) to a moderately low 20.5 ± 8.02 mg/kg DM on 5 March 2015 (Stage 7-8). In the second regrowth period coumestrol increased (P = 0.005) from a moderate 42.7 ± 3.31 mg/kg DM by 2 May 2015 (Stage 1) to a moderately high 56.2 ± 3.24 by 17 May 2015 (Stage 2).

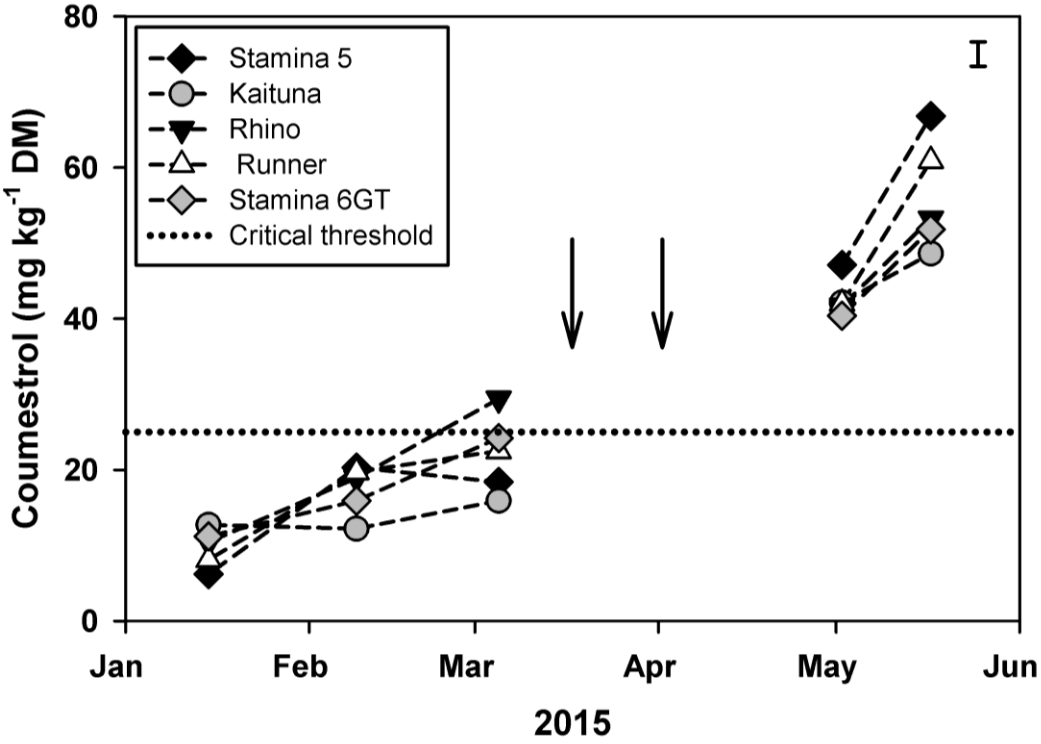

Figure 5.15 Coumestrol content (mg/kg DM) of 'Stamina 5' (◆), 'Stamina 6GT' (◆), 'Grasslands Kaituna' (●), 'Rhino' (▼) and 'Runner' (△) at Ashley Dene H7. Dashed (- - -) lines are the 95% confidence interval. Error bar is the standard error of the mean for date. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

In the newly established stand at Lincoln University HRA9 tested in Experiment 7b, there was no effect (P = 0.106) of date (7 March 2016 vs. 20 April 2016) on coumestrol content. There was an effect (P = 0.024) of cultivar on coumestrol content. Venus had higher (P < 0.05) coumestrol content (14.0 ± 2.58 mg/kg DM, n = 4), than all cultivars except Force 7 (10.7 ± 2.48 mg/kg DM), Torlesse (10.1 ± 3.53 mg/kg DM) and Takahe (9.9 ± 0.88). In contrast, Force 4 had a lower (P < 0.05) coumestrol content (3.2 ± 0.54 mg/kg DM) than Venus, Force 7, Torlesse and Takahe. SFR27-018, with a coumestrol content of 4.7 ± 1.1 mg/kg DM, had a lower (P < 0.05) coumestrol content than Venus and Force 7. All coumestrol levels were low or moderately low.

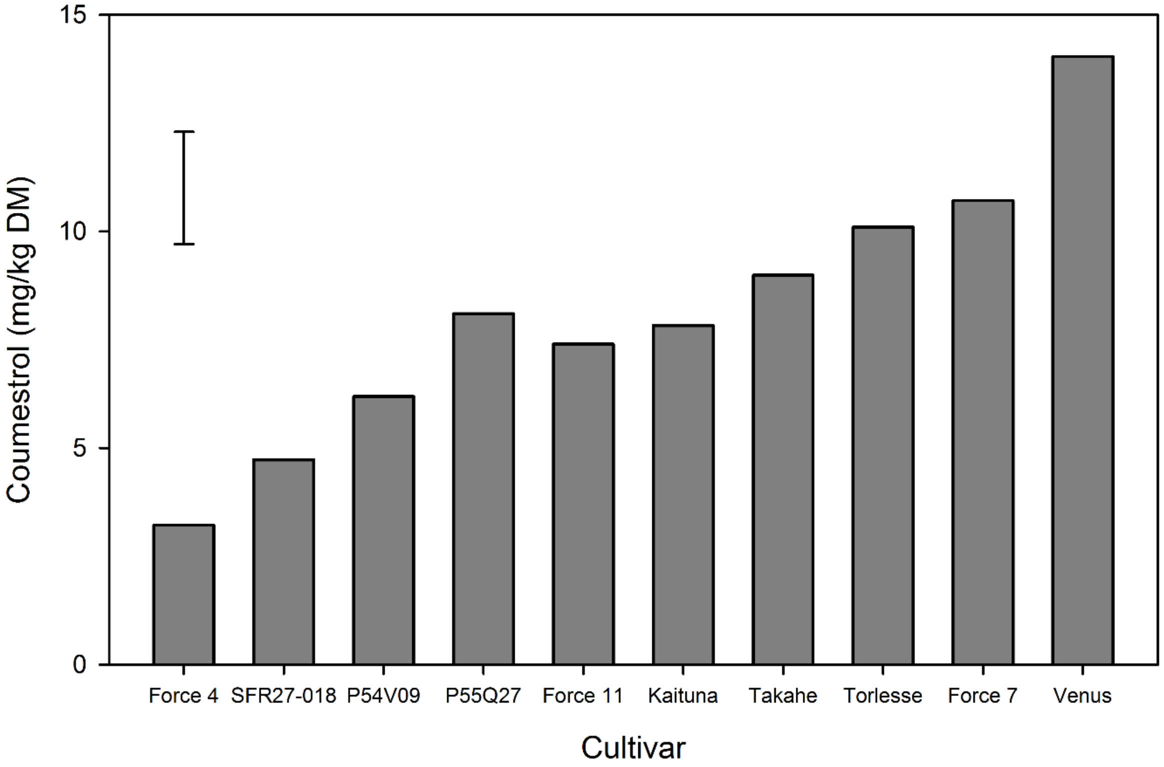

Figure 5.16 Mean coumestrol (mg/kg DM) of 10 lucerne cultivars from HRA9, Lincoln sampled on 7 March and 20 April 2016 (n = 4). Error bar is the standard error of the mean for cultivar.

5.2.3.3 Fungal Damage vs coumestrol content

In Experiment 7a, coumestrol content of lucerne was correlated ($P < 0.001$; $r = 0.766$) with fungal damage score (Figure 5.17). Coumestrol content was greater ($P < 0.001$) in lucerne with damage scores of 3 (a moderate 48.8 ± 12.61; n = 15) or 4 (a moderately high 52.8 ± 15.24; n = 12), than lucerne with a fungal damage score of 1 (a moderately low 14.4 ± 2.27; n = 12) or 2 (a moderately low 13.4 ± 1.76; n = 21). Lucerne with no fungal damage (a score of 1) was not different ($P > 0.1$) from lucerne with a slight fungal damage score of 2. There was also no difference ($P > 0.1$) in the coumestrol content between fungal damage scores of 3 and 4. There was no effect ($P = 0.976$) of cultivar on the damage score with an average damage score of 2.3 ± 0.12 across the experiment (n = 75).

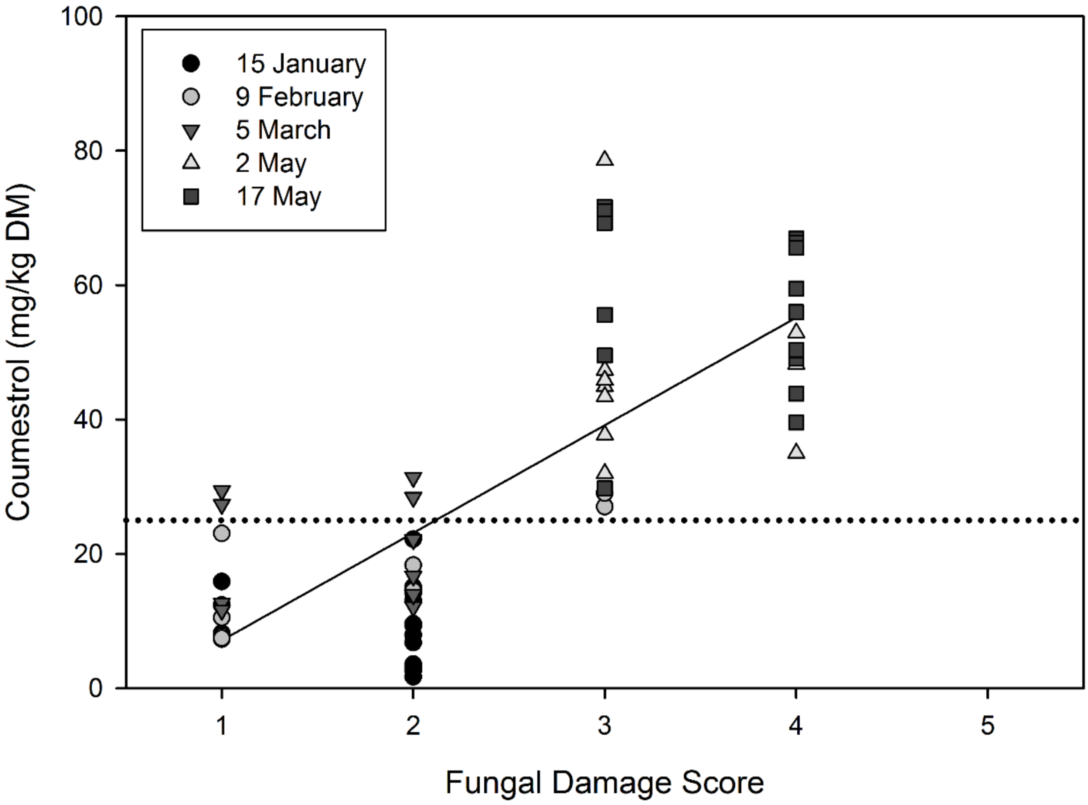

Figure 5.17 Coumestrol content (mg/kg DM) of lucerne against (r = 0.766) fungal damage score in summer and autumn 2015 at Ashley Dene H7. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

5.2.4 Discussion

During Experiment 7a, the autumn season was dry during the first regrowth period with low rainfall (Figure A.5) and a soil moisture deficit above 80 mm, compared with the maximum available water content of 100 mm (Section 4.3.5). This meant that the lucerne would have been under considerable water stress, and explains the low yield (< 0.4 t DM/ha) on the sampling dates from January to March 2015. A rainfall event on 28 April 2015 decreased the soil moisture deficit to 2 mm, near field capacity. Lucerne had a greater, though still low, yield during May 2015 (approximately 0.8 t DM/ha).

Coumestrol content of the five cultivars compared at Ashley Dene in Experiment 7a was affected by sampling date (Figure 5.15). The lucerne sampled on 2 and 17 May 2015 had a moderate to moderately high coumestrol content (43 and 56 mg/kg DM, respectively) while the lucerne sampled between January and March 2015 had moderately low coumestrol (10 to 21 mg/kg DM). The moderately low coumestrol from January to March 2015, while the lucerne was under water stressed, provides evidence that water stress does not directly cause a coumestrol response and this is further investigated in Experiment 10 (Section 5.5).The correlation of coumestrol with the fungal damage score (Figure 5.17) supports prior research showing an effect of disease on coumestrol content (Section 2.6.1.2) and is consistent with the findings from Experiment 6. However, unlike in prior research (Loper *et al.*, 1967; Purves *et al.*, 1981) an effect of cultivar was not observed, and there was no difference in symptoms among the cultivars (Section 5.2.3.3).

All of the cultivars in Experiment 7a were capable of producing coumestrol contents over the 25 mg/kg DM suggested to affect ewe reproductive performance (Figure 5.15). This indicates that it is still imperative to breed for disease resistance. The lack of difference between cultivars supports the null hypothesis that no difference would exist. A reason for the lack of difference among cultivars may be because of the way cultivar resistance is measured. In lucerne, resistance to the various pests and diseases is based on what percentage of the stand is resistant. Highly resistant stands have over 51% of plants resistance, resistant (30-50%), moderately resistant (15-30%), low resistant (6-14%) and susceptible (0-5%). This means that even in cultivars highly resistant to a particular pathogen, up to almost half of the plants could still be susceptible to it. In addition, fungal pathogens such as stemphylium, spring black stem and common leaf spot do not appear to have been the focus of most plant breeding efforts. Typically, the cultivar resistance information does not give resistance levels to these, and in cases where they do they fall under a general ‘leaf diseases’ category (Lattimore, 2013). The coumestrol levels measured were also not as high as those measured in 2014 at Lincoln University I12 (Section 5.1). This means that there may not have been sufficient disease pressure on the lucerne to demonstrate differences in cultivar performance.

On the other hand, in Experiment 7b, the six month old lucerne stand with 10 different cultivars at Lincoln University HRA 9 had low to moderately low levels of coumestrol (< 20 mg/kg DM in all samples), but a difference among cultivars was detected (Figure 5.16) which rejects the null hypothesis. This crop was only sampled on two dates and could therefore be an anomaly. As, for example, some individual harvest dates at Ashley Dene appeared to have differences between cultivars, but the highest and lowest coumestrol producing cultivars were inconsistent and when all sampling dates were taken into account there was no difference. On the other hand there may have been less variation in the Experiment 7a cultivars than the Experiment 7b ones.

The lower levels recorded in Experiment 7b could have been due to less susceptibility of the younger plants to fungal damage or the lack of lucerne debris on the ground to act as a source of fungal infection: the stand had previously been comprised of lucerne until September 2010 followed by oats (*Avena sativa* L.), kale (*Brassica oleracea* L.) and ryegrass-white clover pasture until July 2015 before sowing into the current lucerne stand on 21 October 2015. The overall low to moderately low coumestrol content indicates that newly sown stands may have less coumestrol than older ones, which requires further investigation.

5.3 Experiment 8 Fungicide and insecticide treatment

5.3.1 Introduction

Coumestrol has been shown to accumulate in lucerne in response to foliar fungal pathogens (Hanson *et al.*, 1965; Sherwood *et al.*, 1970; Saba *et al.*, 1972) and aphid herbivory (Hanson *et al.*, 1965; Loper, 1968; Kain and Biggs, 1980). Weekly fungicide applications of mancozeb and benomyl have been shown to reduce coumestrol accumulated in lucerne (Hanson *et al.*, 1965; Purves *et al.*, 1981). However, benomyl has been withdrawn from the New Zealand market and mancozeb is non-systemic and requires frequent, often weekly, application with high coverage on all plant surfaces.

Carbendazim has previously been shown to inhibit *S. botryosum* mycelial growth on agar by 60% (Hosen *et al.*, 2009) and control *Phoma* black stem in annual medics (Barbetti, 1989) and could be an appropriate fungicide to test. However, unlike in previous studies, a lower, potentially more economic frequency will be tested. This would simulate a closer and more realistic approximation of what any potential on-farm application frequency would be. Weekly application is not economic in dryland sheep systems and it may enable species to develop fungicide resistance. For example, the manufacturer of the carbendazim fungicide 'Protek', recommends no more than two spray applications per season to prevent resistance building in fungal populations.

For Experiment 8, the control of coumestrol accumulation in lucerne throughout the year was tested by using carbendazim, a broad-spectrum systemic fungicide, was investigated to determine whether coumestrol content was controlled relative to controls. In addition pirimicarb, an aphidicide, was used to determine if control of aphids would reduce coumestrol in the presence and absence of fungal control.

5.3.2 Methods

5.3.2.1 Experimental Site

Experiment 8 took place at I12, Lincoln University, Canterbury, New Zealand between 29 October 2015 and 25 May 2016. A description of this site is given in Section 4.1.

5.3.2.2 Experimental Design

Experiment 8 was a two-factor factorial with a split-split plot design with three blocks and eight harvest dates. 'Stamina 5' lucerne was grown in an established stand and then two fungicide regimes (+/-) and two aphidicide regimes (+/-) were imposed on 8 x 6 m plots. This resulted in four treatments: no spray (N), fungicide spray (F), insecticide spray (I), and insecticide and fungicide spray (IF). The experimental design was 'fungicide x insecticide x block x harvest date' (2 x 2 x 3 x 8, N =96). The fungicide application was 500 mL/ha of 'Protek' containing 500 g/L of the active ingredient carbendazim. The insecticide was 250 g/ha ‘Pirimor 50’ containing 500 g/kg of the active ingredient pirimicarb.

First spray application

Plots were cut at the end of September and sprayed four weeks later on 29 October 2015 at which time lucerne was 35 cm tall and vegetative (development stage 3). Plant samples were taken four days and three weeks after application.

Second spray application

All plots were cut a second time in early December and sprayed five weeks later on 15 January 2016 at which time lucerne was 55 cm tall and with flowers (stage 6-7). Plant samples were collected one week and three weeks after application.

Third and fourth spray applications

Plots were cut in early March and sprayed four weeks later on 4 April 2016 at which time lucerne was 30 cm tall and vegetative (stage 3). Plant samples were collected at one week, three weeks and five weeks later. Plots were again sprayed on 17 May 2016, when lucerne was 30 cm and vegetative to early buds (stage 3-4). Plant samples were collected one week later.

The lucerne samples were processed and extracted as described in Sections 4.4 to 4.6. Coumestrol was measured by HPLC with the methodology described in Section 4.7. The coumestrol rating scale (Table 4.4) was used to rate coumestrol content from negligible to extreme. Statistical analyses performed are described in Section 4.12.1.

5.3.2.3 Meteorological Data

Rainfall and temperature data were recorded for the duration of Experiment 8 at Broadfield Meteorological Station, Lincoln, 2.5 km from the field site.

The total rainfall during the experimental period between 1 October 2015 and 25 May 2016 was 282.4 mm. The total monthly rainfall is given in Table 5.2. The daily rainfall and mean daily temperature for the experimental period are given in Appendix A (Figures A.7 and A.8).

Table 5.2 Total monthly rainfall recorded at Broadfield EWS (NIWA) between 1 October 2015 and 25 May 2016.

	Oct	Nov	Dec	Jan	Feb	March	April	May (1-25)
Total Rainfall (mm)	8.8	13.2	56.8	91.2	24.2	33.8	9.6	44.8

5.3.3 Results

5.3.3.1 Lucerne yield

In Experiment 8 there was no effect of insecticide (P = 0.547) treatment on the yields of lucerne throughout the experiment. There were effects of fungicide (P = 0.002) and date (P < 0.001) on yield (Figure 5.18), and a weak interaction (P = 0.040) between fungicide and date. On average, across the growing season, fungicide treated lucerne had a yield of 2.6 t DM/ha compared (P < 0.001) with a yield of 2.2 t DM/ha in untreated and insecticide-only treated lucerne.

Yield was 4.6 ± 0.149 t DM/ha (n = 12) on 20 November 2015, three weeks after the first spray was applied onto four week old regrowth. There was no fungicide effect (P = 0.250) on this date, with 4.4 ± 0.23 t DM/ha in untreated and insecticide-only treated plots compared with 4.8 ± 0.18 t DM/ha in fungicide-treated plots (n = 6). Lucerne was vegetative (Stage 3) on this date and 66 ± 0.9 cm tall (n =12)

Three weeks after the second spray application onto five week old regrowth on 7 February 2016, yield (n = 12) was lower (P < 0.001) than on 20 November 2015, with an average of 3.78 ± 0.245 t DM/ha. Lucerne was flowering (Stage 8) and 72 ± 1.8 cm tall. Yield (n = 6) was higher (P = 0.008) in fungicide-treated plots with 4.2 ± 0.41 t DM/ha compared with 3.3 ± 0.14 t DM/ha in untreated plots.

One week after the third spray onto four week old regrowth on 9 April 2016, the yield (n = 12) was 1.43 ± 0.114 t DM/ha, which was lower (P < 0.001) than both 20 November 2015 and 7 February 2016 yields. Lucerne was vegetative (Stage 2-3) and 30 ± 2.2 cm tall. There was no difference (P = 0.159) in yield (n = 6) with 1.7 ± 0.12 t DM/ha in treated plots and 1.2 ± 0.15 t DM in untreated plots. Yield (n = 12) decreased (P = 0.017) to 0.87 ± 0.094 t DM/ha on 25 May 2016, seven weeks after the third spray, and one week after the fourth spray. Lucerne was vegetative to early bud (Stage 3-4) and 32 ± 2.0 cm tall. There was no difference (P = 0.242) in yield (n = 6) between treatments, fungicide-treated plots had 1.1 ± 0.134 t DM/ha compared with 0.69 ± 0.080 t DM/ha in untreated plots.

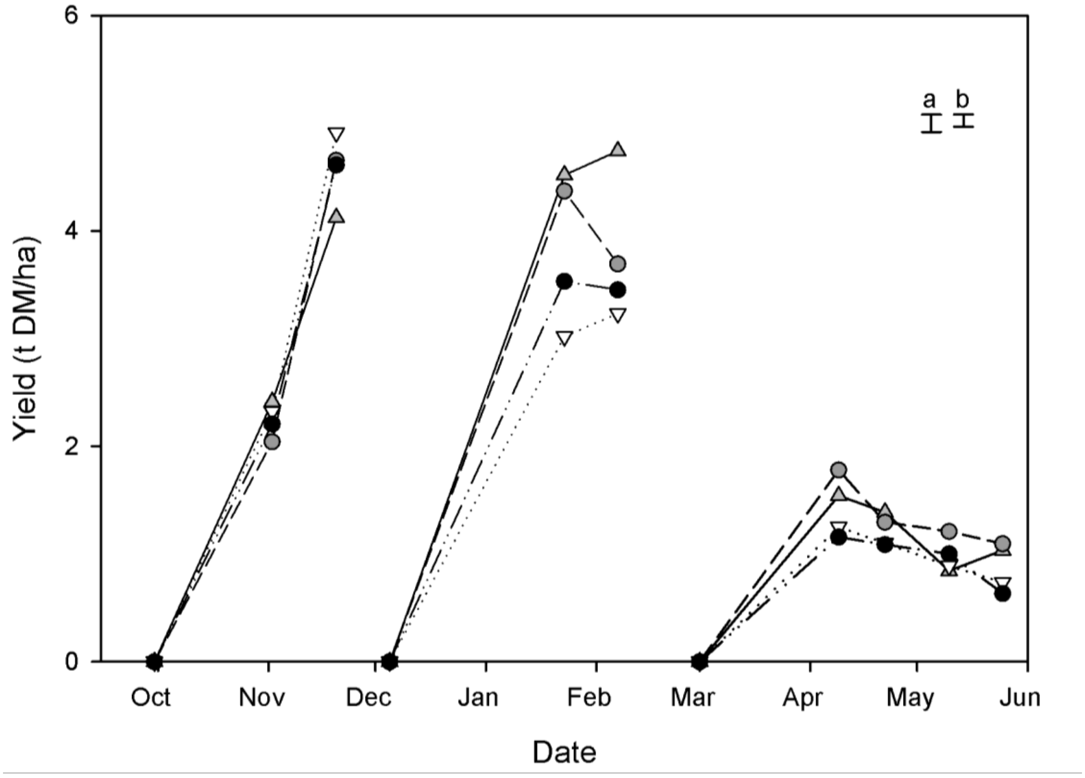

Figure 5.18 Yield of unsprayed (●), fungicide sprayed (▲), insecticide sprayed (▽) and fungicide + insecticide sprayed (●) lucerne between 2 November 2015 and 25 May 2016. Error bars are the standard errors of the mean for a) date and b) fungicide treatment.

5.3.3.2 Coumestrol versus spray treatment

There was no effect of the fungicide (P = 0.279) or insecticide (P = 1.00) treatment on coumestrol content of lucerne across the harvest dates in Experiment 8.

There was an effect (P < 0.001) of date on coumestrol content of lucerne (n = 12) across all treatments (Figure 5.19). In the three weeks following the first spray application onto four week old regrowth on 29 October 2015, there was no difference (P = 0.807) in coumestrol content between four days post-spraying, with a negligible 0.76 ± 0.068 mg/kg DM on 2 November 2015 (Stage 3; 37 ± 0.9 cm), and three-weeks post-spraying, with a low 2.0 ± 0.17 mg/kg DM on 20 November 2015 (Stage 3; 66 ± 0.9 cm).

One week after the second spray application which was applied to five week old regrowth, average coumestrol content of the lucerne crop was higher (P < 0.03) than in November, with a moderately low 13.9 ± 1.62 mg/kg DM on 23 January 2016 (Stage 7; 63 ± 1.4 cm). Coumestrol content then increased (P < 0.001) to a moderately high 54.8 ±6.69 mg/kg DM on 7 February (Stage 8; 72 ± 1.8 cm), three weeks after spray was applied.

In the next regrowth period, one week after the third spray application onto four week old regrowth, average coumestrol content was lower (P < 0.001) than on 7 February 2016, with a moderately low

21.2 ± 1.77 mg/kg DM on 9 April 2016 (Stage 2-3; 30 ± 2.2). Coumestrol content increased (P < 0.001) to a moderate 42.2 ± 4.18 mg/kg DM on 22 April 2016 (Stage 2-3; 29 ± 1.6 cm), three weeks after application, but remained lower (P = 0.016) than on 7 February 2016. Coumestrol further increased (P = 0.003) to a moderately high 57.7 ± 4.50 mg/kg DM on 10 May 2016 (Stage 3-4; 30 ± 2.2 cm), five weeks after application, which was not different (P = 0.578) to 7 February 2016. Coumestrol then declined (P = 0.007) to a moderate 43.5 ± 5.09 mg/kg DM on 25 May 2016 (Stage 3-4; 32 ± 2.0 cm), seven weeks after application (one week after the fourth application).

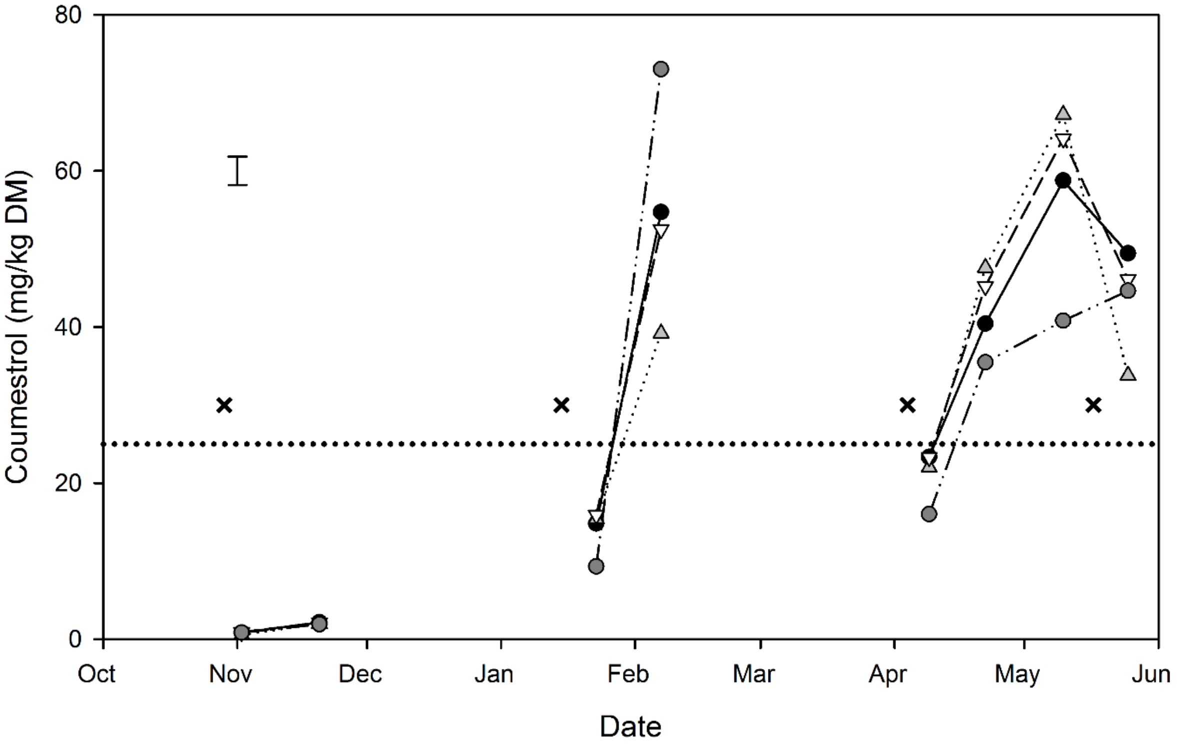

Figure 5.19 There was no difference in mean coumestrol content (mg/kg DM) of lucerne among spray treatments of unsprayed (●), fungicide sprayed (▲), insecticide sprayed (▽), and fungicide plus insecticide sprayed (●). Error bar is the standard error of the mean for date. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance. Dates of spray application are indicated by ‘X’.

5.3.3.3 Fungal pathogen and insect pest presence

There was no effect of fungicide (P = 0.818) or insecticide (P = 0.818) treatment on the fungal damage score. Fungal damage score changed (P < 0.001) throughout the year (Figure 5.20), with lowest (P < 0.001) damage on 2 November 2015, at an average score of 1.1 ± 0.08 (n = 12). The next lowest damage score was on 20 November with 1.9 ± 0.08. Fungal damage was moderate (3.1 ± 0.149) on 23 January 2016 and increased (P < 0.001) to 3.75 on 7 February. On 9 April 2016, fungal damage was not different (P = 0.645) to 23 January 2016 with 3.0 ± 0.123. Damage increased (P < 0.001) to 4 in all plots on 22 April 2016. On 10 May 2016 damage had again increased (P = 0.007), to 4.5 ± 0.20 and was not different (P = 0.645) on 25 May 2016, with a score of 4.6 ± 0.15.

Changes in the abundance of fungal species present over the year are shown in Figure 5.20. The main fungal pathogens present were stemphylium and common leafspot. Low levels of spring black stem were also present in late summer and late autumn.

There were no effects of fungicide (P = 0.094) or insecticide (P = 0.094) on stemphylium. There was an effect (P < 0.001) of date. Stemphylium was lowest (P < 0.001) on 2 November 2015 with a fungal damage score for this species of 1.3 ± 0.14 (n = 12). The next lowest value was on 20 November 2015 with a damage score of 2.1 ± 0.08. In the regrowth on 23 January 2016, there was a higher (P = 0.013) level of stemphylium damage (2.6 ± 0.15), and on 7 February 2016, with a score of 3.5 ± 0.15, damage was higher (P < 0.001) than on 23 January 2016. In the regrowth on 9 April 2016, stemphylium damage was lower (P < 0.001) than on 7 February 2016 with 2.8 ± 0.11. This increased (P = 0.132) to 3.3 ± 0.14 on 22 April 2016 and then further increased (P = 0.004) to 3.9 ± 0.19 on 10 May 2016 which was not different (P = 0.094) to 25 May 2016, with a score of 3.6 ± 0.15.

There were no effects of fungicide (P = 0.477) or insecticide (P = 0.100) on common leafspot. There was an effect (P < 0.001) of date. On 2 and 20 November 2015 there was no common leafspot present (damage score of 1). On 23 January 2016 there was a higher (P < 0.001), but moderate level of common leafspot, with a damage score of 3.4 ± 0.15 (n = 12). This increased to 3.9 ± 0.08 on 7 February 2016. On 9 and 22 April 2016 there was less (P < 0.001) common leaf spot damage, (2.7 ± 0.14 and 2.8 ± 0.21, respectively), with no difference (P = 0.344) between the two dates. Common leafspot damage increased (P < 0.001) to 4.0 in all plants on 10 May 2016, and further increased (P = 0.001) to 4.6 ± 0.15 on 25 May 2016.

There were no effects of fungicide (P = 0.623) or insecticide (P = 0.623) on spring black stem. There was an effect (P < 0.001) of date. No spring black stem was present in November 2015 or April 2016 (damage scores of 1). Slight levels of spring black stem were observed in January and February 2016, with scores of 1.8 ± 0.11 and 2 ± 0.0, respectively. Trace levels were present on 10 and 25 May 2016, with scores of 1.1 ± 0.08 and 1.2 ± 0.11 respectively.

No aphids (< 0.2 aphids per stem) were detected during the growing season. Insect damage throughout the year by non-aphid insects, such as sitona weevil (*Sitona discoideus* Gyllenhal) and lucerne flea (*Sminthurus viridis* L.), was not severe, with maximum overall insect damage scores of three recorded.

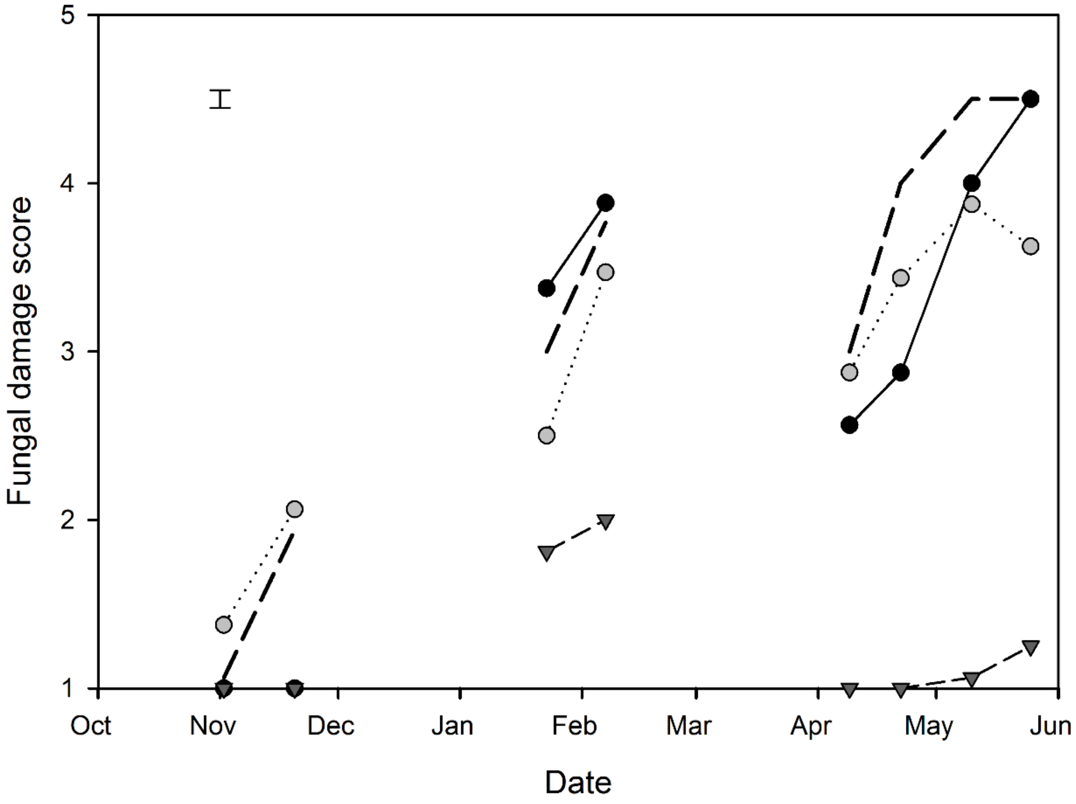

Figure 5.20 The seasonal change in overall fungal damage score (– – –) and individual damage scores for common leaf spot (●), stemphylium (●), spring black stem (▼) averaged across all lucerne plots. Error bar is the standard error of the mean for date.

5.3.3.4 Coumestrol content versus damage

There was a correlation (r = 0.786) between the fungal damage score of lucerne and coumestrol content (Figure 5.21). Lucerne with no damage (a score of 1) was not different (P = 0.537) from lucerne with slight damage (2) with negligible to low average coumestrol contents of 0.81 ± 0.088 mg/kg DM (n = 12) and 3.4 ± 1.14 mg/kg DM (n = 14), respectively. Coumestrol content was higher (P < 0.001) in lucerne with damage scores of 3 or more. Lucerne with a damage score of three had a moderately low coumestrol content of 22.4 ± 2.19 mg/kg DM (n = 24) which was lower (P < 0.001) than the moderate coumestrol content of 45.3 ± 3.36 mg/kg DM (n = 31) in lucerne with a damage score of 4. Lucerne with a damage score of 5 had the highest (P = 0.035) average coumestrol content with a moderately high 55.5 ± 5.94 mg/kg DM (n = 15).

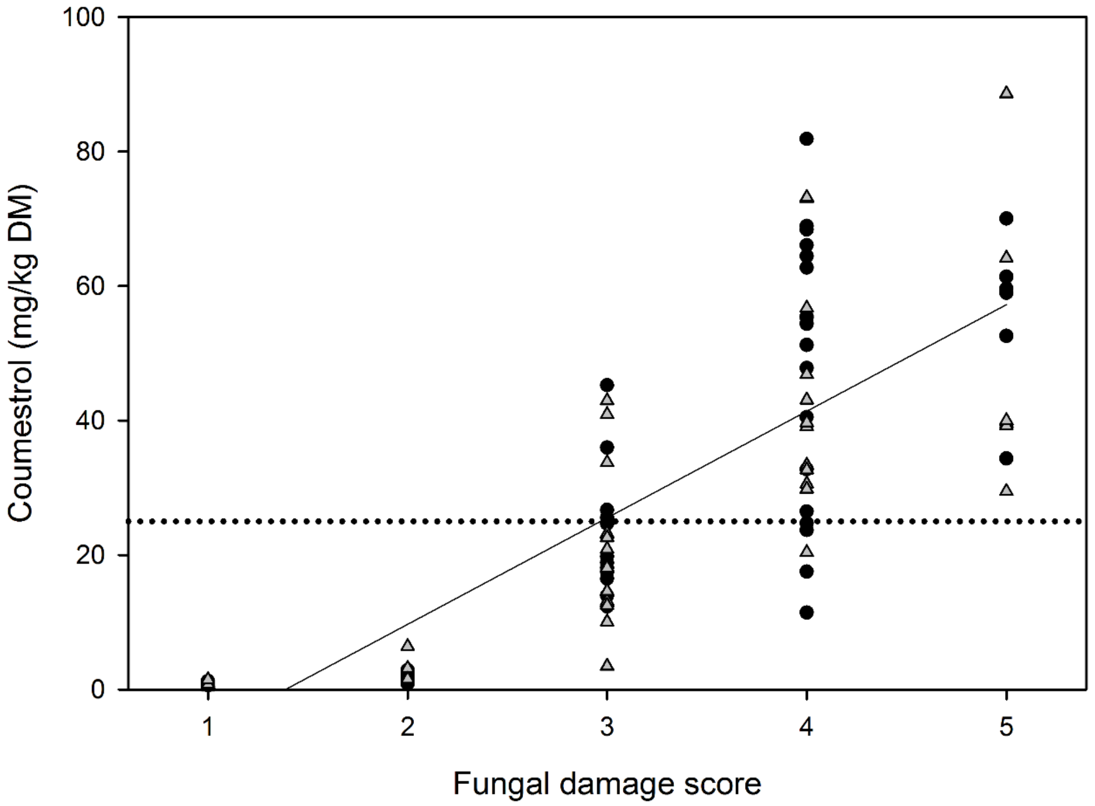

Figure 5.21 Coumestrol content (mg/kg DM) against fungal damage score for fungicide sprayed (▲) and unsprayed (●) lucerne with a split-line relationship (R^2 = 0.631). Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

5.3.4 Discussion

Following the January 2016 and April 2016 sprays, coumestrol level increased regardless of fungicide treatment in the three weeks following spraying despite application occurring while coumestrol was moderately low (Figure 5.19). Coumestrol increased from a moderately low 14 mg/kg DM to a moderately high 55 mg/kg DM after the January application, and increased from a moderately low 21 mg/kg DM to a moderate 42 mg/kg DM after the April application. This is in contrast with previous studies which showed coumestrol accumulation was reduced with weekly fungicide applications of mancozeb and benomyl (Hanson *et al.*, 1965; Purves *et al.*, 1981).

As for Experiment 6 and 7 (Sections 5.1 and 5.2), there was a relationship between the coumestrol content and fungal damage score of lucerne (Figure 5.21). Heightened coumestrol levels coincided with high fungal scores and there was no effect of fungicide on the fungal damage score (Section 5.3.3.3). Moderate fungal symptoms (damage score of 3) were present a week after both the January and April sprays. These symptoms worsened to damage scores of approximately 4, three weeks post-application in both unsprayed and sprayed plots.

Application of fungicide improved yield by an average of 400 kg/DM ha across the dates samples were collected. This suggests that some benefit was gained, despite the lack of difference in visual symptoms or coumestrol contents.

That no difference in visual symptoms or coumestrol contents occurred between fungicide-sprayed and unsprayed plots was unexpected as carbendazim is a protective and curative systemic fungicide and would be expected to reduce the spread of the diseases within the fungicide-sprayed lucerne plants, and therefore the level of coumestrol. This indicates that the fungicide spray regime used was insufficient to control the lucerne disease(s) responsible for the increased coumestrol. This may be due to fungicide resistance, either of the fungal pathogens in general, or the established pathogens. It could also be due to the infrequency of the fungicide applications. Carbendazim has previously been shown to inhibit *S. botryosum* mycelial growth on agar by 60% (Hosen *et al.*, 2009) and control *Phoma* black stem in annual medics (Barbetti, 1989). However, these fungal pathogens, along with common leafspot, were present in the control and sprayed treatments at the same levels visually.

A different fungicide or a pre-emptive application, before any fungal symptoms are present could potentially be more effective and requires further research. Pre-emptive spraying is unlikely to be suitable for most dryland lucerne systems because fungicide applications need to be applied to affected stands to be cost-effective. Historically, frequent spraying of mancozeb and benomyl was effective at decreasing coumestrol accumulation in lucerne (Hanson *et al.*, 1965; Purves *et al.*, 1981). However, frequent use of carbendazim is not recommended and no more than two spray applications per season is advised to prevent resistance (Beresford, 2005). This is likely to also be the case with alternative fungicide options.

