FORMONONETIN CONTENT IN SELECTED RED CLOVER STRAINS AND ITS EFFECTS ON REPRODUCTION IN EWES

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ABSTRACT

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A series of trials were conducted to investigate the oestrogenicity of a low formononetin selection of red clover, 'G27', as compared to the original Pawera red clover. Formononetin concentration was measured in the plants at various stages of their growth. In ewes which grazed Pawera, G27, or Ryegrass-white clover (Control) pastures, comparisons were made of the incidence of ovulation, ovulation rate, and fertility.

As the individual isoflavone level for any one strain may vary with growth stage, a study was conducted to characterize formononetin concentration in various components of G27 red clover and Pawera red clover during different stages of plant growth under field conditions. Mean formononetin concentration (percent dry weight) of leaflets and petioles was lower for G27 than for Pawera ($P<0.05$) at various stages of vegetative leaf development. G27 leaflet concentrations (0.29 ± 0.02) changed little during development compared to Pawera leaflets which declined from 2.16 ± 0.10 in the youngest leaflets to 0.75 ± 0.08 by the end of vegetative leaf development. Formononetin concentration in G27 leaves (leaflet + petiole) at the pre-flowering stage was 0.35% compared to 0.97% in Pawera leaves ($P<0.05$). At early and late-flowering stages, the formononetin concentration in G27 red clover, on a whole plant basis, was 50% of that in Pawera red clover because the formononetin concentration in petioles and stem of G27 did not decline to the same extent as that in the leaflets. When calculated only for the upper parts of the plant, which are usually ingested by sheep, G27 and Pawera red clover contained 0.27% and 0.99% formononetin, respectively, at the early-flowering stage, and 0.19% and 0.53% formononetin, respectively, at the late-flowering stage.

In ewes grazed on various red clover and Control pastures, the main effects studied were ovulation rate and fertility. An investigation was made of the development and the number of follicles in the ovaries and plasma FSH concentrations in ewes grazing

either G27 red clover, Pawera red clover, or Control pasture, close to oestrus. A prostaglandin F2α (PGF) injection was used on day 13 of a synchronized cycle to enhance the synchrony of oestrus in ewes. Mean level of blood equol, which is the main oestrogenic metabolite of formononetin in ewes, was significantly lower on G27 red clover (1.81 ± 0.28 μg/ml) than on Pawera red clover (7.25 ± 1.70 μg/ml) ($P<0.01$). Total number of ovarian surface follicles in Pawera ewes (9.40 ± 1.13) was lower than that in G27 (15.36 ± 1.87) or Control ewes (16.18 ± 2.32) 24 h after PGF injection ($P<0.05$). Histological examination of the left ovaries conducted 72 h after PGF injection showed that the number of healthy follicles with diameter (D) $1mm<D\leq2mm$ was marginally lower in Pawera ewes (2.80 ± 0.66) than that in G27 (5.50 ± 1.04), or that in Control animals (5.18 ± 0.64) ($P<0.06$). Cellular atresia was observed in some of the large follicles ($D>4mm$) in Pawera ewes but not in any of the ewes in the other two treatments. No differences were observed in the mean plasma FSH concentrations between ewes from the three treatments at various sampling times.

Two trials were conducted to compare sperm transport in ewes mated after grazing on Pawera red clover, G27 red clover, or Control pastures. In the first experiment 84 ewes were inseminated each with 500 million spermatozoa at oestrus, after grazing for two oestrous cycles. Mean numbers of spermatozoa in the cranial part of the cervix were not different between various treatments 2 h after insemination. No spermatozoa were recovered from the Fallopian tubes and uteri of many ewes, but this was considered to be due to technical problems. In a second experiment 30 Romney ewes (10 per treatment) were mated to rams after 28 days of grazing either on Pawera red clover, G27 red clover, or Control pastures. The ewes were killed 24 h after service and sperm were recovered from the tract and counted using an improved technique. The number of spermatozoa recovered from different parts of the tract did not differ significantly between treatments, although there was a trend for the low formononetin (G27) ewes to have higher mean sperm numbers than Pawera and Control ewes.

In another two trials, ewes ($n = 16$ per group), that were potential recipients for embryo transfer, grazed on the high oestrogenic red clover (Pawera), low oestrogenic red clover (G27), and Ryegrass-white clover (Control) pastures for 5 weeks around oestrus. In both the trials, the number of ovular ewes and ovulation rate were lower ($P<0.05$) in Pawera ewes. The ovulation rate in Pawera, G27, and Control ewes in trial 1 was 0.62 ± 0.15, 1.62 ± 0.18 and 1.93 ± 0.27; in trial 2 it was 0.31 ± 0.18, 1.17 ± 0.27 and 1.54 ± 0.14 for the three groups respectively. Following the transfer into

suitable recipients of two embryos per ewe, post-mortem examination at 35 days showed a survival rate in Pawera, G27 and Control groups of 50%, 90% and 85% in trial 1, and 50%, 50% and 69% in trial 2.

Fertility, and litter size in ewes when fed on the two types of clovers close to the time of mating were studied in another experiment. The treatment groups (n = 25) and grazing lengths prior to mating were: (1) Pawera, 6 weeks; (2) G27, 6 weeks; (3) G27, 12 weeks; (4) G27 / Ryegrass-white clover (Rg-wc), 6 weeks / 6 weeks; (5) Rg-wc (Control 1), 6 weeks, and (6) White clover (Control 2), 6 weeks. Ewes were mated on non-oestrogenic pasture. Ovulation rates in ewes after the first service were not different for all treatment groups ($P>0.05$). The incidence of returns to service was significantly higher in Pawera ewes (72.7%) than in any of the other groups ($P<0.01$). The return rates for the other groups were 33.3% (G27/6 weeks), 25.0% (G27/12 weeks), 4.8% (G27/Rg-wc), 9.5% (Rg-wc) and 14.3% (white clover). Most ewes which were mated at the next two cycles became pregnant. The litter size was not significantly different between various treatment groups after 3 cycles of matings.

It is concluded that G27 red clover has significantly lower formononetin concentrations than Pawera red clover at different stages of plant growth and development. Follicle growth and ovulation rate in ewes on G27 red clover were not different from those in ewes on non-oestrogenic pasture, and were better than those in ewes on Pawera red clover. The performance of ewes after grazing the low formononetin, G27, red clover was better than that of the ewes that grazed the high formononetin Pawera red clover, because of fewer returns to service and thus earlier mean lambing date. Sperm transport in the reproductive tract, and embryo survival in ewes after transfer of fertilized eggs were also not different in G27 and Control ewes. The study showed that the oestrogenicity of G27 red clover was significantly reduced compared to that of Pawera red clover from which the selection was made.

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The following abbreviations have been used in the text without prior definition:

Units:

°C	degree Celcius
cm	centimetre(s)
g	gram(s) or acceleration due to gravity
h	hour(s)
l.u.	international units
kg	kilogram(s)
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
ng	nanogram(s)
ppm	parts per million
µg	microgram(s)
µl	microlitre(s)

Hormones

FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
oGH	ovine growth hormone
oLH	ovine luteinizing hormone
oPRL	ovine prolactin
PMSG	pregnant mares' serum gonadotrophin

Others

SEM	standard error of the mean
vs	versus
v/v	volume/volume
w/v	weight/volume
NaCl	sodium chloride

CHAPTER I

Introduction

Some of the valuable and widely used pasture species under certain circumstances may cause disorders in grazing animals. One of the animal disorders of this type is depression of fertility in females. Plants containing the compounds, which may cause the reproductive problems, include some of the economically important pasture or forage plants of the Leguminosae family that play a valuable role in the improvement of soil fertility, and animal growth and production. The biologically active compounds have been found to be oestrogenic substances that mimic some of the activity of the animal oestrogens (Shutt, 1976). The main forage species involved are subterranean clover (*Trifolium subterraneum* L.) and red clover (*Trifolium pratense* L.); lucerne (*Medicago sativa* L.) and white clover (*Trifolium repens* L.) may also show appreciable oestrogenic effects in response to fungal infestation (Collins and Cox, 1985). The oestrogenic substances in these plants belong to the isoflavonoid group of compounds (Wong, 1975); the principal phytoestrogens in subterranean clover and red clover are isoflavones, whereas the medics and white clover contain coumestans.

Oestrogenic activity in forage plants and its association with reproductive problems in farm animals has been reported from countries throughout the world, including Australia, Canada, Chile, Germany, Italy, New Zealand, Sweden, the UK, the USA, and the USSR (Bickoff, 1968). The most serious problems have been noted in Western Australia in sheep grazed on certain varieties of subterranean clover. When ewes are grazed on oestrogenic clover prior to, and during mating, they suffer from a temporary type of infertility from which they recover within a few weeks after removal to non-oestrogenic pasture. Prolonged grazing on oestrogenic clover for several years, may result in a permanent and progressive infertility in ewes. Formononetin, an isoflavone, present in oestrogenic subterranean clover and red clover is the substance implicated in these reproductive problems. Formononetin is not oestrogenic itself but it is metabolized, mainly, to equol in the sheep rumen, and equol is oestrogenic. Breeding and use of low formononetin cultivars of oestrogenic clovers is an important method to minimize/control the problem; in addition, a variety of management procedures might also be attempted.

Although subterranean clover is not widely used in pastures in New Zealand, red

clover is often incorporated in pasture mixes, especially in the establishment phase (Shackell *et al.,* 1993). The major red clover cultivars available in New Zealand have been found to be oestrogenic. Recently a low formononetin selection of red clover, G27, has been made from the highly productive and persistent tetraploid 'Pawera' red clover. Extensive and prolonged field testing is crucial in the development of new cultivars (Collins and Gladstones, 1985). Detailed comparisons of the effects of high and low formnononetin red clover strains on various reproductive parameters of ewes have not been made. An effort has been made in the present study to investigate in some detail the formononetin content in G27 and Pawera red clovers, and their effects on ewe reproduction under field conditions.

CHAPTER II

Review of literature

1. Oestrogens and plant oestrogens

An oestrogen can be defined as a compound that acts on the central nervous system and induces oestrous behaviour in female mammals (Shutt, 1976). At least eight oestrogens are secreted by the mammalian ovary (Reeves, 1987) but among these oestradiol-17β is the most important and primary oestrogen. Mammalian oestrogens belong to the steroid group of compounds, and they function as hormones, in the classical sense, being transported via the circulation to act on a wide variety of "target" tissues and organs comprising not only the reproductive system, but also several other organs and systems, such as the central nervous system and musculoskeletal system (Gore-Langton and Armstrong, 1988). The mechanism of action of oestrogens is not precisely understood but it has been suggested that the oestrogen passes via the blood stream and is selectively accumulated in target cells because these cells contain cytoplasmic receptor proteins with a high affinity and specificity toward the hormone (Knowler and Beaumont, 1985); the hormone-receptor complex moves into the nucleus of the cell, binds to the chromatin, and causes changes in the expression of a specific gene. In addition to induction of oestrus in female mammals, oestrogens also stimulate the growth of the reproductive tract and mammary glands (Shutt, 1976). Although the focal point for the hormonal regulation of reproduction is the hypothalamus, the mammalian uterus is the system in which oestrogen receptors have been most intensively studied (Knowler and Beaumont, 1985).

In 1923, Allen and Doisy established a test for oestrogens based on the fact that the occurrence of oestrus in rats and mice was accompanied by characteristic proliferative changes in the vaginal epithelium (vaginal cornification) (described in detail by Emmens, 1950). Using the Allen-Doisy test, the occurrence of oestrogenic substances in plants was first demonstrated by Dohrn *et al.*, in 1926 (see Farnsworth *et al.*, 1975). Further interest in phytoestrogens arose due to the recognition that infertility in animals could follow excessive ingestion of plants rich in compounds possessing oestrogenic activity. A serious breeding disorder of sheep grazed on the Dwalganup cultivar of subterranean clover was first observed in Australia in the early 1940s (Bennetts *et al.*, 1946): the problem was characterized by a failure to conceive, and

was accompanied by varying degrees of dystocia and uterine prolapse in ewes; other effects included enlargement of the bulbo urethral gland in wethers, and lactation in wethers and maiden ewes. Cystic endometrium and mammary gland development provided strong presumptive evidence of the occurrence of oestrogenic activity in the clover. The complex of the abnormalities observed became known as clover disease (Marshall, 1973).

Great interest and, no doubt, alarm was aroused at the height of the problem in 1941-45 both because of the economic loss occurring, and because this was the first recorded example of ill health being, apparently, caused by the presence of oestrogens in animal food (Pope, 1954). Subsequently oestrogenic substances were isolated not only from subterranean clover but also from red clover. The oestrogenic compounds were not steroids but isoflavones which included formononetin, genistein, biochanin A, and daidzein (Guggolz *et al.,* 1961; Wong, 1963); in some cases low concentrations of pratensein were also recorded. The first three isoflavones are often found in high concentration with combined levels of 2.6-5.6% of the leaf dry matter (Beck, 1964). Although only weakly oestrogenic (about 10^{-5} to 10^{-6} times as active as oestradiol), they have a significant biological effect in animals because of the large quantities ingested (Collins and Cox, 1985).

The isoflavones genistein, biochanin A, and daidzein were found to be oestrogenic in mice but formononetin could not be demonstrated to be oestrogenic (Moule *et al.,* 1963), so the first three isoflavones, particularly genistein, were implicated as causal agents of the sheep infertility syndrome until the early 1960s. However, Millington *et al.,* in 1964 showed that oestrogenicity of various strains of subterranean clover in sheep was positively related to the formononetin content of the strains. No such relationship was found for either genistein or biochanin A, the other isoflavones present in appreciable amounts. Later it was proved that conception rates in ewes grazed on subterranean clover were also related to the concentration of formononetin in the clover leaves (Davies *et al.,* 1970). Studies into metabolism of isoflavones in sheep made it clear that genistein and biochanin A were metabolized in the sheep rumen to non-oestrogenic phenols but the major metabolic product of formononetin was equol, which was oestrogenic (Shutt *et al.,* 1970). Thus the oestrogenicity of formononetin in sheep is largely due to equol formed from formononetin in the rumen. While it seems clear that the high oestrogenic activity of subterranean clover is always associated with a high 'formononetin content', the reverse may not always apply

(Beck, 1964); it seems, therefore, desirable not to exclude the possibility that the infertility in ewes grazed on oestrogenic clover is due, in part, to factors other than formononetin.

Another group of chemical compounds found in forage plants that has oestrogenic activity is the coumestans. Coumestrol and 4'-O-methyl coumestrol, together with several other coumestans have been isolated from medics and white clover (Collins and Cox, 1985); they are about 10^{-3} times as active as oestradiol but their levels in plants are usually very low. Wong *et al.*, (1971) observed that coumestans were not detectable, or they were present in trace quantities only (< 2 ppm dry matter) in healthy white clover. Smith *et al.*, (1979) also showed that in the absence of fungal infestation no coumestans were found in lucerne. It has been shown that coumestan concentrations can reach appreciable levels in lucerne and white clover in response to fungal diseases (Loper and Hanson, 1964; Wong *et al.*, 1971; Smith *et al.*, 1979). The coumestan concentration in white clover infested by *Pseudopeziza trifolii* reached 370 ppm (of dry matter) (Wong *et al.*, 1971).

The phytoestrogens have been found to inhibit the binding of oestradiol-17β to sheep and rabbit uterine receptors *in vitro* (Shutt and Cox, 1972; Shemesh *et al.*, 1972). Although phytoestrogens have less affinity for receptors, a full oestrogenic response *in vivo* may be elicited when they are given in repeated frequent doses which may be necessary to maintain a high blood concentration (Shutt and Cox, 1972); thus a maximal oestrogenic response may be obtained even when the rate of breakdown of the oestrogen-protein complex is rapid and the compounds have a short biological half life.

2. Oestrogenic effects in grazing animals

Serious reproductive problems have been noted only in sheep among the domestic animals grazing oestrogenic clover. There are a few reports of hyperoestrogenism in cattle fed oestrogenic forage as detailed below.

2.1. Sheep

Oestrogenic subterranean clover and red clover can cause infertility in ewes by two entirely distinct mechanisms; temporary infertility and permanent infertility (Lightfoot,

1974). Although these two conditions may occur concurrently, they differ in both mechanism and epidemiology (Adams, 1987a).

Exposure to green, oestrogenic subterranean clover for 6 to 8 months annually may produce a permanent, cumulative infertility after two years which persists after removal from oestrogenic pasture (Adams, 1987a). This form of infertility has been most common in Western Australia where sheep are usually mated on dry pastures which have lost their oestrogenicity (Adams *et al.,* 1988). The syndrome, called clover disease, observed in the 1940s included uterine prolapse, dystocia, and permanent infertility with very low lambing rates in ewes (Bennetts *et al.,* 1946). As graziers have become aware of the cause of the trouble and have taken steps to minimize it (by reducing intake of oestrogenic clover by sheep), the more extreme manifestations of the syndrome have almost disappeared (Braden and McDonald, 1970). Now the disease occurs as a comparatively uncomplicated infertility of a more modest nature, and is still widespread in Western Australia and probably also occurs in other parts of Australia (Lightfoot, 1974; Adams, 1990); the permanent infertility in the absence of any obvious clinical problem can be called "clover infertility". The infertility has been induced experimentally in ewes after prolonged grazing on red clover (Barrett *et al.,* 1965; Shackell *et al.,* 1993) or subterranean clover (Davenport, 1967). The main cause of infertility is the lack of ovum fertilization due to impaired transport of spermatozoa through the cervix (Lightfoot *et al.,* 1967). The ewes are not completely infertile but only of reduced fertility (Adams *et al.,* 1988). Repeated injections with diethylstilboestrol, or treatment of ewes with oestradiol-17β implants has produced persistent infertility in ewes similar to permanent infertility produced by the oestrogenic isoflavone compounds found in clover (Underwood *et al.,* 1959; Adams and Sanders, 1988).

Temporary infertility has been recorded in ewes actually grazing oestrogenic red clover or subterranean clover around mating time, and is characterized by a lower ovulation rate and an increase in returns to service (Morley *et al.,* 1966; Lightfoot and Wroth, 1974); fertility recovers by 5 weeks after removal to non-oestrogenic pastures. Reproductive disorders in ewes attributed to the ingestion of coumestans include inhibition of oestrus (Kelly *et al.,* 1976) and a reduction in ovulation rate (Smith *et al.,* 1979). There have been no reports of permanent effects of coumestans on fertility (Collins and Cox, 1985).

There have been no reports of disturbances in fertility of rams grazing oestrogenic clover (Bennetts *et al.*, 1946; Marshall, 1973).