The insecticide did not affect coumestrol content or yield of lucerne. This was because pirimor controls aphids, but aphids were not present during the growing season. Despite a low phyto-toxicity rating (Short and McConnell, 1973), pirimor treated lucerne had yellowing on the leaf margins following the November spray. This did not affect coumestrol and symptoms were not evident following the sprays in subsequent re-growths.

Based on these findings, carbendazim is not recommended for controlling fungal diseases in lucerne as a means to reduce coumestrol content. It is also unlikely to be economic, or worth the risk of developing carbendazim-resistant pathogens, to apply carbendazim for the small yield increase observed here. The use of pirimor to control aphids has not been established, but would likely only be effective in cases when fungal diseases are absent but aphids are present.

5.4 Experiment 9 Lucerne infected with fungal pathogens

5.4.1 Introduction

Results of Experiment 6a (Section 5.1) suggested that stemphylium did not increase the coumestrol content in lucerne. In Experiment 6a, lucerne that only had stemphylium symptoms had low coumestrol (5.9 mg/kg DM) on 24 March 2014 but a high coumestrol content (140 mg/kg DM) on 21 April 2014, four weeks later when symptoms of other fungal pathogens were also present. It is hypothesised that this could be due to the sharp border formed around the cool-type stemphylium lesions, limiting the spread of the pathogen within the leaves. Experiment 9 compared stemphylium and anthracnose with an uninfected control to test whether glasshouse grown lucerne infected with cool-type stemphylium infection caused elevated coumestrol levels relative to control lucerne. In addition, ‘Wairau’ and ‘Stamina 5’ were compared to test whether there was a difference between these old and recent cultivars. The null hypothesis was that infection would not cause coumestrol to differ from the uninfected plants or between cultivars.

5.4.2 Methods

5.4.2.1 Experimental Design

For Experiment 9, two lucerne cultivars, ‘Stamina 5’ and ‘Wairau’ were used, three inoculation treatments (control, stemphylium, and anthracnose), two harvest dates (7 days and 14 days post-inoculation) and three blocks (2 x 3 x 2 x 3, N = 36). This experiment was run at the leaf level with leaves needle damaged six times per leaflet, and at the whole plant level where entire undamaged plants were painted with spore solution. In addition, stemphylium leaves needle damaged three times per leaflet were dissected into diseased material and lesion-free ‘clean’ material for both cultivars and both harvest dates (‘cultivar’ x ‘dissection part’ x ‘harvest date’ x ‘block’, 2 x 2 x 2 x 3, N = 24). In addition, uninfected control lucerne leaflets of both cultivars (N = 6) were sliced to simulate the mechanical damage to the dissected diseased leaves.

5.4.2.2 Isolation of Fungal Species

Lucerne leaf and stem pieces (<5 mm diameter) with fungal symptoms were collected from Iversen 12 in January 2017, surface sterilised in 70% ethanol for 30, 60, 90 or 120 seconds and plated onto V8 agar. V8 agar was prepared with 800 mL distilled water, 200 mL V8® Juice (Campbell Soup Company, New Jersey, USA), 2 g $CaCO_3$ and 15 g agar (Tuite, 1969). Ingredients were mixed, autoclaved and cooled to 55°C. For the plates used to establish primary cultures, ampicillin at a rate of 0.1 g/L and/or penicillin-streptomycin at a rate of 80 units per mL were added to the V8 agar to decrease bacterial contamination. Agar was then poured into 86.5 mm wide, 14.5 mm high Petri dishes.

Agar plates were incubated at 25°C for approximately seven days in the dark before sub-culturing small sections of agar onto clean plates. Sub-cultured plates were incubated and fungal species identified by spore morphology. Plates were sub-cultured until monocultures of the above fungi were obtained. For the final sub-culture plates were incubated for four days in darkness and then exposed to 48 hours of natural day and night cycles for sporulation.

5.4.2.3 DNA identification of fungi

DNA was prepared for sequencing by Dr Hayley Ridgway, Lincoln University. In brief, the protocol was: a small section of mycelium was added to 100 µL of extraction solution (Sigma-Aldrich, Missouri, USA) and incubated at 95°C for 10 minutes. To the solution, 100 µL of dilution solution (Sigma-Aldrich) was added, the sample was vortexed and stored at 4°C. The ribosomal internal transcribed spacer (ITS) was amplified in Extract-N-Amp^{TM} PCR ReadyMix^{TM} (Sigma-Aldrich) with ITS1 and ITS4 primers (White *et al.*, 1990), and the β-tubulin (TUB2) region with T1 (O'Donnell and Cigelnik, 1997) and Bt2b primers (Glass and Donaldson, 1995). DNA was sequenced by the Lincoln University Bio-protection Research Centre and compared to sequences present in the GenBank database using the nucleotide BLAST® algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and a distance tree created with the 'Fast Minimum Evolution' method (Desper and Gascuel, 2004).

5.4.2.4 Application of fungi to lucerne

Lucerne was sown into 18 cm deep x 14 cm wide, 2 L pots (VCE 14, Pöppelmann, Germany) as described in Section 4.1.5. When lucerne plants were one month old, fungal spores were suspended in distilled water containing 0.01% Tween 20 and the spore concentrations of both species were determined with a haemocytometer and then diluted to 5,000 spores/mL. From each pot ($n = 3$), two randomly selected, fully expanded leaves per pot were punctured in six places per leaflet and two leaves were punctured in three places per leaflet. Spore solution or a control solution of distilled water was then liberally applied to lucerne leaves and stem of the entire plant by paintbrush, so that each pot received approximately 4 mL of solution. Plants were sealed in plastic bags for one week, outdoors under shade. Temperature ranged from 8-26°C during this period.

5.4.2.5 Sampling and Processing Lucerne Samples

Plants were harvested one and two weeks after infection. Samples were scored for fungal damage using the criteria described in Section 4.5. Needle-damaged leaflets were photographed, and the damaged area of each leaf was measured with 'Leaf Doctor' (Pethybridge and Nelson, 2015).

Stemphylium leaves with three needle punctures per leaflet were dissected with a scalpel to separate into lesions and visually healthy material. Punctured control leaves were sliced into similar sized pieces to replicate the mechanical damage of the diseased leaves applied by the scalpel. Samples were oven dried at 60°C and material was extracted using the methodology in Section 4.6.

Coumestrol was measured by HPLC with the methodology described in Section 4.7. The coumestrol rating scale (Table 4.4) was used to rate coumestrol content from negligible to extreme. Statistical analyses performed are described in Section 4.12.1.

5.4.3 Results

Nucleotide BLAST® of the ITS sequence of one fungal species (Table D.6; Appendix D) showed the genus to be *Stemphylium,* with 99% shared identity with other species within this genus. The β-tubulin gene sequence (Table D.7) showed the species to most likely be *S. vesicarium*, with 99% sequence match, compared with a 95% shared identity with *S. callistephi* and *S. lycopersici*, and 93% shared identity with *S. solani.* Figure 5.22 shows the relations between these and other species, relative to the species under test. *S. vesicarium,* is a cool temperature biotype of *Stemphylium* spp. that infects lucerne (Irwin and Bray, 1991).

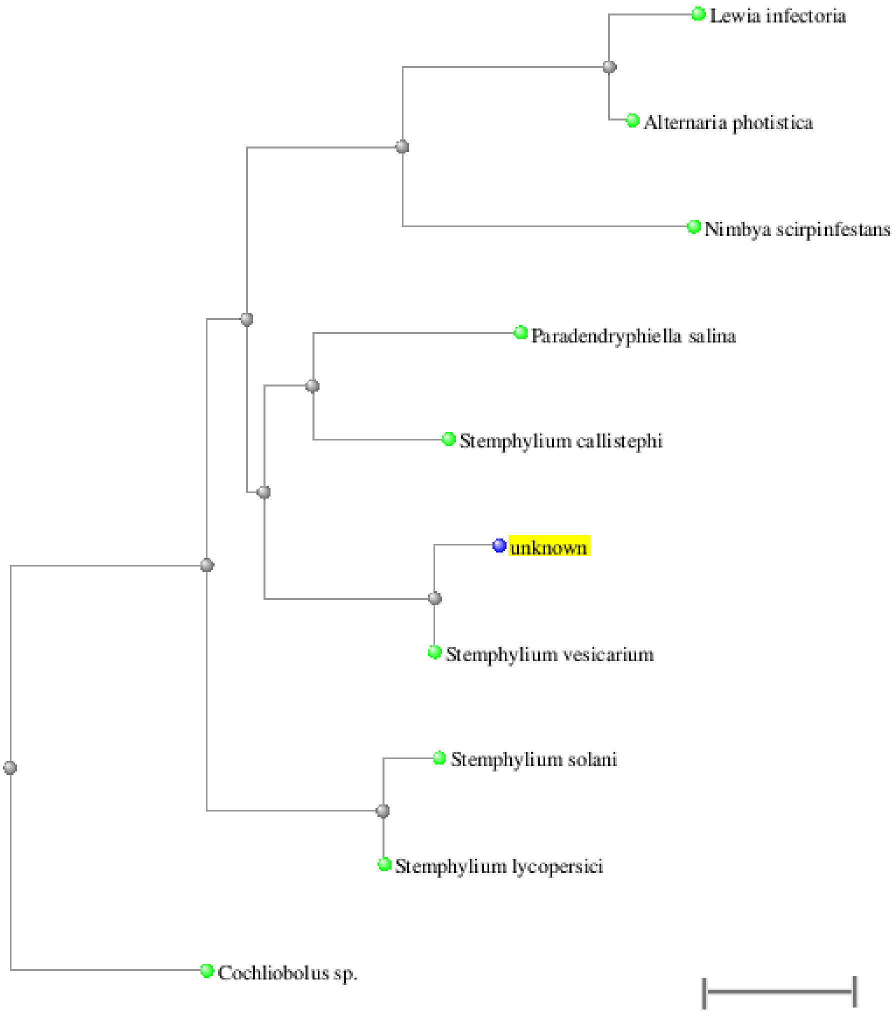

Figure 5.22 Phylogenetic tree based on the β-tubulin gene sequence, showing relations among species including the specimen of interest 'Unknown'. Scale bar is 0.02 nucleotide substitutions per site.

The ITS nucleotide sequence of the other fungal species (Table D.8) showed the genus to be *Colletotrichum*, with 99% identity match to species from this genus. The β-tubulin gene sequence (Table D.9) showed the species to most be part of the *C. destructivum* species complex (Figure 5.23), which is a complex of species identified by Damm *et al.* (2014). Highest identity was with *C. americae-borealis* Damm (100%), *C. linicola* Pethybr. & Laff (99%), *C. tabaci* Böning (98%), *C. utrechtense* Damm (98%) and *C. fuscum* Laubert (98%), all other species shown in Figure 5.23 had at least 95% shared identity with the unknown species. Of these species *C. destructivum, C. americae-borealis* and *C. linicola* have been shown to infect lucerne (Damm *et al.*, 2014; Vasić *et al.*, 2014).

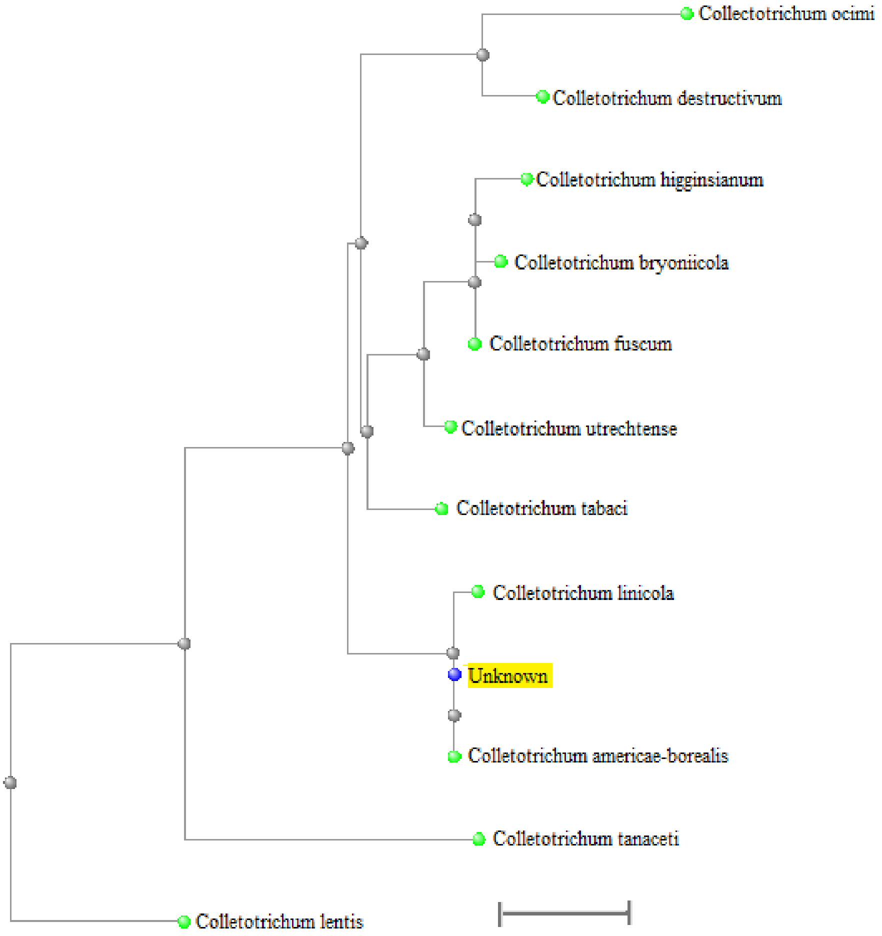

Figure 5.23 Phylogenetic tree based on the β-tubulin gene sequence, showing relations among species including the specimen of interest 'Unknown'. Scale bar is 0.01 nucleotide substitutions per site.

Coumestrol content of whole plants was affected (P < 0.001) by inoculation treatment (Figure 5.24). Lucerne infected with stemphylium had an extreme average coumestrol content of 169 ± 25.1 mg/kg DM (n = 12). In contrast (P < 0.001), the control had a low average coumestrol content of 3.4 ± 0.84 mg/kg DM (n = 12). Coumestrol content of lucerne infected with anthracnose was also low at 7.8 ± 1.6 mg/kg DM, which was lower (P < 0.001) than for stemphylium, but not different (P = 0.398) to the control. There was no effect of cultivar (P = 0.220) or of duration between inoculation and sampling (P = 0.739). Fungal damage score was not affected by cultivar (P = 0.242) but was affected by harvest date (P = 0.035) and disease (P < 0.01). Fungal damage score was 3.1 ± 0.23 in Stemphylium infected plants (n = 12) which was greater than in Anthracnose infected (1.4 ± 0.13; n = 12) or control plants (1.0 ± 0.04; n = 12). Overall, plants had a fungal damage score of 1.7 ± 0.22 (n = 18) one week after inoculation and 2.0 ± 0.28 two weeks after inoculation. Coumestrol content was correlated (r = 0.872; P < 0.001) with the fungal damage score of the entire plants.

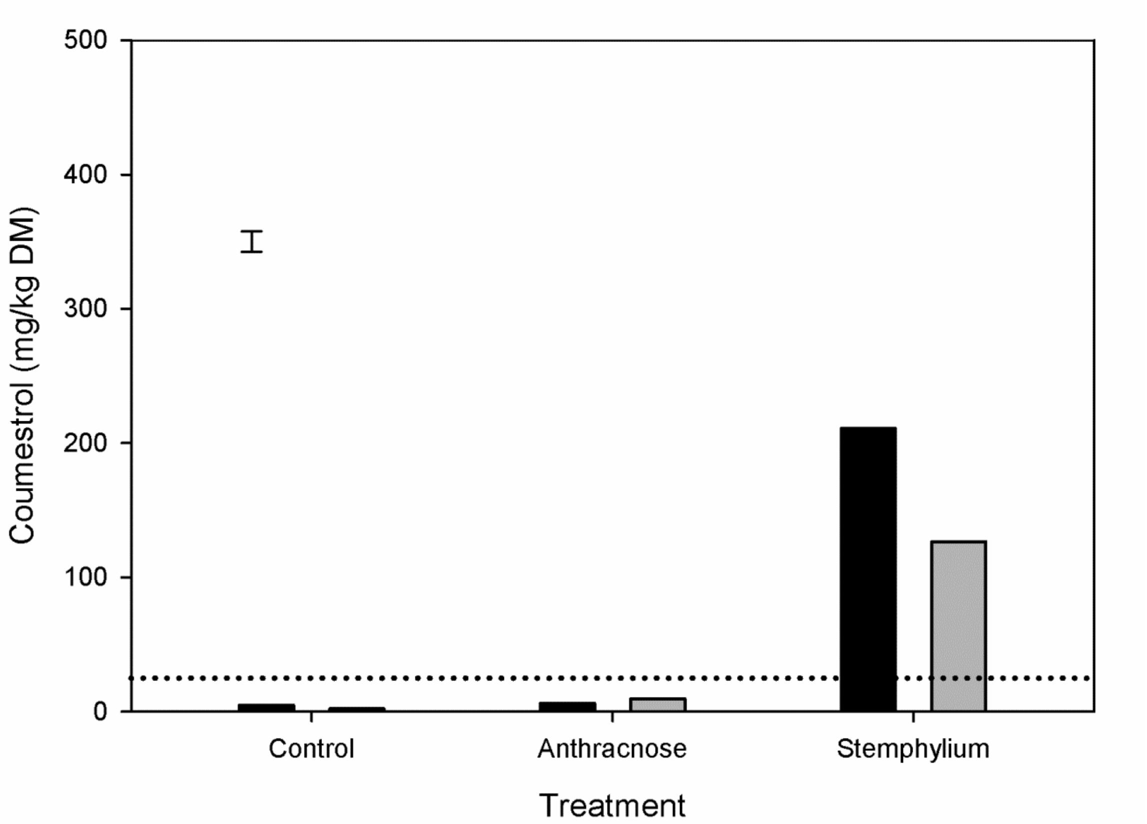

Figure 5.24 Mean coumestrol contents (mg/kg DM) 1-2 weeks after 'Stamina 5' (■) and 'Wairau' (■) lucerne shoots were inoculated with anthracnose or stemphylium, or not inoculated (control). Error bar is the standard error of the mean for infection. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

Leaflets inoculated with stemphylium had a greater (P < 0.001) area of damage (5.3 ± 1.10%; n = 12) than the control (0.5 ± 0.05%; n = 12) or anthracnose (1.0 ± 0.17%; n = 12) leaves when assessed by 'Leaf Doctor'. As pictured in Figures 5.25 and 5.26 areas of damage included the needle punctures and fungal lesions.

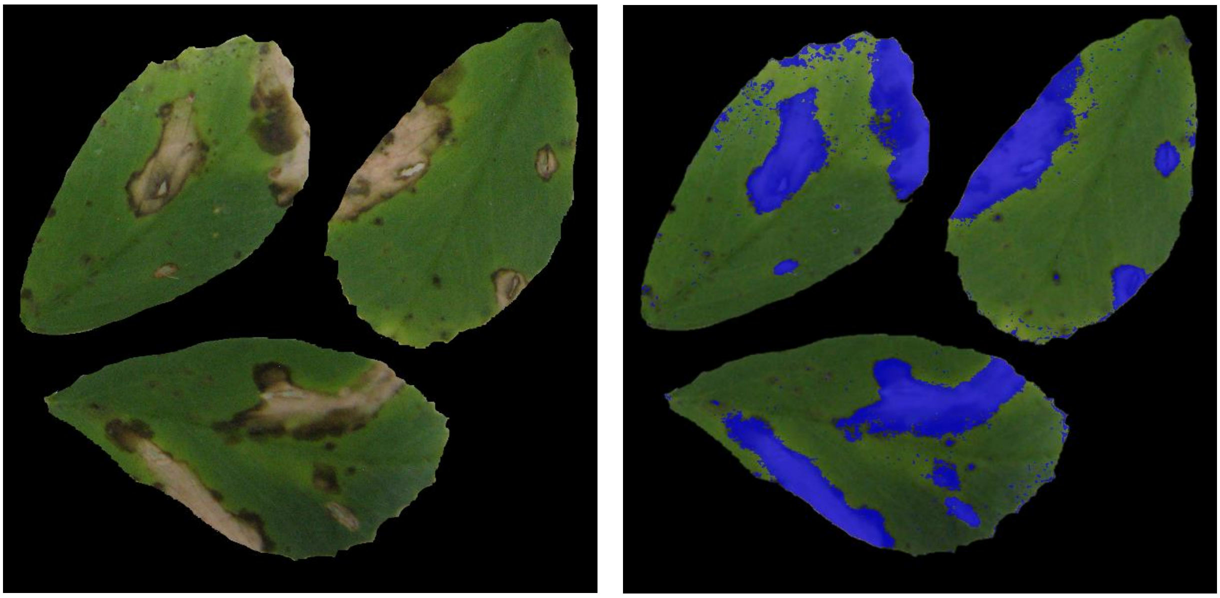

Figure 5.25 Wairau lucerne leaflets from a leaf needle damaged and infected with stemphylium two weeks prior, with and without 'Leaf Doctor' overlay.

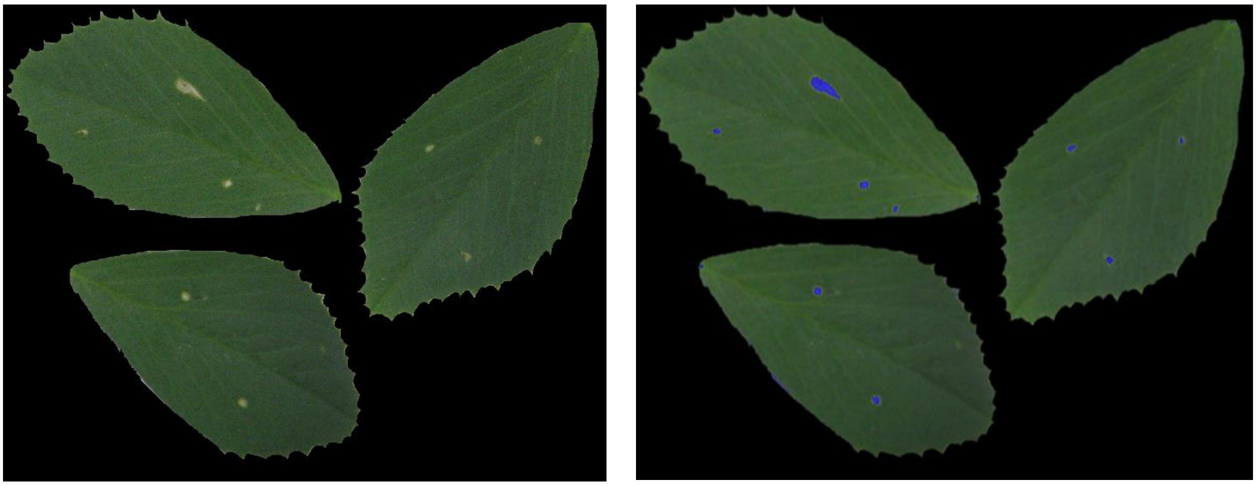

Figure 5.26 Stamina 5 lucerne leaflets from the control treatment, needle damaged two weeks prior, with and without 'Leaf Doctor' overlay.

Coumestrol content of leaves damaged with six needle punctures per leaflet prior to inoculation was affected by an interaction (P < 0.001) of cultivar and fungal treatment (Figure 5.27). 'Stamina 5' leaves inoculated with stemphylium had the highest (P < 0.001) coumestrol content with an extreme level of 396 ± 82.4 mg/kg DM compared with stemphylium-inoculated 'Wairau' leaves with a high level of 143 ± 35.6 mg/kg DM (n = 6). 'Wairau' had higher (P < 0.011) coumestrol content than the anthracnose-inoculated and control leaves. There was also no difference (P > 0.8) in coumestrol content between anthracnose-inoculated and control leaves of either cultivar, with low coumestrol contents of 2.6 ± 0.94 and 3.1 ± 0.48 mg/kg DM for controls of 'Stamina 5' and 'Wairau', respectively, and 5.5 ± 0.65 and 10.7 ± 3.5 mg/kg DM for anthracnose-inoculated leaves of 'Stamina 5' and 'Wairau'. Coumestrol content was not affected (P = 0.685) by duration length.

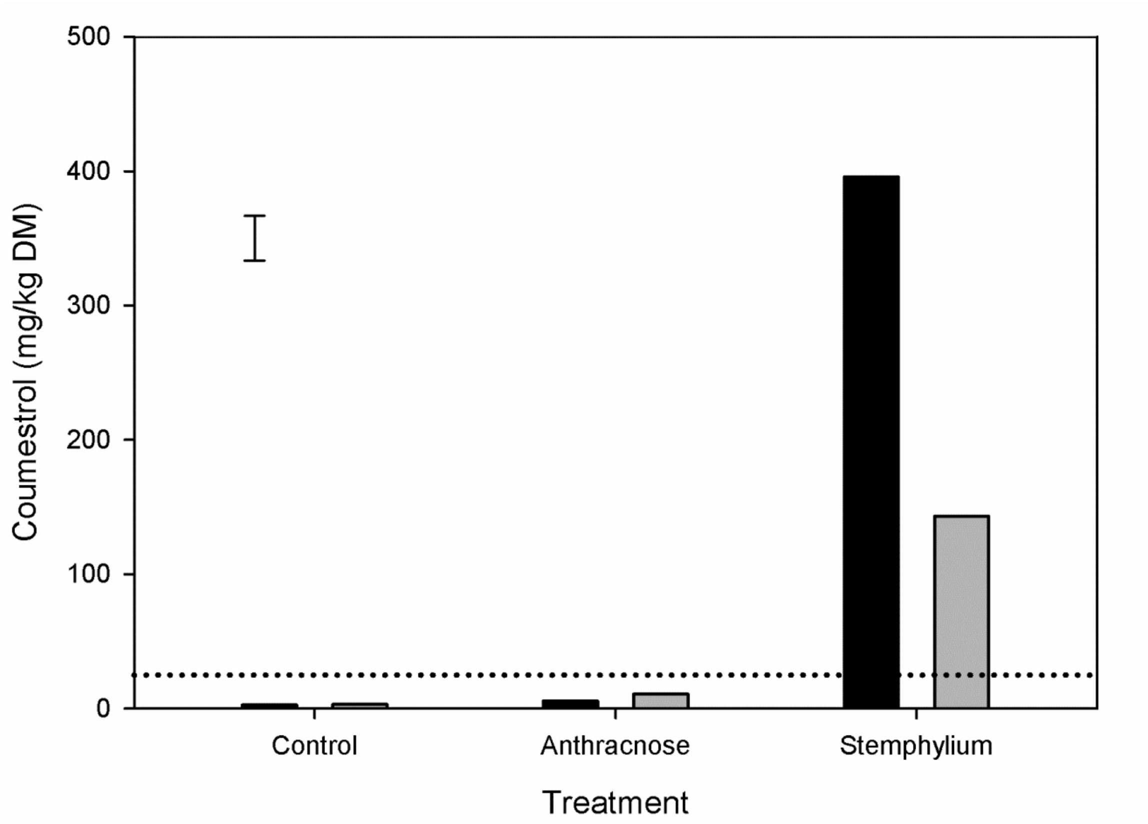

Figure 5.27 Mean coumestrol contents (mg/kg DM) 1-2 weeks after 'Stamina 5' (■) and 'Wairau' (■) lucerne leaves were inoculated with anthracnose or stemphylium, or not inoculated (control), following six needle punctures per leaflet. Error bar is the standard error of the mean for the interaction of treatment and cultivar on coumestrol. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

There was a date x leaf part interaction (P = 0.008) and a cultivar x leaf part interaction (P = 0.005) in pairs of stemphylium-inoculated leaves that were damaged with three needle punctures per leaf, prior to inoculation, and then at harvest one or weeks later, and cut into diseased and visually clean parts.

Figure 5.28 shows the interaction between infection duration and leaf part on coumestrol. Diseased parts had an extreme coumestrol content of 277 ± 61.3 mg/kg DM (n = 6) at the first harvest and increased (P <0.001) to 466 ± 71.5 mg/kg DM at the second harvest, while there was no change (P = 0.882) in coumestrol content of 'clean' parts, which has moderately high coumestrol with 73 ± 20.3 mg/kg DM at the first harvest and 79 ± 18.5 mg/kg DM at the second.

Figure 5.29 shows the interaction between cultivar and leaf part on coumestrol. Diseased parts of 'Stamina 5' had an extreme coumestrol content of 477 ± 49.2 mg/kg DM (n = 6) which was higher (P

< 0.001) coumestrol than diseased parts of ‘Wairau’ at an extreme 252 ± 59.9 mg/kg DM. There was no difference (P = 0.315) in coumestrol between cultivars in visually clean material with a moderately high 55 ± 16.4 mg/kg DM in ‘Wairau’ and a high 101 ± 16.3 mg/kg DM in ‘Stamina 5’

Clean and diseased parts of the leaves had higher (P < 0.001) coumestrol content than control leaves which had been puncture-damaged three times per leaflet, and were sliced into small pieces to replicate the mechanical damage performed on the diseased leaflets. Coumestrol content of the control leaves was negligible at 1.0 ± 0.13 (n = 4) which showed that mechanical damage was not the cause of coumestrol in the clean or diseased portions of the leaflets.

The coumestrol contents of the entire leave punctured three times per leaflet were calculated based on the coumestrol contents and dry weights of the separate ‘diseased’ and ‘clean’ leaf parts. There was no difference (P = 0.869) in coumestrol content between these leaves and the leaves punctured six times, with an average coumestrol content of 246 ± 41.4 mg/kg DM in leaves punctured three times, and 258 ± 56.2 mg/kg DM in leaves punctured six times.

There was no difference (P = 0.701) in the area of damage in leaves punctured three times or six times, and no relationship (P = 0.966; r = -0.01) between the coumestrol content and the area of damage in stemphylium-inoculated leaves punctured three or six times per leaflet. There was also no difference (P = 0.934) in area of damage between stemphylium inoculated ‘Wairau’ and ‘Stamina 5’.

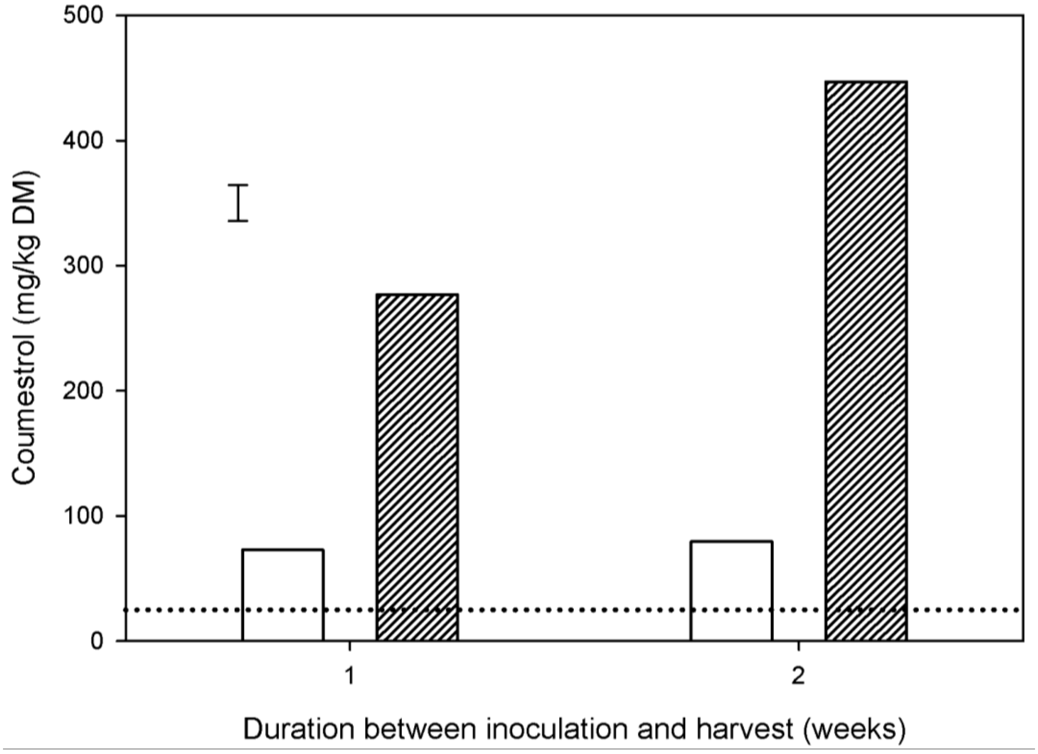

Figure 5.28 Mean coumestrol contents (mg/kg DM) of ‘clean’ (☐) and ‘diseased’ (▨) parts of lucerne leaves, one and two weeks after inoculation with stemphylium. Error bar is the standard error of the mean for the interaction of inoculation duration and leaf part. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

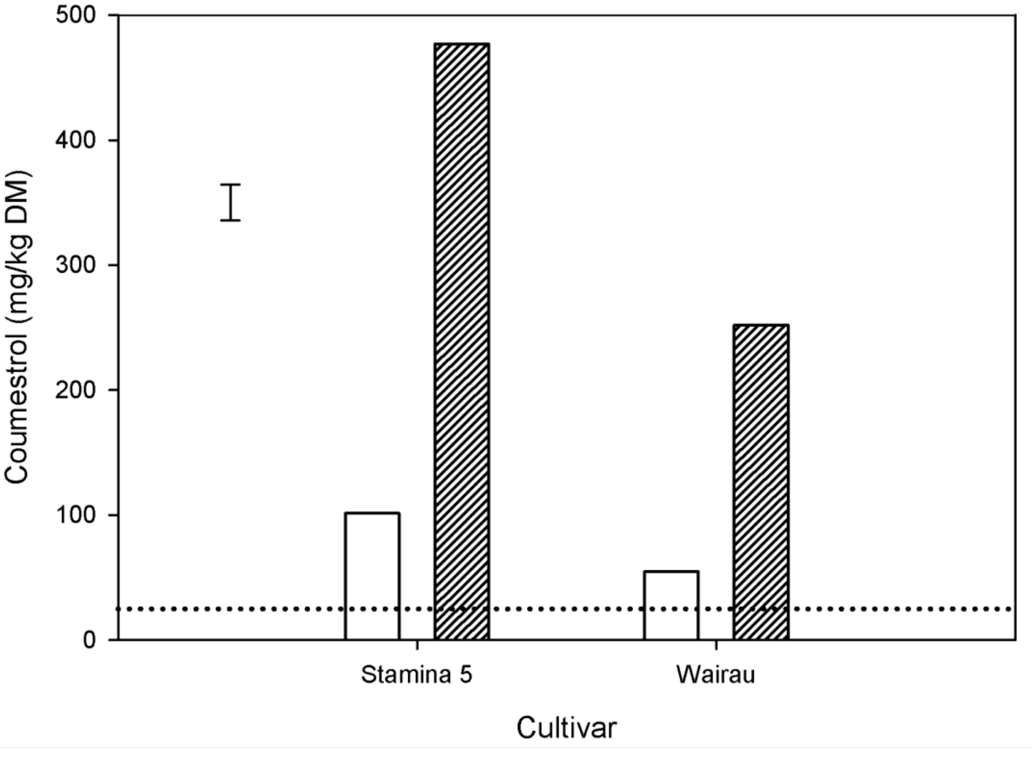

Figure 5.29 Mean coumestrol contents (mg/kg DM) of ‘clean’ (☐) and ‘diseased’ (▨) parts of ‘Stamina 5’ and ‘Wairau’ lucerne leaves, 1-2 weeks after inoculation with stemphylium. Error bar is the standard error of the mean for the interaction of cultivar and leaf part. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance.

5.4.4 Discussion

Presence of stemphylium alone did not affect coumestrol content in Experiment 6a (Section 5.1.3.5). However, lucerne plants artificially inoculated with stemphylium in this experiment had an extreme average coumestrol content of 190 mg/kg DM (Figure 5.24). This rejects the null hypothesis that uninfected and infected plants would have the same coumestrol contents.

The simplest explanation is that like all other fungal pathogens tested (Section 2.6.1.2), including the purportedly warm-biotype symptoms of stemphylium (Hanson *et al.*, 1965), the cool temperature biotype symptoms of stemphylium increase coumestrol content of lucerne. There remains however a possibility that coumestrol accumulation outside the lesion area differed in Experiment 6a from what was observed in Experiment 9. The reason for the low level of coumestrol in the field grown stemphylium affected plants could have been due to compartmentalisation of the pathogen, limiting mycelial growth. In contrast, the lucerne in this experiment was grown under high humidity for a week following inoculation and was also newly sown (in contrast to the established lucerne of Experiment 6a). This could be why the disease was relatively wide spread throughout the plant and why the lesions did not all have the characteristic tan spot with a sharply defined border seen in affected plants in the field. It is possible that the lack of delimiting of the lesion was the cause of the moderately high (70-80 mg/kg DM) coumestrol in the visually unaffected ‘clean’ parts of the leaflets (Figure 5.28). This could also be the cause of the discrepancy in coumestrol content between this experiment and Experiment 6a. Despite the difference in appearance of symptoms (Figure 5.25) compared with the field examples (Figure 2.5), DNA sequencing confirmed the applied pathogen to be *S. vesicarium*, a cause of cool-type stemphylium symptoms (Irwin and Bray, 1991).

Unexpectedly, ‘Stamina 5’ leaves accumulated higher levels of coumestrol than the older ‘Wairau’ in the presence of stemphylium. The null hypothesis was that the two cultivars would have the same coumestrol contents and ‘Stamina 5’ was expected to have greater or at the least equal, resistance to stemphylium, however this does not appear to be the case. There was no effect of cultivar on coumestrol content of entire plants (Figure 5.24), or non-lesioned parts of leaflets (Figure 5.29). However, in whole leaves that were needle damaged prior to inoculation, coumestrol content was 400 mg/kg DM in ‘Stamina 5’, compared with a lower, but still high value of 140 mg/kg DM in ‘Wairau’ (Figure 5.27). This indicates that breeding cultivars for fungal resistance should be a priority.

Inoculation duration affected the coumestrol content within the individual leaves (Figure 5.28), with an increase observed between the first and second weeks. However, duration did not have an effect on the coumestrol content of entire plants.

In contrast, lucerne infected with a purportedly anthracnose-causing fungal species of the *C. destructivum* complex had only slight symptoms of disease and did not have increased coumestrol. The reason for the lack of symptoms may have been due to unsuitable conditions for that pathogen, as anthracnose prefers a warmer climate than stemphylium or lack of virulence. The species was isolated from infected lucerne and had closest shard identity with *C. americae-borealis* and *C. linicola* which have both been shown to infect lucerne (Damm *et al.*, 2014; Vasić *et al.*, 2014).

There was a correlation (r = 0.872) between coumestrol content and fungal damage score of the entire plants. As with Experiments 6-8, lucerne without lesions (score of 1) or slight fungal infection (score of 2) had low coumestrol, while lucerne with moderate to high (scores of 3-4) infection had elevated levels. However, there was no correlation (r = -0.01) between coumestrol content and the area of damage on stemphylium-infected leaves. There was a high variation in coumestrol content observed within elevated leaves, and this result means that it is probably easier to predict that plants with low fungal damage scores will have low coumestrol content and plants with high damage will have high coumestrol content, than it is to predict the actual level of coumestrol in high coumestrol lucerne.

5.5 Experiment 10 Water stress

5.5.1 Introduction

Lucerne is an important crop in dryland pasture systems due to its long taproot and high water use efficiency (Tonmukayakul *et al.*, 2009). This means that it is often exposed to conditions of moderate to severe water deficit in summer/autumn dry periods. These dry periods are typified by a lack of growth in grass pastures, leading to a reliance on lucerne, which due to a long tap root can reach water unavailable to many other pasture species. However, in prolonged dry periods, shallow soils, or situations where the soil has not fully recharged between droughts, lucerne can also become water stressed. These dry periods can continue into the mating period of sheep and wilting lucerne may be the only feed on offer to ewes.

Whether or not lucerne produces coumestrol as a stress response to water deficit has not been investigated. Roots of soybean plants grown in hydroponic systems were found to have increased coumestrol in response to water stress (Tripathi *et al.*, 2015). These findings were described in Section 2.6.4.

Experiment 10 tested whether lucerne produced coumestrol in response to water stress relative to well-watered control lucerne, and whether there was a difference between two cultivars 'Grasslands Kaituna' and 'Wairau'. These cultivars were chosen to represent a modern cultivar ('Kaituna' and an older cultivar 'Wairau') In addition, the previous experiments in this chapter (Experiments 6a, 6b & 7a) showed an increased level of coumestrol after a period of low rainfall followed by a heavy or prolonged rainfall event. This experiment also investigated whether recovery from water stress causes the coumestrol levels in the lucerne cultivars to increase, relative to well-watered and drought-stressed lucerne. The null hypotheses were that there would be no difference between water stressed and well-watered lucerne, no effect of recovery from water stress, and no difference between cultivars.

5.5.2 Methods

5.5.2.1 Experimental design

Experiment 10 took place in an 'Aluminex Glasshouse' at Lincoln University, Canterbury, New Zealand. The mean temperature in the glasshouse during the experiment was 18.5°C (range 15.1 – 24.9°C).

There were three water stress treatments: well-watered, water stressed and re-watered to field capacity following wilting, two cultivars of lucerne ('Grasslands Kaituna' and 'Wairau'), and two harvest dates. On the first harvest date, half of the well-watered and water-stressed plants were harvested. On the second harvested date, the other half of the well-watered and water-stressed, and the re-watered plants were harvested.

Pots (N = 30) were arranged in a randomised block design with three blocks giving an experimental design comparing well-watered and water stressed plants over time of 'cultivar x water regime x harvest date x block' (2 x 2 x 2 x 3, N = 24) and an experimental design comparing the re-watered plants at Harvest 2 with the well-watered and water stressed plants, of 'cultivar x treatment x Harvest 2 x block' (2 x 3 x 1 x 3, N = 18)

Lucerne seeds were sown at a rate of ten per pot in two litre pots of inoculated potting mix nine months prior to the onset of the experiment as described in Section 4.1.5, thinned to three plants per port then cut to 30 mm above soil level every six weeks.

At the beginning of Experiment 10, on 26 August 2016, plants were cut to 30 mm above soil level. The soil was brought to field capacity by saturating for two hours followed by four hours of drainage. Pots were weighed and this weight was deemed 'Field Capacity'. Every 48 hours, for two weeks, each pot was weighed and then watered back to the 'Field Capacity' weight.