2.2. Cattle

The extent to which phytoestrogens may cause deleterious effects in cattle is not clear (Collins and Cox, 1985). Although there are many instances in the literature of reproductive disturbances in cattle receiving oestrogenic forages, a permanent infertility, resulting from prolonged intake of phytoestrogens, does not appear to have been reported (Lightfoot, 1974). Anoestrus and difficulty in the detection of oestrus, and a high incidence of service returns have been described in cows grazing subterranean clover (Thain, 1966). Lactation in maiden heifers, cystic degeneration in the ovaries (Thain, 1965), irregular heats, indeterminate discharges, anovulation, and abortions (Kallela *et al.*, 1984) have also been reported. Kallela *et al.*, (1984) suggested that the effect of plant oestrogens in cattle is generally weaker than that in sheep. In Western Australia, where cows are frequently exposed to oestrogenic subterranean clover, and where problems in sheep are so common, there are no authenticated reports of associated infertility in cows (Adams, 1987b).

3. Mechanism of temporary infertility in sheep

3.1. Oestrous incidence

Disturbances in incidence of oestrus have been recorded in ewes grazing oestrogenic clover in some but not all of the studies. Ch'ang (1958) observed that ingestion of red clover dominant pasture over a three month period after weaning induced oestrus without ovulation in lambs at a relatively early age. A reduction in the incidence of oestrus has been reported in ewes grazing subterranean clover (Lightfoot and Wroth, 1974;). Kelly *et al.*, (1980) found that only 80% of the ewes were marked when mated on Pawera red clover as compared to 100% marking rate in a control group; 20% of the ewes on Pawera red clover showed oestrus without ovulation. Fox *et al.*, (1959) observed that ewes which did not conceive when mated on red clover pasture, exhibited normal oestrous cycles as indicated by acceptance of the ram every 16-20 days during the breeding period. It is evident from these studies that phytoestrogens may affect oestrous behaviour in various ways. They may decrease oestrous incidence, may induce oestrus without ovulation, or the oestrous incidence may remain

unaffected.

3.2. Ovulation rate

A reduced ovulation rate has been reported in ewes grazing oestrogenic pasture during mating, such as subterranean clover (Lightfoot and Wroth, 1974), red clover (Ch'ang, 1961; Kelly *et al.*, 1980), and lucerne (Smith *et al.*, 1979). The lower ovulation response seen in ewes on oestrogenic pasture was due to anovulatory ewes and/or reduced multiple ovulations. This effect may appear after a few days of exposure to oestrogenic pasture. Feeding a coumestan-containing diet for 7 days prior to oestrus reduced multiple ovulation markedly (Smith *et al.*, 1979). The depression in ovulation rate may depend on the degree of oestrogenicity of the pasture (Lightfoot and Wroth, 1974) as a low daily formononetin intake did not reduce ovulation rate in ewes (Holst and Braden, 1972). Fecundity status did not appear to influence ovulation response of ewes to isoflavone-rich Pawera red clover pasture (Kelly and Shackell, 1982). Land (1976) showed that treatment with oestrogen (oestradiol benzoate, 25 or 50 μg) on each of days 3 to 14 inclusive of the oestrous cycle also reduced ovulation rate in ewes.

The deleterious effect of phytoestrogen on ovulation rate was not related to the failure of oestrogenic pasture to sustain body condition, as the ewes grazing such pastures either had live-weights similar to the control ewes (Smith *et al.*, 1979) or they gained significantly more weight than the control animals (Kelly *et al.*, 1980). The lower ovulation rate on oestrogenic pastures may be due to disturbances in follicle development in sheep ovaries (Kelly *et al.*, 1976; Adams, 1977). Adams (1977) observed that ewes grazing subterranean clover developed excessive numbers of small and medium sized ovarian follicles, in many of which antrum formation was deficient. This abnormal development was accompanied by early atresia of the follicles. This author also observed changes in the pituitary gland indicating that gonadotrophin metabolism might be altered in ewes on oestrogenic pasture. These changes, taken together with the changes in the ovary, suggest that in ewes grazing oestrogenic pasture, there might be an alteration in the function of the pituitary/ovarian axis and this would account for the reduced incidence of oestrus and the decreased ovulation rate in these ewes.

3.3. Ovum fertilization and gamete transport

Few studies have been conducted to investigate ovum fertilization rate and gamete transport in ewes mated on oestrogenic clover. Lightfoot and Wroth (1974) showed that ovum fertilization was significantly depressed in ewes grazing oestrogenic subterranean clover. Pelleted red clover, fed to ewes during the mating period (2.4 g formononetin per ewe daily), appeared to lower the egg fertilization rate (Holst and Braden, 1972). Only a few eggs recovered from the oestrogenic treatment were found to have sperm on the zona pellucida (Lightfoot and Wroth, 1974) suggesting that reduced fertilization in ewes mated on oestrogenic clover was due, at least in part, to inefficient sperm transport. It has been suggested that sperm transport might be affected due to disturbances in cervical mucus production in ewes grazing oestrogenic clover (Adams, 1977).

Holst and Braden (1972) suggested that consumption of phytoestrogens during the mating period might also disturb normal ovum transport as observed in ewes during 60 hours after ovulation. In these ewes, there was retention of some ova in the ampulla with acceleration of others into the uterus; however this increased dispersion was not significantly different from the control ewes. Furthermore a low proportion of ewes yielded eggs (54-79 h after the onset of oestrus) after mating on oestrogenic subterranean clover (Lighfoot and Wroth, 1974) . Thus both reduced sperm transport and disturbances in egg transport may contribute to ewe infertility on oestrogenic pasture.

3.4. Embryo mortality

Detailed studies determining the effect of grazing oestrogenic pasture on embryo mortality in ewes are lacking from the literature. Lightfoot and Wroth (1974) checked fertilization rate in a group of ewes 54-79 h after mating, and pregnancy rate in a second group of ewes 21-25 days after mating on oestrogenic subterranean clover. They estimated embryo mortality from the difference between fertilization rate and pregnancy rate but could draw no conclusion due to large differences in ovulation rate between oestrogenic and control ewes. In a study with mice fed the phytoestrogen coumestrol, Fredericks *et al.*, (1981) found that the incidence of degenerate embryos increased with elevated levels of coumestrol in the diet. Evidence in sheep regarding additional embryonic mortality induced by phytoestrogens is inconclusive, although it

has been shown that grazing ewes on oestrogenic clover may cause oviductal hyperplasia and uterine oedema (Adams, 1977).

3.5. Returns to service, and lambing performance

Oestrogenic clover fed to ewes prior to and during mating may result in increased returns to service and a reduction in the number of lambs born. Morley *et al.*, (1966) observed a reduction in the number of multiple births in ewes grazing red clover for 21-33 days before and for 10 days after mating; returns to service remained high at least three weeks after removal from red clover. Thomson (1975) also recorded a reduction in twin births, and an increase in the incidence of barrenness in ewes fed red clover diets for three weeks prior to and for five weeks during mating. The grazing of Pawera red clover pasture for 8 days before and during the first cycle of mating resulted in a higher returns to service (Kelly *et al.*, 1980); only 25% of ewes lambed after one cycle of mating on red clover compared to 75% of control ewes.

3.6. Corpus luteum function

There is an indication of a disturbed luteal function in ewes consuming plant oestrogens. Lightfoot and Wroth (1974) reported that ewes grazing oestrogenic clover prior to and during joining had lighter corpora lutea as compared to the control animals. In ewes failing to conceive on oestrogenic clover, progesterone concentrations began to fall at day 11-12, to reach oestrous levels at days 13-14, indicating a shortened period of corpus luteum function (Obst and Seamark, 1975): lower plasma oestrogen levels were found in infertile matings as compared to fertile matings.

3.7. Recovery period

Morley *et al.*, (1966) showed that the effects on ewes grazing an oestrogenic pasture containing red clover for 21-33 days, persisted for at least three weeks with respect to reduced fertility, but for less than three weeks with respect to decreased proportion of multiple births; fertility and fecundity of ewes had returned to normal five weeks after removal from oestrogenic pasture. Kelly *et al.*, (1980) also observed that ovulation rates recovered within a cycle of removing ewes to non-oestrogenic pasture after 25 days grazing on Pawera red clover but returns to service were still high. Removal of

adult ewes, grazed for 12 weeks on a Pawera red clover dominant pasture, one month before joining was not enough for the animals to return to normal reproduction levels (Shackell and Kelly, 1984), although one month was a sufficient recovery time for ewes which had grazed swards in which red clover was not a dominant species. The same study showed that grazing ewe lambs on red clover dominant swards had no deleterious effects on the animals reproductive performance as 18-month old ewes.

An earlier recovery of ovulation rate, compared to fertility, after removal from oestrogenic pasture suggests that phytoestrogens affect establishment of pregnancy through a different pathway than that determining the proportions of multiple ovulations (Morley *et al.,* 1966); it is possible that the reduced incidence of multiple ovulation is a result of the action of oestrogens directly on the ovary.

4. Mechanism of permanent clover infertility in sheep

4.1. Oestrous incidence

The failure to come into oestrus was not a primary cause of permanent clover infertility (Underwood and Sheir, 1951) as incidence of oestrus, and cycle length were found to be normal in ewes after a prolonged grazing on oestrogenic clover. However, Adams (1979) reported that the incidence of oestrus was depressed in ewes with permanent clover infertility running on non-oestrogenic pasture in the first part of the breeding season. Duration of oestrus (approximately 23 h) was found to be normal in affected ewes (Lightfoot *et al.,* 1974).