After two weeks of re-growth, watering was ceased for the water-stressed and re-watered treatments, and maintained at field capacity for the well-watered plants. When the plants were wilted without recovery to turgidity overnight, randomly selected pots (n = 3) of the well-watered and wilted treatments were harvested from each block.

Following the first harvest the re-watered treatment was watered to and maintained at field capacity. Fifteen days later the well-watered, water stressed, and re-watered treatments were harvested.

Data collection

At each harvest, during the early afternoon, the weight of each pot was recorded and the middle leaflet from the first fully expanded leaf was taken from two stems per pot. The leaflets were weighed together to give fresh weight (FW) and submerged in water for 24 hours in the dark. The surfaces of the leaflets were dried with a paper towel and the turgid weight (TW) of the leaf was then measured. The leaflets were then oven dried and the dry weight (DW) recorded. These measurements were used in the following equation to give the relative water content (RWC) of the leaflets:

$$\mathrm{RWC}\ (\%) = \left[\frac{(\mathrm{FW}-\mathrm{DW})}{(\mathrm{TW}-\mathrm{DW})}\right] \mathrm{x}\ 100$$

Plants were scored for fungal and insect damage (Section 4.5) and harvested to 30 mm height. Fresh weight of the harvest material was recorded. Material was dried at 60°C and dry weight recorded. Material was then extracted using the methodology given in Section 4.6 and coumestrol was measured by HPLC with the methodology described in Section 4.7. The coumestrol rating scale (Table 4.4) was used to rate coumestrol content from negligible to extreme. Statistical analyses performed are described in Section 4.12.1.

5.5.3 Results

5.5.3.1 Water stressed vs. well-watered lucerne

Wilting occurred in all plants in the water stressed treatment on 21 September 2016, twelve days after watering had ceased (Figure 5.30). Two weeks later, on 6 October 2016, the above-ground material of the water stressed plants had senesced (Figure 5.31).

Average dry weights were 8.3 ± 0.50 g (n = 12) for well-watered and 4.5 ± 0.11 g (n = 12) for water stressed plants ($P < 0.001$), respectively. There was an interaction ($P < 0.001$) between harvest date and treatment on dry matter content (%DM). Well-watered plants did not have a change ($P = 0.077$) in %DM with an average %DM of 19 ± 0.9%. Water stressed plants had an increase ($P < 0.001$) in %DM from 38 ± 3.6% to 80 ± 1.6%. There was an interaction ($P < 0.001$) between treatment and harvest date on RWC. RWC did not change ($P = 0.449$) in well-watered plants between the two harvest dates (mean of 86 ± 1.2%, n = 12) and was higher ($P < 0.001$) than the RWC of water stressed plants. The RWC of the water stressed plants decreased ($P < 0.001$) from 49 ± 5.0% (n = 6) at harvest one to 25 ± 1.1% (n = 6) at harvest two.

Coumestrol content was low in all lucerne samples. Coumestrol was affected by the interaction ($P = 0.001$) between water stress and harvest date (Figure 5.32). On the first harvest date there was no difference ($P = 0.105$) in coumestrol content between well-watered and water stressed plants. Between the first and second harvests, coumestrol increased ($P = 0.005$) in water stressed plants (n = 6) from 1.3 ± 0.43 mg/kg DM on the first harvest date, where plants were wilted but green, to 3.0 ± 0.57 mg/kg DM on the second harvest date when foliage had senesced. On the other hand, coumestrol content of well-watered plants (n = 6) decreased ($P = 0.034$) from 2.2 ± 0.59 mg/kg DM to 0.95 ± 0.107 mg/kg DM between harvests. At the second harvest date senesced plants had a higher ($P = 0.002$) coumestrol content than well-watered plants.

Coumestrol content was also affected by the interaction ($P = 0.010$) between cultivar and water stress (Figure 5.32). Water stressed 'Wairau' had higher ($P = 0.006$) coumestrol (2.8 ± 0.71 mg/kg DM; n = 6) than well-watered 'Wairau' (1.1 ± 0.15 mg/kg DM; n = 6) and this was higher ($P = 0.037$) than in stressed 'Kaituna' (1.6 ± 0.44 mg/kg DM; n = 6) but not different ($P = 0.220$) to well-watered

‘Kaituna’ (2.1 ± 0.62 mg/kg DM; n = 6). There was no difference (P = 0.324) between well-watered and stressed ‘Kaituna’ (2.1 vs. 1.6 mg/kg DM). Light fungal symptoms (<1% of total leaf area affected) were observed in the well-watered lucerne at the second harvest, no fungal symptoms were observed at the first harvest or in the water-stressed plants.

Figure 5.30 Lucerne from Harvest 1 of Experiment 10 on 21 September. From left: ‘Grasslands Kaituna’ at field capacity; ‘Grasslands Kaituna’ at permanent wilting point. ‘Wairau’ at field capacity; ‘Wairau’ at permanent wilting point. Photo: R. L. Fields, 2016.

Figure 5.31 'Wairau' at field capacity (left) vs 'Wairau' at senescence (right) at Harvest 2 of Experiment 10 on 6 October 2016, following four weeks of no water. Photo: R. L. Fields, 2016.

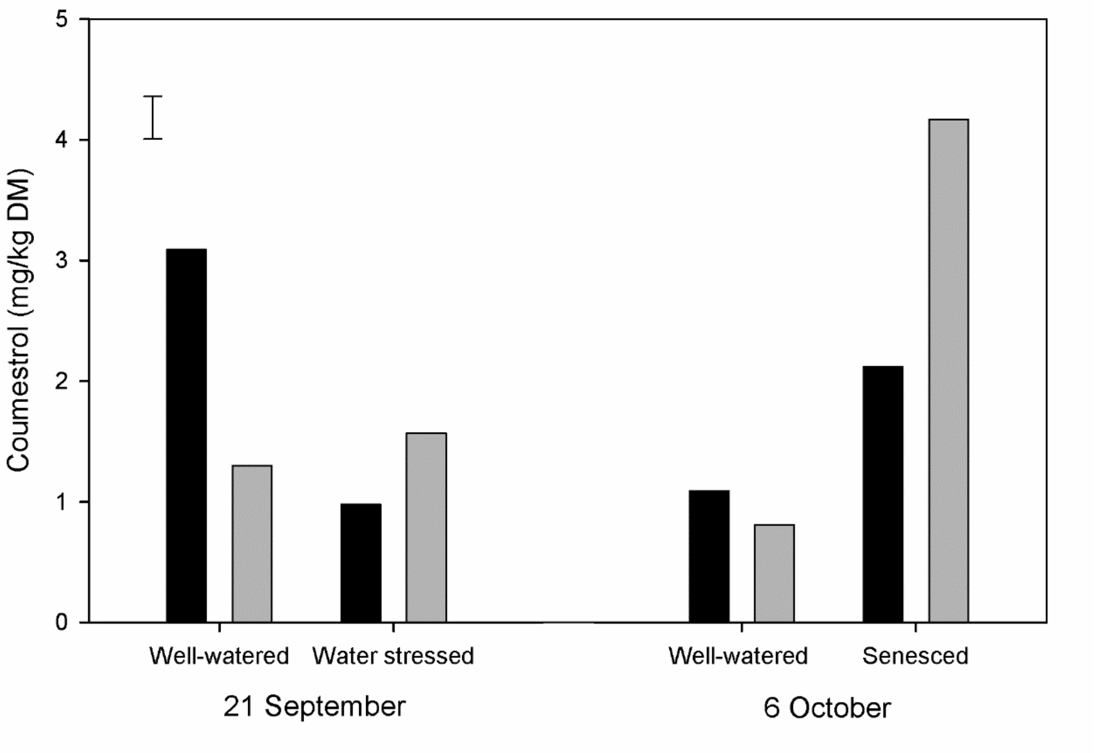

Figure 5.32 Mean coumestrol (mg/kg DM) of glasshouse grown 'Grasslands Kaituna' (■) and 'Wairau' (■) lucerne under well-watered and water stressed treatments on two dates. Error bar is the standard error of the mean for the interactions between cultivar x treatment and date x treatment.

5.5.3.2 Re-watered treatment

After water-stressed plants were re-watered at the first harvest, RWC increased (P < 0.001) from 50 ± 4.3% (n = 6) to 79 ± 1.1% two weeks later. This increased RWC was lower than the well-watered plants (84 ± 2.1%) but higher (P < 0.001) than the water stressed plants (24.7 ± 1.1%). Dry weight at the second harvest was 6.8 ± 2.25 g (n = 6) and was lower (P < 0.001) than in the well-watered plants (9.1 ± 0.39 g; n = 6). Dry weight of the water stressed plants was lowest (P < 0.001) at 4.4 ± 0.18 g (n = 6).

The re-watered plants had a low average coumestrol content of 1.1 ± 0.13 mg/kg DM (n = 6), which was not different (P = 0.712) from well-watered plants (0.95 ± 0.107 mg/kg DM), but was lower (P = 0.002) than for water stressed plants (2.9 ± 0.63 mg/kg DM). There was no difference (P = 0.513) between re-watered 'Kaituna' and re-watered 'Wairau'. Coumestrol content of re-watered plants had not increased (P = 0.691) relative to the content of the water stressed plants two weeks prior (1.3 ± 0.43 mg/kg DM).

Figure 5.33 'Wairau' at field capacity (left) vs. 'Wairau' re-watered after water stress induced wilt (right) at Harvest 2 on 6 October 2016. Photo: R. L. Fields, 2016.

5.5.4 Discussion

Coumestrol content was low (0.6 - 4.2 mg/kg DM) across all plants in Experiment 10, below the levels (ca. 25 mg/kg DM) reported (Smith *et al.*, 1979) to affect ewe reproductive performance. When the lucerne was wilted but green, coumestrol content was not heightened relative to the well-watered plants. Severe drought conditions that resulted in foliage death did coincide with slight coumestrol elevation from 1.3 to 3.0 mg/kg DM. The increase in coumestrol in senesced lucerne may have been due to non-symptomatic fungal infection of dying material or a late stage stress response of the senesced plant cells.

Lucerne that wilted and was then re-watered recovered, albeit with less dry weight than the well-watered plants. The re-watered lucerne did not have increased coumestrol relative to the well-watered plants. This suggests that in isolation the recovery of drought stressed plants after a rainfall event does not cause increased coumestrol levels.

Overall these results for Experiment 10 indicate that water stress alone is not an important cause of increased coumestrol levels in lucerne crops in terms of increased risk to ewes. Even in senesced 'Wairau' plants, coumestrol levels were below those which would be a risk to reproductive performance of ewes. It is contended that wilted lucerne which is only suffering from water stress, but otherwise disease and aphid free, can be used safely in sheep farming systems during the mating period.

5.6 Experiment 11 Aphids

5.6.1 Introduction

Research has indicated that aphid herbivory can cause increased coumestrol levels in lucerne (Loper, 1968; Kain and Biggs, 1980). However, it is unclear whether coumestrol levels increase as a direct response to the aphids, or due to the damage caused by aphids to leaves and stem that enables fungal infections to occur.

As described in Section 2.6.2, aphid-susceptible lucerne cultivars accumulated coumestrol in response to aphids, while a cultivar bred for aphid resistance produced less (Loper, 1968). The resistant cultivar described, ‘Nevada Syn T-P’ is not on the New Zealand market but it could be expected that today’s cultivars would have improved performance and pest and disease resistance relative to older cultivars. It was therefore important to investigate the coumestrol response to aphids in current commercial cultivars.

Experiment 11 investigated the effect of pea aphids on coumestrol levels in lucerne. In Experiment 11a, three cultivars were compared at a whole plant level, with aphids free to reproduce and move among each of the cultivars within large insect cages.

In Experiment 11b, the ‘Leaf Level’, four cultivars were compared using one pea aphid in a clip cage on a lucerne leaf over a five day period to determine whether this was sufficient pest pressure to produce a response. This was also used to determine whether or not secondary fungal infection occurred at the sites of aphid damage.

These experiments tested whether aphids caused coumestrol to increase relative to un-infested controls, and also tested whether there was a difference in the response of coumestrol content in lucerne to aphids between different cultivars. The null hypotheses were that aphids would not affect coumestrol content and no difference would occur between cultivars.

5.6.2 Methods

The following experiments were performed in an 'Eden 8 Single Door' glasshouse at Lincoln University.

5.6.2.1 Experiment 11a

In Experiment 11a, the effect of aphids on lucerne was investigated on whole plants. The average maximum and minimum temperatures during this period were 27 ± 0.6°C and 15 ± 0.2°C respectively. For this experiment three cultivars of lucerne were either exposed, or not exposed, to pea aphids with four blocks of cages in side-by-side +/- aphid pairs ('cultivar x treatment x block': 3 x 2 x 4, N = 24).

The three cultivars used were 'Force 4', 'Wairau', 'and 'Grasslands Kaituna'. Seeds from these cultivars were planted in two litre pots of potting mix and inoculant added, as described in Section 4.1.5. Lucerne plants were grown for nine months with cutting to approximately 4 cm height every six weeks before the experiment commenced. After three weeks of re-growth, plants were put into cages, with one pot of each cultivar per cage. To each cage, 0.25 ± 0.033 g of aphids or no aphids were added. Four weeks later, the number of aphids per stem was counted for each of 10 randomly selected stems at harvest.

Plants were scored for fungal and insect damage using the criteria described in Section 4.5. Lucerne was harvested to 30 mm height above the soil surface. Material was dried at 60°C and dry weight recorded. Material was then extracted using the methodology given in Section 4.6 and coumestrol was measured by HPLC with the methodology described in Section 4.7. The coumestrol rating scale (Table 4.4) was used to rate coumestrol content from negligible to extreme. Statistical analyses that were performed are described in Section 4.12.1.

5.6.2.2 Experiment 11b

In Experiment 11b, the effect of a single aphid on a leaf was investigated. The average maximum and minimum temperatures during this period were 23 ± 0.4°C and 13 ± 0.7°C respectively. Leaves on different plants of four cultivars of lucerne were either exposed, or not exposed, to pea aphids. Treatments were replicated three times with a duplicate pair of leaves within each block ('cultivar x treatment x block': 4 x 2 x 3, N = 24). Pots were arranged in a randomised complete block design.

The four cultivars used were 'Torlesse', 'Force 4', 'Wairau' and 'Grasslands Kaituna'. Seeds from these cultivars were sown in four litre pots of inoculated potting mix (Section 4.1.5). Lucerne was grown for six months prior to the experiment, with cutting to approximately 4 cm height every six weeks. On 19 August, after a further three weeks of re-growth, clip-cages were attached to the first fully unfolded leaf of two plants per pot (Figure 5.34), and either one or no pea aphids were added to each duplicate pair of cages.

After five days, the clip-cages and the aphids were removed and leaflets harvested and stored frozen. One leaflet per leaf was stained to measure fungal presence based on the protocol from Tuite (1969). Leaflets were put into a 1:1 glacial acetic acid:95% ethanol mix for 15 minutes until white. They were then soaked for 5 minutes in lactoglycerol cotton blue which stains cell damage and fungi. Leaflets were then rinsed with a 50:50 water:glycerol mix and mounted on a microscope slide.

Remaining leaflets were dried, weighed, ground in liquid nitrogen and extracted in 0.5 mL of methanol in a microcentrifuge tube (Section 4.6). Coumestrol was measured by HPLC (Section 4.7).

Figure 5.34 Aphid clip cages attached to individual leaves of lucerne in a greenhouse at Lincoln University, Canterbury, New Zealand (R. L. Fields, 2015).

5.6.2.3 Statistical analysis

Statistical analyses were performed for the two experiments separately and are described in Section 4.12.1.

5.6.3 Results

For Experiment 11a, the average number of aphids per stem at harvest on the treated pots was 4.6 ± 0.76 (n = 12). There was no difference (P = 0.214) in aphid numbers among the three cultivars. The mean lucerne dry matter production was 9.0 ± 0.24 g per pot. There was no difference in the dry matter production among the cultivars (P = 0.095) or between aphid and control treatments (P = 0.901). Coumestrol levels were low but higher (P < 0.001) in aphid treated lucerne (5.3 ± 0.65 mg/kg DM; n = 12), than the control lucerne (2.4 ± 0.39 mg/kg DM; n = 12). There was no effect of cultivar (P = 0.104) on the coumestrol content. There was no relationship (P = 0.874) between the average number of aphids per stem and the coumestrol content in the aphid-treated lucerne. Regression analyses showed no relationship between fungal (P = 0.689) or insect damage (P = 0.506) and coumestrol content.

For Experiment 11b coumestrol was at a negligible level (< 1 mg/kg DM). Lucerne leaves that contained an aphid within a clip cage had a higher (P = 0.016) coumestrol content than leaves without an aphid. Coumestrol content in aphid-affected leaves was 0.57 ± 0.132 mg/kg DM (n = 12) compared with 0.24 ± 0.029 mg/kg (n = 12) in control leaves. There was no effect (P = 0.218) of cultivar. Stained leaflets had no fungal damage on the leaves, including no secondary infection around the sites of aphid damage.

5.6.4 Discussion

Lucerne produced coumestrol when whole plants were subjected to aphid herbivory (Experiment 11a) thus not supporting the null hypothesis, however levels were low with 5.3 mg/kg DM in aphid-treated and 2.4 mg/kg DM in control lucerne. There was no difference in dry matter production between the treated and untreated pots indicating the aphid levels were below production damaging levels. Coumestrol was also produced in response to damage by a single pea aphid on a leaf for five days (Experiment 11b), however levels were negligible in both aphid-treated (0.57 mg/kg DM) and control leaves (0.24 mg/kg DM). The coumestrol response was not due to a secondary infection by fungi at the site of aphid damage as no hyphae were detected in stained leaves. The low aphid levels that can cause a coumestrol response are important for controlled glasshouse experiments, which could be confounded by very low infestation levels.

Coumestrol was low in all cultivars with no difference in the coumestrol response to pea aphids among the four cultivars at the leaf level (Experiment 11b) or among the three cultivars at the plant level (Experiment 11a). ‘Grasslands Kaituna’, ‘Force 4’ and ‘Torlesse' are considered to be modern cultivars, expected to be aphid-resistant, while ‘Wairau’ is regarded as a susceptible cultivar. A higher aphid population in the lucerne should be tested to further assess whether the cultivars respond differently to aphids and to determine whether lucerne cultivars can produce coumestrol levels at or above the 25 mg/kg DM threshold due to aphids. Kain and Biggs (1980) found that aphid numbers greater than 30 per stem could cause coumestrol to reach unsafe levels while in this experiment aphids were present at a rate of approximately five aphids per stem. However, in this experiment aphids were on lucerne for four weeks, and therefore it is not expected that higher populations will produce a significantly greater coumestrol response. Nonetheless, based on the previous research which has reported coumestrol to exceed 25 mg/kg DM due to aphids (Loper, 1968; Kain and Biggs, 1980), aphids should still be considered a risk factor to farm systems if they are present at production damaging levels during the mating period of ewes. Based on this a clear conclusion cannot be reached and more research is needed.

5.7 Conclusions

The objective of Chapter 5 (Objective 2) was to isolate factors which increase the risk of high coumestrol in lucerne and identify strategies to minimise coumestrol accumulation.

- The main trend that emerged from Experiments 6-8 was that the fungal infected lucerne samples had higher coumestrol content than uninfected samples. When lucerne was inoculated with stemphylium in Experiment 9 coumestrol content was significantly elevated. This effect of fungal infection was in agreement with past research described in the literature (Section 2.6).

- Despite fungi being the major cause, use of carbendazim fungicide (Experiment 8) to control coumestrol was ineffective and cannot be recommended. Instead, the focus should be on the regrowth after diseased material is removed as this tended to contain a low level of coumestrol.

- Aphids at below production damaging levels caused a small increase in coumestrol content but coumestrol remained at negligible to low levels (Experiment 11). Fungal disease was not present indicating aphids to be the sole cause. It is possible that greater degrees of infestation would cause a greater coumestrol response. However aphids were not observed in the field during the experiments of this thesis. Therefore, caution is advised for sheep farmers if high aphid levels are present during the mating period.

- Development stage (Experiment 6) and water stress (Experiment 10) did not affect coumestrol content and therefore lucerne which is wilting or flowering but is not infected can be safely grazed by sheep.

- Cultivars currently on the market (i.e. ‘modern’ cultivars) are capable of producing at-risk levels of coumestrol and therefore breeding effort focusing on low coumestrol levels and fungal resistance is still of importance (Experiment 7).

The next objective (Objective 3) was to develop a predictive tool to estimate coumestrol content of lucerne. The field data from Experiments 6-8 was used for this purpose in Chapter 6.

Chapter 6

Modelling of coumestrol in lucerne

6.1 Introduction

The objective of Chapter 6 was to develop a predictive tool to estimate coumestrol content of lucerne (Objective 3). Ideally this tool could be used to assess the risk of lucerne having heightened coumestrol.

Most of the agronomic factors individually tested in the studies described in Chapter 5 did not have an effect on the coumestrol content of lucerne. However in Experiments 6 to 8 (Sections 5.1 to 5.3) coumestrol content was variable throughout the growing season. Coumestrol ranged from negligible levels below 1 mg/kg DM to high levels above 100 mg/kg DM. This variability could not be explained by the crop development stage, cultivar, or level of water stress. Additionally, aphids caused coumestrol levels to rise, but remain relatively low, from 2.4 to 5.3 mg/kg DM in Experiment 11 (Section 5.6) but were not present during the high coumestrol periods of the field experiments (Experiments 6-8).

In most experiments of Chapter 5 there was one factor that appeared to be most related to the coumestrol content, and this was the fungal damage score. This was in line with many previous publications that have shown a strong link between fungal infection and coumestrol (Hanson *et al.*, 1965; Bickoff *et al.*, 1967; Sherwood *et al.*, 1970). However, the fungal damage score is subjective, based on the perspective of the assessor. Material with no or little fungal disease present was always low in coumestrol during the experiments of Chapter 5. However, it can be difficult to quantify the boundary between negligible and problematic levels of fungal infection. In practice, many of the leaf diseases in lucerne do not appear visually severe. They are often out of sight on the lower leaves, and the majority of an infected leaf surface remains green and healthy. These diseases do not seem to severely affect yield and in farm systems there is usually no application of fungicide for control.

Ideally, a model could be developed based on quantifiable environmental factors such as temperature, rainfall, humidity, and sunlight, rather than a visual score. This would enable prediction of high risk periods for coumestrol during the flushing and mating periods, which in New Zealand vary between regions from late February to early June. In this chapter, the coumestrol contents of lucerne sampled across three years to create a coumestrol prediction model were related to multiple quantifiable agronomic and environmental factors. This model was then tested against independent samples and a risk assessment created for different regions of New Zealand.

6.2 Methods

6.2.1 Predictors

Weather data were recorded at Broadfield EWS (NIWA) for Lincoln University sites (2.5 km away) for temperature (°C), rainfall (mm), relative humidity (%), and solar radiation (MJ/m^2). Broadfield was also used for the Ashley Dene site (12 km away), with the exception of rainfall which was measured at Burnham Sewage Plant (NIWA), 4 km away. Total sunlight hours were measured at the Christchurch Aero Station (Christchurch Airport), located 18 km from the Lincoln site and 25 km from the Ashley Dene site.

A range of calculations for each sample was made using the weather data from the entire regrowth period, and for the range of different fortnightly and monthly periods prior to sampling date:

- For rainfall, total rainfall (mm) was calculated.
- For temperature, the mean daily temperature (°C) was used.
- For relative humidity (RH) data, the number of days where RH was over (or equal to) 70, 80, 90 and 95% at 0900 h were calculated.
- For soil moisture deficit (SMD) the average deficit expressed as a percentage of the maximum potential SMD for the paddock was used (Section 4.3).
- For radiation, average solar radiation (MJ/m^2) and average daily sunshine hours were used.

In addition, the other variables related to the stand at time of sampling, i.e. month, year, age, height, dry matter production and development stage, were included in the model. Fungal damage score was not included in the prediction model due to its subjective nature, but the prediction of coumestrol content by the fungal damage score was compared with the prediction of coumestrol content by the prediction model.

6.2.2 Initial regression model

An initial regression model using all of the predictors described in Section 6.2.1 was performed to select, for further modelling, the variables that were not highly correlated with each other, while also providing a representative range. Correlated predictors were selected by their relative *F*-statistics in the regression model, and by the R^2 value of coumestrol content versus individual predictors, and the poorer performing predictors removed.

6.2.3 Model 1: Estimation of coumestrol content of a lucerne crop

A best subset regression model was used to account for the effect of each of these predictors on coumestrol content. This model could be used to identify whether a stand was likely to have high or low coumestrol level. For the model, lucerne crops with a regrowth age of two or three weeks were excluded as a large proportion of the leafy material was residual from before the mowing, and therefore older than two weeks, particularly in the fortnightly cutting regime plots of Experiment 6a (Section 5.1.3). Lucerne sampled in winter and during the first regrowth of the spring was also excluded, as many of the environmental factors experienced during the 'growing period' of these samples occurred while the plants were dormant.

The adjusted R^2 was reported, to take into account the number of terms in the model. The two best one, two and three-predictor models were compared.

6.2.4 Model 2: Conditions behind increased coumestrol

To determine the conditions that cause an increase in coumestrol content in lucerne, the distribution of the data used in Model 1 is skewed, as once lucerne has high coumestrol it remained in the plant until the material was cut or grazed. It was therefore important to look at the period prior to a coumestrol spike, and disregard the weather conditions following a coumestrol spike. This analysis excluded lucerne that had already accumulated coumestrol and had since plateaued. As described in Section 6.2.3, lucerne less than three weeks old and lucerne during winter and the first regrowth of spring was also excluded.

A best subset regression model was used to analyse the relationship between predictors and coumestrol content. In addition to the predictors used in Model 1 for the entire prior growing period, the sum of rainfall and days above 95% RH data were broken into fortnightly intervals of 0-2, 2-4 and 4-6 weeks prior to each sampling event. The three best one, two and three predictor models were compared.

6.2.5 Testing the prediction model

Independent coumestrol data were sourced from the literature (Hanson *et al.*, 1965; Purves *et al.*, 1981) and additional coumestrol measurements reported in Chapters 7 and 8. Climate data for the regrowth period of the lucerne was sourced from nearby weather stations. The independent coumestrol and climate data are summarised in Table C.4.

6.2.5.1 Logan, Utah

Coumestrol data for untreated with fungicide 'Lahontan' and 'Ranger' lucerne grown in Logan, Utah during summer 1961 were obtained from Hanson *et al.* (1965). The first harvest occurred at 'pre-bud' on 30 June 1961. The final harvest date of 'full bloom plus 25 days' occurred on 21 August 1961. This enabled the determination of harvest dates for 'full bloom plus 10 days' (6 August 1961) and 'full bloom' (27 July 1961). A regrowth start date of 16 June 1961, two weeks prior to the first harvest was assumed. Due to a lack of rainfall in June 1961, the model would not change the coumestrol prediction if the regrowth start date was set at one week or three weeks before the first harvest.

Climate data were sourced from the Utah Climate Center (2017) for the period between 16 June and 21 August 1961. Rainfall data for Logan, Utah was from Logan Radio KVNU (41° 43' 43.416" N, 111° 56' 10.334" W). During this period there was 25.5 mm rainfall. The RH data were sourced from Hill Air Force Base (Hill AFB; 41° 7' 2.604" N, 111° 57' 11.308" W) approximately 80 km away from Logan. When RH was not provided it was calculated (Wanielista *et al.*, 1997) from the dew point (T_D) and temperature (T) data at 0900 h:

$$RH\% = 100\left(\frac{112 - 0.1 * T + TD}{112 + 0.9 * T}\right)^8$$

There were no dates with humidity above 95% at 0900 h at Hill AFB. Recent humidity data (1999-2016) from Logan-Cache Airport (41° 46' 58.678" N, 111° 51' 17.302" W) and Hill AFB were correlated (r = 0.819) for the June to August months. Furthermore, during this 17 year interval the average RH% at Logan-Cache was 45.7 ± 0.39%, and RH at Logan-Cache was above 95% on only one occasion. It is therefore extremely unlikely that a RH over 95% occurred during the experimental period.

6.2.5.2 Brookings, South Dakota

Coumestrol data for untreated with fungicide 'Ranger' lucerne grown in Brookings, South Dakota during summer 1963 was obtained from Hanson *et al.* (1965). Lucerne was reported to be 15 cm high on 29 July 1963. Therefore a regrowth start date two weeks earlier on 15 July 1963 was assumed. There was little effect of setting the start date three weeks prior as there was only 4 mm rainfall in this extra week, and no days over 95% RH. Lucerne was reported to be at 'full bloom plus nine days' on 12 September 1963. Thus, coumestrol content of 'full bloom' lucerne was from 3 September and 'full bloom plus 10 days' was on 13 September.

Rainfall data for Brookings, South Dakota were sourced from Brookings 2 NE (44° 19' 0.012" N, 96° 46' 0.012" W) between 15 July and 13 September, 1963 (Utah Climate Center, 2017). The humidity data for Brookings, South Dakota were sourced from Sioux Falls (43° 33' 41.695" N, 96° 43' 57.004" W) approximately 90 km away from Brookings. Recent humidity data for the July, August and September months between 1 July 2006 and 30 September 2016 were compared from Sioux Falls and Brookings 2 NE. The correlation was not as strong (r = 0.676) as for Utah and therefore RH may be under or overestimated in the prediction model.

6.2.5.3 Lincoln, New Zealand

Data from Lincoln were obtained from Purves (1981) for lucerne sampled between 12 February and 17 April 1980. Coumestrol levels of untreated for fungicide lucerne from cut and uncut plots were estimated from Fig.4 of Purves (1981). Meteorological data were from Lincoln weather station (43° 38' 50.654"S, 172° 27' 49.182"E; CliFlo, NIWA).

Coumestrol data from the lucerne used in Chapter 8 which had regrowth between 7 March and 22 April 2016 were also used. Meteorological data for this site were from Broadfield EWS.

6.2.5.4 Oamaru, New Zealand

Lucerne samples from Oamaru, North Otago were included in the prediction model, with meteorological data from Windsor station 22 km away (CliFlo, NIWA).

6.2.5.5 Statistical analysis

The climate data were used to predict coumestrol content of the independent lucerne samples using the equation produced in Model 1:

$$\begin{aligned} Coumestrol\ (mg/kg\ DM) &\\ &= -13.8\ + 4.80 * \text{'}Days\ in\ growing\ period\ above\ 95\%\ RH\text{'} + 0.252 \\ &\quad * \text{'}Sum\ of\ rainfall\ in\ growing\ period\text{'} \end{aligned}$$

A regression line between predicted and measured values was produced to determine whether the model was a valid predictor of the coumestrol contents of the independent data.

In addition, linear regression with categories for the independent and original data sets (Genstat 16.1) was used to determine whether the parameter estimates for each data set, derived from coumestrol content, rainfall and RH data, differed. The independent data were then added to the main data set to provide a final prediction equation.

6.2.6 Decision tree

Recursive partitioning (R (version 3.3.3) with package ‘rpart’) was used to create a tree model to classify coumestrol content by the rainfall and days over 95% RH variables. The tree was pruned to five terminal nodes. RandomForest was used (R package: ‘randomForest’) to create 1000 different trees from random samples with replacement from the original data and random samples without replacement from predictors.

6.2.7 Regional risk assessment

The prediction model was used to create a risk assessment model using 30-40 years of climate data (0900 h relative humidity and rainfall) from four regions of New Zealand, Lauder, Central Otago; Lincoln, Canterbury; Blenheim, Marlborough; and Napier, East Coast. Climate data were retrieved from CliFlo, NIWA.

In Central Otago, climate data from Lauder Pel (1983-1985) and EWS (1987- 2016) were used (45° 2' 31.165"S, 169° 41' 18.441"E).

In Canterbury, climate data from the Lincoln weather station (43° 38' 52.8''S, 172° 27' 46.799''E) from 1977 to 1987 were used, followed by climate data from Broadfield Edl (1988-1999) and Ews (2000-2016) (43° 37' 34.392'' S, 172° 28' 13.44" E).

In Marlborough, climate data from Blenheim Aero (1976-1986) and Blenheim Aero AWS (1992-2013) were used (41° 31' 14.445"S, 173° 52' 28.208"E).

In Napier, climate data from Napier Aero (1976-1987) and Napier Aero AWS (1992-2012) were used (39° 27' 56.568"S, 176° 51' 21.274"E). This was followed by data from Napier’s Nelson Park (39° 29' 53.599" S, 176° 54' 44.109" E) from 2013 to 2015.

If there were ≥10 days of data missing in a year it was considered incomplete and the year was not included in prediction.

The climate data were used with the prediction model that had been updated with the independent data to predict the coumestrol content, based on the rainfall and days over 95% RH in the six weeks prior to a date of interest, i.e. the mating date, for each year of climate data and for each region. The equation was:

$$\begin{aligned}&Coumestrol\ content\ (mg/kg\ DM)\\&\qquad = -10.9 + 3.77 * \text{‘}Days\ in\ growing\ period\ above\ 95\%\ RH\text{’} + 0.386\\&\qquad * \text{‘}Sum\ of\ rainfall\ in\ growing\ period\text{’}\end{aligned}$$

Mating dates at fortnightly intervals were used, covering the range of lambing dates in New Zealand. The distributions of lambing dates are from the 2014 Sheep and Beef Survey (Beef and Lamb New Zealand), summarised in Table 6.1. Mating dates were based on an estimated gestation length of 147 days. The prediction was based on six weeks of lucerne regrowth, as this is the recommendation for rotationally grazed lucerne in autumn (Moot *et al.*, 2003b)

Once coumestrol content was predicted for each year a cumulative distribution graph was created to compare the four regions and to compare the mating dates. The difference in median lucerne coumestrol content of each region and for the mating dates was tested with Mood's Median Test. The frequency with which the predicted coumestrol content was over the threshold value of 25 mg/kg DM was also calculated.

Table 6.1 Distribution of mating and lambing dates in New Zealand and regions of interest in New Zealand (East Coast, Canterbury and Marlborough, and Otago and Southland). Lambing data are from 2014 Sheep and Beef Survey (Beef and Lamb New Zealand). Mating dates are based on an estimated gestation length of 147 days.

Mating Date	Lambing Date	Lambing date distribution (Cumulative percent)			
		New Zealand	East Coast	Canterbury/ Marlborough	Otago/Southland
23 February	20 July	1.2	2.8	2	0
9 March	3 August	3.7	6.1	4.6	0
23 March	17 August	11.1	16.4	13.6	0
6 April	31 August	25.8	37.1	29.6	0.8
20 April	14 September	49.2	69.4	51.2	14.7
4 May	28 September	74.3	89.1	69.6	54.2
18 May	12 October	90.4	96.8	84.9	84
1 June	26 October	97	99.2	94.9	94.9
15 June	9 November	99.2	99.9	98.5	98.6

6.3 Results

6.3.1 Selection of predictors

An initial regression analysis of the coumestrol contents of 289 lucerne samples with all predictor terms included had high multicollinearity, with variance inflation factor (VIF) of range 12 to 4500. The main predictors that were correlated were the different RH thresholds, and the weather data that considered the whole and partial growth period of lucerne regrowth prior to sampling. For example, rainfall in the month prior to sampling was highly correlated with rainfall for the whole growing period prior to sampling. The weather data (rainfall and RH) for the whole growth period were better predictors of coumestrol, with higher *F* values and R^2 values, than the partial growth period data and therefore only whole growth period weather terms were included.

Removal of the partial growth period terms decreased the VIF slightly (Table C.1), with a new range of 4.8 to 335, which was still high. The high VIF was due to collinearity between the RH predictors. For example, the relationship between days over 90% RH and days over 95% RH had a correlation coefficient (r) of 0.933, and the correlation between days over 80% RH and days over 95% RH was r = 0.780. The days above 95% RH during the growing period was the strongest predictor of coumestrol ($F_{1,288} = 27.5$; $P < 0.001$; $R^2 = 0.733$), compare with, for example, days with humidity above 80% ($F_{1,288}$

= 1.1; P = 0.306; R^2 = 0.461) or 90% ($F_{1,288}$ = 5.1; P = 0.025; R^2 = 0.658). Therefore the strongest predictor ‘Days above 95% RH’ was retained while the days above 70, 80%, 85% and 90% RH predictors were removed.

Height ($F_{1,288}$ = 5.45; P = 0.020; R^2= < 0.001) was removed due to correlation (r = 0.905) with, but worse prediction of coumestrol content than, yield ($F_{1,288}$ = 10.3; P = 0.001; R^2 = 0.025). Average daily sunshine hours and sunlight radiation (MJ/m^2) were correlated (r = 0.885) and were both weak ($F_{1,288}$ = 1.48; P = 0.225; R^2 = 0.308 vs. $F_{1,288}$ = 3.81; P = 0.05; R^2 = 0.209) predictors of coumestrol content. The average daily sunshine hours factor was retained due to the higher R^2 value.

Removal of the aforementioned terms resulted in all predictors having a VIF between 3 and 8.5; with levels above 5 indicating that some multicollinearity was still present (Table 6.2). The remaining variables included the strongest predictors of coumestrol and also represented a range of the agronomic and environmental factors present. Days above 95% RH and the sum of rainfall over the growing period were collinear (r = 0.842), but were both strong predictors of coumestrol content. Therefore they were both considered potential candidates for further modelling and not removed.

Table 6.2 The refined list of coefficients used to predict coumestrol content of a lucerne crop, following removal of obsolete, collinear parameters. *F*-statistic, *P*-values and VIF are from the ANOVA of a regression model which contained all terms. The R^2 of the regression (0.766) is the adjusted R^2 and includes all terms. The individual predictor R^2 values are based on the regression between each predictor and coumestrol content. Coefficients are ranked by F-value.

Term	DF	*F*- statistic	*P*-value	VIF	R-Sq
Regression	10	95.60	<0.001		(0.766)
Days above 95% RH	1	85.96	< 0.001	5.70	0.736
Sum of rainfall (mm)	1	21.44	< 0.001	8.24	0.653
Development stage	1	12.99	< 0.001	6.10	0.009
Soil moisture deficit (%)	1	11.03	0.001	6.53	0.115
Average temperature (°C)	1	8.20	0.004	7.28	0.057
Days in growing period	1	6.54	0.011	5.72	0.129
Average daily sunshine hours	1	2.48	0.117	5.83	0.308
Dry matter yield (t DM/ha)	1	1.51	0.221	3.57	0.025
Month	1	0.24	0.623	5.54	0.002
Date of harvest	1	0.10	0.751	3.13	0.085
Error	278				
Total	288				

6.3.2 Model 1

The first model was created to estimate the coumestrol content of a lucerne crop. Best subsets regression analysis (Table C.2) predicted a moderate relationship ($R^2_{adj.}$ = 0.735; RMSD = 23.6 mg/kg DM) between coumestrol content and the number of days above 95% RH over the growing period that a lucerne stand had been exposed to (Figure 6.1). A weaker relationship ($R^2_{adj.}$ = 0.652; RMSD = 27.6 mg/kg DM) was predicted between coumestrol content and the sum of rainfall. Inclusion of the sum of rainfall over the growing period, which was correlated with days above 95% humidity in the model, increased the adjusted $R^2_{adj.}$ to 0.754, with RMSD of 22.7 mg/kg DM. The VIF for these coefficients was 3.3. Inclusion of additional predictors did not increase the R^2 sufficiently (<1%) to warrant their inclusion.

Rainfall and days above 95% humidity were stronger predictors of coumestrol than the lucerne fungal damage score (R^2 = 0.388; Figure 6.2). The variation in coumestrol content increased with fungal damage score. At a damage score of 1, the mean was 10.2 mg/kg DM and standard deviation was 8.5 mg/kg DM. At a score of 3 the mean was 35.0 mg/kg DM and standard deviation was 30.2 mg/kg DM, and at a score of 5, the mean was 82.0 mg/kg DM and standard deviation was 55.0 mg/kg DM. The skewed distribution of coumestrol content shows the fungal damage score to not be a reliable predictor.

Based on the results from the best subsets regression analysis, the following equations were produced. Equation 6.1 describes the relationship ($R^2_{adj.}$ = 0.735; $R^2_{pred.}$ = 0.730) between coumestrol content and the number of days above 95% RH during the growing period. Equation 6.2 describes the relationship ($R^2_{adj.}$ = 0.652 $R^2_{pred.}$ = 0.646) between coumestrol content and the sum of rainfall over the growing period. Finally, Equation 6.3 describes the relationship between coumestrol content and both days above 95% and the sum of rainfall during the growing period. These equations could be applied to determine whether a particular lucerne crop can be expected to produce a high or low coumestrol content.

Equation 6.1 The relationship ($R^2_{adj.}$ = 0.735; $R^2_{pred.}$ = 0.730) between coumestrol content of lucerne and the number of days above 95% RH during the growing period. Standard errors of the coefficients are ± 2.35 and ± 0.227, respectively.

$$Coumestrol\ (mg/kg\ DM) = -13.3 + 6.51 * \text{‘}Days\ in\ growing\ period\ above\ 95\%\ RH\text{’}$$

Equation 6.2 The relationship ($R^2_{adj.}$ = 0.652; $R^2_{pred.}$ = 0.646) between coumestrol content of lucerne and the sum of rainfall over the growing period. The standard errors of the coefficients are ± 2.51 and ± 0.0327, respectively.

$$Coumestrol\ (mg/kg\ DM)\ = -2.58\ + 0.745 * \text{‘}Sum\ of\ rainfall\ in\ growing\ period\text{’}$$

Equation 6.3 The relationship ($R^2_{adj.}$ = 0.754; $R^2_{pred.}$ = 0.749) between coumestrol content of lucerne and days above 95% RH and the sum of rainfall during the growing period. Standard errors of the coefficients are ± 2.26, ± 0.398 and ± 0.0489, respectively.

$$\begin{aligned} &Coumestrol\ (mg/kg\ DM) \\ &\quad = -13.8\ + 4.80 * \text{‘}Days\ in\ growing\ period\ above\ 95\%\ RH\text{’} + 0.252 \\ &\quad * \text{‘}Sum\ of\ rainfall\ in\ growing\ period\text{’} \end{aligned}$$

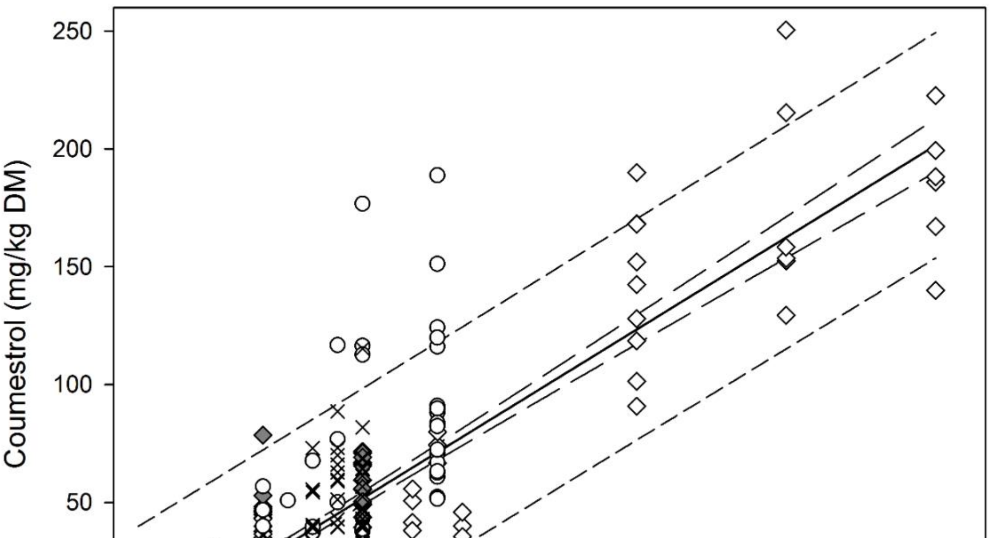

Figure 6.1 Coumestrol content (mg/kg DM) of lucerne against days above 95% relative humidity that the lucerne crop had been exposed to. The regression line (R^2_{adj} = 0.735) is described by the equation: y = = 6.51x −13.3 (± 0.227; ± 2.35), with 95% confidence (— — —) and prediction (- - -) intervals. Data from Lincoln in 2014 (◇), 2015 (○) and 2016 (X), and from Ashley Dene in 2015 (◆).