4.2. Ovulation rate and corpus luteum function

Ovarian activity, as assessed by follicle development and ovulation rate was not affected in ewes of reduced fertility associated with the prolonged grazing of oestrogenic clover (Lightfoot *et al.,* 1967). Adams *et al.,* (1979) observed that ewes which had been exposed to oestrogenic clover for 3 years showed an elevated ovulation rate due to an increase in the frequency of twin ovulations. It has been suggested that the increased ovulation rate in affected ewes might be due to a change in hypothalamic control of the ovary.

No differences have been found in the levels or patterns of production of progesterone

between fertile ewes and ewes with the 'permanent' type of infertility due to ingestion of phytoestrogens (Smith, 1975).

4.3. Lambing rate

A progressive decline in lambing rate has been recorded in successive years in ewes grazed on high oestrogenic cultivars of subterranean clover and red clover (Bennetts *et al.*, 1946; Barrett *et al.*, 1965; Davenport, 1967). Over a period of 6 years, the proportion of ewes lambing fell progressively from 87 to 25% in ewes grazing red clover for 8 months per year, compared with a decline from 87 to 65% over the same period in ewes grazing non-oestrogenic pasture (Barrett *et al.*, 1965).

4.4. Sperm transport and cervical mucus

Failure of fertilization due to an extremely low number of sperm reaching the Fallopian tube has been considered the most important reason for infertility in sheep grazed on oestrogenic pasture for several years (Bennetts *et al.*, 1946; Turnbull *et al.*, 1966; Lightfoot *et al.*, 1967; Kaltenbach and Davies, 1970). The primary failure of sperm transport occurred when sperm did not enter the cervix in adequate numbers following service (Lightfoot *et al.*, 1967). The rheological properties and macro molecular structure of mucus are of functional importance with regard to the establishment and maintenance of a cervical population of spermatozoa in the ewes (Gibbons and Mattner, 1966). It has been shown that ewes suffering from permanent clover infertility produce a thin and watery mucus around oestrus (Smith, 1971). The total amount of mucus is not increased but it has a significantly decreased spinnbarkeit (Adams, 1976a) reflecting an absence of the normal molecular structure which prevents the effective orientation for sperm migration through the cervix (Adams, 1981).
Moreover, severe changes occur in the cervix of clover affected ewes. The cervical folds fuse together, the lamina propria becomes thicker and much more cellular, and coiled tubular glands similar to uterine glands develop (Adams, 1990); in all the cervix takes on the appearance of endometrium.

Therefore an abnormal cervical mucus, and changes in microanatomy of the cervix might interfere with orientation and progression of spermatozoa in the cervix, reducing the chances of the ovum being fertilized.

4.5. Embryo mortality

Turnbull *et al.*, (1966) indicated that the major cause of infertility in ewes after prolonged grazing on oestrogenic clover was non-fertilization due to a failure in sperm transport to the Fallopian tube, also death of embryos in the first 60 days *post coitum* contributed to the low fertility in ewes. But Kaltenbach and Davies (1970) observed that early embryonic mortality was not an additional source of reproductive failure in clover affected ewes. The variability between studies might result from irregular bacterial invasion of the uterus through the damaged cervix (Adams, 1975).

4.6. Changes in hypothalamus and pituitary gland

Gardiner and Nairn (1969) found pathological changes in the hypothalamus of sheep suffering from permanent oestrogenic infertility. But some other histopathological studies carried out on the ewes affected with clover disease showed no consistent changes in pituitary gland tissue (Hearnshaw *et al.*, 1972; Adams, 1976b).
Exogenous oestradiol tends to suppress the concentrations of both FSH and LH followed by an oestrous-like peak in both gonadotrophins (Goding *et al.*, 1969; Scaramuzzi *et al.*, 1971; Bolt, 1981). Findlay *et al.*, (1973) observed that the hypothalamus of ovariectomized ewes that had been exposed to oestrogenic clover for long periods, were unable to show 'preovulatory type' releases of luteinizing hormone in response to oestradiol-17β treatment. Pituitaries of the clover affected ewes were capable of a full scale discharge of LH in response to synthetic GnRH which strongly suggest that the failure of oestradiol to evoke an LH release in these ewes was due to a long term interference with the neural mechanism for oestradiol involved in the preovulatory surge of LH. Adams and Martin (1983) confirmed that clover affected ovariectomized ewes were less able to give an increase in LH after treatment with oestrogen, both in the normal breeding season and in the anoestrous season. Chamley *et al.*, (1981) showed that LH concentrations in the clover infertile ewes remained static throughout the breeding and non-breeding season. Phytoestrogens when ingested over a long period, bring about a permanent change in the binding parameters of oestradiol-17β receptors in the pituitary gland and probably also in the hypothalamus of the ewe, and thus may alter the response of the pituitary gland and hypothalamus to oestradiol (Tang and Adams, 1978).

5. Metabolism of isoflavones in sheep

Subterranean clover and red clover varieties contain isoflavones of the 5-deoxy group, formononetin and daidzein, and of the 5-hydroxy group, biochanin A and genistein (Figure 1) (Cox *et al.,* 1984). In the plant, these occur largely as glycosides (Beck and Knox, 1971) which are rapidly hydrolyzed by glycosidases when the leaf cell structure is ruptured. In-vitro studies using ovine rumen fluid, and in-vivo studies with sheep have shown that formononetin and biochanin A are demethylated, resulting in the formation of daidzein and genistein (Nilsson *et al.,* 1967; Lindner, 1967). It has also been observed that demethylation of formononetin and biochanin A may take place both in the rumen and after absorption into the general circulation (Lindner, 1967; Batterham *et al.,* 1971). Shutt *et al.,* (1967) showed that sheep grazing oestrogenic subterranean or red clover contained substantial amounts of plasma daidzein in addition to formononetin indicating that demethylation of formononetin had taken place in sheep, since only traces of daidzein were found in the plant material from these clover species. They observed that levels of biochanin A and genistein in the plasma were lower relative to the levels in clover. When administered by intraruminal infusion, genistein and biochanin A were found to be largely excreted as para-ethylphenol, but equol was observed as the major metabolite in the urine of sheep given formononetin (Batterham *et al.,* 1965; Braden *et al.,* 1967). In-vitro studies showed that the formononetin-daidzein-equol pathway was present in the rumen of sheep, while biochanin A was converted to genistein and some simple phenolics (Nilsson *et al.,* 1967). So biochanin A and genistein which are known to be oestrogenic in mice (Wong and Flux, 1962), are degraded to simple phenols in the sheep rumen, thus becoming inactive. Equol was shown to be present in relatively high concentrations in the plasma of sheep grazing oestrogenic subterranean or red clover (Shutt *et al.,* 1967). Later studies confirmed that equol was the major metabolite produced by degradation of formononetin (Shutt and Braden, 1968; Shutt *et al.,* 1970; Davies and Hill, 1989), although O-desmethyl angolensin is another metabolite of formononetin, produced by a different pathway from that by which equol is produced (Shutt and Braden, 1968; Batterham *et al.,* 1971). Equol is probably the oestrogen responsible for clover disease as it has been shown to be oestrogenic in mice, and its content in the uterine tissues of ewes grazing red clover greatly exceeded that of the other known phytoestrogens (Shutt and Braden, 1968). In a study with ewes fed red clover, Shutt *et al.,* (1970) found that despite a negligible excretion of formononetin, daidzein and O-desmethyl angolensin in the urine, almost 70% of the formononetin

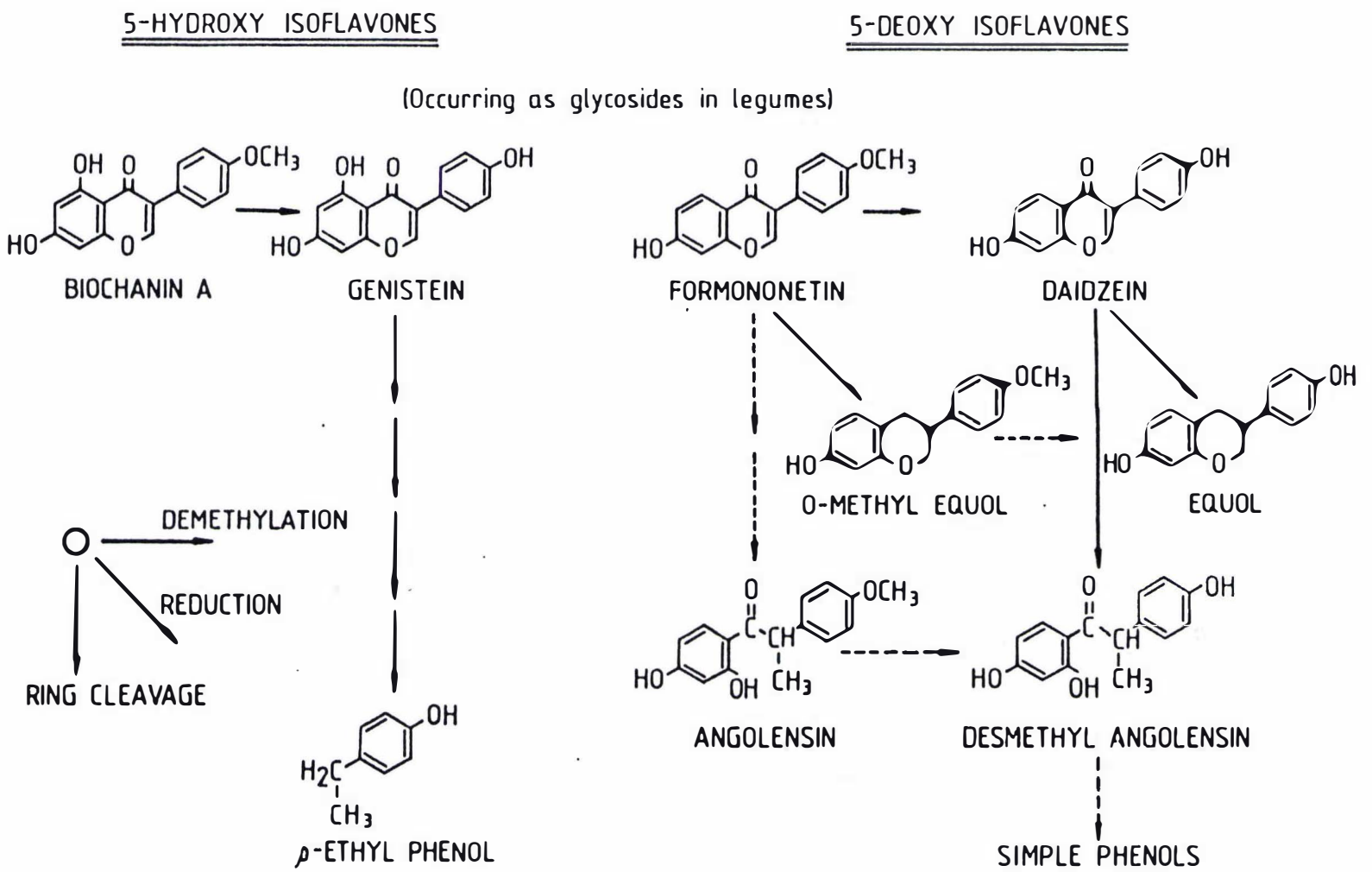


Figure 2.1. Metabolic conversions of isoflavones in the sheep. Broken arrows indicate probable changes. The reactions in the metabolic lattice are shown in the insert. (Courtesy, Cox *et al.*, 1984).

intake appeared as equol in urine. It was also observed that very little formononetin passed in the digesta from the rumen indicating that equol production occurred almost entirely in the rumen (Shutt *et al.,* 1970; Davies and Hill, 1989). Equol did not appear to undergo any further extensive metabolism in the rumen, from where it was readily absorbed (the mean residence time for equol in the rumen was estimated to be 1.7 h) (Shutt *et al.,* 1970).

The rate of metabolism of labelled isoflavones has been found to be highly dependent upon the diet eaten by sheep (Davies, 1989) as the type, number, and concentration of rumen organisms and their fermentative activities change in response to changes to diet.

The isoflavones and equol are present in blood plasma predominantly (more than 90%) in a conjugated form (Shutt *et al.,* 1970; Braden *et al.,* 1971; Lundh *et al.,* 1990), and the bulk of the conjugated fraction consists of glucuronides (Shutt *et al.,* 1967) and small amounts (5-15%) occur as sulphoconjugates (Cox *et al.,* 1984). Only 1-2% of isoflavones in the plasma of sheep grazing various oestrogenic clovers is present in the "free" (i.e. unconjugated) form (Shutt *et al.,* 1967). Equol can reach levels of 300-500 µg/100 ml in blood plasma of which about 1% is present in a free form (unconjugated) (Shutt *et al.,* 1970; Lundh *et al.,* 1990). The "free" fractions probably represent the biologically active form of the compound (Shutt, 1976). A small proportion of sulphoconjugate may also be biologically active (Cox *et al.,* 1984). Conjugation is probably one of the most important mechanisms in most animal species to detoxify different foreign substances ingested, including plant oestrogens (Lundh *et al.,* 1990). Conjugation of isoflavones and their metabolites may take place in the liver and in the gastrointestinal epithelium of sheep (Collins and Cox, 1985; Lundh, 1990).

The phytoestrogens and their metabolites are excreted, mainly in the urine (in contrast to the largely faecal excretion route for metabolites of the endogenous steroid oestrogen) (Cox, 1978). Davies and Hill (1989) observed that most of the equol (80%) produced was excreted in 48 hours; it was also noted that excreted equol was predominantly unconjugated during the first 24 hours and conjugated during the second 24 hours indicating that conjugation may be a mechanism involved in the detoxification of isoflavones.

In addition to equol, another metabolite of formononetin, 4'-methyl equol may be

present in appreciable amounts in some conditions, indicating that there may be direct reduction of formononetin to 4'-methyl equol in the rumen without prior demethylation to daidzein (Cox and Braden, 1974b; Nottle and Beck, 1974). Possibly a change in rumen microbial activity may be required to give a lessening of demethylation relative to reduction; under field conditions sheep variability and seasonal factors may also be involved. The mouse assay indicated that 4'-methyl equol was slightly less (0.7) oestrogenic than equol when administered subcutaneously (Nottle and Beck, 1974). Davies and Hill (1989) indicated that there might be other metabolites present which may or may not be involved in the oestrogenicity of clover. More detailed measurements for isoflavones and their metabolites in sheep are overdue in order to define further metabolic sequences (Cox and Davies, 1988).

To summarize, formononetin is metabolized in the sheep rumen mainly to equol with some 4'-**O**-methylequol and small amounts of daidzein and **O**-desmethyl angolensin, all of which are oestrogenically active. Genistein and biochanin A which are both oestrogenic when given parenterally, are largely catabolized by rumen micro organisms to **p**-ethylphenol and other simple phenols which are oestrogenically inactive. It has been indicated that the effective degradation of genistein and biochanin A to para-ethylphenol takes place only after a short period of adaptation and during this period of a few days, these isoflavones may exert oestrogenic effects (Morley *et al.*, 1969; Lindsay and Kelly, 1970); the demethylation of formononetin to its main active metabolite, equol, apparently does not require a period of adaptation.

6. Factors affecting phytoestrogen concentration in legumes

6.1. Genetic factor

Genetic variance appears to be responsible for a significant portion of the isoflavone variation in clover (Braden and McDonald, 1970; Nicollier and Thompson, 1982). The isoflavone pattern or proportion of three major isoflavones vary greatly in different subterranean clover strains (Francis and Millington, 1965b). The clover cultivars also differ in their oestrogenicity and the effects on sheep reproduction (Davies and Bennett, 1962; Davies *et al.*, 1970); thus the use of low oestrogen cultivars, if available and having suitable agronomic characters, is a powerful tool to minimize clover infertility.

6.2. Plant growth stage

Ontogenetic factors may be responsible for within strain variation of isoflavone levels (Rossiter, 1970). Rossiter and Beck (1967) studied in detail the effect of growth stage on isoflavone concentration in leaves of subterranean clover. Appreciable differences were found in formononetin concentrations between leaves at a specified date or plant age; concentration tended to fall in older leaves, however, the absolute amounts of formononetin per leaf were similar. Concentration of formononetin in the individual leaf also decreased from emergence to senescence. The total amount of formononetin per leaf reached maximum amounts early in the life of the leaf when the leaf became fully expanded. The isoflavone daidzein was detected at the early stages of senescence along with a marked decline in formononetin concentration in the leaves. Formononetin concentrations averaged over the total expanded leaf population were shown to decrease during plant growth for the Dwalganup strain but to increase for the Yarloop strain of subterranean clover. It shows that the effect of plant maturity on formononetin concentration may be a strain dependent phenomenon.

Isoflavone levels also vary markedly with maturation stage in red clover leaves (Wong, 1963): in broad red clover ('Grasslands Hamua'), formononetin concentration decreased during flowering, but in Montgomery red clover ('Grasslands Turoa') it was found to be higher during flowering than at a pre-flowering stage. Dedio and Clark (1968) noted a high isoflavone concentration in red clover leaves in earlier stages of growth until about flowering followed by a rapid decline. McMurray *et al.*, (1986) also showed that formononetin concentration in red clover declined with plant age in the plant parts tested (laminae, petioles and stem): the decline was greater in stems (67%) than in laminae or petioles (48 and 54% respectively); formononetin concentration of total harvested herbage decreased from the late vegetative stage to the dying head (flower) stage by 57%.

Francis and Millington (1965a), and Davies and Dudzinski (1965) showed that relative to green clover, the mature, naturally dry subterranean clover had negligible oestrogenic activity, but in contrast, the perennial legume red clover was found to be oestrogenic throughout the year (Kelly *et al.*, 1979).

6.3. Nutrients

Moderately severe deficiencies of the major nutrients (phosphates, sulphur, and nitrogen) have been shown to almost double the concentrations of formononetin in subterranean clover (Rossiter, 1970); potassium, copper, and zinc deficiencies had negligible effects. McMurray *et al.*, (1986) showed that the concentration of formononetin in red clover was affected significantly only when deficiency of phosphorus was sufficient to markedly reduce growth; a level which slightly reduced growth did not significantly increase formononetin concentration. They found that the dry weight of the red clover shoots when no phosphate was applied in the potting medium was 13 and 11% of the shoots receiving 96 kg and 23 kg phosphate per hectare. The formononetin concentration increased by 43% in the shoots which received no phosphate. This shows that the severe phosphate deficiency had a bigger effect on plant growth than on formononetin concentration in the plant.