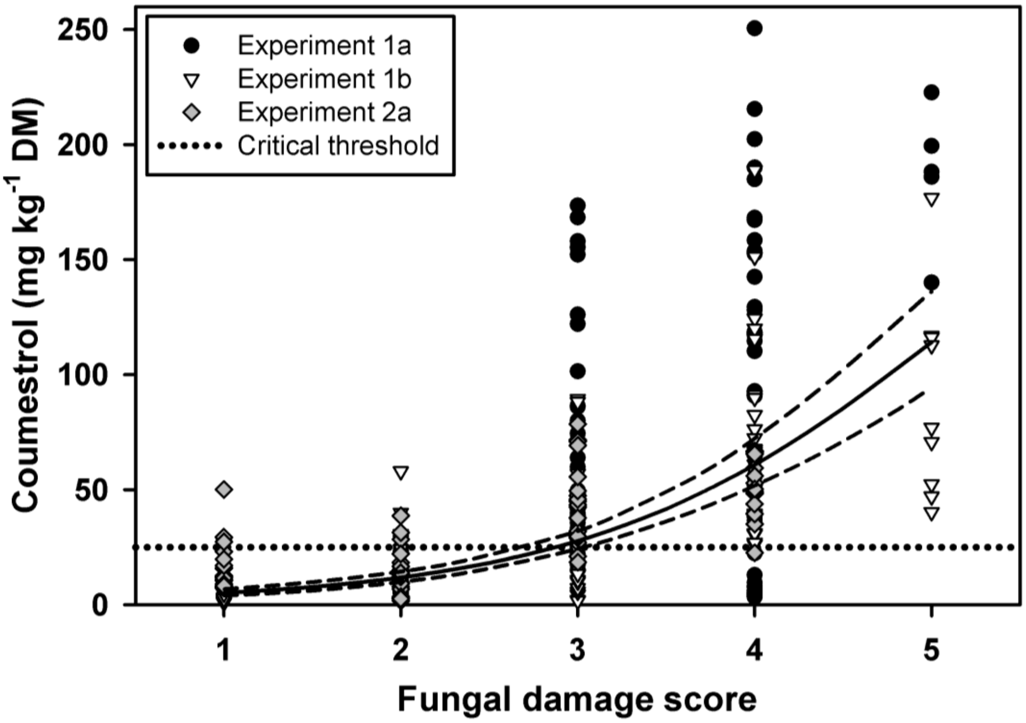

Figure 6.2 Coumestrol content (mg kg^{-1} DM) against fungal damage score in field samples alfalfa sampled in Experiments 1a, 1b, and 2a. The relationship (P < 0.001; R2 = 0.388) is described by the equation: y = $10^{0.45 + 0.31x}$. Dashed lines around the regression line are the 95% confidence interval. Dotted line is the critical level (25 mg kg^{-1} DM) above which alfalfa is reported to be a risk for ewe reproductive performance.

6.3.3 Model 2

6.3.3.1 Entire growing period prior to sampling

In the second model (Model 2) the entire growing period and also the 14 days before sampling were examined, but samples taken after a coumestrol spike were not included. Best subsets regression analysis (Table C.3) predicted that the strongest single variable relationship ($R^2_{adj.}$ = 0.600) was between coumestrol content and the sum of rainfall over the growing period (Equation 6.4). There was also a relationship ($R^2_{adj.}$ = 0.577) between coumestrol content and the days over 95% RH during the growing period (Equation 6.5). A regression model of both the sum of rainfall and days over 95% RH during the growing period gave an $R^2_{adj.}$ of 0.685 (Equation 6.6).

Equation 6.4 The relationship ($R^2_{adj.}$ = 0.600) between coumestrol content in lucerne and the sum of rainfall during the growing period. The standard errors of the coefficients are ± 2.75 and ± 0.0547, respectively.

$$Coumestrol\ (mg/kg\ DM) = -9.018 + 0.994 * Sum\ of\ rainfall\ in\ growing\ period'$$

Equation 6.5 The relationship ($R^2_{adj.}$ = 0.577) between coumestrol content in lucerne and days above 95% RH during the growing period. The standard errors of the coefficients are ±3.03 and ± 0.374, respectively.

$$Coumestrol\ (mg/kg\ DM) = -14.1 + 6.66 * 'Days\ in\ growing\ period\ above\ 95\%\ RH'$$

Equation 6.6 The relationship ($R^2_{adj.}$ = 0.685) between coumestrol content in lucerne and days above 95% RH and the sum of rainfall during the growing period. The standard errors of the coefficients are ± 2.69, ± 0.462 and ± 0.0683, respectively.

$$Coumestrol\ (mg/kg\ DM) = -18.8 + 3.82 * 'Days\ in\ growing\ period\ above\ 95\%\ RH' + 0.591 * Sum\ of\ rainfall\ in\ growing\ period'$$

Addition of a third term, predicted as the average soil moisture deficit over the growing period, increased the $R^2_{adj.}$ to 70.7 but is probably meaningless biologically.

6.3.3.2 Two weeks prior to sampling

The relationship between coumestrol and the sum of rainfall during the 14 days prior to sampling gave an $R^2_{adj.}$ of 0.501. This was the third strongest single term prediction, after those for the entire growing period.

The second strongest ($R^2_{adj.}$ = 0.637) two term prediction was with the days above 95% RH over the entire growing period, and the sum of rainfall in the 14 days before harvest, while the third strongest ($R^2_{adj.}$ = 0.631) used the sum of rainfall over the entire growing period and days over 95% RH in the prior fortnight.

A three term model of days over 95% RH during the growing period, sum of rainfall during the growing period, and days above 95% RH in the prior 14 days, increased $R^2_{adj.}$ to 0.691 (compared with $R^2_{adj.}$ of 0.685 in Equation 6.6 which did not use the predictor of days above 95% RH in the prior 14 days).

6.3.3.3 Two to four weeks and four to six weeks prior to sampling

Best subsets regression analysis did not predict a model using the rainfall or humidity data in the period two to four weeks or the period four to six weeks prior to sampling.

6.3.4 Prediction model validation

When the prediction model (Model 1) using the RH and rainfall coefficients was used for independent data there was a linear relationship ($R^2 = 0.664$) between the reported and predicted coumestrol levels (Figure 6.3) and a RMSD of 24.9 mg/kg DM, similar to the RMSD of 22.7 mg/kg DM in the original data set. The slope of the independent reported coumestrol data against the predicted coumestrol content was 1.4 compared to the identity line (slope of 1). Most of the variation was from to the results reported in Purves *et al.* (1981) which had higher than predicted coumestrol.

Rainfall and RH of the independent data set were used to produce an independent regression ($R^2_{adj.} = 0.648$; $R^2_{pred.} = 0.633$) relationship. The R^2 was lower than in the original model, indicating a poorer fit. However, there was no significant difference in the coefficients and y intercept of the independent equation compared to the original equation. The y intercept of the original model was -13.8 ± 2.26 which was not different (P = 0.678) from the independent y intercept of -10.9 ± 7.26. The original coefficient of days above 95% RH was 4.8 ± 0.398 compared (P = 0.055) to 7.3 ± 1.34 in the independent model. The original coefficient of rainfall was 0.252 ± 0.0489 compared (P = 0.231) to the independent value of 0.346 ± 0.0661.

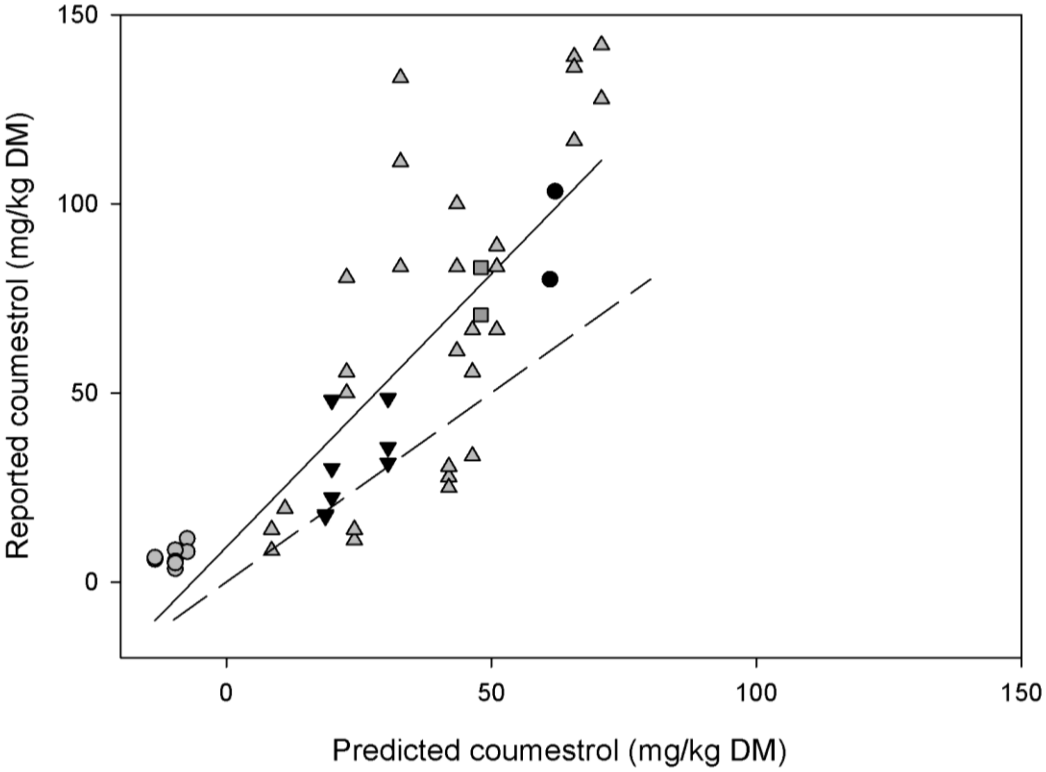

Figure 6.3 Independent reported coumestrol content of lucerne (mg/kg DM) against predicted coumestrol content (mg/kg DM) from Model 1. Solid line is the relationship ($R^2 = 0.664$) between reported and predicted coumestrol as described by: $y = 1.44x + 9.26$. Dashed line is the identity line. Data sourced from Logan, Utah 1961 (●); Brookings, South Dakota 1963 (●); Lincoln, Canterbury, NZ 1980 (▲); Oamaru, North Otago, NZ 2015 (■); and Lincoln, NZ 2016 (▼).

The data set was added to the initial prediction model producing a model ($R^2_{adj} = 0.717$; $R^2_{pred} = 0.712$; RMSD = 24.1; VIF = 2.0), as described in Equation 6.7.

Equation 6.7 The relationship ($R^2_{adj.} = 0.717$; $R^2_{pred} = 0.712$) between coumestrol content of lucerne and days above 95% RH and the sum of rainfall during the growing period. The standard errors of the coefficients are ± 2.22, ± 0.310 and ± 0.0354, respectively.

$$\begin{aligned} &Coumestrol\ content\ (mg/kg\ DM) \\ &\qquad = -10.9 + 3.77 * \text{‘}Days\ in\ growing\ period\ above\ 95\%\ RH\text{’} + 0.386 \\ &\qquad * \text{‘}Sum\ of\ rainfall\ in\ growing\ period\text{’} \end{aligned}$$

This new equation requires validation with a further independent data set, however a graph is provided (Figure 6.4) of predicted against actual coumestrol content using the data set which created the model.

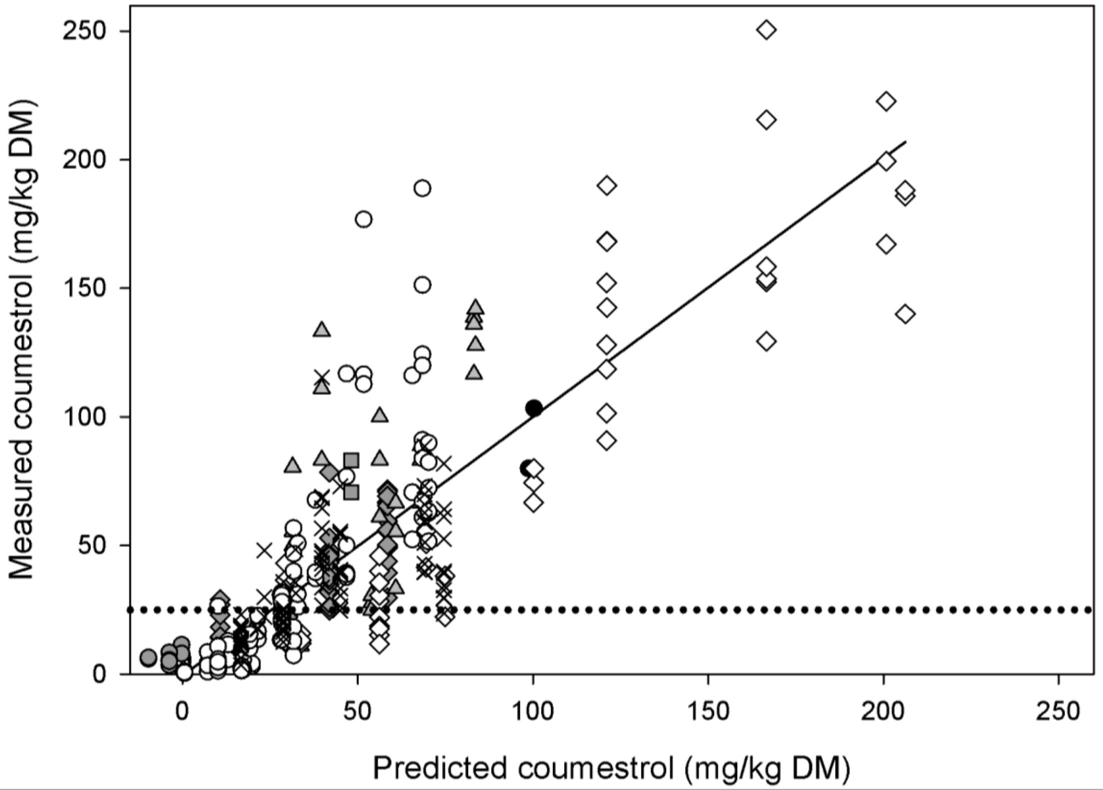

Figure 6.4 Measured coumestrol content (mg/kg DM) against (r = 0.847) predicted coumestrol content of lucerne from the second iteration of Model 1. Dotted line is the level (25 mg/kg DM) above which lucerne is a risk for ewe reproductive performance. Data sourced from Logan, Utah 1961 (●); Brookings, South Dakota 1963(●); Lincoln in 1980 (▲), 2014 (◇), 2015 (○) and 2016 (X); Oamaru, North Otago, NZ 2015 (■) and Ashley Dene in 2015 (◆).

6.3.5 Decision Tree

A decision tree (Figure 6.5) was created in R using the complete data set (n = 353) and the variables identified as predictors in Model 1 (total rainfall during the growing period and days above 95% RH.

The decision tree showed that when rainfall was greater than 131 mm during the regrowth period, average coumestrol would likely be high (139 ± 8.1 mg/kg DM, n = 34). This was further split by the days above 95% RH. When there were fewer than 17 days above 95% RH mean coumestrol was 100 ± 7.9 mg/kg DM (n = 13), when there were more than 17 days above 95% RH, mean coumestrol was 163 ± 8.7 mg/kg DM (n = 21). In addition, a moderately high coumestrol content could be expected if rainfall was between 61 and 131 mm, with a mean of 58.4 ± 2.98 mg/kg DM (n = 116) in this model. However, when rainfall was below 61 mm coumestrol content was low to moderate depending on the number of days above 95% RH (Figure 6.7). When there were fewer than 5.5 days above 95% RH the mean coumestrol content was 7.91 ± 0.749 mg/kg DM (n = 115), when there were more than 5.5 days above 95% RH, the mean coumestrol content was 31.7 ± 1.93 mg/kg DM (n = 88).

The coumestrol data and coumestrol means for these rainfall thresholds are shown in Figure 6.6. The data for the days above 95% RH thresholds, when rainfall was less than 61 mm are shown in Figure 6.7. RandomForest with 1000 trees showed 83.9% of variance explained by the model. Permutation increased MSE of rainfall by 70.3% and increased MSE of days above 95% RH by 61.8%.

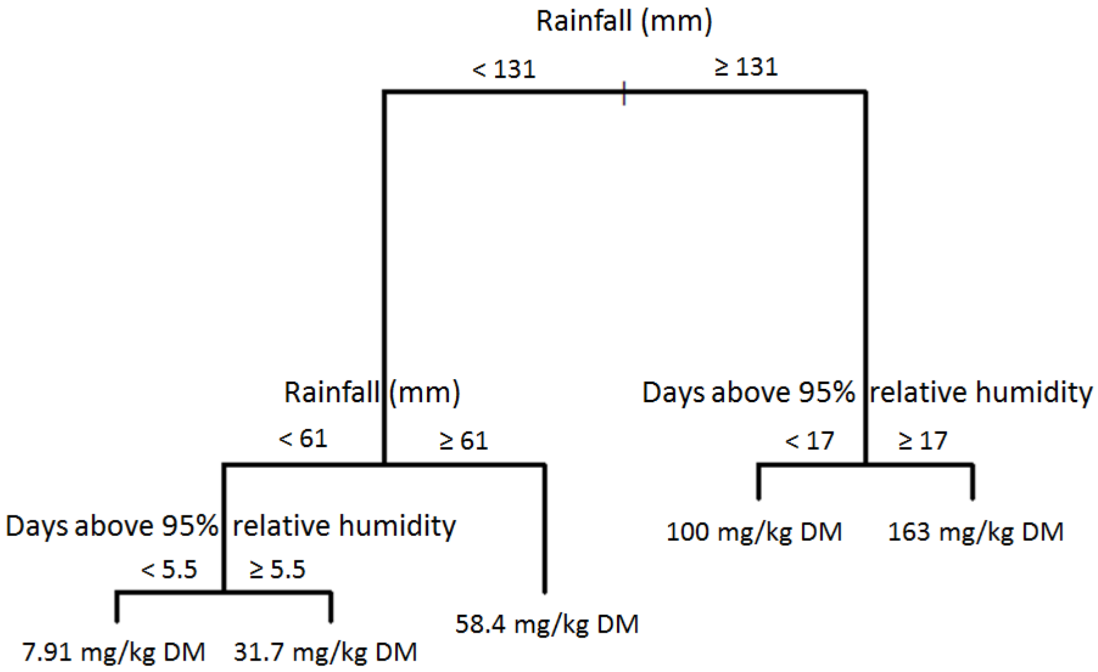

Figure 6.5 A decision tree to estimate mean coumestrol content based on rainfall (mm) and days above 95% relative humidity.

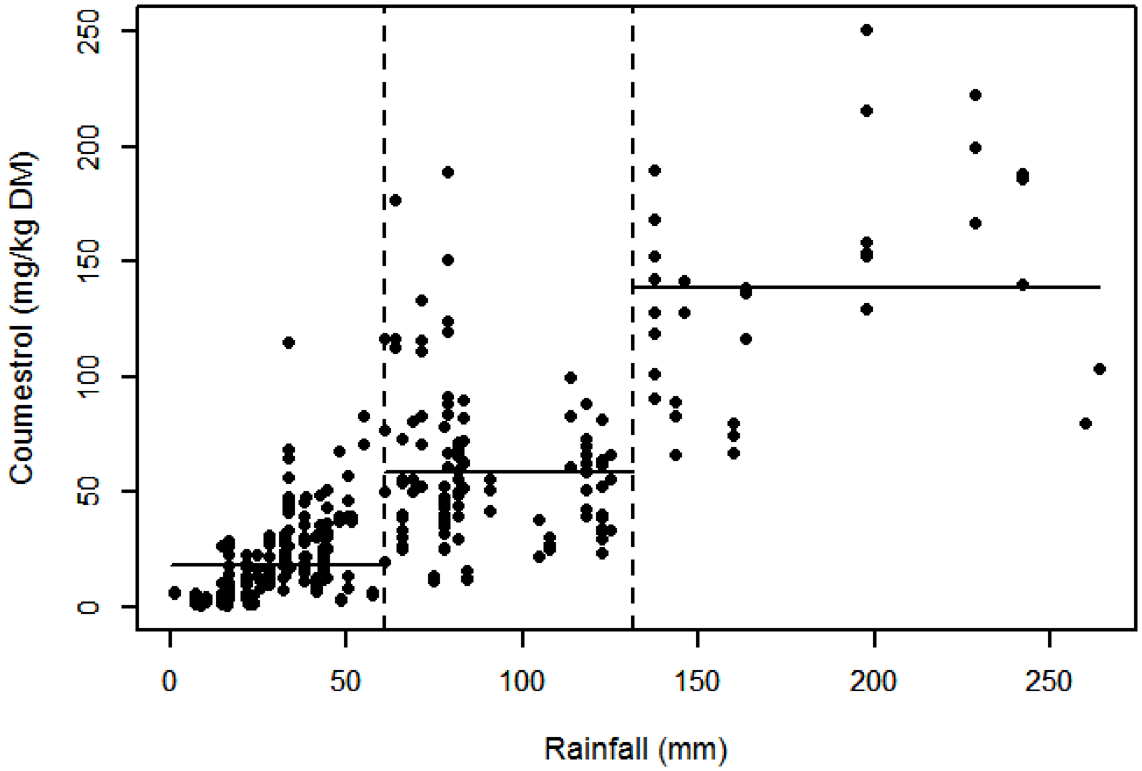

Figure 6.6 Coumestrol against the sum of rainfall (mm) during the regrowth period of lucerne samples. From left: horizontal lines are mean coumestrol for rainfall less than 61 mm, rainfall between 61 and 131 mm, and rainfall above 131 mm.

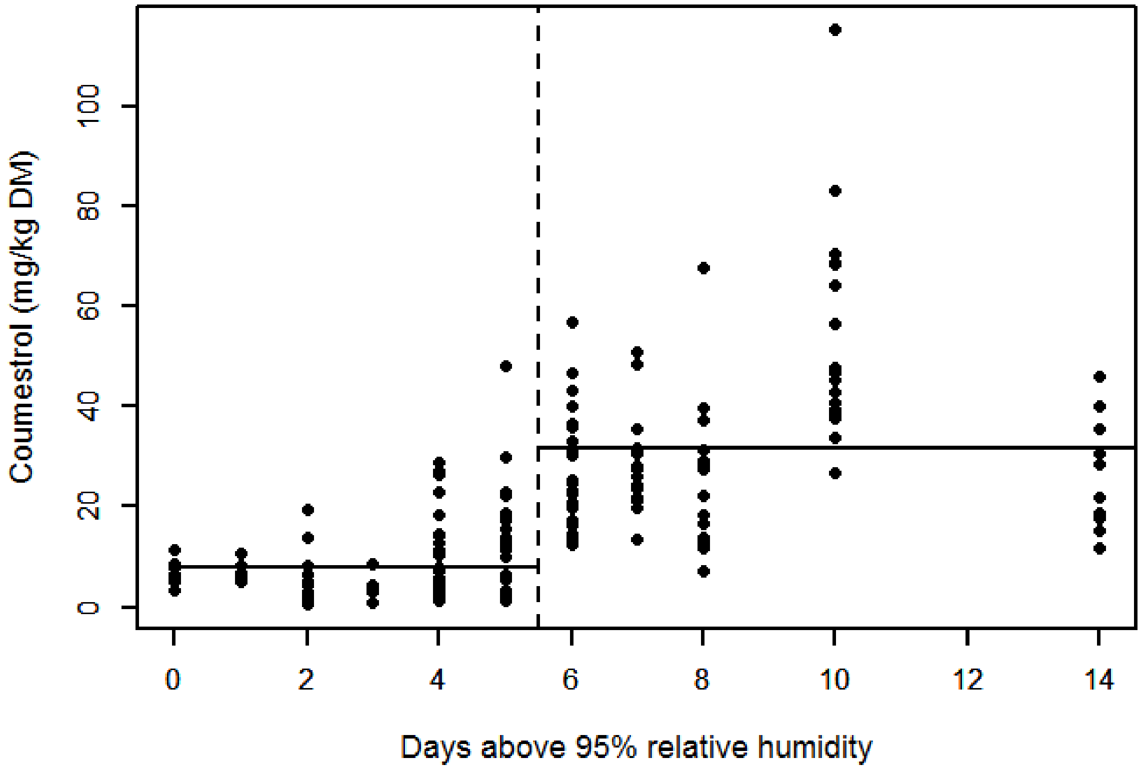

Figure 6.7 Coumestrol against days above 95% relative humidity for lucerne samples with rainfall less than 61 mm during the regrowth period. From left: horizontal lines are mean coumestrol for less than 5.5 days over 95% RH and greater than 5.5 days.

6.3.6 Regional risk assessment

The predicted coumestrol on nine dates, each 14 days apart, after the standard six weeks regrowth or a shortened four week old regrowth, were assessed. The four locations were Lauder, Lincoln, Blenheim and Napier.

Mood's median test showed an effect ($P < 0.001$) of regrowth age on predicted coumestrol content. Across nine dates and four locations, lucerne regrown for six weeks had a moderate median coumestrol content of 30.8 mg/kg DM. This was higher than in lucerne grown for four weeks which had a moderately low median value of 19.5 mg/kg DM.

Mood's median test showed effects ($P < 0.001$) of date on the median predicted coumestrol content of six week old lucerne regrowth, and of four week old regrowth, at Lauder, Lincoln and Blenheim. There was no effect of date on median predicted coumestrol content of four week ($P = 0.574$) or six week ($P = 0.935$) lucerne regrowth at Napier. The overall median predicted coumestrol content for six week old lucerne in Napier was moderate (34 mg/kg DM) and for four week old lucerne was moderately low (19.4 mg/kg DM).

Regression analysis of median predicted coumestrol in six week old (Figure 6.8) and four week old (Figure 6.9) lucerne regrowth over successive mating dates showed median coumestrol content of six week old lucerne regrowth at Lincoln increased ($P < 0.001$; $R^2 = 0.899$) at a rate of 0.34 ± 0.043 mg/kg DM/d (4.8 mg/kg DM per fortnight) from a moderate 25 mg/kg DM on 23 February to a moderately high 67 mg/kg DM on 15 June. Median coumestrol content of four week old lucerne regrowth at Lincoln increased ($P < 0.001$; $R^2 = 0.942$) at a rate of 0.27 ± 0.025 mg/kg DM/d (3.8 mg/kg DM per fortnight) from a moderately low 12 mg/kg DM on 23 February to a moderate 42 mg/kg DM on 15 June. The rate was not different ($P = 0.163$) from the six week old regrowth,

Median predicted coumestrol content of six week old lucerne at Lauder increased ($P < 0.001$; $R^2 = 0.874$) at a rate of 0.25 ± 0.035 mg/kg DM/d (3.5 mg/kg DM per fortnight) from a moderately low 22 mg/kg DM on 23 February to a moderate 47 mg/kg DM on 15 June. Median coumestrol content of four week old lucerne at Lauder increased ($P = 0.008$; $R^2 = 0.659$) at a rate of 0.13 ± 0.034 mg/kg DM/d (1.8 mg/kg DM per fortnight) from a moderately low 11 mg/kg DM on 23 February to a moderately low 24 mg/kg DM on 15 June. This rate was similar ($P = 0.082$) to six week old regrowth,

Median predicted coumestrol content of six week old lucerne at Blenheim increased ($P < 0.001$; $R^2 = 0.938$) at a rate of 0.18 ± 0.018 mg/kg DM/d (2.5 mg/kg DM per fortnight) from a moderately low 16 mg/kg DM on 23 February to a moderate 34 mg/kg DM on 15 June. Median coumestrol content of four week old lucerne at Blenheim increased ($P < 0.001$; $R^2 = 0.911$) at a rate of 0.17 ± 0.020 mg/kg

DM/d (2.4 mg/kg DM per fortnight) from a low 3 mg/kg DM on 23 February to a moderate 26 mg/kg DM on 15 June. This rate was not different (P = 0.761) from the six week old regrowth.

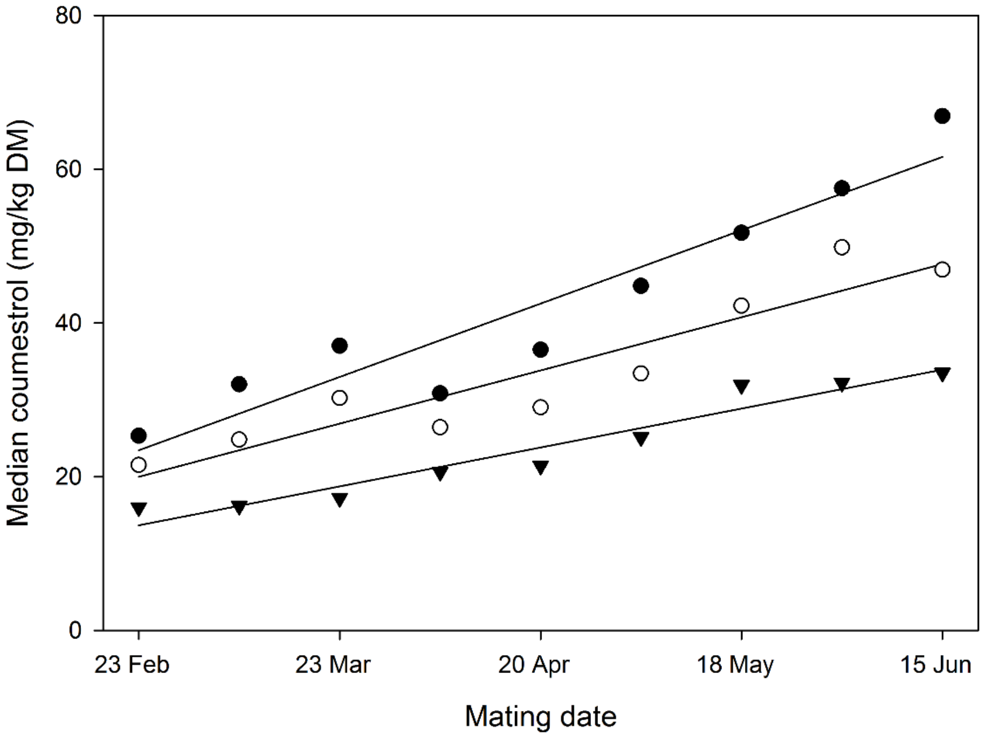

Figure 6.8 Predicted median coumestrol content (mg/kg DM) of six week old lucerne regrowth over successive mating dates for Lincoln (●), Lauder (○), and Blenheim (▼).

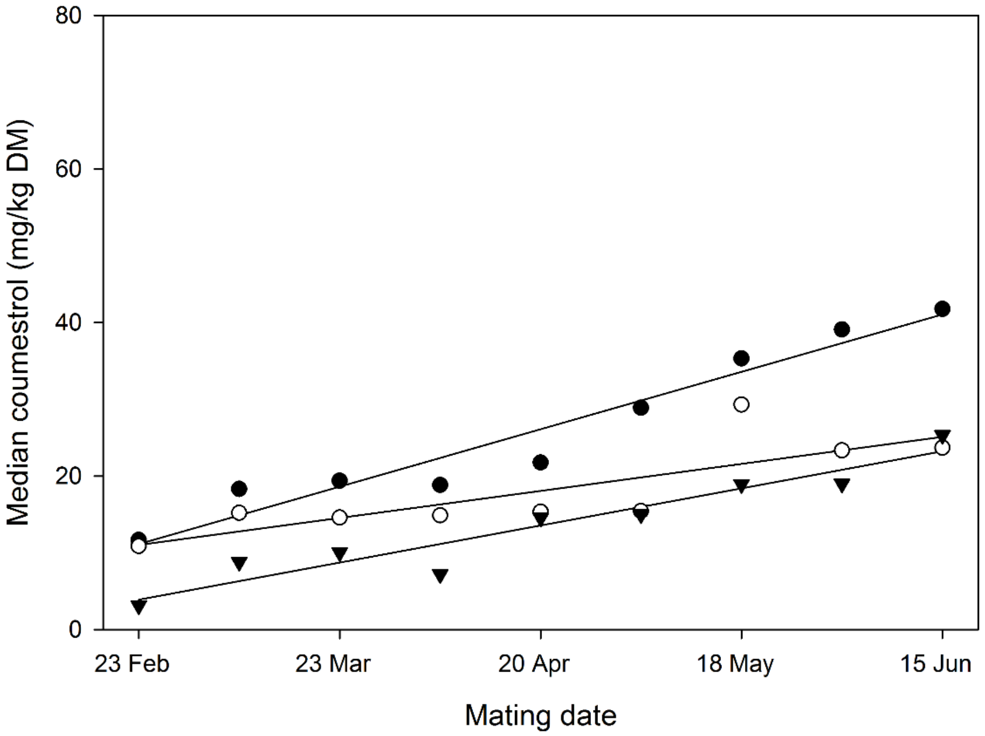

Figure 6.9 Predicted median coumestrol content (mg/kg DM) of four week old lucerne regrowth over successive mating dates for Lincoln (●), Lauder (○), and Blenheim (▼).

The cumulative distribution functions for five of the nine dates averaged across the four regions are shown for six weeks regrowth in Figure 6.10, and for the shorter four weeks regrowth in Figure 6.11. Across the four regions, prior to the mating period on 23 February (1.8% of ewes mated nation-wide by this date), the probability of a coumestrol content above the 25 mg/kg DM threshold was 45% in six week regrowth and 25% in four week regrowth. On 23 March (11.1% mated cumulatively), the probability was 59% in six week regrowth and 30% in four week regrowth. On 20 April (50% mated), the probability was 65% in six week regrowth and 36% in four week regrowth. On 18 May (90% mated) the probability was 74% in six week regrowth and 50% in four week regrowth. At the end of the mating period on 15 June (99% mated) the probability was 79% in six week regrowth and 58% in four week regrowth.

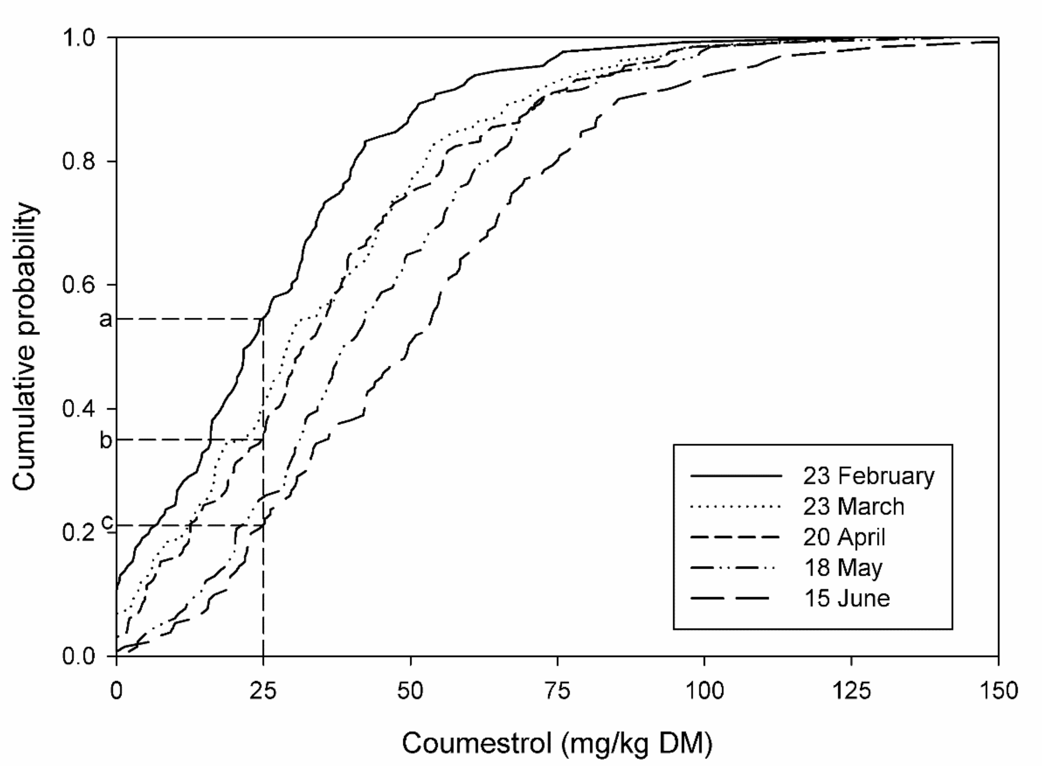

Figure 6.10 Cumulative distribution functions for predicted coumestrol content (mg/kg DM) in six week lucerne regrowth for five mating dates based on a simulation analysis using long-term meteorological data from four areas of New Zealand (Lauder, Lincoln, Blenheim & Napier). Drop-lines are the probability of lucerne not exceeding 25 mg coumestrol/kg DM on 23 February (a: 0.55), 20 April (b: 0.35), 15 June (c: 0.21).

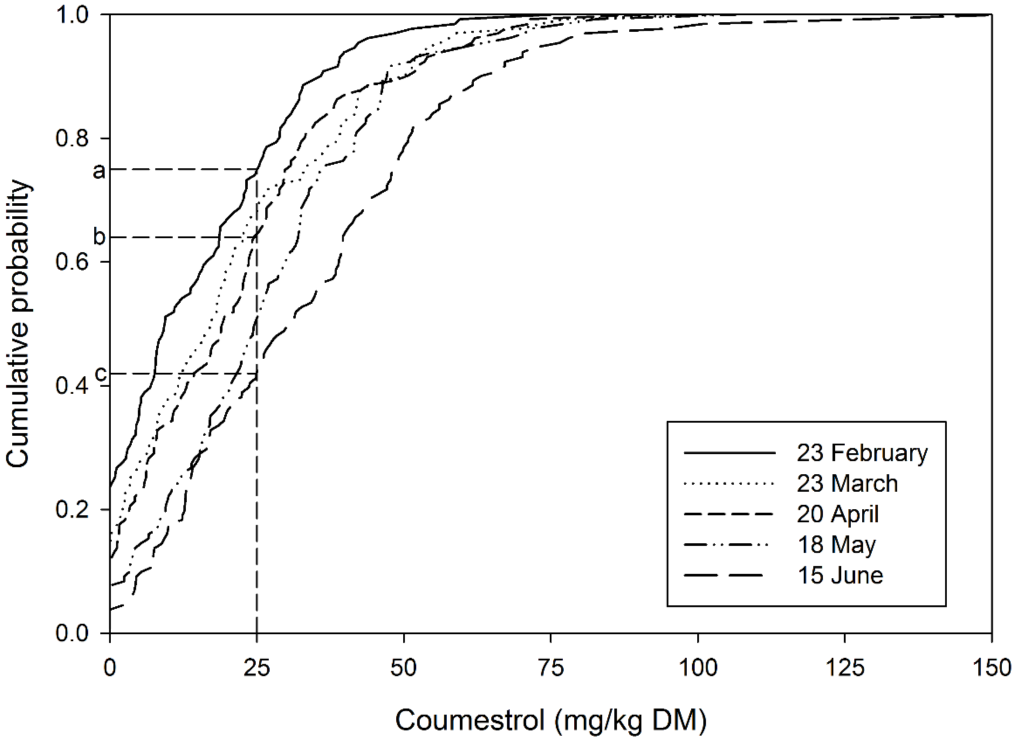

Figure 6.11 Cumulative distribution functions for predicted coumestrol content (mg/kg DM) in four week lucerne regrowth for five mating dates based on a simulation analysis using long-term meteorological data from four areas of New Zealand (Lauder, Lincoln, Blenheim & Napier). Drop-lines are the probability of lucerne not exceeding 25 mg coumestrol/kg DM on 23 February (a: 0.75), 20 April (b: 0.64), 15 June (c: 0.42).

Mood's median test showed an effect of location (P < 0.001) on the median predicted coumestrol content in six week old lucerne regrowth across the nine dates. Six week old lucerne at Blenheim had the lowest (P < 0.05) median level at a moderately low 24 mg/kg DM. Six week old lucerne at Lincoln had the highest (P < 0.05) predicted median coumestrol level (a moderate 41 mg/kg DM). Six week old lucerne at Lauder and Napier had moderate median values in between, with 33 and 34 mg/kg DM, respectively.

Four week old lucerne showed a similar effect (P < 0.001) of location. Four week old lucerne at Blenheim had the lowest (P < 0.05) median value with a moderately low 13 mg/kg DM. Four week old lucerne at Lincoln had the highest (P < 0.05) coumestrol at a moderate 25 mg/kg DM and Napier and Lauder had moderately low values in between of 19 and 20 mg/kg DM respectively.

The cumulative distribution functions for four regions in New Zealand using data taken over nine dates between 23 February and 15 June are shown for the standard six weeks regrowth in Figure 6.12 and the shortened four weeks regrowth in Figure 6.11. The likelihood of a coumestrol content above or equal to 25 mg/kg DM in four and six week old lucerne, respectively, were 29% and 50% in Blenheim, 36% and 66% in Lauder and Napier, and 50% and 75% in Lincoln.

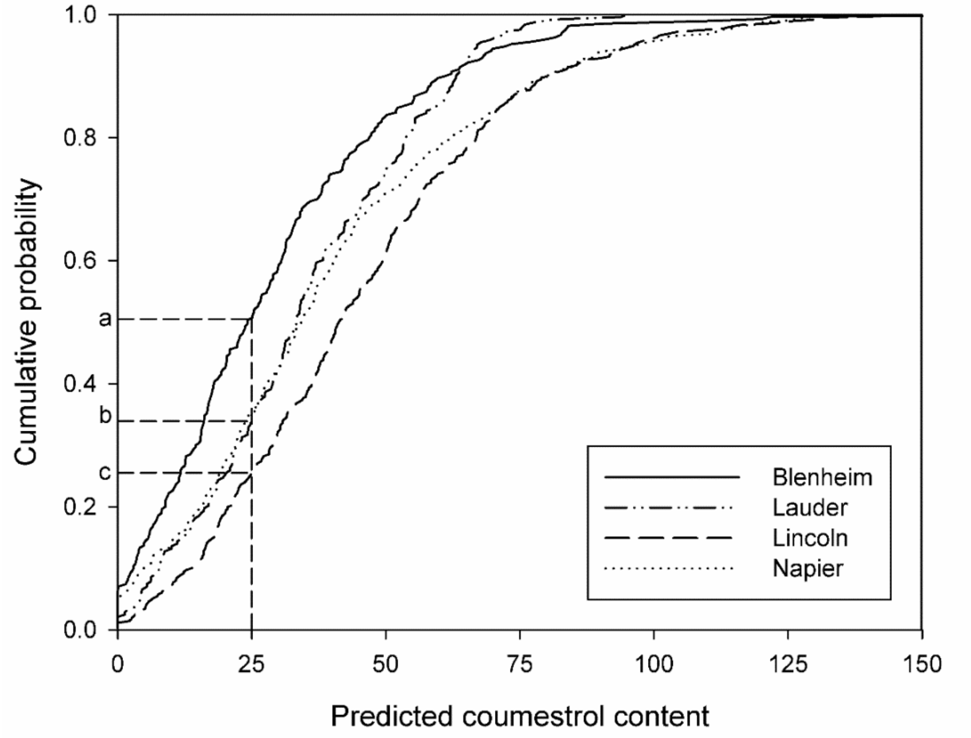

Figure 6.12 Cumulative distribution functions for predicted coumestrol content (mg/kg DM) in six week lucerne regrowth at four sites (Blenheim, Lauder, Lincoln and Napier) based on a simulation analysis using long-term meteorological data of nine dates between February and June. Drop-lines are the probability of lucerne not exceeding 25 mg coumestrol/kg DM in Blenheim (a: 0.51), Lauder and Napier (b: 0.34), and Lincoln (c: 0.26).

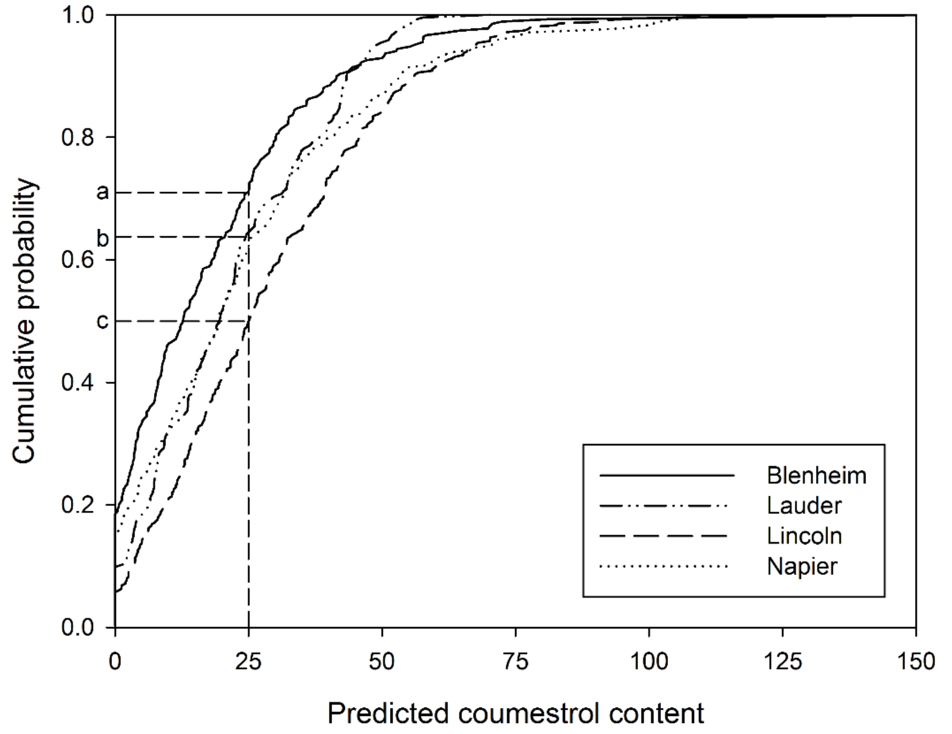

Figure 6.13 Cumulative distribution functions for predicted coumestrol content (mg/kg DM) in four week lucerne regrowth at four sites (Blenheim, Lauder, Lincoln and Napier) based on a simulation analysis using long-term meteorological data of nine dates between February and June. Drop-lines are the probability of lucerne not exceeding 25 mg coumestrol/kg DM in Blenheim (a: 0.71), Lauder and Napier (b: 0.64), and Lincoln (c: 0.50).

6.3.6.1 Otago/Southland

In Otago/Southland (Figure 6.14), 94% of ewes lamb between 1 September and 26 October. Peak lambing is in the fortnight ending on 28 September and the fortnight ending 12 October with 40% and 30% of ewes lambing in this period, respectively. In Lauder, likelihood of coumestrol content over 25 mg/kg DM in both four and six week old lucerne increased ($P < 0.001$; $R^2 = 0.898$) through the corresponding mating period. There was no difference ($P = 0.101$) in the rate of increase over time (0.43 ± 0.048%/d) but the constant, and therefore overall risk, was lower ($P = 0.005$) in the four week old lucerne than in six week old crops, with average predicted risks of 36 ± 5.0% in the four week regrowth compared ($P = 0.003$) with 65 ± 6.9% in the six week regrowth ($n = 9$). Risks for four and six week regrowth respectively were 30% and 52% on 6 April (31 August lambing), 37% and 70% on 4 May (28 September lambing), 60% and 85% on 18 May (12 October lambing), and 48% and 95% on 1 June (26 October lambing).

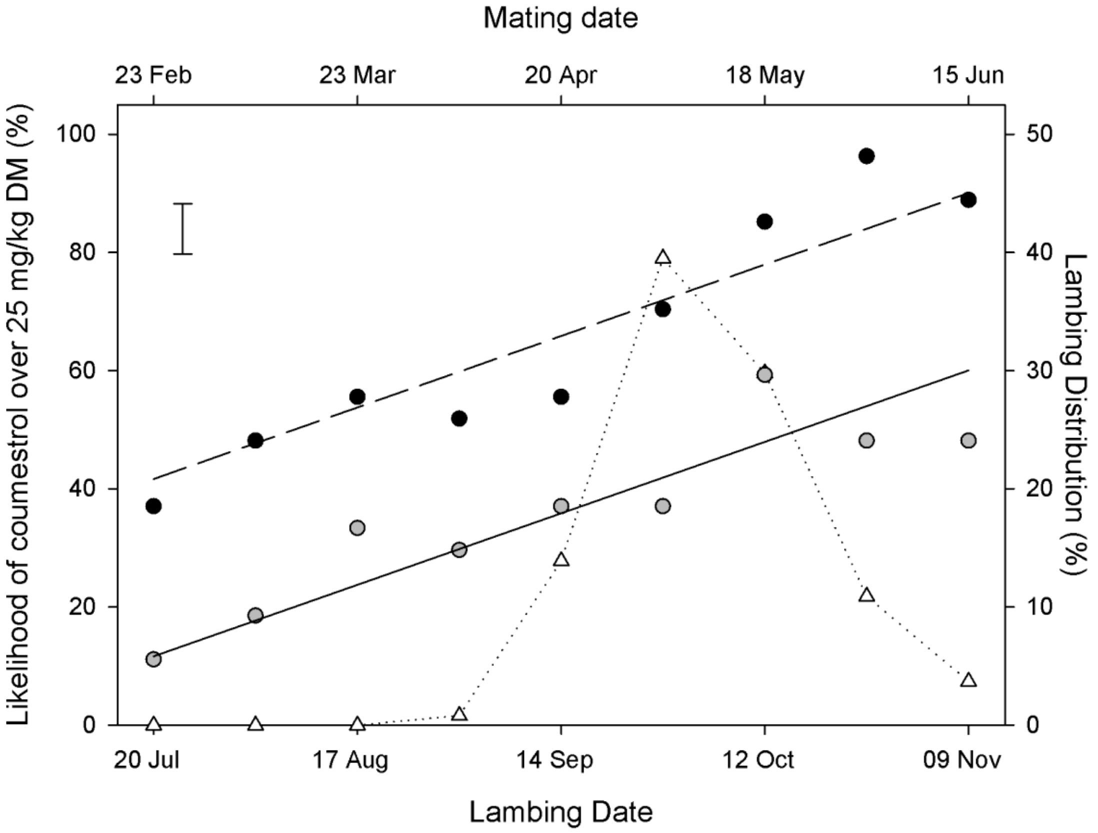

Figure 6.14 The distribution of lambing date (△) in Otago/Southland and the likelihood (%) of coumestrol content over 25 mg/kg DM in four week old (●) and six week old (●) lucerne on corresponding mating dates in Lauder based on a simulation analysis using long-term meteorological data. Regression lines ($R^2 = 0.898$) are parallel ($P = 0.101$) lines of slope '0.43 ± 0.048%/d' for the relationship between date and risk for the four (dashed line) and six (solid line) week old lucerne. Error bar is the standard error of the mean for regrowth age.

6.3.6.2 Canterbury/Marlborough

In Canterbury/Marlborough (Figures 6.15 & 6.16), the lambing date has a wider distribution, with 90% from 4 August to 26 October. Highest rates of lambing were the fortnights ending 31 August (16% of lambing), 14 September (22%), 28 September (18%) and 12 October (15%).

In Lincoln (Figure 6.15), the likelihood of coumestrol content over 25 mg/kg DM in both four and six week old lucerne increased ($P < 0.001$; $R^2 = 0.922$) through the corresponding mating period. There was no difference ($P = 0.482$) in the rate of increase over time (0.45 ± 0.040%/d) but the constant, and therefore overall risk, was lower ($P < 0.001$) in the four week old lucerne than in six week old crops, with average predicted risks of 50 ± 6.4% in the four week regrowth compared ($P = 0.011$) with 75 ± 5.6% in the six week regrowth ($n = 9$). Risks for four and six week regrowth respectively, were 33% and 59% on 9 March (3 August lambing), 41% and 74% on 20 April (14 September lambing), 69% and 87% on 18 May (12 October lambing), and 77% and 100% on 1 June (26 October lambing).

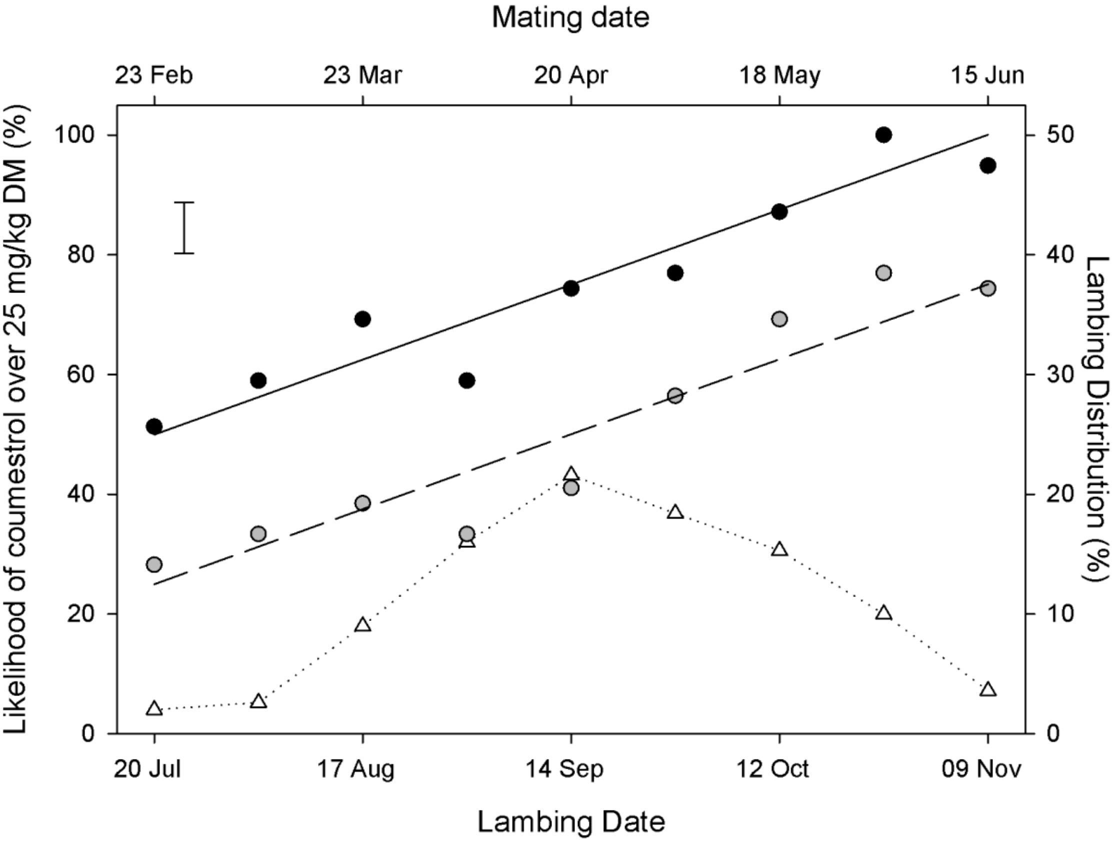

Figure 6.15 The distribution (%) of lambing date (△) in Canterbury/Marlborough and the likelihood (%) of coumestrol content over 25 mg/kg DM in four week old (●) and six week old (●) lucerne on corresponding mating dates in Lincoln based on a simulation analysis using long-term meteorological data. Regression lines ($R^2 = 0.922$) are parallel ($P = 0.482$) lines of slope '0.45 ± 0.040%/d' for the relationship between date and risk for the four (dashed line) and six (solid line) week old lucerne. Error bar is the standard error of the mean for regrowth age.

In Blenheim (Figure 6.16), the likelihood of coumestrol content over 25 mg/kg DM in both four and six week old lucerne increased ($P < 0.001$; $R^2 = 0.857$) through the mating period. There was no difference ($P = 0.399$) in the rate of increase over time (0.27 ± 0.039%/d) but the constant, and therefore overall risk, was lower ($P < 0.001$) in the four week old lucerne than in six week old crops, with average predicted risks of 29 ± 3.7% and 50 ± 4.1% respectively ($n = 9$). In the six week old lucerne, there was a 40% risk of coumestrol values over 25 mg/kg DM between 9 March and 6 April (3 to 31 August lambing). In contrast, in the four week old lucerne there was a 27% risk on 9 March (3 August lambing) and a 17% risk on 20 March and 6 April (17 and 31 August lambing). Risks for four and six week lucerne respectively, were 23% and 47% on 20 April (14 September lambing), 37% and 57% on 18 May (12 October lambing), and 40% and 70% on 1 June (26 October lambing).

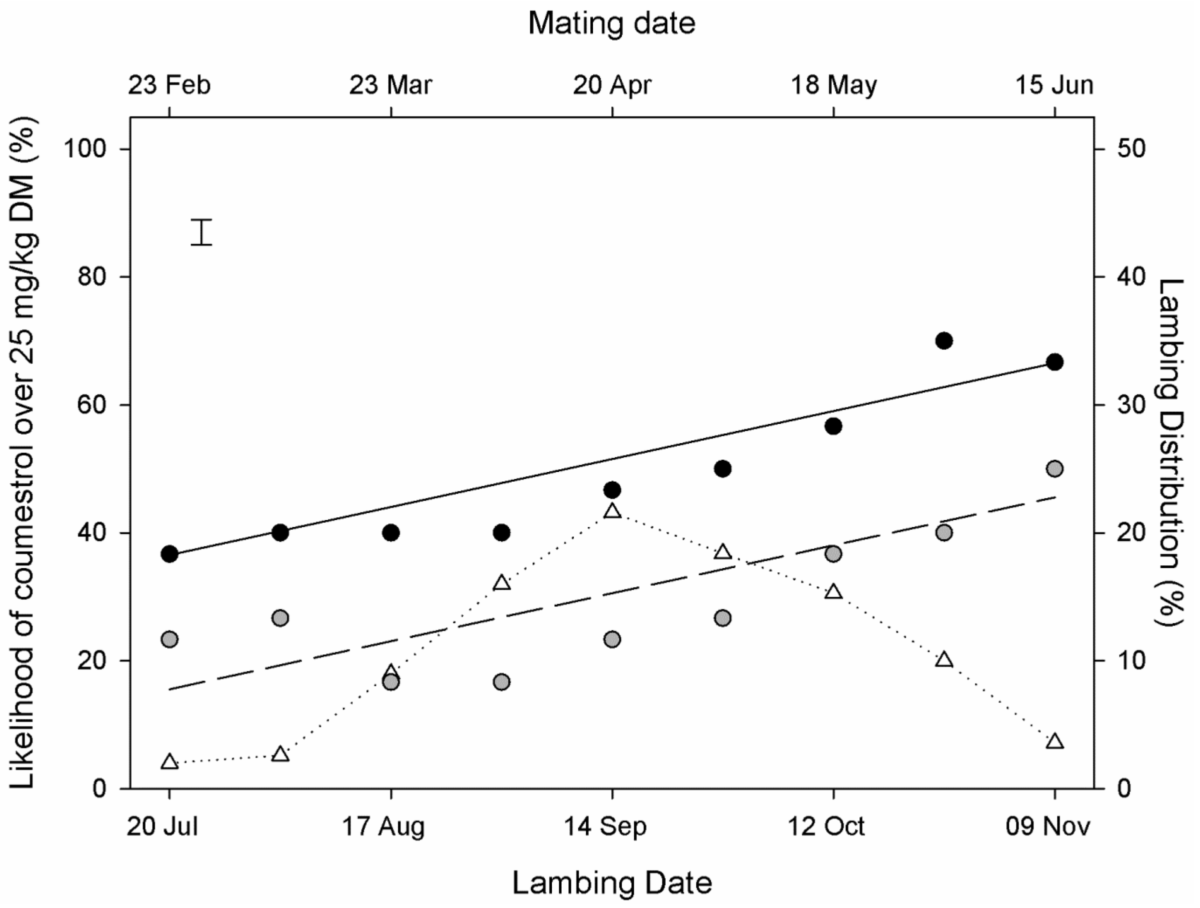

Figure 6.16 The distribution of lambing date (△) in Canterbury/Marlborough and the likelihood (%) of coumestrol content over 25 mg/kg DM in four week old (●) and six week old (●) lucerne on corresponding mating dates in Blenheim based on a simulation analysis using long-term meteorological data. Regression lines ($R^2 = 0.857$) are parallel ($P = 0.399$) lines of slope '0.27 ± 0.039%/d' for the relationship between date and risk for the four (dashed line) and six (solid line) week old lucerne. Error bar is the standard error of the mean for regrowth age.

6.3.6.3 East Coast

On the East Coast of the North Island (Figure 6.17), 91% of ewes lamb from 4 August to 12 October. Highest rates of lambing were the fortnights ending 31 August (21%), 14 September (32%) and 28 September (20%). Six week old lucerne had a greater ($P < 0.001$) likelihood of coumestrol content over 25 mg/kg DM than four week old lucerne, with predicted risks of 65 ± 2.0% and 38 ± 2.4% respectively. The likelihood of coumestrol over 25 mg/kg DM did not increase through the mating period in six week old ($P = 0.172$) or four week old lucerne ($P = 0.080$).

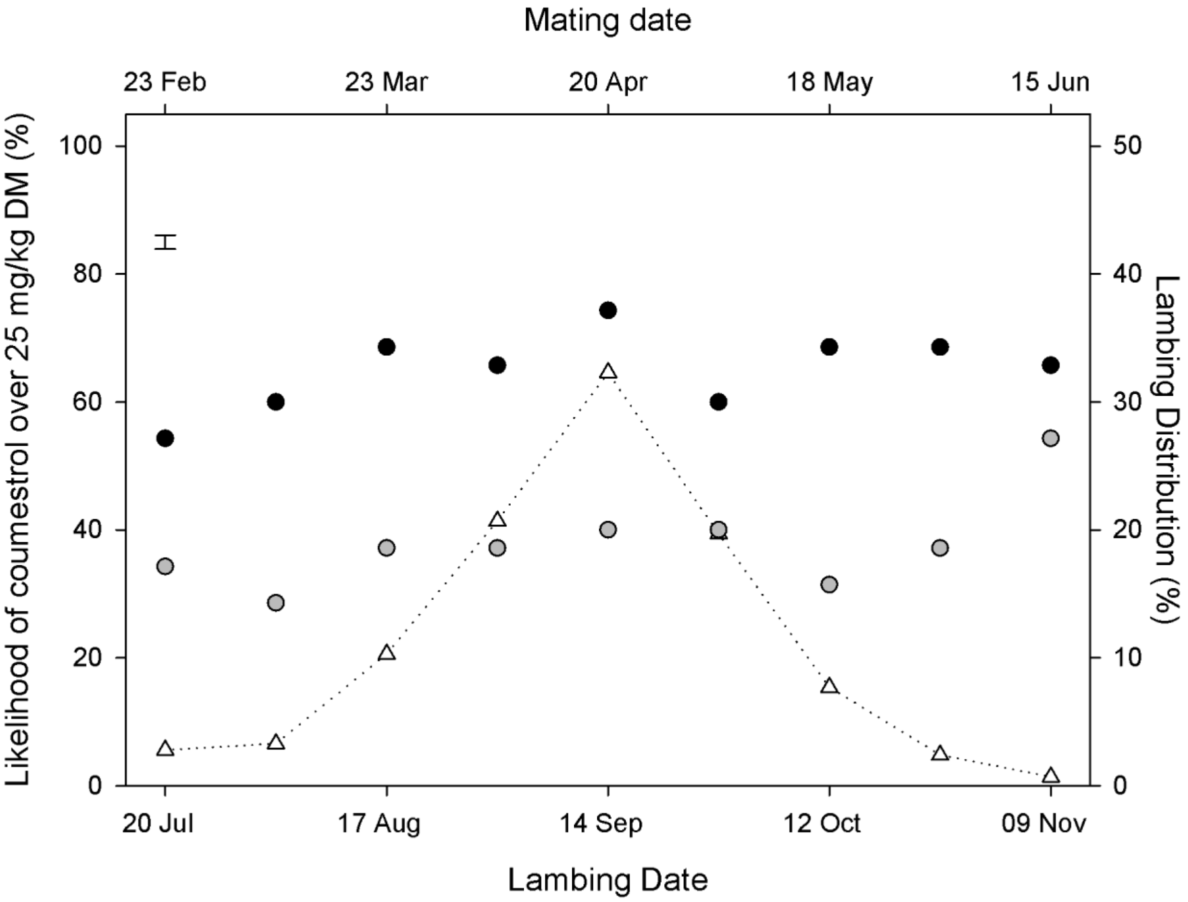

Figure 6.17 The distribution of lambing date (△) on the East Coast and the likelihood (%) of coumestrol content exceeding 25 mg/kg DM in four week old (●) and six week old (●) lucerne on corresponding mating dates in Napier, based on a simulation analysis using long-term meteorological data. Error bar is the standard error of the mean for regrowth age.

6.4 Discussion

6.4.1 Prediction of coumestrol in a lucerne crop

The objective of Chapter 6 was to develop a predictive tool to estimate coumestrol content of lucerne (Objective 3). Best subsets regression showed days above 95% RH and sum of rainfall to be the strongest predictor of coumestrol content in lucerne over three weeks old during the late spring to autumn period. These factors were able to predict coumestrol content with greater precision than the fungal damage score which was a relatively weak predictor ($R^2 = 0.388$). The equation (Equation 6.3) for coumestrol content using days above 95% and sum of rainfall was further validated by the independent data set. This produced Equation 6.7.

An equation is however difficult to implement for on-farm decision making. The decision tree (Figure 6.5) could be used by farmers to determine whether coumestrol content of a particular crop is likely to be high or low. When rainfall during the growing period of the lucerne was greater than 61 mm coumestrol was likely to be high, and ewes should be removed prior to mating (Chapter 8). When coumestrol was below 61 mm coumestrol was likely to be low or moderate. Low coumestrol content was likely when there were five or less days above 95% RH and moderate coumestrol levels were likely when there were 6 or more days.

When using these models care should be taken that if one predictor occurs without the other a significant response of coumestrol may still occur. In Model 1, RH ≥95% best explained coumestrol content, while Model 2 which does not take into account post-spike coumestrol data, considers rainfall data to best explain coumestrol. In addition, the decision tree showed rainfall to be the best way of splitting coumestrol into high and low groups. It is also possible that in situations with days of humidity between 90 and 95% RH, but not above 95% RH, coumestrol content may be underestimated. This may have been the case with the data from Purves *et al.* (1981). By the final sampling date of the experiment, only 10 days had been recorded with humidity over 95% while 25 over 90% were recorded. This may contribute to the underestimation of coumestrol for this data with the original prediction model.

Rainfall and humidity do not directly cause the coumestrol levels to increase in lucerne. Instead they create the environmental conditions suitable for fungal pathogens (Section 2.6.1.1). The fungal pathogens are what the coumestrol responds to (Sections 2.6.1.2 and 5.3). The coumestrol levels from experiments in Utah, South Dakota and Oamaru fitted well into the prediction model but it is possible that other pathogens, not encountered in the study sites, could respond to different environmental conditions. Therefore it is still important to check for fungal disease symptoms on plants. In addition, further research should be done to validate the conditions that predict coumestrol in different regions.

Ideally, more independent data from the literature would have been used but this was limited by a requirement to know the mean coumestrol content, the sampling date, and the age of the lucerne regrowth. Many prior experiments gave only development stage at sampling, with no sampling date or regrowth duration, or they gave the range of coumestrol contents over a month or period of interest. In addition, RH nor dew point are common measurement at meteorological stations, particularly over 30 years ago; this further limited the availability of suitable coumestrol data. This also meant that often the humidity data were taken from a site a considerable distance from the lucerne crops, which may contribute to error in the predictions. For this reason, recent humidity data now available at nearby meteorological stations were correlated with recent data from the further afield station. Correlations were not strong (r = 0.819 for Logan, Utah; r = 0.676 for Brookings, South Dakota), indicating some error was contributed by this factor. This was unlikely to be a problem for Utah, which over the last 17 years had a low 0900 h RH (45.7 ± 0.39%), above 95% on only one occasion during the months of the experimental period. On the other hand, it may have produced some error at South Dakota, with a recent average 0900 h RH closer to the 95% threshold.

In regions without access to humidity data it may be possible to use the single coefficient of rainfall to predict coumestrol content (Equation 6.2); however the accuracy will be reduced.

6.4.2 Requirements for a coumestrol spike

When only environmental factors prior to a coumestrol spike were taken into account, rainfall during the entire growing period was the best predictor of coumestrol content ($R^2 = 0.600$), followed by days above 95% RH ($R^2 = 0.577$). As with Model 1, the two variables together were the best predictor of coumestrol content ($R^2 = 0.685$), superior to either individually. The next best single predictor was the rainfall in the fortnight prior to coumestrol measurement: 50% of variation was predicted by this factor. This suggests that a coumestrol response by the plant is rapid, within two weeks of suitable environmental conditions.

6.4.3 Regional risk of heightened coumestrol

The coumestrol threshold of 25 mg/kg DM chosen for the regional risk assessment was based on the coumestrol levels reported in Chapter 8 and previous research by Smith *et al.* (1979). Coumestrol at approximately this level was shown in both cases to cause an effect on ewe reproductive performance. However, it is probable that levels below this will also have an effect and so a true safe value has not yet been determined. The threshold and median predicted coumestrol level do, however, provide an indication of the differences between regions, with Blenheim likely to have lower coumestrol levels in any given autumn than Lincoln, Lauder or Napier (Figures 6.12 & 6.13), and therefore less likely to have reduced reproductive performance. The risk assessment model also showed an effect of delaying the mating period on the prevailing coumestrol content of lucerne (Figures 6.10 & 6.11). It was predicted that the later into autumn that ewes are mated, the more likely they are to be consuming high coumestrol lucerne. The exception was Napier which tended to have a consistent median predicted coumestrol (19 and 34 mg/kg DM for four and six week old lucerne, respectively), and a constant likelihood of exceeding the 25 mg coumestrol/kg DM threshold (38 and 65% for four and six week old lucerne, respectively; Figure 6.17), throughout the mating period.

In Lauder, Lincoln and Blenheim, the lowest risk of heightened coumestrol values was for lambing dates between 15 July and 1 September (Figures 6.14 to 6.16). However, data from The Sheep and Beef Farm Survey (Beef and Lamb New Zealand, 2014) showed that across New Zealand only 2% of ewes lamb before 3 August and only 17% by the end of August. There was a regional difference. In Marlborough and Canterbury, lambing dates were spread out with approximately 20% of ewes lambed by the end of August, 40% by mid-September, 60% by the end of September, and 90% by the end of October. In contrast, in Otago and Southland there is a lot less spread with no lambing in August, approximately 30% of lambing completed by the end of September and 90% by the end of October.

The lambing date distributions (Figures 6.15 & 6.16) mean that in Canterbury and Marlborough there may be lee-way for farmers to reduce the fecundity impairing risk of coumestrol by mating ewes earlier in the season. However, the spread in lambing date distribution could also indicate a greater variation in suitable lambing date between areas within Canterbury and Marlborough. On the other hand in regions such as Central Otago (Lauder), ewes are mated in late-autumn when coumestrol is most at risk of being high (Figure 6.14), and it is unlikely to be viable to move outside of this norm and mate ewes earlier in the season. The best way to reduce the risk from coumestrol in lucerne across all regions would be to mate ewes on four week old regrowth rather than on six week old regrowth as the risk is substantially reduced. In addition, at the end of a period of high rainfall and/or high humidity lucerne allocated for mating ewes could be grazed off by non-mating livestock or cattle, which are unlikely to be affected by coumestrol in the lucerne. This would enable new regrowth to utilise the improved soil moisture from this rainfall, and as long as no further substantial rainfall and few high humidity days occur in the crop, and it is grazed while still relatively young (i.e. three or four weeks), it should be safe for ewes to return to it.

Chapter 7

Experiment 12 Morphological response of ewes to coumestrol

7.1 Introduction

Experiment 12 investigated the morphological response of ewes exposed to coumestrol as part of Objective 4 which was to quantify animal responses to elevated coumestrol. Teat growth has previously been reported in wethers grazing oestrogenic lucerne (Newton and Betts, 1968) but not in ewe lambs. This chapter reports a serendipitous incident (Experiment 12a) where ewe lambs grazing lucerne on a commercial sheep farm were observed to have premature mammary and teat development. The farmers contacted Lincoln University about this observation and further investigation was undertaken as part of this PhD research. Specifically, this spontaneous on-farm event provided an opportunity to investigate the occurrence of mammary development in ewe lambs grazing an oestrogenic lucerne crop and to determine if these effects had consequences for reproductive performance after removal from the crop. The null hypothesis was that there would be no difference in mammary development between ewe lambs on grass or lucerne.

Where there is uncertainty about whether a lucerne crop is sufficiently oestrogenic to cause impairment of fecundity in breeding sheep it would be useful to have a simple means to monitor the crop for this propensity. For farmers the occurrence of mammary and/or teat development in female sheep may be an easily recognisable indicator of oestrogenic activity in forage crops. However, if there is a difference in sensitivity of sheep that is dependent on their age, it may be necessary to use immature females for this purpose. This chapter also includes a comparison of coumestrol responsiveness between ewe lambs and adult ewes that was designed to address this question (Experiment 12b). In the study, ewe lambs and adult ewes were injected with coumestrol for nine days and teat growth and mammary development were monitored in comparison with those of animals receiving control (saline) injections. The hypothesis under test was that ewe lambs are more responsive to the effects of an exogenous coumestrol than adult ewes. The null hypothesis was that ewe lambs were not more responsive than adult ewes to coumestrol.

7.2 Methodology

7.2.1 Experiment 12a: Ewe lambs exposed to an oestrogenic lucerne pasture (an on-farm study)

In Experiment 12a sheep were cross-bred ewe lambs of predominantly Texel-East Friesian-Coopworth ancestry held on a commercial farm, ‘Creedmoor’, located 17 km southwest of Oamaru, North Otago, New Zealand. They were born in spring 2014 and at weaning on 27 November 2014 any with a live weight below 32.5 kg were allocated to a lucerne crop (n = 36). Those with higher live weights were returned to a predominantly ryegrass/white clover pasture (n = 22). The average live weights were 25.1 ± 0.84 kg for sheep on lucerne and 34.7 ± 0.41 kg for the grass-based pasture. This allocation was a commercial management decision intended to provide a superior nutrient supply to the lighter lambs. Lambs assigned to lucerne were nine days younger ($P < 0.001$) than lambs assigned to grass. The average date of birth (± SEM) was 3 September 2014 ± 0.9 days and 12 September 2014 ± 1.6 days for grass- and lucerne-fed lambs, respectively.

Throughout the post-natal growth period the lambs received routine husbandry treatments for endo- and ecto-parasites, anti-clostridial vaccinations and vitamin supplements. Their fleeces were shorn on 2 March 2015. Live weight was measured with electronic scales at weaning (27 November 2014), pre-shearing (2 March 2015), on 12 March 2015, and at commencement of mating (20 April 2015).

On 12 March and 16 April 2015, all ewe lambs were checked by palpation for protruding mammary glands. Measurements of teat length, teat width at the base and mammary gland diameter were recorded using a digital calliper for 22 of the lucerne-fed ewe lambs and for 10 of the grass-fed lambs selected randomly. For each lamb, data for right and left teat measurements were averaged to give a single value.

On 20 March 2015, sheep were removed from the lucerne crop and re-united with their grass-fed cohorts on the grass-based pasture. A vasectomised ram was placed with the ewe mob on 9 April 2015 and remained there for two weeks before being replaced with a crayon-harnessed entire ram from 20 April 2015. Crayon marking of the ewes was recorded and the number of fetuses present was determined by transabdominal ultrasound recording carried out by a commercial operator on 24 June 2015.

7.2.1.1 Lucerne coumestrol measurement

Plant samples were collected on 12 March 2015 from the grass-based pasture and the lucerne paddocks used in the grazing rotation and analysed by HPLC for coumestrol content using the methodology described in Sections 4.4 to 4.7.

7.2.1.2 Statistical analysis

Data in Experiment 12 (Chapter 7) were analysed in Minitab 17. For Experiment 12a two-sample *t*-tests were used to compare the two treatment groups for live weight at each weighing, teat width, teat length, and scanning rate. Regression models were used to account for the effects of diet, live weight, growth rate, birth rank and age on mammary presence, teat width and teat length. These models were built with terms through order two and stepwise regression (α to enter = 0.15). Ordinal regression ((R (version 3.3.3) with 'polr' function from package 'MASS') was used to account for the effects of diet and live weight on the number of fetuses per ewe at scanning.

7.2.2 Experiment 12b: Response of ewe lambs and mixed age ewes to coumestrol

Experiment 12b was conducted under protocols approved by the Lincoln University Animal Ethics Committee in accordance with the New Zealand Animal Welfare Act (1999). For this experiment, five month old Coopworth ewe lambs and mixed age (MA) ewes were allocated (n = 8) to coumestrol or control (vehicle only) treatments. The injection vehicle was a 1:4 ratio dimethyl sulfoxide (DMSO):saline solution. For the first nine days of the study the ewe lambs were given a daily intramuscular injection of 0 or 3 mg coumestrol and MA ewes a daily injection of 0 or 7 mg coumestrol. The adult dose rate was based on that used by Shutt *et al.* (1969) who reported near maximal uterine growth stimulation with 7.5 and 25 mg/day injections over four days. The ewe lamb dose was based on the relative average live weights of the two age classes. The average live weight (± SEM) at commencement was 36.1 ± 0.63 kg for ewe lambs and 82.9 ± 2.25 kg for MA ewes. On average ewes received 0.084 ± 0.0030 mg coumestrol/kg live weight and ewe lambs received 0.085 ± 0.0013 mg coumestrol/kg live weight, based on their initial live weight.

During the study period the sheep were grazed *ad libitum* on irrigated ryegrass pasture at the Johnstone Memorial Laboratory Research Farm, Lincoln University. The animals were monitored prior to commencement of the study and at day 12 for live weight, teat size (length and width) and occurrence of mammary gland development (diameter).

7.2.2.1 Statistical analysis

For Experiment 12b, ANOVA was used to analyse the effects of age and coumestrol treatment on teat size.

7.3 Results

7.3.1 Experiment 12a: Ewe lambs exposed to an oestrogenic lucerne pasture

7.3.1.1 Pasture coumestrol levels

On 12 March 2015, lucerne in non-grazed stands of the crop was mature, at a late flowering/early seed pod development stage (Stage 7-8). The pre-grazing lucerne dry matter was 2.0 ± 0.36 t DM/ha and the dry matter of the post-grazing residual stems was 0.9 ± 0.15 t DM/ha. Fungal diseases, namely spring black stem and stemphylium were present in the stands. Coumestrol content of the lucerne was moderately high (77.1 ± 2.2 mg/kg DM) for the material being grazed by the ewe lambs in this study on 12 March 2015. In the residual stems of the previously grazed lucerne stand, which the ewe lambs were removed from on 6 March 2015, the coumestrol content was a moderately high

55.8 ± 2.9 mg/kg DM. In the lucerne stand the ewe lambs were moved to, on 13 March 2015, the coumestrol content was a moderately high 76.9 ± 6.3 mg/kg DM. The grass-based pasture had a negligible 0.2 mg/kg DM.

7.3.1.2 Live weight

At weaning (27 November 2014), the lambs about to be transferred to lucerne were lighter ($P < 0.001$) than the lambs remaining on grass (25.1 ± 0.84 kg vs. 34.7 ± 0.41 kg). The lambs on lucerne remained lighter ($P < 0.001$) than lambs on grass with mean live weights of 45.4 ± 0.98 kg and 53.2 ± 0.58 kg on 12 March 2015, which was towards the end of the lucerne grazing period (20 March). Between weaning and shearing (2 March 2015) the average daily live weight gains for lucerne and grass-fed lambs were 215 ± 4.5 g and 198 ± 6.1 g per day, respectively ($P = 0.032$).

Between 12 March 2015 and mating (20 April 2015) ewe lambs fed lucerne for the first eight days followed by 31 days of grass had a higher ($P < 0.001$) gain in live weight than the grass-fed lambs. During this period live weight gain was 195 ± 7.4 g per day for lucerne-fed lambs moved to grass and 110 ± 4.5 g per day for grass-fed lambs.

At mating, lambs previously fed lucerne were still lighter ($P < 0.01$) than grass-fed lambs (53.0 ± 1.01 kg vs. 57.5 ± 0.60 kg).

7.3.1.3 Mammary development

On 12 March 2015, there was substantial mammary and teat development in some of the ewe lambs that had grazed lucerne (Figure 7.1). Protruding mammary glands were present in 19 of the 36 lucerne-fed lambs and in none of the grass-fed lambs. Mammary gland protrusion was related to grazing treatment ($P < 0.001$) and not related to birth rank, age, current live weight or growth rate between weaning and 12 March 2015. The mean diameter of protruding mammary glands was 61.1 ± 2.8 mm.

Figure 7.2 shows lucerne-fed ewe lambs had a larger ($P = 0.004$) mean teat width than grass-fed lambs (17.9 ± 0.53 mm vs. 13.4 ± 0.78 mm) respectively. There was a weak ($P = 0.06$) relationship between weaning to 12 March 2015 growth rate and teat width, where lambs that had put on more weight during this period tended to have greater teat widths. Mean teat length of the lambs was 22.1 ± 0.50 mm and there was no effect of grazing treatment ($P = 0.783$). Teat length was related to the growth rate of lambs between weaning and 12 March 2015 ($P = 0.001$).

Three weeks after lucerne-fed lambs were moved to grass (16 April 2015) there had been no change ($P = 0.104$) in mammary size compared with 12 March 2015. Grass-fed lambs had a higher increase in teat width between 12 March and 16 April 2015 than ex-lucerne-fed lambs (5.5 ± 0.81 mm vs. 0.9 ±

0.51 mm, P < 0.001). Mean teat widths of the grass-fed and ex-lucerne-fed lambs were no longer different (19.0 ± 0.93 and 18.9 ± 0.58 mm respectively; P = 0.966).

On 16 April 2015, the mean teat length had decreased (P = 0.039), relative to 12 March 2015, to 20.4 ± 0.49 mm and there was still no difference (P = 0.116) in mean teat length between ex-lucerne-fed and grass-fed lambs.

In this study there was no effect (P > 0.100) of birth rank, age or live weight at time of measurement on teat size.