6.4. Other factors

At extreme levels, some environmental factors such as temperature, light, moisture stress, water logging, and defoliation might influence formononetin level (Rossiter, 1970); insect and virus damage, leaf detachment, prolonged darkness, and herbicides did not have significant effects on formononetin concentration in subterranean clover.

7. Conservation procedures and oestrogenicity of clover

When oestrogenic subterranean clover was made into hay, there was a marked diminution in the oestrogenic potency (Davies and Dudzinski, 1965). However, hay made to ensure a high content of green leaf following very rapid curing and storing, retained all its activity. Kelly *et al.*, (1979) also showed that when hay was made from Pawera red clover, the concentration of formononetin in the leaf material fell from 0.95% in the green pasture to 0.43% in the hay. It is not clear how far the endogenous β-glucosidase (which is needed for the hydrolysis of formononetin from the glycoside during analysis) is affected during hay making. Thomson (1975) observed that drying at a high temperature and processing by grinding and pelleting reduced the level of formononetin by 75% in a red clover diet, but direct ensiling of the unwilted crop resulted only in a 25% reduction in the level of formononetin; when fed to ewes around mating, the silage led to a marked reduction in the number of twin births, and

an increase in the incidence of barrenness. So ensiling will not appreciably reduce the effects of oestrogenic clover on the fertility of ewes. Effects of feeding hay made from oestrogenic clover on the fertility of ewes have not been studied in detail.

8. Performance of ewes on high and low formononetin cultivars

Cultivars of subterranean clover have been shown to differ in oestrogenic potency which is related mainly to the formononetin content (Millington *et al.*, 1964). Davies *et al.*, (1970) confirmed that subterranean clover cultivars also differed in their effects on sheep fertility, and these effects were also mainly related to the formononetin content of clover; they observed that the proportion of ewes which conceived declined to 61% on the high oestrogen cultivars (leaf formononetin concentration = 0.86-1.36% of dry matter) compared with 85% on the low oestrogen strains (leaf formononetin concentration = 0.06-0.17% of dry matter) during four-years of grazing. Differences in conception rates of ewes were not significant between high and low oestrogen cultivars during the first year of grazing but they were from the second year onward. Other studies have shown spectacular reductions in the proportion of ewes lambing on high formononetin clovers compared to those on low formononetin ones (Neil *et al.*, 1974; Rossiter and Marshall, 1974; Marshall, 1974).

Davies and Maller (1970) investigated the effect of dilution of oestrogenic subterranean clover (cultivar Yarloop) with non-oestrogenic grass on ewe-fertility after a three-year grazing period. They found that in the fourth year, the percentage of pregnant ewes on 100% clover was 53%; on 60% clover - 63%; on 30% clover - 77%; and on grass (no clover) control 88%. This indicated that in long term grazing situations, reduction in fertility might occur even when the oestrogenic clover constituted only 30% of the feed on offer. The Yarloop cultivar has been shown to have a formononetin concentration of 1.9% of dry matter (Francis and Millington, 1965b). Marshall (1973) suggested that in the field situation, especially at moderate to high stocking rates, pastures containing as little as 20% of oestrogenic subterranean clover (like Yarloop cultivar) might not be completely safe.

9. Ewes immune to clover infertility

In practically all flocks which become affected with clover infertility, some ewes maintain normal fertility while others in the flock become infertile (Marshall, 1973).

Despite identical histories of grazing and management, these fertile ewes are comparable to control ewes with regard to number of sperm on zona pellucida and egg fertilization rate (Lightfoot *et al.*, 1974). It is possible that resistant ewes are representative of a class that are capable of grazing selectively to achieve a diet which contains less phytoestrogen, or they are able to conjugate and detoxify plant oestrogens more effectively. A suggestion (Obst *et al.*, 1971) that resistance to clover infertility was associated with blood haemoglobin type was not supported by a subsequent study (Wroth *et al.*, 1973) involving a larger number of ewes.

Croker *et al.*, (1989) examined the hypothesis of whether ewe progeny of rams derived from ewes which consistently lambed while grazed on oestrogenic pasture had a better fertility than ewe progeny of rams derived from unselected ewes, when they were grazed on highly oestrogenic subterranean clover pastures. It was noted that the ewe progeny from ewes that sustained fertility when grazed on oestrogenic pastures did not develop clover infertility to the same extent as did ewe progeny from unselected ewes grazed on non-oestrogenic pasture. However, neither the vulvar appearance (which was masculinized) nor mucus spinnbarkeit (which was decreased) indicated that resistant ewes were protected. The study suggested that resistance to the development of clover infertility might be present in the ewes and this resistance might be genetically controlled. There seem to be no reports as to a possible physiological basis for the resistance mechanism.

10. Control of phytoestrogen induced infertility

Marshall (1973) and Lightfoot (1974) have listed a number of recommendations for the control of infertility due to oestrogenic clover. The most obvious and important way of avoiding clover infertility is to prevent sheep having access to potent pastures. When establishing new pastures this can be achieved by sowing strains with low formononetin content (Marshall, 1973).

10.1. Breeding and use of low oestrogenic cultivars

Analytical techniques that permit rapid determination of phytoestrogen levels in plants (Beck, 1964; Francis and Millington, 1965b; Gosden and Jones, 1978) have been developed and have proved very useful in plant breeding programmes to obtain cultivars free of, or low in formononetin. A clover breeding programme to develop

cultivars of subterranean clover with low oestrogenicity has been in progress in Australia since the 1960s (Collins and Cox, 1985). A maximum formononetin concentration of 0.2% of the leaf dry weight is used as a guide to selection in the programme, but levels of 0.1% or lower are preferred (Collins and Gladstone, 1985). Moseley *et al.,* (1984) indicated that even a 0.21% formononetin level in the diet was able to cause oestrogenic effects in ewes. It is still not known how low a level is needed before all adverse effects are eliminated (Collins and Gladstone, 1985).

Although serious reproductive problems in animals have not occurred in countries other than Australia, a low formononetin level has been included as one of the objectives in the subterranean and red clover breeding programmes in other countries. Low formononetin red clover cultivars are being developed in New Zealand (Hay and Ryan, 1989) and Britain (Gosden *et al.,* 1984).

10.2. Other measures

Other measures proposed to prevent/minimize the problem are changes in grazing management of the flock, dilution of oestrogenic cultivars in the pasture, and optimum fertilizer applications to the pasture (Lightfoot, 1974). In addition to agronomic measures some other potential measures may include immunization of the ewes against phytoestrogens, and alteration of the metabolism of phytoestrogens in animals (Cox, 1978).

11. Oestrogenic clover and New Zealand

Disturbances in reproductive function in sheep grazing red clover have been recorded in New Zealand since the 1950s (See Pope, 1954; Ch'ang, 1958; Ch'ang, 1961), although these have been neither permanent nor as serious as those occurring in Australia. Two red clover types which have long been used in farming practice in New Zealand are 'Grasslands Hamua' and 'Grasslands Turoa' (Anderson, 1978). Both of these contain high levels of formononetin (0.76-0.99%) (Kelly *et al.,* 1979). A study conducted in Australia showed that ewes mated on a mixed pasture containing Hamua red clover suffered from temporary infertility characterized by high returns to service and a low percentage of multiple births (Morley *et al.,* 1966).

A late flowering tetraploid red clover, 'Grasslands Pawera', was released in New

Zealand in 1974 (Anonymous, 1982). Pawera red clover, which was derived largely from seed of Turoa origin but included some material of Swedish origin (Anderson, 1973a), had a greater persistence and production than the diploid red clovers Hamua and Turoa (Anderson, 1973b). Hay and Ryan (1989) also showed that Pawera was more productive and persistent than other red clover cultivars, and that its strong summer growth met the need for heavy-weight lamb feed and high quality forage for conservation in intensive sheep farming systems in Southland. But this cultivar was also shown to contain high levels of formononetin, and to be highly oestrogenic to sheep (Kelly *et al.*, 1979). Pastures dominant in Pawera red clover were found to be unsuitable for grazing by ewes at or about mating as the grazing of Pawera pasture for 8 days before and during the first cycle of mating resulted in a reduction in the incidence of oestrus, low ovulation rate and high returns to service (Kelly *et al.*, 1980). Furthermore, as the formononetin concentration in Pawera red clover was found to be high throughout the year (0.64-1.38%), it was suggested that even a 30% red clover dilution in non-oestrogenic pasture might not be 'safe' for sheep fertility in the long term (Kelly *et al.*, 1979). Under typical New Zealand pastoral conditions, permanent clover infertility is unlikely to occur but Shackell *et al.*, (1993) showed permanent infertility could be induced in ewes after prolonged grazing on Pawera red clover.

It has been indicated that red clover should only contribute from 10-15% of the sward in New Zealand (Jagusch, 1983), and thus low lambing percentage due to the presence of phytoestrogens in New Zealand pastures might not be a national problem. However, it is essential that phytoestrogen activity be considered in plant breeding programmes. Subclinical problems due to oestrogenic clover may be present on individual farms in New Zealand. Hay and Ryan (1989) commented that oestrogenic activity of Pawera red clover has deterred many farmers from realising the potential benefits of red clover in their farming systems.

Grasslands Division of the New Zealand DSIR (now incorporated in AgResearch Grasslands) initiated a programme to select within Pawera red clover for a low formononetin population (Hay and Ryan, 1989). Selection was based on decreasing the formononetin concentrations of the leaflets of Pawera red clover. After 6 generations of selection, a potential new cultivar, G27, was put under evaluation. A preliminary study showed that reproductive performance of ewes, grazed for 6 weeks prior to and for 12 days of the first mating cycle on G27 red clover, was comparable to those grazed on non-oestrogenic pasture (Williams, 1988). In another study, conception rate

in ewes mated on G27 red clover was found to be higher than that in ewes mated on Pawera or on non-oestrogenic pasture (R. Keogh and M.F. McDonald, personal communication).

12. Purpose and scope of the study

The purpose of the present study was to investigate and measure the decrease brought about in the oestrogenic effects of the low formononetin selection, 'G27' red clover, in ewes as compared to those of Pawera red clover. In a detailed comparison of the formononetin content between the two clover strains, differences between G27 and Pawera red clovers were studied at various stages of plant growth and in various parts of the plant (Chapter III). Effects of grazing G27 and Pawera red clover on various reproductive parameters in ewes were studied and the results are presented in separate chapters.

The specific objectives of the experiments involved in this programme were:

1) To determine differences in formononetin content between G27 and Pawera red clover at different stages of plant growth and between various plant parts.

2) To study the effects on ovarian follicle development in ewes grazed on both types of red clover and in Control ewes.

3) To compare ovulation rate, fertility, and litter size in ewes when fed on the two types of clovers close to the time of mating.

4) As fertility differences were apparent, the effects of grazing low and high formononetin red clovers were studied on sperm transport and embryo survival in ewes. For this latter study, to overcome potential fertilization problems, egg transfer techniques were used to provide embryos to the animals that grazed on the clover or the Control pastures.

CHAPTER III

Comparison of formononetin concentration and yield between 'Grasslands' Pawera red clover and G27 red clover, at different stages of plant development

1. Abstract

The study was conducted to characterize formononetin concentration in various components of a newly selected G27 red clover, and Pawera red clover during different stages of plant growth under field conditions. Mean formononetin concentration (percent dry weight) of leaflets and petioles was lower for G27 than for Pawera ($P<0.05$) at various stages of vegetative leaf development. G27 leaflet concentrations (0.29 ± 0.02) changed little during development compared to Pawera leaflets which declined from 2.16 ± 0.10 in the youngest leaflets to 0.75 ± 0.08 by the end of vegetative leaf development. Formononetin concentration in G27 leaves (leaflets + petioles) at pre-flowering stage was 0.35% compared to 0.97% in Pawera leaves ($P<0.05$). At early and late-flowering stages, the formononetin concentration in G27 red clover, on a whole plant basis, was 50% lower than Pawera red clover because the formononetin concentration in petioles and stems of G27 did not decline to the same extent as in the leaflets. When calculated only for the upper parts of the plant which are usually ingested by sheep, G27 and Pawera red clover contained 0.27% and 0.99% formononetin, respectively, at the early-flowering stage, and 0.19% and 0.53% formononetin, respectively, at the late-flowering stage. The results of this study indicate that due to a lower formononetin concentration at various stages of plant growth, G27 red clover might be a safer feed for breeding ewes than Pawera red clover.

2. Introduction

A permanent or temporary infertility may occur in ewes following the excessive ingestion of oestrogenic subterranean clover or of red clover (Bennetts *et al.*, 1946; Barrett *et al.*, 1965; Morley *et al.*, 1966; Kelly *et al.*, 1980; Shackell *et al.*, 1993). The substances considered responsible for the oestrogenic activity are the isoflavones, formononetin, daidzein, biochanin A, and genistein, and possibly pratensein (Davies, 1987). The evidence indicates that formononetin plays a major role in the oestrogenic activity of subterranean clover and red clover to sheep (Millington *et al.*, 1964; Morley

et al., 1968; Davies *et al.*, 1970). The problem can be minimized by induction of qualitative changes in the pasture and thus reducing the quantity of phytoestrogen ingested by sheep (Lightfoot 1974). The most important control measure is the breeding and use of cultivars low in formononetin. For plant breeding purposes where single plant performance is the criterion of initial selection, techniques suitable for measuring formononetin concentration in a large number of small samples have been developed (Francis and Millington, 1965b; Gosden and Jones, 1978). In Australia where subterranean clover has been the cause of the most damaging oestrogenicity, low formononetin cultivars of subterranean clover have been bred and distributed (Collins and Cox, 1985).

In New Zealand, a very productive tetraploid red clover, 'Grasslands Pawera', has been shown to contain high concentrations of the isoflavone formononetin, and to be highly oestrogenic to sheep (Kelly *et al.*, 1979; Kelly *et al.*, 1980). Pawera is not unique in this characteristic as other New Zealand red clovers (e.g. Hamua and Turoa) also contain relatively high concentrations of formononetin, and it would appear to be the exception rather than the rule to find red clovers with low formononetin concentration, unless they have been bred for this purpose (Kelly *et al.*, 1979).

In an endeavour to produce plant material with low formononetin (concentration), Pawera red clover plants were screened for formononetin concentration by the method of Gosden and Jones (1978). The aim was to produce (after a number of generations) a late-flowering, palatable, tetraploid red clover with agronomic attributes similar to those of Pawera red clover, but without the associated oestrogenicity problems. G27 red clover was a 6th generation selection based on decreasing the formononetin concentration of plant leaflets.

Variations in formononetin concentrations have been noted between different plant components and at different stages of development in both red and subterranean clover. Changes in formononetin concentration with plant maturity have been shown to be strain dependent in red clover (Wong, 1963) and in subterranean clover (Rossiter and Beck, 1967). Studies with subterranean clover have shown relatively low concentrations of formononetin in the petiole portion (Francis and Millington, 1965a). The concentration of formononetin in subterranean clover usually decreases with development of individual leaves from emergence to senescence (Rossiter and Beck, 1967). McMurray *et al.*, (1986) also showed that formononetin concentration in red

Figure 3.1 Pawera and G27 red clover plants in pots

clover declined with plant age in all plant parts.

The present study was carried out to characterize the formononetin concentrations in various plant components of G27 and of Pawera red clovers during different stages of vegetative and reproductive growth under field conditions. Specific objectives were to compare the changes in the formononetin concentration and yield (1) in the leaves of Pawera and G27 red clover during a developmental sequence from leaf emergence to its senescence, (2) in pre-flowering shoots of the two strains, (3) in various parts of the shoots at early-flowering stage, and (4) in various parts of the shoots from two strains at late-flowering stage. Dry weights of various plant components of G27 and Pawera red clover were also compared.

3. Materials and methods

The terminology used in this chapter is defined below.

Formononetin concentration; Percent formononetin on a dry weight basis per component.

Formononetin yield; Formononetin (mg) per component. Calculated from percent formononetin and dry weight of the component.

Part of a shoot; Shoots were dissected into various parts i.e. leaves (further split into leaflets and petioles), axillary shoots, internodes, flower and bract. In the following text, parts of the shoots are numbered from youngest to the oldest e.g. leaf1 is the youngest leaf on the shoot.

3.1. Plant sampling

Pawera and G27 red clover plots established on the Sheep and Beef Cattle Research Unit at Massey University were used for plant sampling. These were on a soil type classified as Tokomaru Silt Loam. Collection of leaf and shoot samples at various stages of plant growth was done as described below.

3.1.1. Formononetin in vegetative (developing) leaves

The leaves of the Pawera and G27 red clover were sampled during a developmental sequence starting with leaflet emergence and finishing at leaf senescence. Leaf sampling was done during spring (September-October) as well as summer (December-January). Following is the description for the spring sampling. More than 500 newly emerging leaflets (still folded) were marked on different plants with a red dye on 23rd of September. The area was well grazed by sheep two days before the leaf marking. Marked leaves were sampled on day 1 (marking day), day 4 and day 9 of the regrowth periods and then at weekly intervals until day 37 when about 60% of leaves were senescent. Four samples were collected on each occasion. On day 1 and day 4, the number of leaves per sample was 20 and 15 respectively. On subsequent days 10 leaves were taken per sample. The fresh leaves in each sample were dissected into leaflets and petioles (Figure 3.2a), put in separate plastic vials and stored at -15°C until required for freeze-drying, and subsequent processing in the formononetin assay. The summer sampling started on 22nd of December. Newly emerging leaves were marked, and sampled at day 1, and day 6 of growth, and then at weekly intervals until day 34 of leaf age.