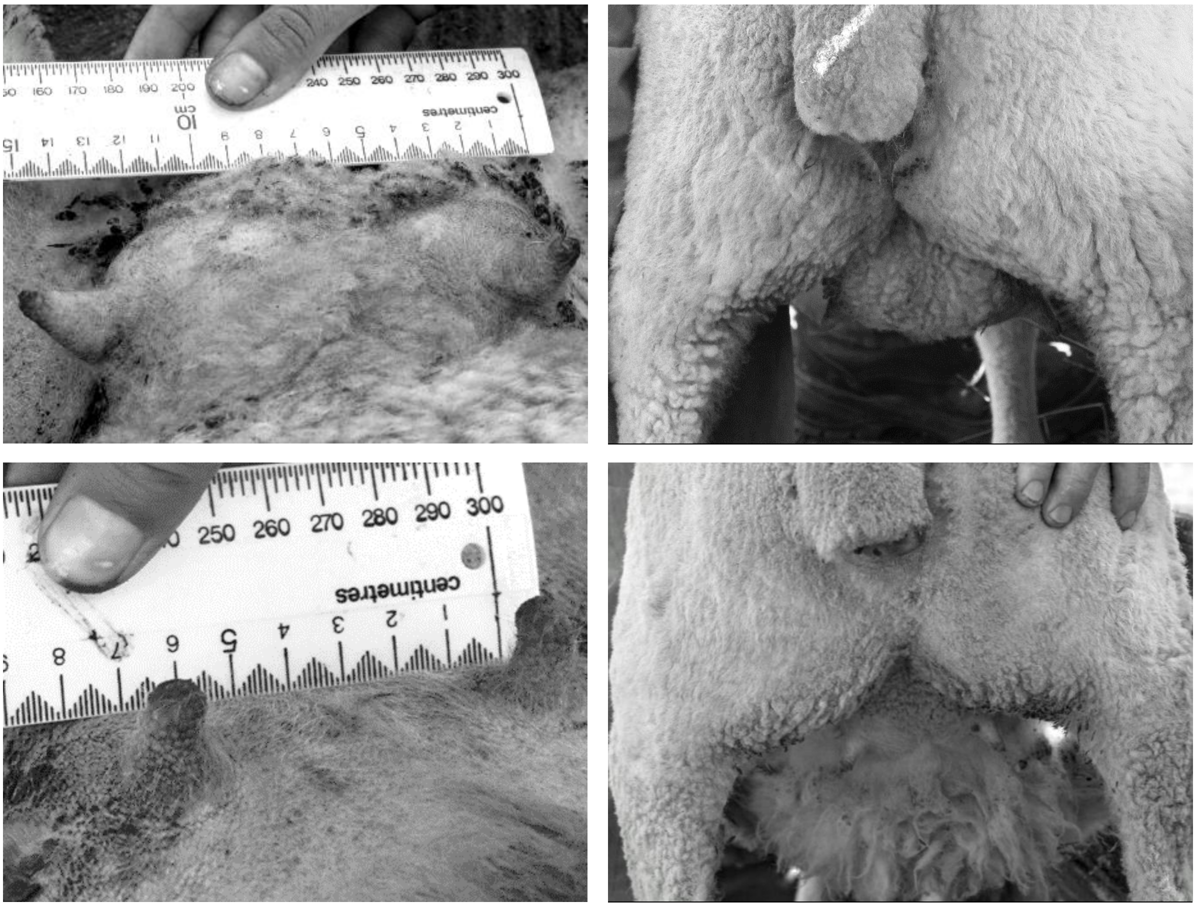

Figure 7.1 Mammary gland and teat development of a ewe lamb that had grazed lucerne (top) in comparison with one that had grazed a ryegrass/white clover pasture (lower). Photographs taken 12 March, 2015.

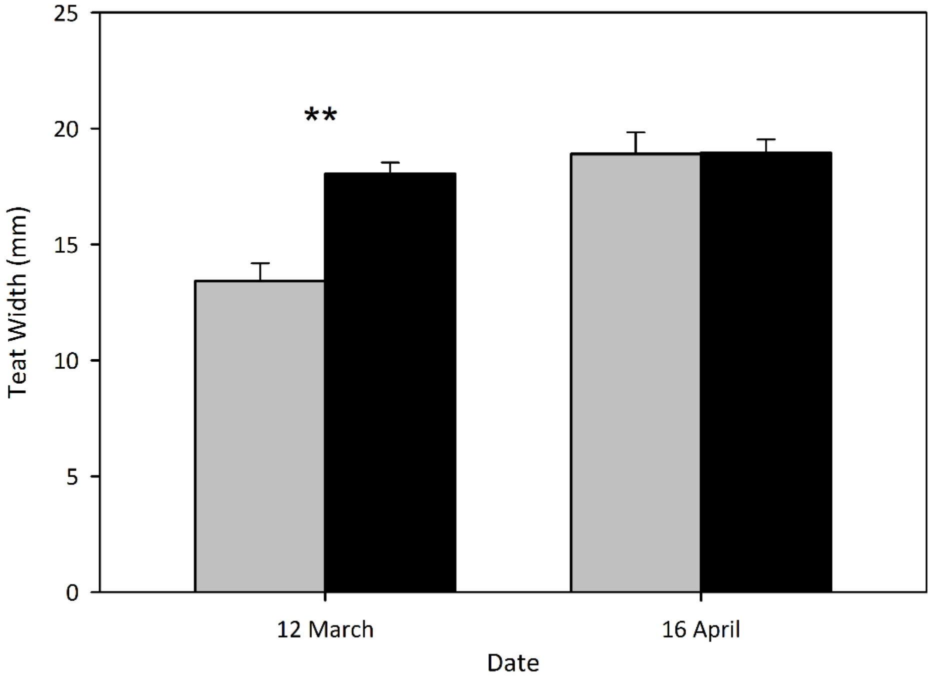

Figure 7.2 Mean teat width of ewe lambs grazing grass (▨) or lucerne (■) up to 12 March 2014, then grass only until 16 April 2014. Vertical bars represent SEM, ** indicates the significance (P = 0.004) between treatment groups on 12 March.

7.3.1.4 Reproductive performance

All animals were marked by the crayon-harnessed ram during the mating period, which indicates that there was no effect of the earlier grazing of lucerne by post-weaning pre-pubertal lambs on mating activity compared with those that grazed grass.

The grass-fed ewe lambs were on average 4.5 kg heavier in live weight and had a greater number of fetuses per ewe compared with the ex-lucerne-fed animals (2.05 ± 0.10 vs 1.66 ± 0.10; $P = 0.026$). Ordinal regression showed the number of fetuses per ewe was affected ($P < 0.001$) by live weight and not affected ($P = 0.559$) by diet.

From the estimated regression coefficients of fetus number against live weight, a matrix of predicted logits were calculated for each kilogram increment in mating weight from 40 to 65 kg. From this, cumulative probabilities were calculated and a table of probabilities produced (Table 7.1).To interpret this table, consider a ewe with a 50 kg mating weight. The ewe has a 4% change of being barren, a 35% change of having a single, a 59% change of having twins and a 1.8% chance of triplets. This probabilities for each number of fetuses across the live weight range is also represented in Figure 7.3.

Table 7.1 The probabilities of 0, 1, 2 and 3 fetuses per ewe at scanning for ewe live weights between 40 and 65 kg as predicted from Creedmore data with logistic regression.

No. of	Ewe mating weight (kg)

fetuses	40	45	50	55	60	65
0	0.318	0.123	0.040	0.012	0.004	0.001
1	0.560	0.560	0.352	0.150	0.051	0.016
2	0.121	0.312	0.590	0.781	0.778	0.582
3	0.002	0.005	0.018	0.057	0.167	0.401

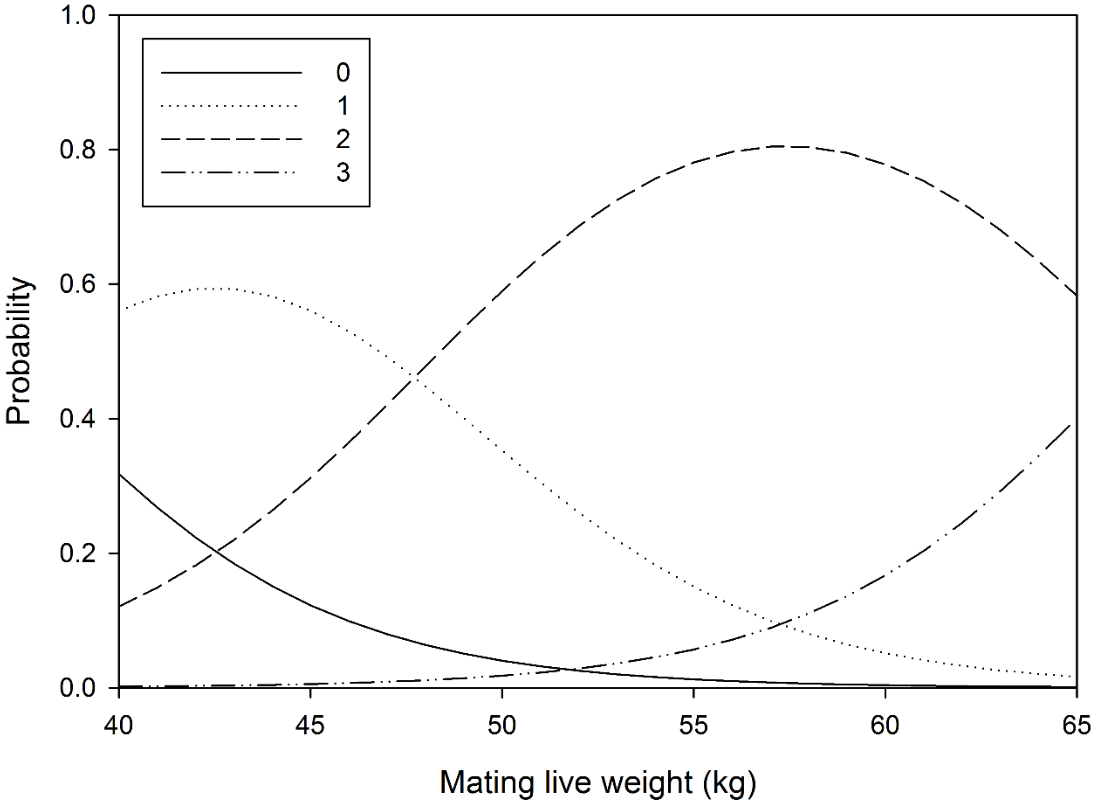

Figure 7.3 The probabilities of 0, 1, 2, and 3 fetuses per ewe at scanning for ewe live weights at mating between 40 and 65 kg as predicted from Creedmore data with logistic regression.

All grass-fed animals (n = 22) had a live weight above 53 kg. When results from the fetal scanning data for the grass-fed animals were compared with those of the ex-lucerne-fed animals weighing over 53 kg (n = 19) there was no difference (P = 0.736) in the mean live weight of the selected animals (57.5 ± 0.6 kg and 57.8 ± 0.7 kg, for grass- and ex-lucerne-fed ewes respectively). There was no difference (P = 0.834) in the proportion of multiple births between grazing treatments with 89% of ex-lucerne-fed and 91% of grass-fed ewes having two or more fetuses present. There was no difference (P = 0.581) in the total number of fetuses present per ewe (1.95 ± 0.14 and 2.05 ± 0.10 fetuses for ex-lucerne- and grass-fed animals respectively). There was also no relationship (P = 0.894) between 12 March to 20 April 2015 animal live weight gain and number of fetuses present in pregnant animals.

Table 7.2 Mean (± SEM) number of fetuses (from ultrasonic scanning), percentage of ewes with at least two fetuses, and live weight of ewes with live weight above 53 kg (lightest grass-fed ewes).

Herbage	n	Live weight (kg)	Ewes with at least	Number of

			two fetuses	fetuses
Grass	22	57.5 ± 0.6	91%	2.05 ± 0.10
Lucerne	19	57.8 ± 0.7	89%	1.95 ± 0.14

7.3.2 Experiment 12b: Response of ewe lambs and mixed age ewes to coumestrol

On Day 1, the average ewe lamb weight was 36.1 ± 0.6 kg and the mean live weight of the MA ewes was 82.9 ± 2.2 kg. On Day 12 the ewe lambs weighed 38 ± 0.7 kg and the ewes weighed 78.2 ± 1.9 kg. There was a difference ($P < 0.001$) in weight change between lambs and MA ewes. Lambs gained 180 grams per day while MA ewes lost 400 grams per day. There was no effect ($P = 0.376$) of coumestrol on live weight gain.

Coumestrol increased teat length ($P = 0.001$) in both MA ewes and ewe lambs (Figure 7.4). There was an effect ($P = 0.028$) of age, with ewe lambs having a greater change in teat length than MA ewes. There was no interaction ($P = 0.696$) between age and treatment. Between Day 1 and Day 12 ewe lambs injected with coumestrol had mean teat length growth of 2.69 ± 0.57 mm while lambs injected with vehicle only had an average teat length growth of 0.75 ± 0.53 mm ($P = 0.011$). Over this time-frame MA ewes injected with coumestrol had a mean teat length increase of 1.53 ± 0.82 mm while control ewes had a change in teat length of -0.879 ± 0.43 mm ($P = 0.021$).

Coumestrol did not cause a difference ($P = 0.574$) in teat width growth relative to that of controls in either MA ewes or ewe lambs (Figure 7.5). There was also no effect of age on teat width growth ($P = 0.190$). No mammary tissue growth was observed during the experiment.

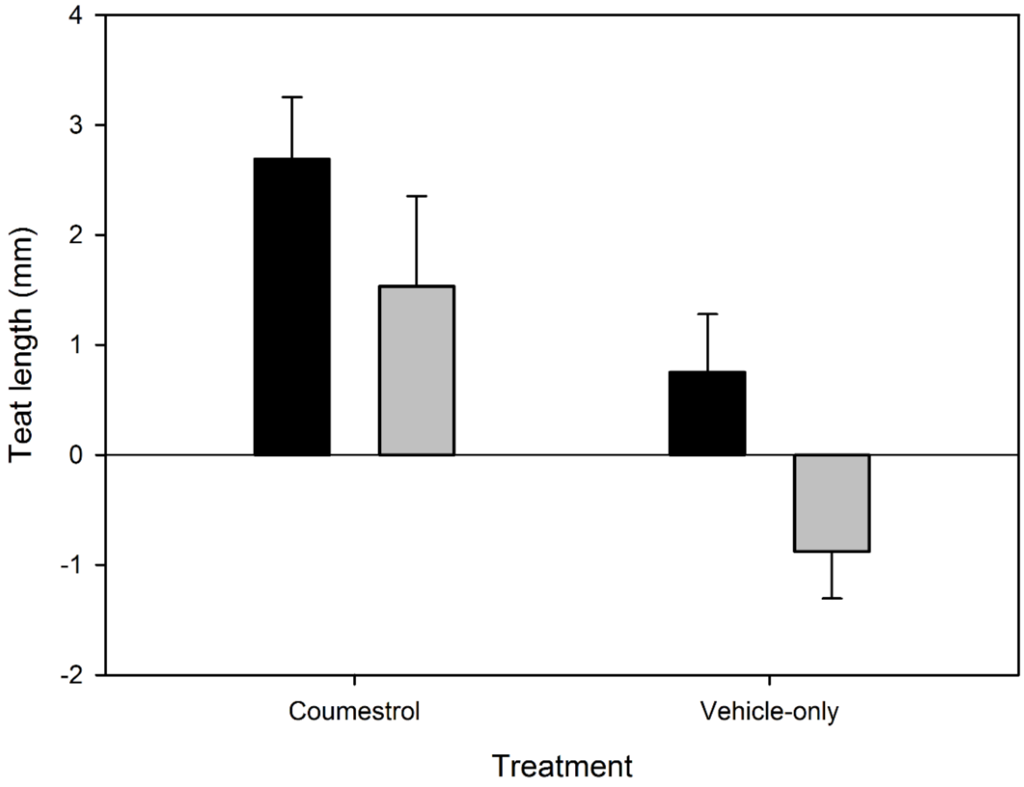

Figure 7.4 Mean change in teat length (mm) of ewe lambs (■) and mixed aged ewes (■) between Day 1 and Day 12 after nine days of injection with either coumestrol or vehicle only. Error bars are SEM.

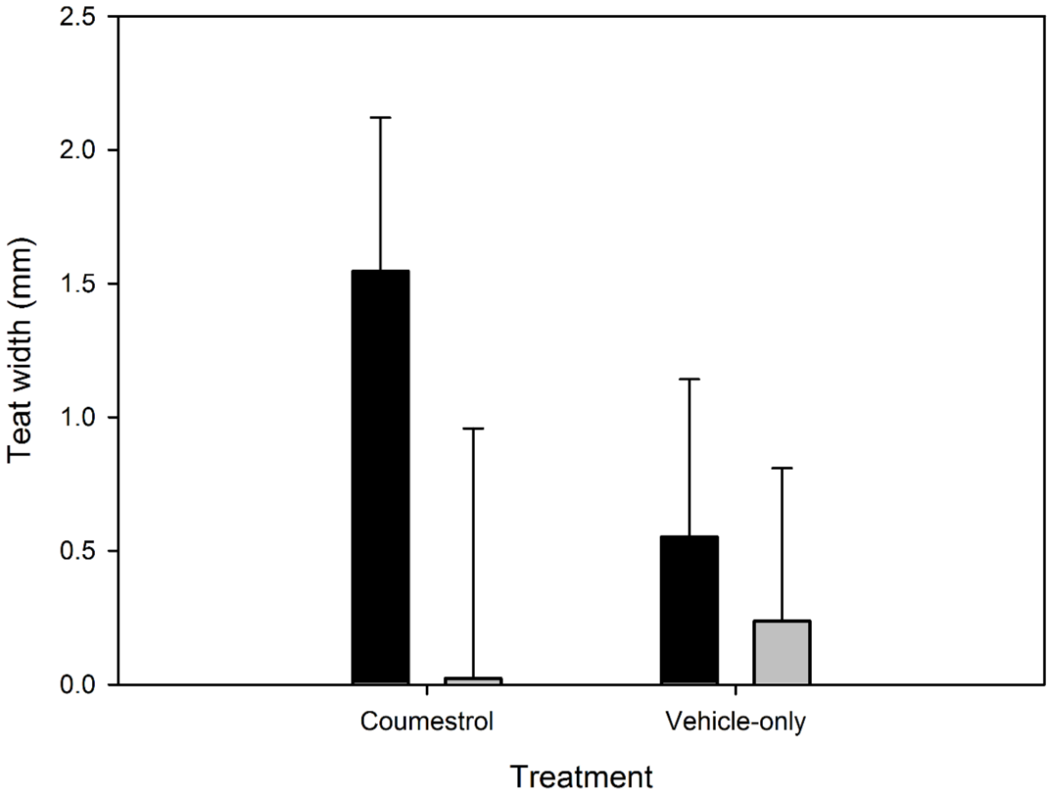

Figure 7.5 Mean change in teat width (mm) of ewe lambs (■) and mixed aged ewes (■) between Day 1 and Day 12 after nine days of injection with either coumestrol or vehicle only. Values were not significantly different. Error bars are SEM.

7.4 Discussion

Experiment 12 investigated the morphological response of ewes exposed to coumestrol as part of Objective 4 which was to quantify animal responses to elevated coumestrol. Spontaneous occurrence of mammary development in ewe lambs grazing an oestrogenic lucerne crop was investigated to determine if these effects had consequences for reproductive performance after removal from the crop. This was followed by a comparison of the responsiveness of ewe lambs and adult ewes to coumestrol.

In Experiment 12a, mammary gland protrusion and increased teat width were observed in ewe lambs grazed on lucerne pastures, while their cohorts that grazed on grass did not show these developments (Figures 7.1 & 7.2). Animals were allocated to grazing treatments at weaning as a commercial management decision to provide the lighter ewe lambs a higher quality diet than the heavier lambs. Between 27 November 2014 and 2 March 2015 the ewe lambs on the lucerne had a 17 g/hd greater average daily live weight gain than the ewe lambs on grass. Despite this, the grass-fed ewe lambs remained heavier throughout the observational period.

This between-group difference in live weight could affect teat and mammary development. However, mammary development is typically isometric with live weight in non-pregnant ewe lambs (Johnsson and Hart, 1985). A previous study that measured mammary development in four- and eight-month-old pre-pubertal lambs showed greater mammary fat pad mass in the heavier animals (Johnsson and Hart, 1985). This means that in the present on-farm study the heavier lambs on grass would be expected to have larger teats and greater mammary development than the smaller lambs on lucerne. However, the reverse was observed. Age and birth rank also did not account for the observed mammary gland and teat development. Therefore, the mammary gland and teat development of the ewe lambs grazing lucerne, and the lack of this development in lambs grazing grass, was most likely due to the presence of the biologically active phyto-oestrogens in the grazed lucerne.

The lucerne had a coumestrol content above the level of 25 mg/kg DM reported as sufficient to cause a negative impact on ovulation rate (Smith *et al.*, 1979). Removal of the putatively oestrogen-affected ewe lambs from the lucerne crop and their re-location to a grass-based pasture four weeks before mating appeared to allow recovery from any potential negative impact of the coumestrol on the subsequent numbers of fetuses produced when animals within a similar live weight range were compared. However, removed animals also had greater live weight gain in the five weeks prior to mating than the grass-fed animals and this may account for some of the similarity in the numbers of fetuses produced by each group.

In Experiment 12b, the hypothesis that ewe lambs are more responsive to the effects of an exogenous coumestrol than adult ewes was tested. In this experiment both ewe lambs and MA ewes exhibited a teat length growth response to coumestrol injections (Figure 7.4). The response to coumestrol was larger in the ewe lambs than for the MA ewes and there was less variation. This indicates that ewe lambs were a more reliable indicator of oestrogenic lucerne than older ewes. Unlike in Experiment 12a, teat width did not respond to coumestrol. There was also no mammary tissue development. This lack of development may have been due to the short duration of the experiment, this means that ewes could be grazing an oestrogenic pasture for some time before mammary development becomes noticeable, but that a teat length response to oestrogenic lucerne may be detectable reasonably promptly.

To monitor the oestrogenicity of a lucerne crop, the findings of Experiment 12b show that teat length growth is more likely to be detected in ewe lambs than in MA ewes. This rejects the null hypothesis that ewe lambs would not be more responsive than MA ewes. Any mammary development is also more likely to be detected in the ewe lambs, which normally do not have protruding udders, than in ewes that have previously lambed and already have udder development. Monitoring udder and teat growth to detect elevated coumestrol levels in lucerne may simply mean running a few ewe lambs with the older ewes. These animals should be compared with same-age cohorts that are not on lucerne, as normal growth of the ewe lambs over time will cause an isometric increase in teat size.

Based on the results of Experiment 12a and 12b it is concluded that premature mammary and teat development in ewe lambs is an indicator of oestrogenicity of grazed lucerne. If increases in teat or mammary growth are detected in co-grazed ewe lambs, this would serve as an indicator signal for removal of ewes from the lucerne. Research to determine how long animals should be removed from an oestrogenic pasture prior to mating is described in Chapter 8.

Chapter 8

Experiment 13 Ewe fecundity after removal from lucerne

8.1 Introduction

Experiment 13 investigated the recovery of ewe reproductive performance following coumestrol consumption as part of Objective 4 which was to quantify animal responses to elevated coumestrol. Removal of ewes from lucerne-based forages prior to the mating period allows them to recover from any negative effects of coumestrol on reproductive performance (Coop, 1977) but the duration of the period that is required for full recovery from such effects has not been determined. Because the length of time prior to mating that the animals should be removed from lucerne is unknown, farmers are currently simply advised not to feed ewes on lucerne in the weeks prior to and during mating. The objective of this study was to determine the stand-off period required to reverse any effects of grazing high coumestrol lucerne.

Specifically, Experiment 13 involved transferring ewes from a lucerne pasture to a grass-based pasture to measure the rate of reduction of coumestrol levels in the blood and to determine the duration of the period in which negative effects of coumestrol on ovulation rate persisted. Identification of the duration that animals should be removed from high coumestrol lucerne pastures would allow farmers to maximise lucerne grazing days in autumn in order to take advantage of its high quality and yield whilst minimising the risk to fecundity by removing the animals from lucerne early enough to allow full recovery from any negative impacts. The purpose of the present study was to determine the duration of time needed to overcome these effects.

8.2 Methodology

8.2.1 Experimental design

This experiment was conducted under protocols approved by the Lincoln University Animal Ethics Committee in accordance with the New Zealand Animal Welfare Act (1999). It was located at Iversen Field, Lincoln University near Christchurch, New Zealand and ran from the start of April to mid-May 2016. Rainfall and temperature data (Figures A.9 & A.10) were recorded for the duration of the experiment at Broadfield Meteorological Station, Lincoln; 2.5 km from the field site. Sixty 18-month old Coopworth ewes (mean live weight 60.9 ± 0.83 kg, range 49 to 76 kg) were allocated randomly, but balanced for live weight, to four grazing treatments (n = 15). The treatments were six weeks of grass-based pasture, three weeks of lucerne followed by three weeks of grass, five weeks of lucerne followed by one week of grass, and six weeks of lucerne. Lucerne was a five-year-old pure stand of

‘Stamina 5’. The grass-based pasture was comprised of ‘Arrow’ AR37 perennial ryegrass and ‘Tribute’ white clover sown 18 months earlier. Pastures were irrigated prior to onset of the experiment. The ewes were break-fed with a rear fence on both the lucerne and grass pastures so that they grazed a defined segment of each crop that shifted progressively across the whole field. In the grass pasture, five breaks were used of 0.16 ha each, and in the lucerne, eight breaks of 0.22 ha each.

8.2.2 Pasture sampling

Lucerne and grass were sampled pre and post-grazing in each break with three randomly placed 0.2 m^2 quadrats for measurement of yield, botanical composition, utilisation (%), metabolisable energy (ME), protein content, neutral detergent fibre (NDF) and acid detergent fibre (ADF) using near infra-red spectroscopy (NIRS), and for measurement of coumestrol by HPLC (Section 4.7).

8.2.3 Control of ovulation and measurement of reproductive performance

To induce ovulation, ewes were synchronised with intravaginal progesterone-releasing devices (Eazi-BreedTM sheep CIDR®, Zoetis Inc, Kalamazoo, MI, USA), containing 0.3 g progesterone per CIDR® that were inserted for 12 days. CIDR® induces ovulation approximately 60 hours after removal (Shackell, 1991). Eight days following CIDR® removal, ovulation rate was determined by intra-abdominal laparoscopy using 10 mL acetyl promazine (10 mg/mL, Acezine 10, Ethical Agents Limited, Auckland, NZ) delivered by intramuscular injection for sedation plus a local anaesthetic of 2 mL on each side of lignocaine hydrochloride (20 mg/mL, Lopaine 2%, Ethical Agents Limited, Auckland, NZ) delivered subcutaneously and 4 mL of an antibiotic (300 mg/mL, procaine penicillin, Depocillin®, Schering-Plough Animal Health Ltd, Upper Hutt, NZ) delivered intramuscularly to enable counting of the total number of corpora lutea. This procedure was carried out by an experienced commercial operator.

8.2.4 Plasma sampling and extraction

Blood samples (10 mL) were obtained by venepuncture from an external jugular vein whilst the sheep were manually restrained using evacuated plastic tubes containing sodium ethylene diamine tetra acetic acid (BD 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. Blood samples were centrifuged for 10 minutes at 1300 x *g* to obtain plasma which was transferred to glass vials and stored frozen (at -20 °C).These samples were collected from four ewes per treatment at the beginning of the experiment, at the time points when a group was transferred from lucerne to grass and three and seven days after transfer. Coumestrol was extracted from plasma and measured by HPLC via the methodology described in Sections 4.10 and 4.11.

8.2.5 Statistical analyses

In Experiment 13 (Chapter 8), statistical analyses were performed using Genstat 16.1.

Regression analysis was used to examine the effect of sampling date on yield, quality and coumestrol content of the pastures.

One-way analysis of variance (ANOVA) was used to assess the effect of treatment on live weight gain during the experiment, mean live weight at ovulation. Fisher's protected least significant difference *post hoc* test was used to compare means when the ANOVA was significant (α = 0.05).

An exponential regression analysis was used to test the relationship between ovulation rate and 'grass days' (days on grass rather than lucerne). Ordinal regression was performed to account for the effects of grass days, live weight and change in live weight on the number of corpora lutea per ewe. Live weight and change in live weight were found to be very non-significant so the analysis was continued to study the effect of grass days alone. Preliminary graphs of mean ovulation rate suggested that an exponential model would most accurately describe the response. In place of 'grass days' the transformed variate 'exp(-0.11*grass days)' was used. Graphs were drawn with the independent variable back transformed to days. From the estimated parameters and their respective standard errors, the probabilities and 95% confidence intervals of a ewe having 0, 1, 2 or 3 corpora lutea were calculated for a range of 'grass days' (0 - 42, n = 22) prior to ovulation as detailed in the supplementary material. From the probabilities of each corpora luteal value the expected number of corpora lutea were calculated for the range of 'grass days'.

8.3 Results

8.3.1 Pasture analyses

Mean coumestrol content of the herbage was moderate at 29.1 ± 2.88 mg/kg DM (n = 27) in lucerne and a negligible 0.50 ± 0.11 mg/kg DM (n = 12) in grass. The levels of coumestrol recorded in each break for the duration of the experiment are given in Figure 8.1. There was no effect of date on coumestrol content of lucerne (P = 0.371) or grass (P = 0.992). White clover has previously been reported to produce low levels of coumestrol (Wong *et al.*, 1971). There was a linear relationship (P = 0.006, R^2 = 0.635) between white clover content sampled in the grass pasture and coumestrol, indicating white clover to be the probable source.

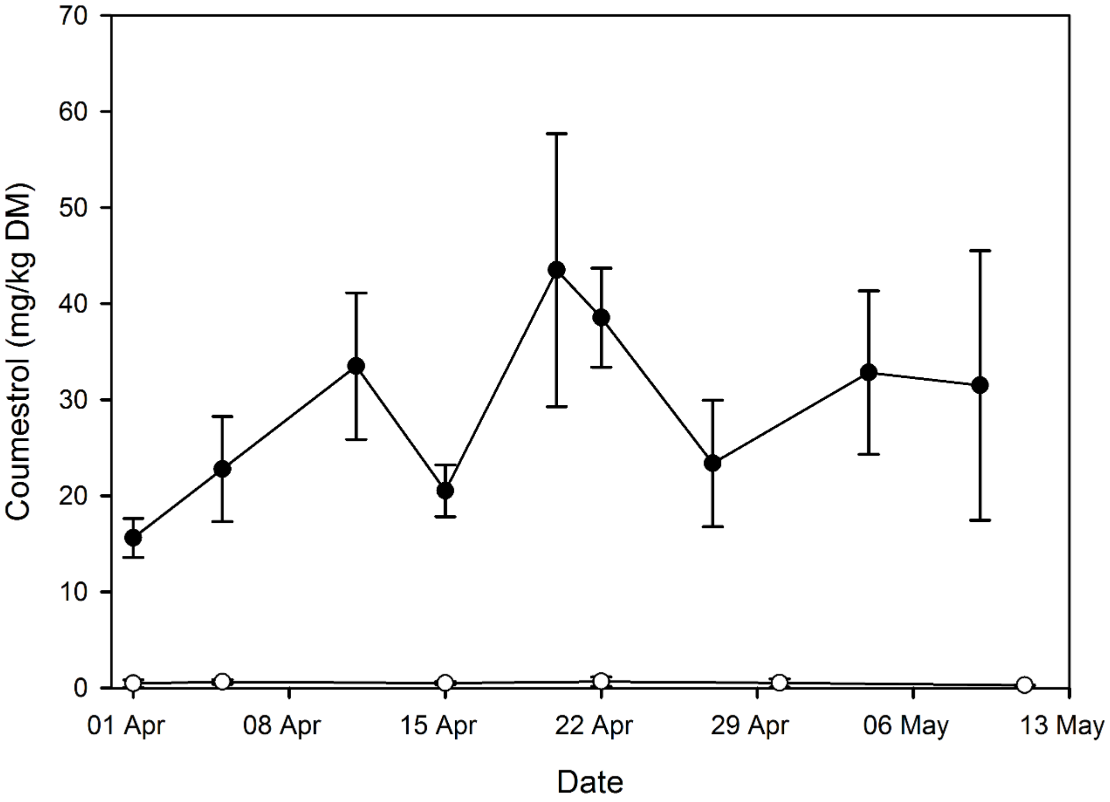

Figure 8.1 Mean coumestrol content of lucerne and grass sampled from 1 April to 9 May 2016. Samples were taken pre-grazing for each pasture break. Vertical bars represent ± SEM.

Average botanical composition of the lucerne crop (n = 24) was 96 ± 1.1% lucerne, 4 ± 0.9% dead material and 0.4 ± 0.42% weeds. Lucerne was 66 ± 0.9% leaf and 34 ± 0.9% stem. Lucerne was mid to late vegetative throughout the experiment, with a developmental stage between 2 and 3. Average pre-grazing lucerne dry matter yield was 1.13 ± 0.094 t DM/ha (n = 24), with a decline ($P = 0.003$, $R^2 = 0.349$) of 0.024 t DM/ha/d in lucerne over time, from 1.6 t DM/ha in the first break to 0.83 t DM/ha in the final break. Average pre-grazing dead material yield was 0.03 ± 0.006 t DM/ha, with no change ($P = 0.712$) over time. Post-grazing, lucerne residual was 0.12 ± 0.018 t DM/ha and dead residual was 0.06 ± 0.012 t DM/ha. Average utilisation of lucerne in each block (n = 8) was 88 ± 2.9% with ewes consuming an average of 1.2 ± 0.14 kg/head/d.

In the grass pasture (Figure 8.2), average botanical composition was 55 ± 5.3% ryegrass, 23 ± 6.9% white clover, 22 ± 3.6% dead material and 0.1 ± 0.07% weeds. Average pre-grazing green dry matter was 1.8 ± 0.12 t DM/ha (n = 15) with no relationship ($P = 0.417$) between date and dry matter yield. There were also no relationships between date and grass yield ($P = 0.462$), with an average grass yield of 1.3 ± 0.10 t DM/ha, or between date and clover yield ($P = 0.305$), with an average clover yield of 0.51 ± 0.17 t DM/ha. There was an effect ($P = 0.039$) of date on dead material yield, with more ($P < 0.05$) dead material (1.2 ± 0.42 t DM/ha) on 5 April 2016 than on the 22, 15 and 30 April 2016, with 0.30 ± 0.059 t DM/ha, but this was not different ($P = 0.274$) to 11 May 2016 with 0.84 ± 0.225 t DM/ha.

Post-grazing, the dry matter yield of the non-dead residual was 0.54 ± 0.047 t DM/ha, with no differences (P = 0.843) among the breaks. There was no relationship (P = 0.506) between date and post-grazing grass yield with an average yield of 0.53 ± 0.046 t DM/ha. Average post-grazing clover yield was 0.008 ± 0.0032 t DM/ha with no relationship (P = 0.246) with date. There was no relationship (P = 0.066) between date and dead material yield with an average of 0.68 ± 0.112 t DM/ha. Average utilisation of non-dead material in each block was 72 ± 5.4% (n = 5), with ewes consuming an average of 0.96 ± 0.110 kg/head/d.

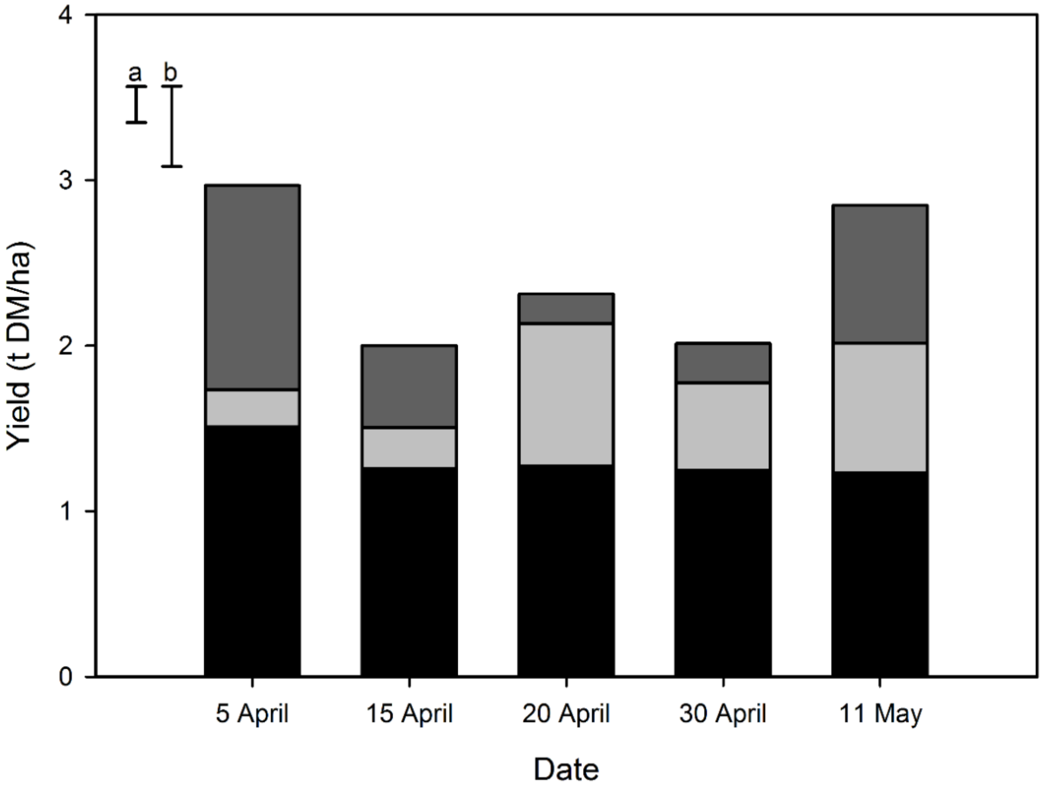

Figure 8.2 Pre-grazing yields (t DM/ha) of ryegrass (■), white clover (□) and dead material (■) in the grass pasture at the onset of each break from 5 April 2016 to 11 May 2016. Error bar (a) is the standard error of the effect (P = 0.039) of date on dead material yield. Error bar (b) is the standard error of the date versus (P = 0.417) the combined yield of ryegrass and white clover.

Nutritional quality of the pastures is provided in Table 8.1. ME was different (P < 0.001) among species. Fishers LSD showed that the highest (P < 0.002) ME levels were in ryegrass and lowest (P = 0.014) levels in white clover. There was a difference (P < 0.001) in CP among species with highest (P < 0.022) protein in lucerne, and lowest (P < 0.001) in ryegrass. There was also a difference (P < 0.001) in NDF among species. Ryegrass had a higher (P < 0.001) NDF content than lucerne or white clover. There was no difference (P = 0.292) in ADF among species. In lucerne, ryegrass and white clover there were no relationships (P > 0.1) between sampling date and ME, CP, ADF, or NDF and therefore mean values are reported.

Table 8.1 Nutritional data for the lucerne, ryegrass and white clover components of the pastures. Means within a column with letter subscripts in common were not significantly different ($\alpha = 0.05$).

Species	n	Metabolisable energy (MJ/kg DM)	Crude protein (%)	Neutral detergent fibre (%)	Acid detergent fibre (%)
Lucerne	24	11.4 ± 0.08 b	26.0 ± 0.82 a	29.6 ± 0.65 b	23.9 ± 0.60 a
Ryegrass	15	12.0 ± 0.14 a	13.8 ± 0.43 c	46.2 ± 1.16 a	25.4 ± 0.78 a
White clover	7	10.6 ± 0.54 c	22.2 ± 2.03 b	29.7 ± 1.68 b	24.6 ± 1.47 a

8.3.2 Ewe reproductive performance

Overall the ewes (n = 59) had a 5.2 ± 0.27 kg average live weight gain ($P < 0.001$) during the six weeks of the experimental period with no difference ($P = 0.431$) among treatments. At the predicted time of ovulation, 60 hours after CIDR® removal, their mean live weight was 66.1 ± 0.96 kg with no difference ($P = 0.942$) among treatments.

Due to the nature of the response variable (0, 1, 2 or 3 corpora lutea per ewe) it was considered that the appropriate method to analyse the ovulation rate data was an ordinal logistic regression (McCullagh and Nelder, 1989). An initial analysis of ovulation rate against ’grass days’, live weight at mating and change in live weight data showed no effects of live weight ($P = 0.746$) or change in live weight ($P = 0.357$) on ovulation rate, while ’grass days’ was significant ($P = 0.007$), with longer periods on grass corresponding to higher ovulation rates.

From the estimated regression coefficients of corpora lutea number against days on grass, a matrix of predicted logits were calculated for days 0 to 42. From this, cumulative probabilities were calculated and a table of probabilities produced (Table 8.2). These probabilities are also represented in Figure 8.3. Based on the predicted probabilities, ewes had a 52% chance of a single corpus luteum when on lucerne for the six weeks prior to ovulation (zero grass days). This decreased exponentially to 38% with three grass days, to 25% with seven grass days and to 16% with 14 grass days. The probability then levelled off with 11% at 42 grass days. In contrast, the probability of two corpora lutea was predicted to increase with the duration of time on grass. The probability of two corpora lutea was 42% with zero grass days, 56% with three grass days, and levelled off with 65% with seven grass days and 70% at 42 grass days. The probability of two corpora lutea levelled out due to both a levelling off in ewes expected to have a single corpus luteum and an increased probability of three corpora lutea. The probability of three corpora lutea was 2% with zero grass days, 8% with seven grass days, 16% with 21 grass days and 20% with 42 grass days.

Table 8.2 The probabilities of 0, 1, 2 and 3 corpora lutea per ewe against days on grass prior to ovulation as predicted with logistic regression.

No. of corpora lutea	Days on grass						
	0	7	14	21	28	35	42
0	0.029	0.021	0.015	0.011	0.008	0.006	0.004
1	0.403	0.333	0.267	0.210	0.161	0.122	0.091
2	0.524	0.587	0.637	0.670	0.686	0.682	0.658
3	0.043	0.059	0.080	0.108	0.145	0.190	0.246

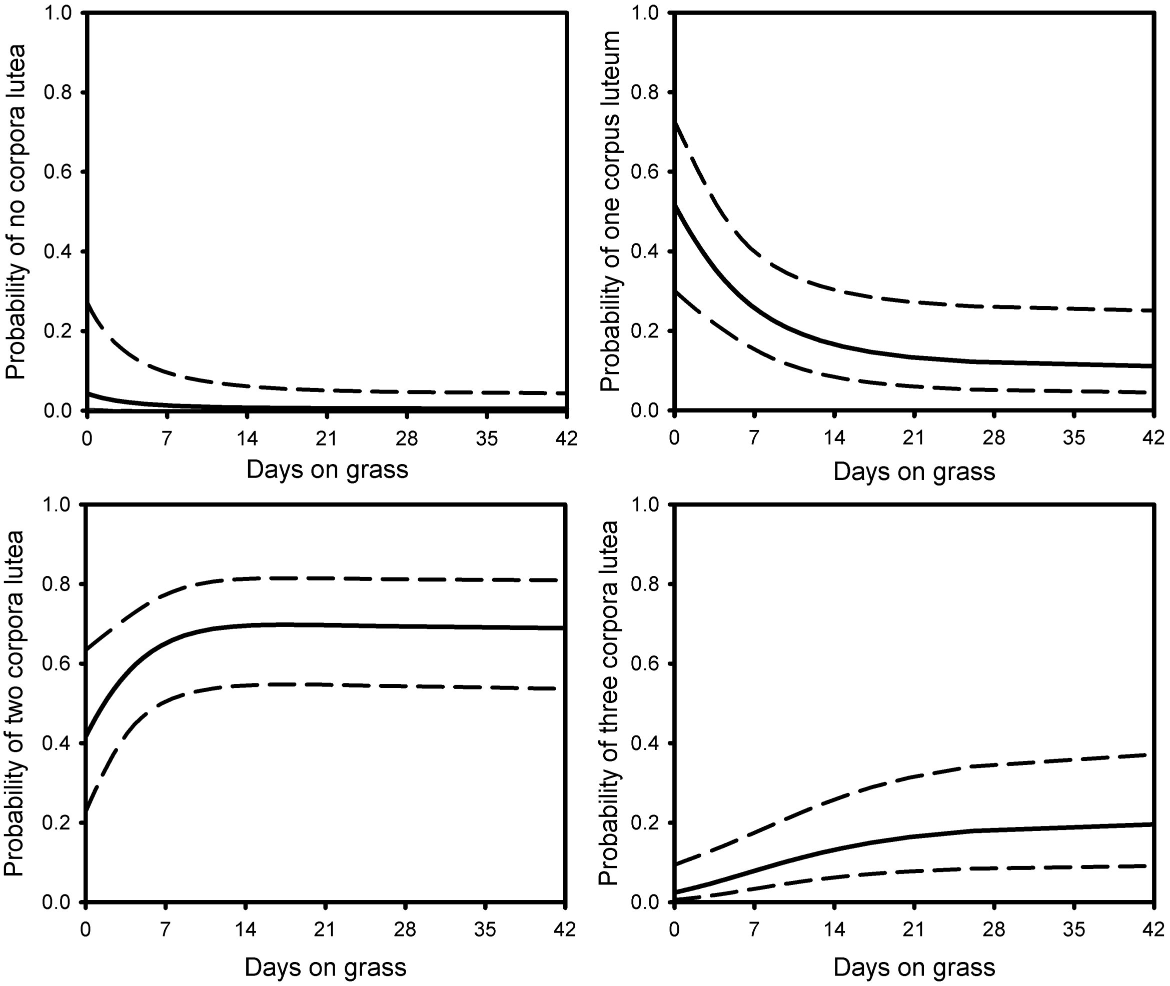

Figure 8.3 The probabilities of 0, 1, 2 and 3 corpora lutea per ewe against days on grass prior to ovulation as predicted with ordinal logistic regression. Dashed lines are 95% confidence intervals.

Overall the expected number of corpora lutea per ewe increased with time on grass (Figure 8.4). The expected number of corpora lutea increased sharply at first, followed by a levelling off. When only moderately oestrogenic lucerne was grazed, 1.42 corpora lutea per ewe were expected. This increased to 1.77 with seven grass days, and 1.93 with 14 grass days. Further time on grass had diminishing returns, with 2.01 corpora lutea per ewe expected with 21 grass days, 2.04 with 28 days, and 2.06 with 42 days.

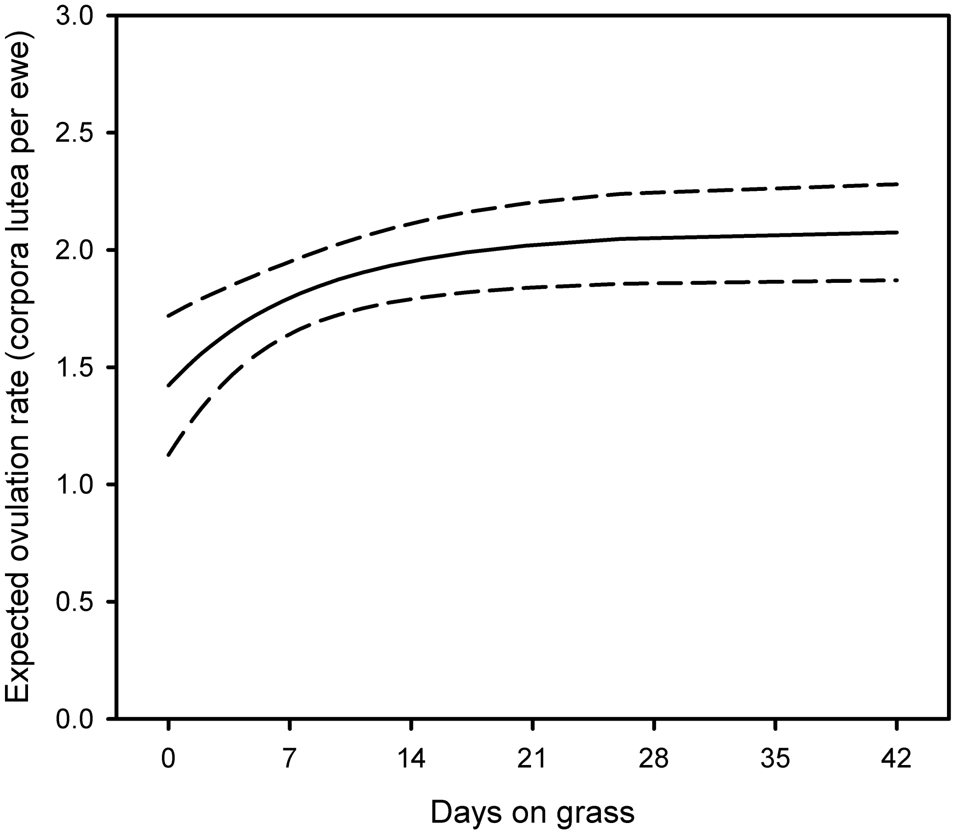

Figure 8.4 The expected number of corpora lutea per ewe against the number of days on grass prior to ovulation. The change in expected ovulation rate with days on grass is described by the equation y = 1.42 + 0.65(1 - 0.896^x). Dashed lines are 95% confidence intervals.

8.3.3 Plasma analysis

Plasma samples did not give conclusive results. Pre-treatment, free coumestrol was present in the plasma of ewes at 5 ± 1.6 µg/L (n = 8) with no difference (P = 0.187) among treatment groups. Conjugated coumestrol was present at 31 ± 11.7 µg/L (n = 12) also with no difference (P = 0.690) among groups. The standard error of conjugated coumestrol was high due to two animals with plasma levels of 98 and 127 µg/L, respectively. Without these individuals (n = 10), the mean was 15 ± 4.3 µg/L.

Following three weeks of lucerne or control treatment, on 22 April 2016 there was no difference in free coumestrol (P = 0.480) or conjugated coumestrol (P = 0.103) concentration in plasma between ewes on grass (n = 3) and lucerne (n = 9), with a mean conjugated coumestrol concentration of 24 ± 13.6 µg/L on grass and 52 ± 8.3 µg/L on lucerne. The lack of difference was again due to the wide range of conjugated coumestrol concentrations in plasma which were 5.7 - 51 µg/L in ewes on grass diets and 22 - 87 µg/L in ewes on lucerne diets. Likewise, free coumestrol concentration in plasma ranged from 2 to 3 µg/L in grass ewes and from 1 to 12 µg/L in lucerne ewes.

On 26 April, four days after the three weeks lucerne treatment were moved to grass pasture, both the grass controls and the ewes moved to grass had lower ($P < 0.05$) conjugated coumestrol concentration in plasma than the lucerne animals (16 ± 6.9 and 13 ± 10.5 µg/L (n = 3) versus 61 ± 12.5 µg/L (n = 6)). There was no difference ($P = 0.590$) in free coumestrol levels of plasma.

On 5 April, there was no difference in free ($P = 0.671$) or conjugated ($P = 0.238$) coumestrol concentrations between the ewes on lucerne and ewes moved to grass or on grass throughout. On 9 April, four days after the five week lucerne treatment were moved to grass, there remained no difference ($P = 0.823$) in the free or conjugated ($P = 0.442$) coumestrol concentrations between treatment groups. At mating, one animal on lucerne had a high plasma conjugated coumestrol concentration of 272 µg/L while the other animals had levels of 33 and 48 µg/L. There was no difference in free ($P = 0.450$) or conjugated ($P = 0.260$) coumestrol concentrations among treatment groups.

8.4 Discussion

The purpose of Experiment 13 was to determine the duration of time needed to overcome the effects of coumestrol on ewe reproductive performance. The results show that removal of ewes from an oestrogenic lucerne pasture two weeks before the mating period allowed full recovery from any negative effects of the forage on reproductive performance. Specifically, it provided data to show how rapidly this recovery occurred and, thus, the duration of time required to overcome this impairment of fecundity. These data can inform farmers who have to make management decisions about ewes grazing potentially oestrogenic lucerne in the period leading up to mating, when little other feed may be available.

Ordinal logistic regression predicted the number of corpora lutea per ewe would increase with the duration ewes were on grass prior to ovulation. This was most pronounced over the range of 0 to 14 days, when a sharp increase in the probability of multiple corpora lutea was predicted (Figure 8.4). In contrast, the probabilities levelled off when ewes were on grass for durations longer than approximately two weeks. Changes in corpora lutea probabilities as duration on grass increased were from a decreased chance of one corpus luteum and increased chance of two corpora lutea, followed by a levelling off of two and an increase in three. The results of the ordinal logistic regression indicate that recovery of ewes from the effects of lucerne is rapid, and that two weeks removal prior to mating should be sufficient, while prolonged time off lucerne (i.e. more than three weeks) is unnecessary.

Coumestrol content of the lucerne remained constant throughout the experiment (Figure 8.1). This was important as it meant that the results were not due to a sudden increase in coumestrol experienced by only a portion of the treatment groups. The coumestrol content of the lucerne crop was moderate, with approximately 30 mg/kg DM. This is above the level of 25 mg/kg DM previously reported to decrease ovulation rate in grazing ewes (Smith *et al.*, 1979) but below the levels greater than 100 mg/kg DM which have been measured in other lucerne crops (Loper *et al.*, 1967; Scales *et al.*, 1977). This means that the duration that ewes should be removed from a crop with higher levels of coumestrol may be longer and requires further research. It also highlights the importance of determining the lower threshold of coumestrol that can affect ewe reproductive performance. In addition, this experiment used 18-month-old ewes, but whether ewe lambs and older ewes are affected to the same extent by coumestrol is uncertain. A comparison of the effect of coumestrol on different age classes of ewes could benefit grazing management decisions by determining which age classes should be prioritised for removal from the lucerne.

The ryegrass-white clover pasture had a relatively constant pre-grazing dry matter yield (Figure 8.2) while lucerne yield declined over time (Section 8.3.1). The lack of growth may have been due to fall dormancy (FD) which would also have reduced shoot growth in the lucerne crop (Brown *et al.*, 2006). The lucerne cultivar ‘Stamina 5’ has a FD rating of 5 and this experiment was run from mid to later autumn. It would be expected that during this period an FD5 cultivar of lucerne would be partitioning a higher proportion of metabolites to roots than to shoot (Brown *et al.*, 2006). The legumes had a higher protein content and as shown by NDF, greater digestibility than the grass (Table 8.1). In addition, the intake of lucerne per ewe per day was greater than the intake of the ryegrass-white clover. This meant that it was possible for a difference in live weight among treatment groups to occur, affecting ovulation rates (Thompson *et al.*, 1990). However, this possibility can be dismissed as there were no treatment-related effects on live weight of the ewes in this study (Section 8.3.2). In addition, the ewes on lucerne had a lower ovulation rate than other treatments, as opposed to the higher ovulation rate that would be expected on a higher quality pasture (Thompson *et al.*, 1990). This means that the ovulation rate differences recorded here can be ascribed entirely to a non-nutritional component of the lucerne pasture. Thus, the coumestrol content of the lucerne could fully explain the lowered ovulation rate of ewes on this pasture.

Most coumestrol concentrations measured in plasma samples were in a similar range to those of previous studies. Kelly and Lindsay (1978) reported average free coumestrol of 3.7 and 8.1 µg/L in plasma of ewes on two different diets and Shutt *et al.* (1969) reported values of 5 to 7 µg free coumestrol and 12 to 40 µg conjugated coumestrol. However, in contrast to these earlier studies, the coumestrol measurements from Experiment 13 appeared to be unreliable with high variation among animals within the same group. Some levels were well above those of the other animals within a treatment group and in the previous studies, e.g. 272 µg/L in one ewe, and there was coumestrol present in the plasma of ewes grazing on the ryegrass-white clover pasture. The grass-based pasture itself had low coumestrol content, even in areas of the samples with high (> 60%) white clover presence. The variation in plasma levels was likely to be due to technical limitations either with the extraction methodology not effectively yielding the coumestrol contained in the plasma or contamination of the samples. Further research is therefore required for reliable measurement of the changes in coumestrol in plasma over time.

In this experiment, pastures were initially irrigated prior to onset of the experiment, meaning that adequate supplies of both ryegrass-white clover and lucerne were available. However, in cases where the alternative feed source is dead or of low quality, it may be preferable to retain the ewes on lucerne to maintain or increase live weight, as live weight has beneficial effects on ovulation rate that can mitigate the detriment arising from ingestion of coumestrol (Thompson *et al.*, 1990; King *et al.*, 2010).

Results from this experiment agree with previous literature containing findings that lucerne reduces ovulation rate in ewes (Scales *et al.*, 1977; Smith *et al.*, 1979; Ramòn *et al.*, 1993). The results are also in agreement with published research showing the effect on ewes to be temporary (Coop, 1977). This experiment demonstrated that the risk of impaired lambing performance due to moderately oestrogenic lucerne consumption decreased with time on grass prior to ovulation. Removal of ewes two weeks prior to ovulation sufficed to mitigate the risk of decreased lambing performance in this situation. Such knowledge may encourage farmers to use lucerne as forage for ewes closer to the onset of the mating period than has previously been considered safe. This should allow more flexible options for maintaining, or increasing, ewe live weight without compromise to their fecundity during dry seasons or in rain-fed farming environments.

Chapter 9

General Discussion

9.1 Introduction

As outlined in Section 1.1 the main aim of the studies described in this thesis was to identify the factors that increase the occurrence of lucerne with a high coumestrol content. A second aim was to develop management strategies to mitigate the risk of depressed ovulation from ewes grazing lucerne before and during mating. Therefore these studies have investigated the factors that affect coumestrol accumulation and then considered management of ewes during the mating season to avoid the suppression of ovulation rate. The practical outcome is to provide sheep farmers with confidence to incorporate lucerne into dryland farming systems.

This chapter draws the results from each of the experiments together to determine their contribution to the scientific knowledge underlying the oestrogenicity of lucerne crops in relation to ewe fecundity. The application of these results to manage the risk of coumestrol for sheep grazing in dryland systems is discussed and areas in which require further research may be of merit are suggested.

9.2 Measurement of coumestrol

Objective 1 was to refine the tools used to measure coumestrol, to ensure the accuracy of Objectives 2-4. Results of the experiments undertaken to meet Objective 1 (Chapter 3) decreased the length of time required to extract coumestrol from lucerne samples (Experiments 1-4). The results also confirmed HPLC as a precise and accurate tool to use for coumestrol measurement, with intra and inter assay coefficients of variance of 1.05% and 1.4%, respectively (Experiment 5; Section 3.3.5). HPLC directly quantified coumestrol content which allowed for results obtained in the subsequent experiments to be compared to those described in the literature (Sections 2.6 and 2.7). However, the expense of the equipment and the lack of on-demand availability would limit its use as a rapid screening tool for farmers.

A yeast bioassay, considered as a cost-effective alternative to HPLC, was capable of measuring oestrogenicity of the lucerne, but although it correlated (r = 0.873) with HPLC measurements of coumestrol (Figure 3.9), the high inter- and intra-assay coefficients of variation (56% and 26%, respectively) meant this method was unsuitable for use in Objectives 2-4.

The yeast assay may still have relevance for preliminary and rough approximations of oestrogenicity of lucerne. However, the yeast bioassay does not directly quantify coumestrol, but rather measures the oestrogenicity of the tested material. This means it would include the contribution of other oestrogenic molecules such as genistein, which is metabolised in ruminants to the non-oestrogenic compound 4-ethylphenol (Batterham *et al.*, 1965). The yeast assay would also underestimate oestrogenicity of some plant material such as subterranean and red clover because these contain formononetin which has low oestrogenicity until it is metabolised to equol (Nilsson *et al.*, 1967; Dickinson *et al.*, 1988). A further limitation of the yeast bioassay is that due to the genetically modified status of the yeast, laboratory containment is required in New Zealand, which would restrict its use as a commercial tool.

These experiments have ensured that the extraction and measurement methodology is suitable for obtaining reliable coumestrol results in Objectives 2-4. However, neither HPLC nor the yeast bioassay is sufficiently suitable for rapid turnaround of coumestrol content which is required for farmers to make everyday management decisions regarding lucerne grazing during the mating season. Therefore, determination of the agronomic factors which cause coumestrol to be elevated in lucerne (Section 9.3), and indirect management tools to predict coumestrol content of a lucerne crop or identify high crop oestrogenicity are required (Section 9.4).

9.3 Agronomic factors that increased coumestrol

The first part of Objective 2 was to isolate factors which may increase the risk of high coumestrol in lucerne. Isolation of agronomic factors in Chapter 5 confirmed that, aphids and fungi increased coumestrol content. The results also suggested that neither flowering (Experiment 6b; Section 5.1.4) nor water stress (Experiment 10; Section 5.5) cause elevation of coumestrol content in lucerne.

9.3.1 Fungal pathogens

Coumestrol content in lucerne was predominantly elevated in response to fungal infections; this was particularly of note in Experiments 6 to 8 (Sections 5.1 to 5.3). Fungal pathogens, in particular stemphylium, common leaf spot and spring black stem were present on the plants when spikes in coumestrol content occurred (Figures 5.5, 5.13, 5.17 & 5.21). In contrast, when pathogens were not present (damage score of 1) or only present at low levels (damage score of 2) the coumestrol contents were low. This was in line with previous studies in which coumestrol content tended to increase in response to fungal infection (Loper and Hanson, 1964; Hanson *et al.*, 1965; Bickoff *et al.*, 1967; Sherwood *et al.*, 1970).

The presence of the cool biotype stemphylium alone, in Experiment 6a (Section 5.1), did not appear to affect coumestrol content (Figure 5.6). In contrast, lucerne plants inoculated with stemphylium in Experiment 9 (Section 5.4) had an extreme mean coumestrol content of 190 mg/kg DM (Figure 5.24), which was higher than that observed in the majority of field samples measured in this thesis

(Sections 5.1 to 5.3). The large discrepancy between Experiment 6a and Experiment 9 could be due to differences in the severity and symptoms of the fungal infection. The inoculated lucerne of Experiment 9 had fewer delimited lesions and more widespread disease than the field grown lucerne. The coumestrol status of field-grown lucerne infected with the cool biotype stemphylium requires further research.

The level of fungal pathogen that caused at-risk coumestrol contents in lucerne (for sheep) was difficult to quantify consistently. The fungal damage score uses the pathogen n risk assessment key from James (1971) to estimate the extent of disease (Section 4.5). However, this damage score is subjective and will vary among observers. Overall, coumestrol contents measured in Experiments 6 to 8 did not strongly relate ($R^2 = 0.388$) to fungal damage scores (Figure 6.2). Based on the relationship between coumestrol content and fungal damage score, lucerne with less than 5% of area infected (damage score of 1 or 2), was consistently safe for ewes grazing during the mating season. Across all field grown lucerne with a damage score of 1 or 2 the mean coumestrol content was 12 mg/kg DM with a standard deviation of 10.1 mg/kg DM. However, lucerne with damage scores of 3 or more (over 5% area affected) may or may not be safe as the variation around the mean is large. For example, across all field grown lucerne samples with a damage score of 5 (>50% area affected) the coumestrol content had a moderately high mean of 82 mg/kg DM, but there was also a standard deviation of 55.0 mg/kg DM. Results from this research highlight the difficulty of predicting coumestrol from visual scores but also show that disease free lucerne appears to be safe to graze. As stemphylium has been shown to increase coumestrol under controlled conditions, the safest option for farmers is to consider lucerne infected by stemphylium, and all other fungal pathogens, to have heightened coumestrol content. Methods of coumestrol content prediction to supplement visual scoring are discussed in Section 9.4.

9.3.2 Aphids

Lucerne produced a low level of coumestrol (5 mg/kg DM, vs 2.4 mg/kg DM in controls) when plants were subjected to aphid herbivory for four weeks (Experiment 11; Section 5.6.3). This was in line with previous field and glasshouse studies that showed a response to aphid herbivory (Loper, 1968; Kain and Biggs, 1980). Coumestrol was sensitive to aphids with a measurable, though negligible (<1 mg/kg DM) response, due to damage by a single pea aphid on a leaf for five days. The coumestrol response was not due to a secondary infection by fungi at the site of aphid damage as no hyphae were detected in stained leaves. The low aphid levels that can cause a measureable coumestrol response are important for controlled glasshouse experiments, which could be confounded by low infestation levels. The increase in coumestrol content observed in Experiment 11 when aphid numbers were low, with either one aphid on a leaf or approximately five per stem, was not to levels that are of concern for ewe reproduction. Research from Kain and Biggs (1980) indicated that aphid numbers greater than 30 per stem could cause coumestrol to reach unsafe levels. In cases where aphid numbers are high (>30 per stem), farmers may consider removing ewes from lucerne prior to mating, even in the absence of fungal disease. However, four weeks exposure to aphids did not cause coumestrol levels of concern to sheep, which is a promising indication that aphid herbivory alone was not as important of a factor as previously thought (Kain and Biggs, 1980).

9.3.3 Development stage

That maturity of the lucerne crop did not have an effect on coumestrol content in Experiment 6b (Section 5.1) was in contrast to previous field research which has shown that flowering and seed set stages of lucerne coincide with heightened coumestrol content (Bickoff *et al.*, 1960a; Hanson *et al.*, 1965; Seguin *et al.*, 2004) and supports growth-chamber research in which coumestrol levels in lucerne were low across development stages (Loper and Hanson, 1964). The prior field research followed stands through the growing season, but did not isolate the effect of regrowth age or developmental stage. This may incorrectly have given the appearance that coumestrol was elevated during flowering. In Experiment 6a, when young and old vegetative regrowth were simultaneously compared over time in the field, coumestrol content increased at the same time (Figure 5.2). For example, between 14 March and 21 April 2014, coumestrol increased from a moderate 25 mg/kg DM to a high 140 mg/kg DM in six week old lucerne regrowth, and to a high 145 mg/kg DM in two week old lucerne regrowth (Section 5.1.3.2).

Experiment 6b showed that lucerne at vegetative or flowering stages could contain either low or high levels of coumestrol (Figures 5.3 & 5.11). Vegetative lucerne increased coumestrol content at the same time as reproductive age lucerne in stands on 25 March 2015 (Figure 5.10). There was no difference in the amount of coumestrol among plots of vegetative, flowering and seed pod lucerne, with an average coumestrol content of a moderately high 70 mg/kg DM (Section 5.1.4.2). This confirmed that the elevation was independent of development stage or crop regrowth duration.

The results of Experiment 6b mean that farmers can safely graze mating ewes on lucerne that is flowering. This is important as late summer to early autumn is not only the beginning of the mating season of sheep, but also a time when farmers are recommended to spell lucerne until flowering in order to build root reserves to improve stand persistence (Moot *et al.*, 2003b). However, it is important to bear in mind that older lucerne crops are more likely to have experienced the conditions (i.e. rainfall, high humidity) suitable for an increase in coumestrol content during the regrowth duration, and once this coumestrol is in the plant it remains there until the herbage is removed (Figure 5.2; Section 5.1).

9.3.4 Water stress

When the lucerne plants were wilted due simply to water stress under controlled conditions in Experiment 10 there was no effect on coumestrol content (Section 5.5). Severe drought conditions that resulted in complete foliage death did coincide with slightly increased coumestrol content (3.0 mg/kg DM) but this was low and well below the levels of risk to ewe reproductive performance.

A lack of coumestrol response to water stress in lucerne was observed in Experiments 6 to 8 (Sections 5.1 to 5.3). Periods and locations expected to be dry, based on soil moisture deficit models (Section 4.3), such as during the summer at Ashley Dene, did not coincide with heightened coumestrol levels. Rather, it was observed that coumestrol content sometimes increased following rainfall events. In addition, the model produced to predict coumestrol content (Section 6.3) identified the amount of rainfall during a regrowth period as a moderate predictor ($R^2_{adj.} = 0.652$) of coumestrol content. The increase in coumestrol was not due to recovery from drought. In Experiment 10 (Section 5.5), lucerne that was re-watered after it had wilted did not have increased coumestrol relative to the well-watered or water stressed plants, with negligible coumestrol levels of approximately 1 mg/kg DM (Section 5.5.3.2). This means that the recovery of water stressed plants after a rainfall event was not the cause of the increased coumestrol content. However, rainfall events can trigger germination of fungal spores of lucerne pathogens (Stuteville and Erwin, 1990), and so lucerne should be observed for disease symptoms of fungal infection following rainfall.

The finding that wilted lucerne did not have elevated coumestrol content is noteworthy, as NZ dryland farms often have a significant water deficit at the end of summer, in the lead up to the mating period. Lucerne has greater drought tolerance than other alternative pasture species; however it is not uncommon in New Zealand dryland systems for lucerne to be wilted by the end of summer, but also the only green forage available. The results of this study show that wilted lucerne which is only suffering from water stress can safely be used by ewes during the mating period.

The approach used in Objective 2 in which the different agronomic factors were isolated to determine whether that affected coumestrol content, has furthered the understanding of the processes which elevate coumestrol content of lucerne. This should encourage the incorporation of lucerne into sheep grazing systems and also enables the provision of improved management advice to farmers.

9.4 Prediction of coumestrol content

For Objective 3, a model was created to predict coumestrol content of a crop in response to different agronomic and environmental factors (Equation 6.7; Chapter 6). This model showed that coumestrol content could be estimated based on the relative humidity and/or rainfall that the crop had experienced throughout its regrowth period (Figure 6.4). This was expected as the fungal pathogens of lucerne typically infect plants when humidity is heightened (Stuteville and Erwin, 1990). However, the model was also a stronger predictor (R^2_{adj} = 0.717) of coumestrol content than the fungal damage score (R^2 = 0.388). This model may not be practical or intuitive for on-farm use, and therefore a decision tree model was created to indicate whether coumestrol was low, medium or high based on rainfall and relative humidity data (Figure 6.5). This tree model showed that when rainfall during the regrowth was less than ~60 mm and there were five or fewer days above 95% relative humidity, coumestrol content would be expected to be low. In contrast, when there were six or more days above 95% relative humidity, a moderate coumestrol content could be expected, and when rainfall during the growing period was greater than 60 mm, a moderately high to high coumestrol content could be expected.

A limitation of these models was that fungal pathogen species that were not observed or were uncommon in the experimental component of this thesis, such as rust, the warm biotype of stemphylium, anthracnose and lepto leaf spot, could have different requirements for infecting lucerne plants. For example rust, anthracnose and the warm biotype of stemphylium generally require a warm climate while lepto leaf spot, common leaf spot and spring black stem grow in cool climates (Stuteville and Erwin, 1990). These models should therefore be used in conjunction with monitoring for fungal infection on the leaves or stem of the lucerne. In addition, these models should be calibrated with data from different regions.

An additional method for predicting coumestrol levels in lucerne was identified from the results of Objective 4, which was to test the response of ewes to coumestrol. The results of this objective in Experiment 12 showed that coumestrol can cause increased teat size in ewe lambs (Figures 7.1, 7.2, & 7.4; Chapter 7). Increased teat lengths of wethers grazing lucerne and ewes grazing subterranean clover have previously been reported (Adams, 1995), but this phenomenon had not been shown in entire ewes. Mammary gland protrusion and increased teat widths were observed in Experiment 12a ewe lambs grazed on lucerne pasture with moderately high coumestrol (Figures 7.1 & 7.2; Chapter 7). Experiment 12b (Chapter 7) showed both ewe lambs and MA ewes exhibited a teat size response to coumestrol. The response to coumestrol was larger in the ewe lambs with less variation (Figure 7.4; Section 7.3.2). Therefore, to monitor the oestrogenicity of the lucerne, ewe lambs are a more reliable indicator of oestrogenic lucerne than MA ewes. Mammary development is also more likely to be detected in the ewe lambs than in ewes that have previously lambed, and therefore already have udder development. The use of this method to monitor lucerne may mean running a few ewe lambs with older ewes. Animals should be compared to cohorts that are not on lucerne, rather than observing only a group on lucerne over time, as normal growth of the ewe lambs over time produces an isometric increase in teat size (Johnsson and Hart, 1985).

Whether or not the greater response in the ewe lambs to coumestrol means that the fecundity of younger ewes is more detrimentally affected than the MA ewes is unknown and requires further investigation. This is an important avenue to explore, as it would allow farmers to prioritise which stock classes should be removed from the lucerne.

The main factors identified by the model were used to meet Objective 3, plus the insight gained from the animal studies conducted here for Objective 4, have improved the ability to make qualified judgements about managing the grazing of sheep on lucerne crops and have provided new tools for decision-making.

9.5 Mitigation of the effects of coumestrol in ewes

9.5.1 Risk reduction

The second part of Objective 2 was to identify strategies to minimise coumestrol accumulation. This was tested in field and glasshouse experiments in Chapter 5 and with the regional risk assessments created with the prediction model in Chapter 6. Possible strategies that could be implemented to minimise coumestrol accumulation are discussed in this section.

9.5.1.1 Regional strategies to reduce risk

The regional risk assessment model identified Blenheim (Marlborough) as more likely to have low coumestrol compared with Napier (Hawkes Bay), Lauder (Central Otago) and Lincoln (Canterbury) throughout the late summer to late autumn months in both four and six week regrowth lucerne (Figures 6.12 & 6.13). This shows that some regions are more or less likely to experience effects of lucerne on ewe reproductive performance, which will play a role in decision making.

For regions such as Marlborough with lower risk of heightened coumestrol and a drier autumn climate, it is likely to be more beneficial to graze ewes on lucerne for the mating period, than to move them to an alternative pasture. The normally low summer and autumn rainfall in Marlborough means that any alternative pasture at this time of year may be of poor quality. In regions with moderate to high risk, but adequate autumn rainfall such as Hawkes Bay, it may be beneficial to have alternative pasture available for mating ewes each year. The higher rainfall in Hawkes Bay means that it is less likely for an alternative feed source to be poor quality and compromise live weight. In intermediate locations, where rainfall is unpredictable or low, but risk is moderate to high, it is less likely that a high quality alternative pasture will be reliably available, and additional management is required.

Where possible, rams should be put out as early as possible in the mating season. As the autumn months progressed, median predicted coumestrol content of lucerne increased in Central Otago, Canterbury and Marlborough (Figures 6.8 & 6.9), in both four and six week old regrowth. This means that the earlier ewes are mated, the less likely they are to be affected by coumestrol. In Hawkes Bay, coumestrol risk was the same throughout the autumn period (Figure 6.17) and therefore early mating dates may not be as effective. Lambing distribution dates showed that in Canterbury and Marlborough there may be greater lee-way for farmers to mate ewes earlier in the season (Figures 6.15 & 6.16). In contrast, mating dates in Southland and Otago were concentrated late in the season (Figure 6.14). This means that earlier mating is unlikely to be feasible for these regions. However, the effect of the timing of mating is confounded by research which has shown ewe ovulation rate to increase with successive oestrous cycles in a season (Thompson *et al.*, 1990) and so the later mating date in Central Otago may not have as large of an effect on ovulation rate as expected.

9.5.1.2 Utilisation of new lucerne regrowth to reduce risk

To lower the risk of high coumestrol in lucerne, breeding ewes should be grazed on young lucerne regrowth of up to four weeks rather than the standard six weeks. Regional risk assessments (Section 6.3.6) created with the prediction model showed that the best way to reduce the risk associated with high coumestrol lucerne was by grazing when the regrowth was up to four weeks old. Across nine grazing dates and four locations, predicted coumestrol content based on long term weather data was substantially lower in four week old regrowth (20 mg/kg DM) than six week old regrowth (31 mg/kg DM) of lucerne (Figures 6.14 to 6.17). Based on the ovulation rate results of Experiment 13 (Section 8.3.2), the most important lucerne crops to have at a low-risk four weeks regrowth are the first 17 days (one oestrous cycle) after the rams are put out and the two or three weeks prior to this. It is also essential, at some point during the late summer or autumn, to give the lucerne an opportunity to recharge root reserves for spring regrowth and persistence (Moot *et al.*, 2003b). Therefore it seems sensible for a longer regrowth cycle to be used for either a prior or subsequent regrowth period.

At the end of a period of rainfall or high humidity over multiple days (Section 9.4; Equation 6.7), or when soil moisture is adequate but lucerne plants have fungal infection (Section 9.3.1), lucerne allocated for future mating ewes could be grazed off and the subsequent young regrowth instead used for mating ewes. After high coumestrol lucerne crops were mown or grazed coumestrol content was, in almost all cases, low in the regrowth in the subsequent fortnight (Figures 5.2 & 5.10). This has also been observed in previous studies (Loper and Hanson, 1964; Sherwood *et al.*, 1970). As long as little rainfall and few high humidity days subsequently occur in the crop, the regrowth should be safe for ewes. Removal of high coumestrol herbage could be by non-mating livestock or cattle. Cattle are unlikely to be affected, as coumestrol causes ovulation rate, and thus proportion of multiple births, to decrease, but does not appear to increase barrenness (Scales *et al.*, 1977; Ramòn *et al.*, 1993). However, this strategy is not recommended if soil moisture is inadequate to support new lucerne regrowth. In addition, due to the slowed growth rate of lucerne in the autumn period, the amount of feed produced in the regrowth may be compromised late in the season (Brown *et al.*, 2006), as seen in Experiment 6 (Figures 5.1 & 5.9). This should be taken into account as it is better for the ewes to have adequate lucerne available to maintain or gain weight, than for ewes to lose weight on crops with an inadequate quantity of lucerne regrowth (Thompson *et al.*, 1990). This technique is therefore most likely to be effective in the late summer and early autumn months and with adequate soil moisture.

9.5.1.3 Young lucerne crops

New lucerne crops, sown in the absence of infected lucerne debris may further reduce the risk of elevated coumestrol. In a six-month old lucerne crop, coumestrol levels ranged from 3 – 14 mg/kg DM (Figure 5.16), which was below the 25 mg/kg DM risk threshold. This was also lower than the levels measured in lucerne simultaneously growing nearby (Section 8.3) which had approximately 30 mg/kg DM throughout this period. However, this observation requires further research for confirmation.

9.5.1.4 Fungicide application

Carbendazim fungicide is not recommended for reducing the risk of coumestrol in lucerne as it was unable to control either fungal damage (Figure 5.20) or reduce the coumestrol content of lucerne (Figure 5.19) in Experiment 8 (Section 5.3). Sprays were applied while the coumestrol content was moderately low but the fungicide failed to prevent subsequent increases in coumestrol levels (Figure 5.19). In contrast, weekly fungicide applications of mancozeb and benomyl have previously been shown to reduce coumestrol accumulated in lucerne (Hanson *et al.*, 1965; Purves *et al.*, 1981). However, benomyl has been withdrawn from the New Zealand market and mancozeb is non-systemic and requires frequent, often weekly, application with high coverage on all plant surfaces. A different fungicide or a pre-emptive application, before any fungal symptoms are present could potentially be more effective and merits further research. However, this is unlikely to be suitable for most dryland lucerne systems, where confining applications only to affected stands on an as-needed basis would be more cost-effective and reduce the risk of the pathogens gaining fungicide resistance.

9.5.1.5 Cultivar

The use of newer lucerne cultivars currently on the market is unlikely to reduce the risk of heightened coumestrol. There was no effect of the five cultivars compared at Ashley Dene on coumestrol content in Experiment 7a (Section 5.2). All cultivars produced moderate to moderately high coumestrol levels, above the 25 mg coumestrol/kg that is considered detrimental to mating performance (Figure 5.15). Of interest was Experiment 9 where ‘Wairau’ and ‘Stamina 5’ plants were inoculated with stemphylium (Section 5.4). There was no difference in entire ‘Stamina 5’ and

‘Wairau’ shoots, with an extreme average coumestrol content of 169 mg/kg DM compared with a low 3 mg/kg DM in uninfected controls (Figure 5.24). However, in leaves which were needle-damaged prior to inoculation, ‘Stamina 5’ had an extreme coumestrol content of 400 mg/kg DM compared with ‘Wairau’ levels which were lower, but still high, at 140 mg/kg DM (Figure 5.27). This result was despite the fact that ‘Stamina 5’ is regarded as a resistant cultivar to disease and aphids (Table 5.1) while ‘Wairau’ is regarded as a susceptible. This was unexpected as prior studies have reported less coumestrol accumulation in resistant than susceptible cultivars (Loper *et al.*, 1967; Loper, 1968; Purves *et al.*, 1981).

In response to aphids in Experiment 11 (Section 5.6), there was no difference in coumestrol content among the different cultivars. ‘Grasslands Kaituna’ and ‘Force 4’ are generally regarded as resistant cultivars (Table 5.1) relative to ‘Wairau’. Coumestrol levels were relatively low (5 mg/kg DM) however and thus a higher aphid pressure may produce a differential response.

The six month old lucerne stand with 10 different cultivars in Experiment 7b (Section 5.2) had low to moderately low levels of coumestrol but a difference was detected (Figure 5.16), with higher coumestrol levels in ‘Venus’, and lower coumestrol in ‘Force 4’. This stand was only sampled on two dates and thus the cultivar difference could be an anomaly, but means that there is possibly genetic potential to reduce coumestrol content.

Data from cultivars grown in New Zealand and the United States in the 1960s and 1980s, which were used to validate the prediction model, had reasonable correlation and fit (r = 0.813; RMSD = 24.9 mg/kg DM) with the model. Despite underestimation of independent coumestrol data by a factor of approximately 1.4 compared with the prediction model created with recent cultivars, the independent values were not dissimilar to the spread within the data set used to create the original model. This provides further evidence that the current cultivars still produced a comparable coumestrol response to the older ones.

That modern cultivars continued to produce at-risk levels of coumestrol highlights the imperative for crop breeders to reduce the risk of a coumestrol response by focusing on resistance to leaf spot and stem diseases, such as stemphylium, common leaf spot, and spring black stem. These diseases may have been neglected from previous breeding efforts as regrowth yield tends to be unaffected by their presence and stand persistence is not substantially hampered. Most cultivars do not have resistance reported for these pathogens, or simply have resistance to ‘leaf diseases’ reported (Table 5.1). It may be that these diseases are not perceived to cause major effects on plant production, although yield losses of over 40% have been previously reported with both common leaf spot (Morgan and Parbery, 1977) and spring black stem (Hijano, 1981). The present results can be used to highlight that the effect of coumestrol on ewe production alone warrants the inclusion of resistance to these diseases in lucerne breeding programmes.

9.5.2 Risk removal

As part of Objective 4, which was to test the response of ewes to coumestrol, the recovery of reproductive performance of ewes following ingestion of lucerne was assessed in Experiment 13 (Chapter 8).

Results from Experiment 13 showed that when the coumestrol content of lucerne was predicted to be elevated (as described in Sections 9.3 & 9.4) removal of ewes from moderately oestrogenic lucerne two weeks prior to ovulation will avoid the risk of decreased ovulation rates in ewes (Section 8.3.2).

The levels of coumestrol in the lucerne crop for this experiment were a moderate 30 mg/kg DM (Figure 8.1). This was similar to the level of 25 mg/kg DM reported by Smith *et al.* (1979) to cause decreased reproductive performance, but below the extreme levels of 150 – 200 mg/kg DM measured in the autumn 2014 cutting frequency experiment (Figure 5.2). Further research to determine whether the withholding period should be increased if the lucerne is at higher levels, and the effect of coumestrol on different age classes of ewes is required to provide further benefit for grazing management decisions.

From a pragmatic point of view, ewes should be removed from lucerne only if there is a sufficient quantity of a quality alternative feed available. This is because it is likely to be more beneficial for ewes to gain or maintain weight on oestrogenic lucerne pastures, rather than losing weight on a lower quality feed (King *et al.*, 2010). There is a strong relationship between live weight and ovulation rate (Figure 7.3) which means that a gain in live weight could compensate for the potential negative effects of coumestrol.

9.6 Recommendations for future research

The findings of the studies presented in this thesis highlighted areas that will require further research to understand the factors causing elevation of coumestrol levels in lucerne and its effects on ewe reproductive performance. These include:

- Determine the length of time ewes should be removed from lucerne with coumestrol over 100 mg/kg DM.
- Determine the effects of coumestrol on the reproductive performance of different age classes of ewes.
- Improve calibration of the coumestrol prediction model for different regions by collecting data across New Zealand and abroad.
- Isolate the effect of stand age on coumestrol content.
- Isolate the cultivar response using a wider range of cultivars.
- Develop lucerne cultivars resistant to fungal pathogens such as spring black stem and common leaf spot.

9.7 Conclusions

The research described in this thesis has identified when coumestrol is likely to be elevated in lucerne. The research has also provided guidelines on how long ewes should be removed from lucerne to recover from the detrimental effects of coumestrol on ovulation rate. Specific conclusions include:

- HPLC is a more reliable method than the yeast assay for determining coumestrol content.
- Fungal diseases are the main cause of increased coumestrol content, and fungal diseases appear to occur following at least six days with average humidity above 95% during the re-growth period of a crop.
- Current lucerne cultivars on the New Zealand market remain capable of producing high levels of coumestrol in the presence of fungal pathogens.
- Lucerne development stage does not affect coumestrol content.
- Water stress and recovery from water stress do not affect coumestrol content.
- High coumestrol lucerne can be identified by observing ewe lambs on the pasture and seeing if udder protrusion occurs, or if teat size is larger than equivalent animals on grass based pasture.
- Ewes should be mated early in autumn on young lucerne regrowth to reduce the likelihood of being affected by elevated coumestrol levels.
- In cases where the coumestrol content of lucerne is elevated to a moderate level, removal of the ewes two weeks before mating is sufficient for them to recover from the ovulation supressing effects of coumestrol.

Appendix A

Meteorological conditions

A.1 Weather during Iversen 12 cutting frequency experiment (first season)

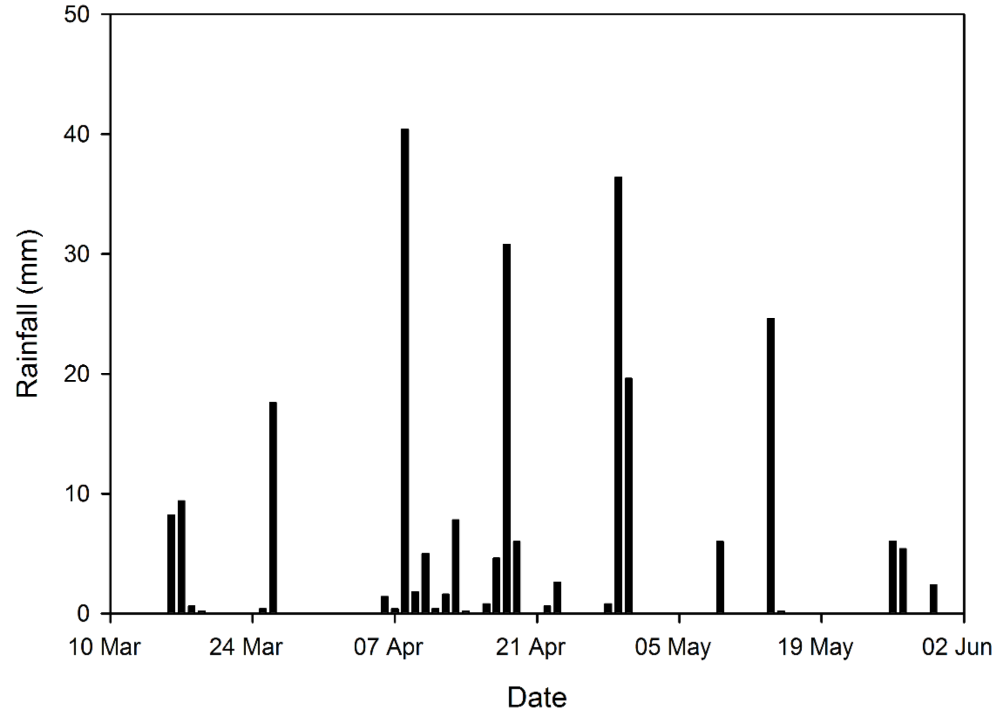

Figure A.1 Rainfall (mm) in Iversen 12 during the first cutting frequency experiment from 10 March to 2 June 2014 recorded at Broadfield EWS (NIWA).

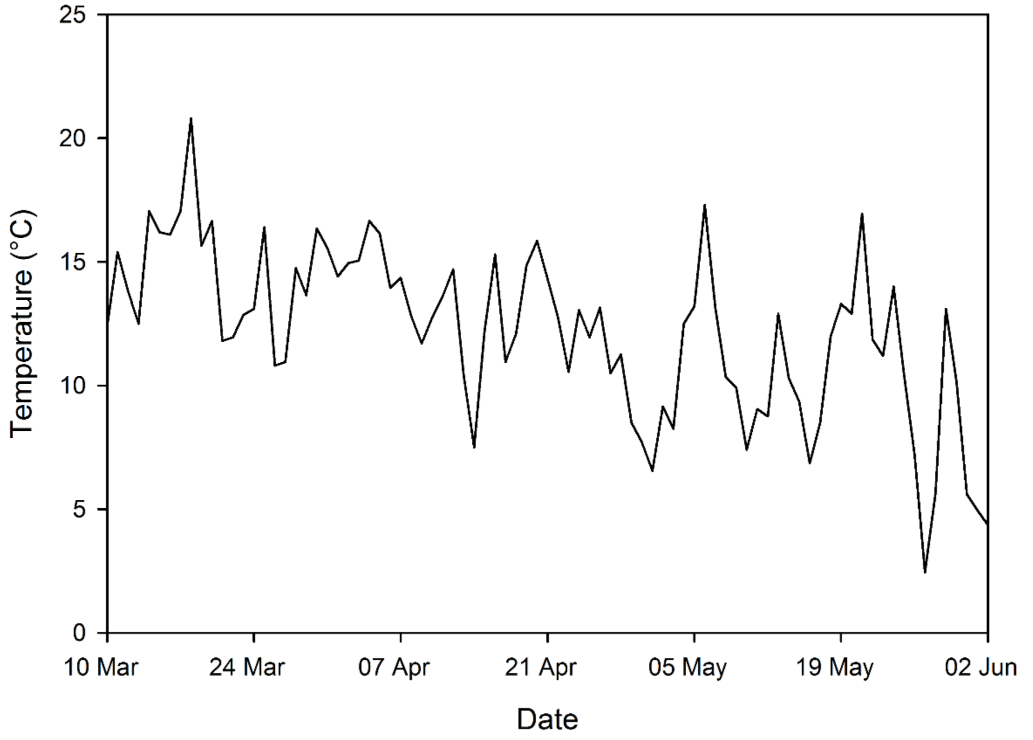

Figure A.2 Mean daily temperature (°C) in Iversen 12 during the first cutting frequency experimental period from 10 March to 2 June 2014 recorded at Broadfield EWS (NIWA).

A.2 Weather during Iversen 12 cutting frequency experiment (second season)

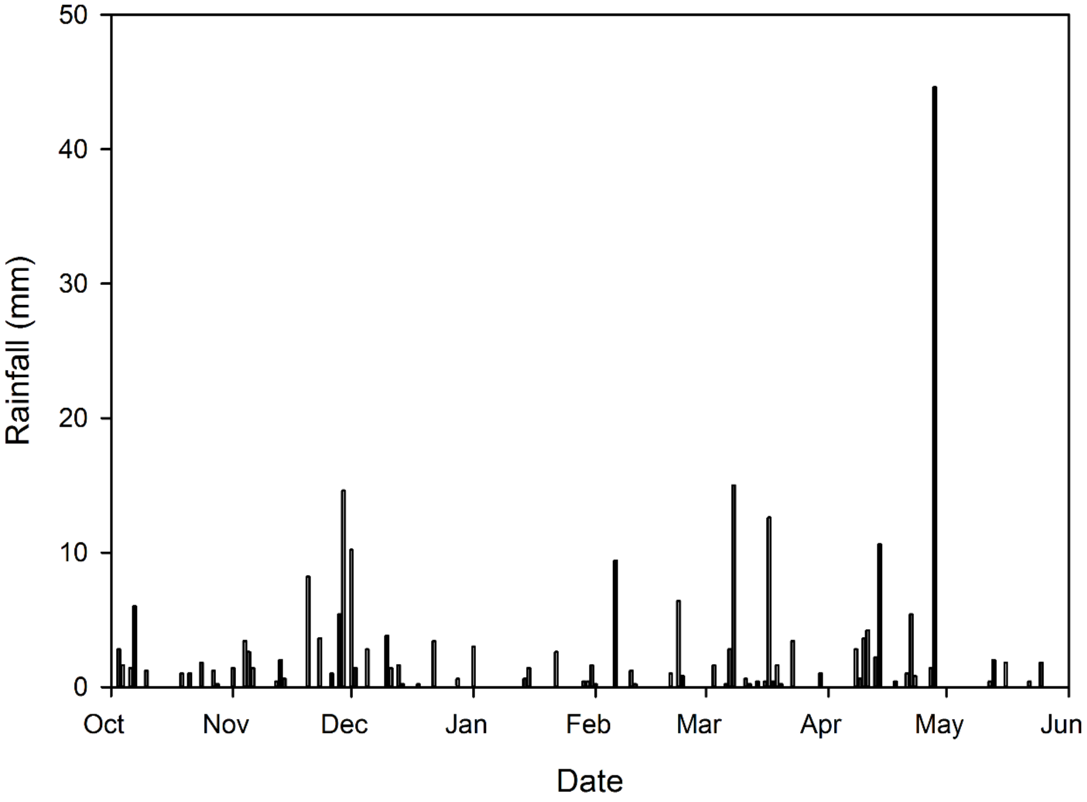

Figure A.3 Rainfall (mm) in Iversen 12 during the second cutting frequency experiment from 1 October 2015 to 31 May 2015 recorded at Broadfield EWS (NIWA).

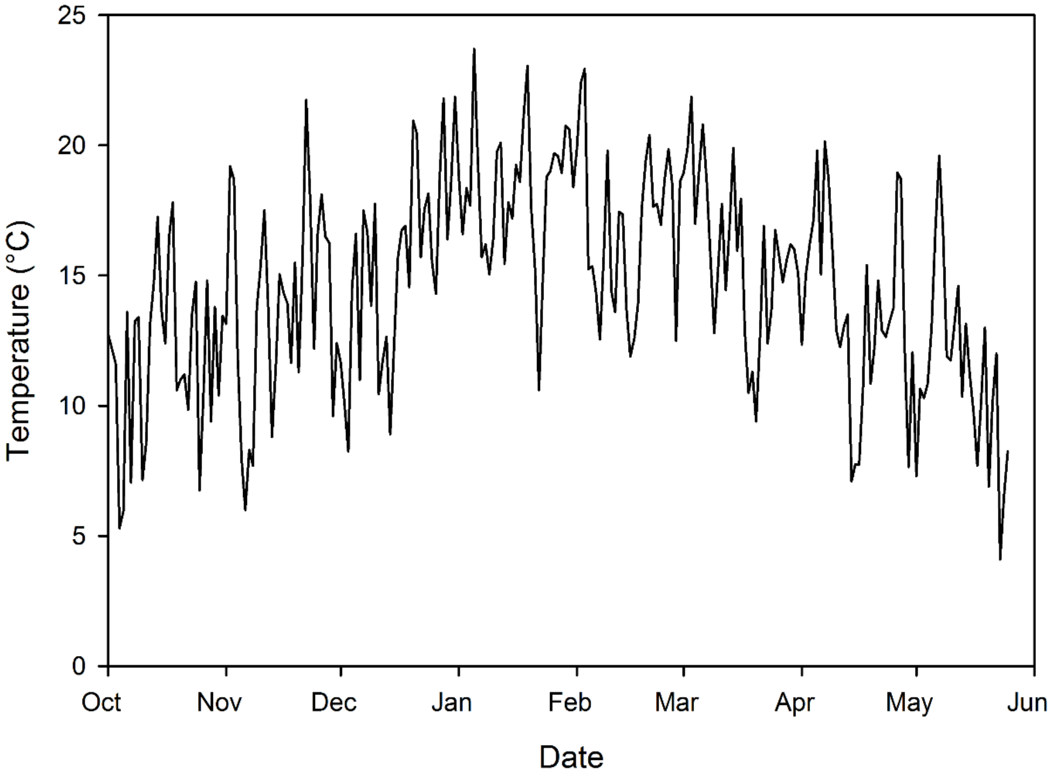

Figure A.4 Mean daily temperature (°C) in Iversen 12 during the second cutting frequency experiment from 1 October 2015 to 31 May 2015 recorded at Broadfield EWS (NIWA).

A.3 Weather during Ashley Dene cultivar experimental period

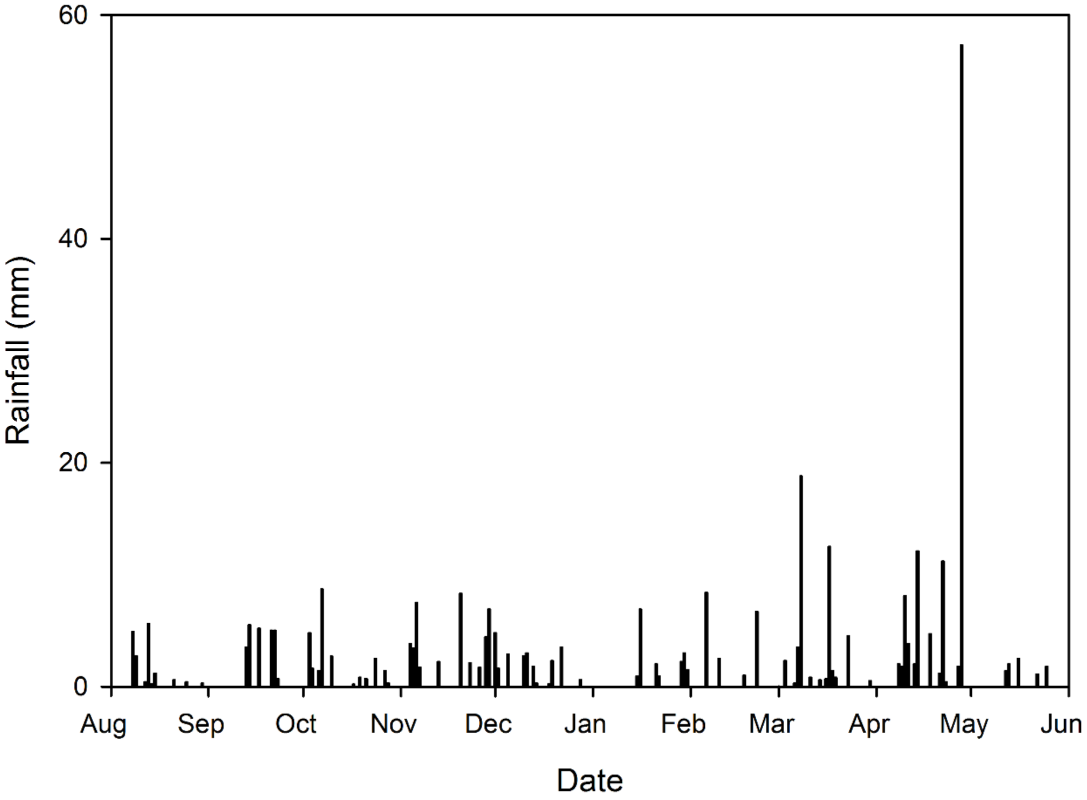

Figure A.5 Rainfall (mm) during the Ashley Dene cultivar experiment from 1 August 2014 to 25 May 2015 recorded at Burnham Sewage Plant (NIWA).

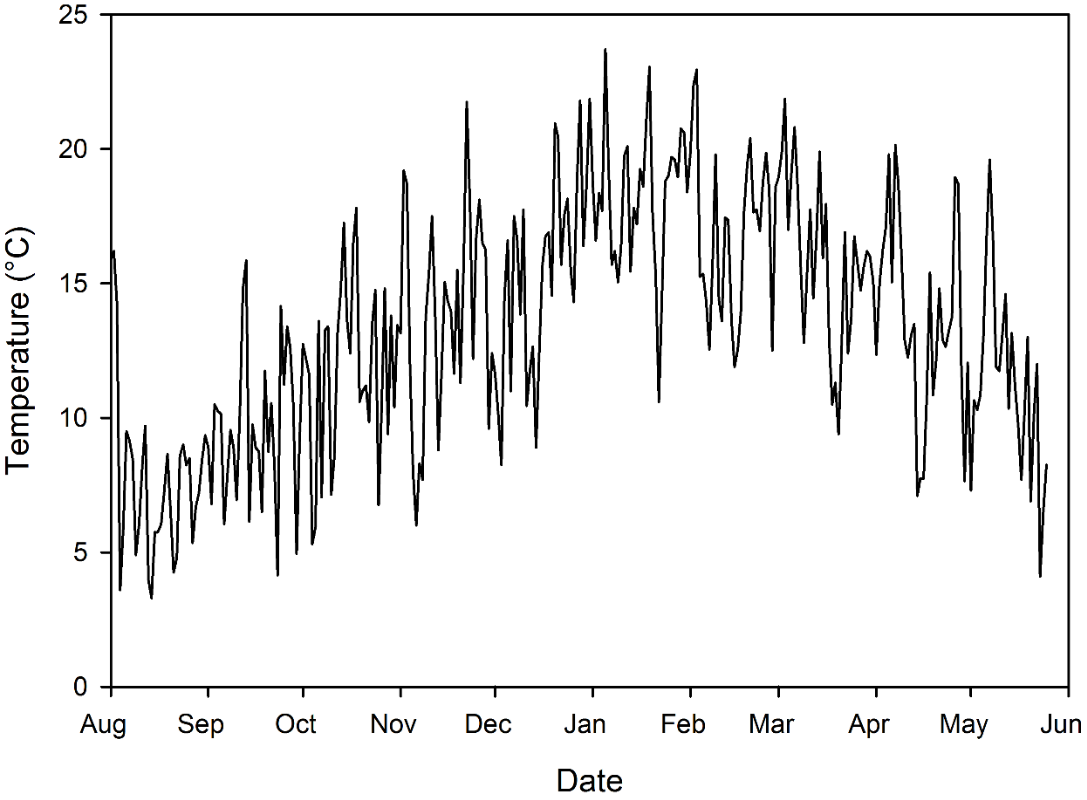

Figure A.6 Mean daily temperature (°C) during the Ashley Dene cultivar experiment from 1 August 2014 to 25 May 2015 recorded at Broadfield EWS (NIWA).

A.4 Weather during Iversen 12 fungicide and insecticide experimental period

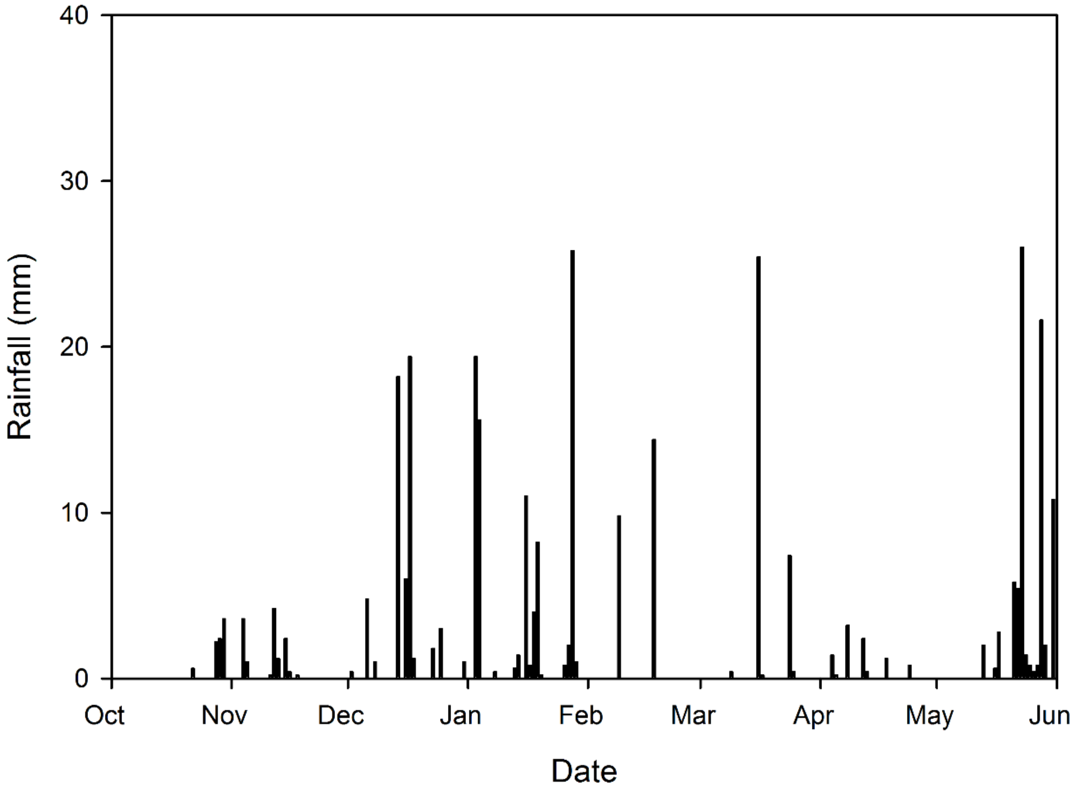

Figure A.7 Rainfall (mm) during Iversen 12 fungicide and insecticide experiment from 1 October 2015 to 31 May 2015 recorded at Broadfield EWS (NIWA).

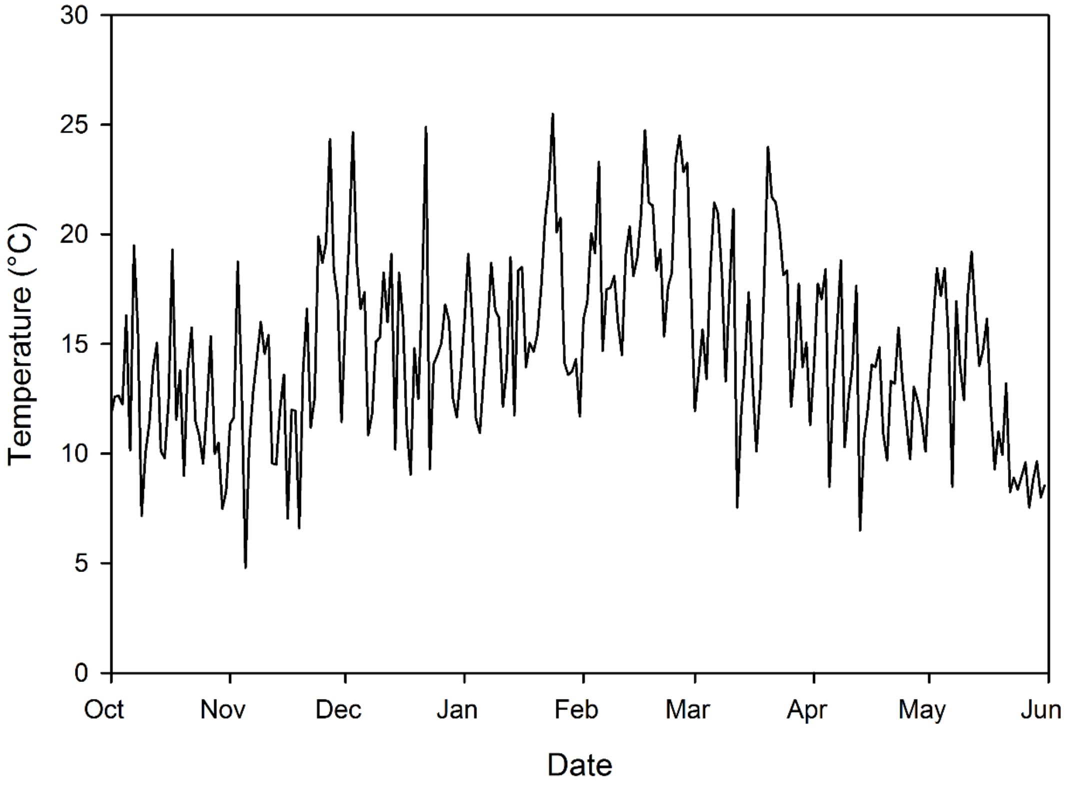

Figure A.8 Mean daily temperature (°C) during Iversen 12 fungicide and insecticide experiment from 1 October 2015 to 31 May 2015 recorded at Broadfield EWS (NIWA).

A.5 Weather during Iversen Field ewe fecundity grazing experiment

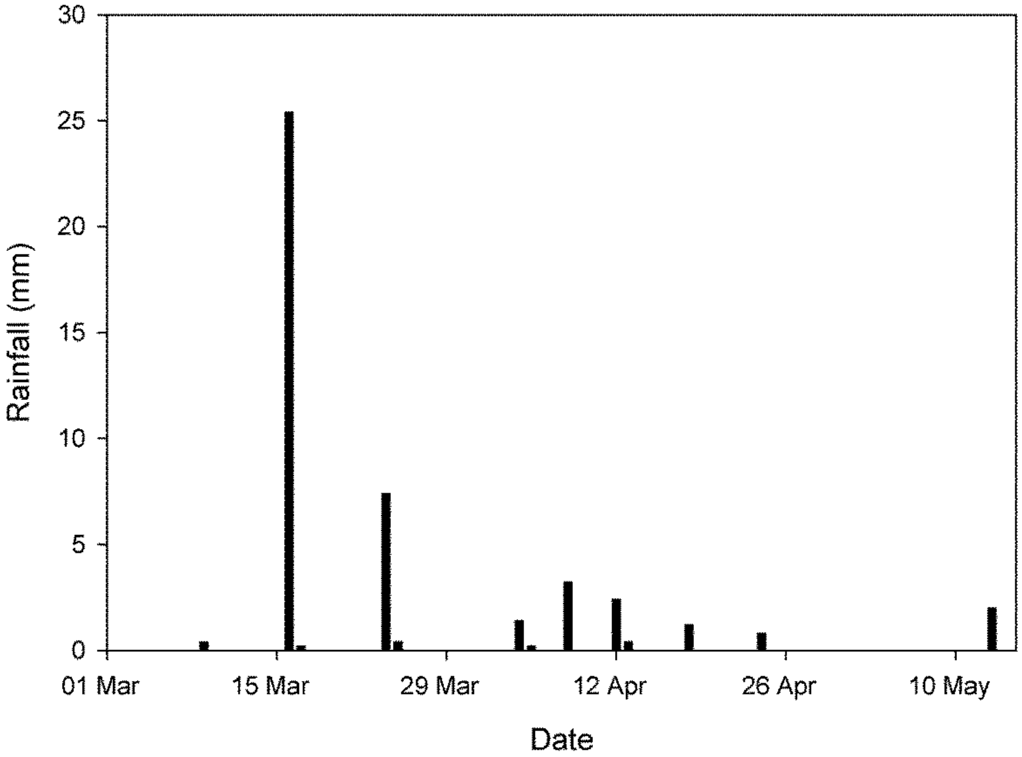

Figure A.9 Rainfall (mm) in Iversen Field during the ewe fecundity grazing experiment from 01 March to 15 May 2016 recorded at Broadfield EWS (NIWA).

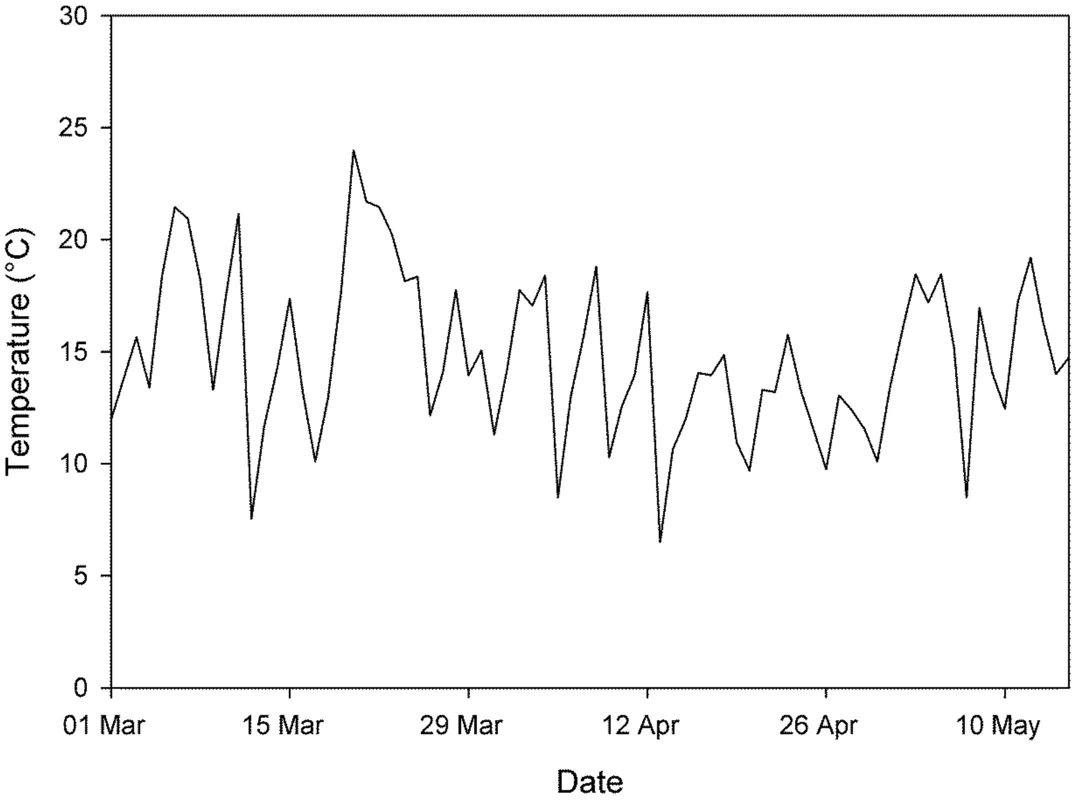

Figure A.10 Mean daily temperature (°C) in Iversen Field during the ewe fecundity grazing experiment from 01 March to 15 May 2016 recorded at Broadfield EWS (NIWA).

Appendix B

Growth media for yeast assay (Experiment 5)

B.1 Media solution

The media solution was made of six solutions, stored separately to lower contamination risk and allow replacement of the individual parts in the event of contamination. The solutions were combined at the time of inoculation with yeast and as required to subculture the yeast. The solutions and the volume of each used in the growth media are:

- 22.5 mL of minimal media. The minimal media contains nutrients N, P, K and S, Mg and Fe, and a range of amino acids,
- 2.5 mL of 20% w/v glucose solution
- 625 µL 30 mM L-aspartic acid solution
- 250 µL 0.2 M L-threonine solution
- 200 µL of a vitamin solution containing thiamine, pyridoxine, HCl, D-panthothenic acid, myo-inositol, and biotin (Section B.1.2),
- 62.5 µL 20 mM $CuSO_4$ solution, which acts as a selective agent.

This was the volume that yeast was grown in prior to assays and was also equivalent to just over one 96 well plate assay.

All laboratory chemicals for the yeast assay were analytical grade from Sigma-Aldrich (Missouri, USA).

B.1.1 Minimal media

To make the minimal media 800 mL of DI water was placed on a heated stirrer and the chemicals given in Table B.1 were dissolved. Final volume was made up to 1 L, and media was autoclaved and stored at room temperature.

Table B.1 Chemicals in minimal media

Chemical	Amount	Concentration	Chemical	Amount	Concentration
KH_2PO_4	13.6 g	100 mM	L- methionine	20 mg	20 µg/mL
KOH (pellets)	4.2 g	75 mM	L-tyrosine	30 mg	30 µg/mL
$(NH_4)_2SO_4$	1.98 g	15 mM	L-isoleucine	30 mg	30 µg/mL
$MgSO_4.7H_2O$	0.41	1.7 mM	L-lysine.HCl	30 mg	30 µg/mL
2 mM $Fe_2(SO_4)_3^*$	1.0 mL	2 µM	L-phenylalanine	25 mg	25 µg/mL
L-leucine	50 mg	50 µg/mL	L-glutamic acid	100 mg	100 µg/mL
L-histidine	50 mg	50 µg/mL	L-valine	150 mg	150 µg/mL
adenine	50 mg	50 µg/mL	L-serine	375 mg	375 µg/mL
L-arginine	20 mg	20 µg/mL			

*2mM $Fe_2(SO_4)_3$ solution made with dissolved $Fe_2(SO_4)_3$ in sterile DI water at ratio of 20 mg per 25 mL. Solution was used to make minimal media and then discarded.

B.1.2 Vitamin solution

Vitamin solution was made with 8 mg thiamine, 8 mg pyridoxine. HCl, 8 mg D-panthothenic acid, 40 mg myo-inositol in 180 mL DI water and 20 mL of 20 µg/mL biotin. The vitamin solution was filter sterilised and stored as 10 mL aliquots at 4°C.

B.1.3 Other solutions

- The 20% (w/v) glucose solution was made with 20 g of D(+) glucose dissolved in 70 mL DI water. This was then made up to 100 mL, autoclaved and stored at room temperature.

- The 30 mM L-aspartic acid solution was made with L-aspartic acid at a ratio of 4 mg per mL of DI water, autoclaved and stored at room temperature.

- The 0.2 M L-threonine solution was made with L-threonine at a ratio of 2.4 g per 100 mL, autoclaved and stored at room temperature.

- The 20 mM $CuSO_4$ solution was made with 0.5 g of $CuSO_4.5H_2O$ dissolved in 100 mL of DI water. The solution was filter sterilised and stored at 4°C.

Appendix C

Supplementary information for Chapter 6

C.1 Coefficient selection for coumestrol model

Table C.2 The initial coefficients used to predict coumestrol content of lucerne. *F*-statistics, *P*-values and VIF are from the ANOVA of a regression model which contained all terms. The R^2_{adj} of the regression is 0.779 and includes all 16 terms. Degrees of freedom was 288 total, with error of 272. The predictor R^2 values are based on the regression between each individual predictor and coumestrol content. Coefficients are ranked by F-value.

Term	*F*-statistic	*P*-value	VIF	R-Sq
Regression	62.90	< 0.001		(0.779)
Days above 95% RH	27.52	< 0.001	36.47	0.733
Sum of rainfall (mm)	26.41	< 0.001	11.71	0.635
Development stage	15.97	< 0.001	9.41	0.009
Soil moisture deficit (%)	11.42	0.001	7.76	0.115
Dry matter yield (t DM/ha)	10.30	0.001	9.34	0.025
Days above 70% RH	5.25	0.023	132.10	0.205
Days above 90% RH	5.05	0.025	209.42	0.658
Height	5.45	0.020	11.89	<0.001
Average sunlight radiation (MJ/m^2)	3.81	0.052	35.43	0.209
Average temperature (°C)	1.56	0.213	15.54	0.057
Average daily sunshine hours	1.48	0.225	21.53	0.308
Month	1.72	0.191	4.89	0.002
Days above 80% RH	1.05	0.306	334.98	0.461
Days above 85% RH	1.03	0.312	303.04	0.519
Date of harvest	0.46	0.496	6.47	0.085
Days in growing period	0.57	0.451	50.72	0.129
Error	272			
Total	288			

C.2 Model 1 Best subsets regression analysis

Table C.3 The first four levels of predictors identified via best subsets regression as best relating to coumestrol content in lucerne. For clarity, terms that were not identified by best subsets regression were not included in this table.

Number of coefficients	R-Sq (adj.)	R-Sq (pred.)	Mallows CP	Standard error of regression	SMD (%)	Days >95% RH	Days in growing period	Sum of rainfall (mm)	Average temperature (°C)	Daily sunshine hours
1	73.5	73	41.3	23.88		X				
1	65.2	64.6	143.4	27.37				X		
2	75.4	74.9	18.0	22.98		X		X		
2	73.8	73.3	38.3	23.74		X				X
3	75.8	75.2	14.4	22.80	X	X		X		
3	75.5	74.9	18.0	22.94		X		X	X	
4	75.9	75.3	13.7	22.74	X	X	X	X		
4	75.8	75.1	14.9	22.79	X	X		X		X

C.3 Model 2 Best subsets regression analysis

Table C.4 The first three levels of predictors identified by best subsets regression as relating to coumestrol content in lucerne. For clarity terms not identified by best subsets regression are not included in this table.

Variables	R-Sq (adj.)	R-Sq (pred.)	Mallows CP	S	SMD (%)	Days >95% RH	Sum of rainfall (mm)	Days above 95% RH (Fortnight prior)	Sum of rainfall (Fortnight prior)
1	60.0	59.0	93.6	24.09			X		
1	57.7	56.7	112.0	24.78		X			
1	50.1	49.2	172.4	26.92					X
2	68.5	67.5	27.8	21.40		X	X		
2	63.7	62.7	64.9	22.94		X			X
2	63.1	62.1	69.9	23.15			X	X	
3	70.7	69.7	10.9	20.62	X	X	X		
3	69.1	68.1	23.5	21.18		X	X	X	
3	69.0	68.0	24.2	21.21	X		X	X	

C.4 Independent data used to validate prediction model

Table C.5 The independent data used to validate the prediction model from a range of different locations, years, publications and regrowth ages. The weather stations used were the nearest from the site that recorded relative humidity and/or rainfall. This table provides the approximate distance from the field site to the weather station(s), the climate data for number of days above 95% relative humidity, sum of rainfall during the regrowth period, predicted coumestrol and average coumestrol content for that date. These climate data were used with Equation 6.3 to predict coumestrol.

Location	Harvest date	Growth days	Weather station[3] (RH/Temp)	Station distance (km)	Sum Rainfall (mm)	Days of 0900 h humidity ≥95%	Predicted coumestrol content (mg/kg DM)	Average reported coumestrol (mg/kg DM)
Logan, Utah[1]	30/06/1961	14	Hills AFB/ Logan-Cache	80/near	1.3	0	-12.5	6.3
Logan, Utah[1]	27/07/1961	42	Hills AFB/ Logan-Cache	80/near	16.5	0	-8.4	4.3
Logan, Utah[1]	6/08/1961	52	Hills AFB/ Logan-Cache	80/near	16.5	0	-8.4	7
Logan, Utah[1]	21/08/1961	67	Hills AFB/ Logan-Cache	80/near	25.5	0	-5.9	9.8
Brookings, South Dakota[1]	3/09/1963	50	Sioux Falls/ Brookings NE	90/near	260	2	66.3	80
Brookings, South Dakota[1]	13/09/1963	60	Sioux Falls/ Brookings NE	90/near	263.9	2	67.4	103.3
Lincoln, NZ[2]	7/03/1980	24	Lincoln	<5	74.6	4	25.7	12.0
Lincoln, NZ[2]	14/03/1980	31	Lincoln	<5	107.4	6	43.7	27.8
Lincoln, NZ[2]	21/03/1980	38	Lincoln	<5	113.5	6	45.3	81.5
Lincoln, NZ[2]	28/03/1980	45	Lincoln	<5	125.2	6	48.5	51.9
Lincoln, NZ[2]	28/03/1980	20	Lincoln	<5	50.6	2	10.0	10.2
Lincoln, NZ[2]	3/04/1980	51	Lincoln	<5	143.4	6	53.4	79.6
Lincoln, NZ[2]	3/04/1980	26	Lincoln	<5	60.7	2	12.7	19.4
Lincoln, NZ[2]	11/04/1980	59	Lincoln	<5	163.4	8	68.0	130.6
Lincoln, NZ[2]	11/04/1980	34	Lincoln	<5	68.8	4	24.1	62.0
Lincoln, NZ[2]	17/04/1980	65	Lincoln	<5	145.7	10	72.4	134.9
Lincoln, NZ[2]	17/04/1980	40	Lincoln	<5	71.1	6	33.9	109.3
Oamaru, NZ	12/03/2015	56	Windsor	22	55	10	48.0	76.9
Lincoln, NZ	1/04/2016	25	Broadfield EWS	2	33.8	5	19.3	17.6
Lincoln, NZ	11/04/2016	35	Broadfield EWS	2	38.6	5	20.6	33.5
Lincoln, NZ	22/04/2016	46	Broadfield EWS	2	42.6	7	30.9	38.5

Coumestrol data sourced from: [1]Hanson *et al.* (1965), [2]Purves (1981).

[3]Meteorological data from New Zealand retrieved from CliFlo, US data from Utah Climate Center.

Appendix D

Sequence data from Experiment 9

Table D.6 ITS nucleotide sequence from *Stemphylium* sp.

```
1       CAATATGAAA GCGGGGTTGG GACCTCACCT CGGTGAGGGC TCCAGCTTGT
51      CTGAATTATT CACCCATGTC TTTTGCGCAC TTCTTGTTTC CTGGGCGGGT
101     TCGCCCGCCA CCAGGACCAA ACCATAAACC TTTTTGTAAT TGCAATCAGC
151     GTCAGTAAAC AATGTAATTA TTACAACTTT CAACAACGGA TCTCTTGGTT
201     CTGGCATCGA TGAAGAACGC AGCGAAATGC GATACGTAGT GTGAATTGCA
251     GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC CCTTTGGTAT
301     TCCAAAGGGC ATGCCTGTTC GAGCGTCATT TGTACCCTCA AGCTTTGCTT
351     GGTGTTGGGC GTCTTTGTCT CTCACGAGAC TCGCCTTAAA ATGATTGGCA
401     GCCGACCTAC TGGTTTCGGA GCGCAGCACA ATTCTTGCAC TTTGAATCAG
451     CCTTGGTTGA GCATCCATCA AGACCACATT TTTTTCAACT TTTGACCTCG
501     GATCAGGTAG GGATACCCGC TGAACTTAAG C
```

Table D.7 β-tubulin gene sequence from *Stemphylium* sp.

```
1       GTTGTCGGGG CGGAACAGCT GGCCGAAGGG GCCAGCGCGG ACGGCGTCCA
51      TGGTACCGGG CTCGAGGTCA ACGAGAACGG CACGGGGCAC GAACTTGTTG
101     TTGGAGGCCT GCTGTATATC AGTATTGGTC TTTTATCTGT TGCATGCGTA
151     GTCGAGTGAC ATACTTCGTT GAAGTAGACG TTCATGCGCT CGAGCTGGAG
201     GTCCGAGGTA CCATTGTAGA CACCGGAGCC GTCGAGGCCA TGCTCGCCGG
251     AGATGGTCTG CCAGAAGGCG GCACCGATTT GGTTACCCTG CGATGGTGGT
301     TAGCAATCGT CTCCATGGCG CCGTGTGAAG GGGGGAAGAG CTTACGCATT
351     GACCGGTCTG AAGGTGAACC TGTGGGAGAG AATCGTCGTT AGCTGTCTGG
401     TTCGCAATCT CTTGTTGTCC CTGAATGCAG CCTATCGCGC GTTGTTGCCC
451     CGCGCCCCGC
```

Table D.8 ITS nucleotide sequence from *Colletotrichum* sp.

```
1       CCCTTTGTGA CATACCTTAA CTGTTGCTTC GGCGGGCAGG AGGACAACCC
51      CCCCTCGGGG GGGCGGTCCC CCTCCCGGCC GCGCCCTCAC GGGCGTGGCG
101     CCCGCCGGAG GATACCAAAC TCTATTTTAA CGACGTTTCT TCTGAGTGGC
151     ACAAGCAAAT AATTAAAACT TTTAACAACG GATCTCTTGG TTCTGGCATC
201     GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG
251     TGAATCATCG AATCTTTGAA CGCACATTGC GCCCGCCAGC ATTCTGGCGG
301     GCATGCCTGT TCGAGCGTCA TTTCAACCCT CAAGCCCGGC TTGGTGTTGG
351     GGCCCTACGG TCGACGTAGG CCCTTAAAGG TAGTGGCGGA CCCTCCCGGA
401     GCCTCCTTTG CGTAGTAACT TAACGTCTCG CACTGGGATC CGGAGGGACT
451     CTTGCCGTAA AACCCCCAAA ACTTTTACAG GTTGACCTCG GATCAGGTAG
501     GAA
```

Table D.9 β-tubulin gene sequence from *Colletotrichum* sp.

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1       TTGTCGGGGC GGAAGAGCTG GCCGAAGGGG CCAGCACGGA CAGCGTCCAT
51      GGTACCGGGC TCCAAGTCGA CGAGGACAGC GCGGGGGACA TACTTGTTGC
101     CGGAAGCCTG GTTAAGGGAG AAGGTCAGTA TTCGTCAATA GGTTTGTCTG
151     CACACTGGGG GACTAGGATG ACAAACTTCG TTGAAGTAGA CGCTCATGCG
201     CTCGAGCTGG AGCTCAGAGG TGCCGTTGTA CCTGTTGGGG GGATTAGCGG
251     TGAATTCGTG GTCAGGGCCT GATCAGGAGG TCGACAACAT ACACGCCATT
301     GCTGTCAAGG CCGTGCTCGC CAGAGATGTT TTGCCTACGC CACAGTCAAT
351     GAGTTGTTTC CTTTCGAGGT CGATATCGTC GAGCCGTTGG CGTCGGTCTG
401     GCTACGCACC AAAAGGCAGC ACCAATCTGG TTACCCTATT CGAAGAAAGG
451     TTAGCCCCCG CACTGGGATT CATTGGGTCG GCAGCATTCA TCAGGAGAGA
501     CTTACGCACT GGCCGGTCTG AAGGTGAACC TATCGAAAGA GGAAGAAAAA
551     AAAGTCAGCA AGATATCCCC AAGGTGAGCG CAGTTGGTGA TGGTGTCGCG
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