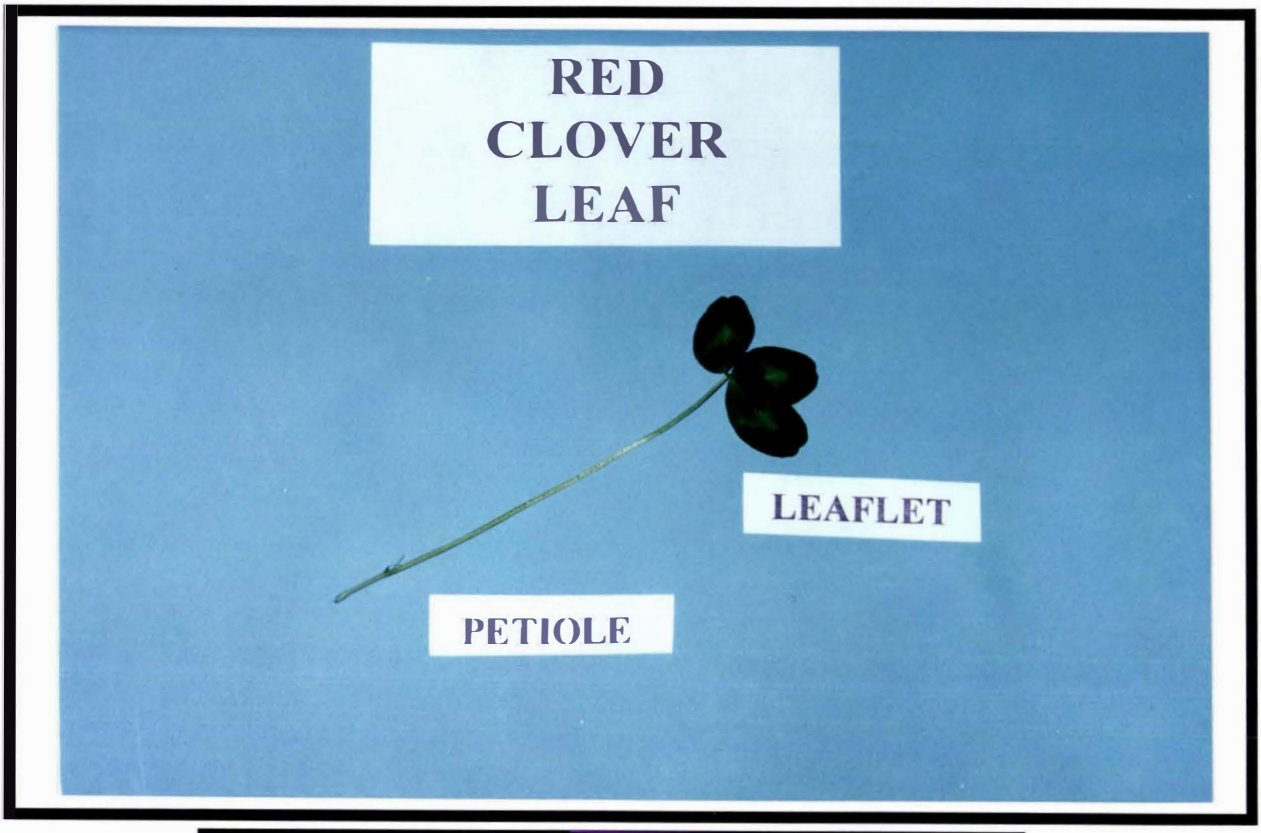
3.1.2. Formononetin in pre-flowering shoots

Four samples of 10 shoots each were collected from different parts of G27 and Pawera paddocks in the second week of November. It was the 53rd day after the last grazing on the paddocks. The shoots sampled were at a comparable stage of growth, each having four leaves on it (see Figure 3.2b). The youngest leaf in each case had emerged from stipule and unfolded. In the laboratory, the leaves in each sample were removed from the stem and grouped according to age. They were further dissected into leaflets and petioles, put in separate containers, and stored at -15°C until required for further processing.

3.1.3. Formononetin in plants at an early-flowering stage

Four samples per strain with 5 shoots per sample were collected at a comparable stage of growth during the last week of December. Each shoot had one newly emerging flower on the top (Figure 3.2c). In the laboratory, the shoots in each sample were dissected into individual leaves, axillary shoots, internodes, flower bracts and flowers.

Figure 3.2 a & b Parts of the red clover plant.



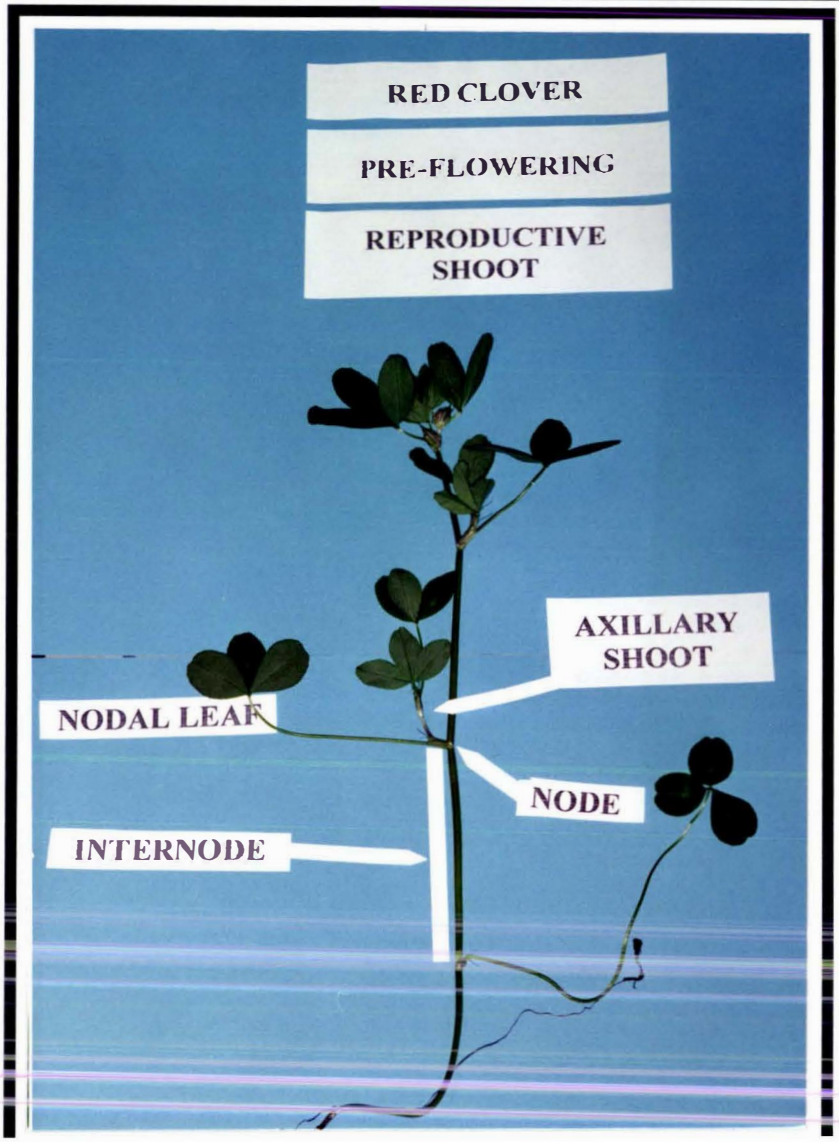


Figure 3.2c Parts of the red clover plant

Different parts were stored separately at -15°C until required for further processing.

3.1.4. Formononetin in plants at a late-flowering stage

Shoots, with one fully opened flower (that had not withered or browned off) were sampled, during the first week of February.
Four samples per strain and 5 shoots (at similar stage of growth) per sample were collected. Each shoot was dissected into various parts and stored at -15°C.

3.2. Formononetin assay

Formononetin concentration was determined by a modification of the fluorimeteric assay described by Gosden and Jones (1978). The method uses the fluorescence of a crude clover extract. The fluorescence determinations are carried out as solutions in ethanol containing 2% aqueous ammonia.

The frozen samples were freeze dried, weighed and then finely ground. 25 mg of the ground sample was put in a glass vial. Distilled water (1.5 ml) was added to the sample which was incubated overnight at room temperature. In the morning 3.5 ml ethanol was added per vial. The mixture was shaken and left for two hours when a sub-sample (20 µl) of the supernatant was placed in ethanolic ammonia (2 ml) and the fluorescence compared to that of a formononetin standard using a fluorimeter. The fluorimeter scale ranged from 1-100. Scale reading for the formononetin standard (20 µl of which was dissolved in a 2 ml ethanolic ammonia) was set at 100. Formononetin percentage was calculated from the fluorimeteric reading by the following formula.

$$\frac{\textbf{Read x St x Vol x 100}}{\textbf{100 x WT}} = \textbf{Formononetin (\% dry weight)}$$

Where
Read = Fluorimeter Scale reading for a sample
St = Formononetin standard used to compare fluorescence (µg/ml)
Vol = Volume of extraction fluid (5 ml in the present experiment)
Wt = Weight of ground sample used for formononetin extraction
(25 x 1000 µg in the present experiment).

Overnight incubation was employed after addition of water, in the present study, compared to 0.5 h incubation described in the original method. Moreover, after the addition of ethanol, extraction was allowed to occur for two hours compared to one hour. The modification was necessary to ensure complete hydrolysis of formononetin glycoside especially in plant components such as stem and petiole which contain relatively small amounts of β-glucosidase.

3.3. Statistical analyses

Analysis of variance was used to compare formononetin concentration, yield and dry weight between various plant parts of the two strains. The means were compared by Duncan's multiple range test. The level of statistical significance was set at $P<0.05$. The data were analysed using the Statistical Analysis System computer package (SAS Institute Inc., 1988). The data are expressed as mean ± SEM.

4. Results

4.1. Formononetin during leaf development

Formononetin concentrations in G27 leaves were not very different during spring and summer, although the concentrations in Pawera leaves were slightly higher in spring than those during summer sampling. The concentration pattern for leaflets and petioles of G27 was similar (Figure 3.3a and 3.3b) during the two seasons. Therefore only the spring results will be presented in this section. The summer results are presented in Appendix 1.

4.1.1. Formononetin concentration

Table 3.1 and Figure 3.3a summarize the concentration of formononetin during vegetative leaf development in spring.

Mean formononetin concentration in G27 leaflets and petioles was significantly lower ($P<0.05$) than that in Pawera leaflets and petioles respectively at all stages of development except for petioles on day 16 when the difference failed to reach significance.

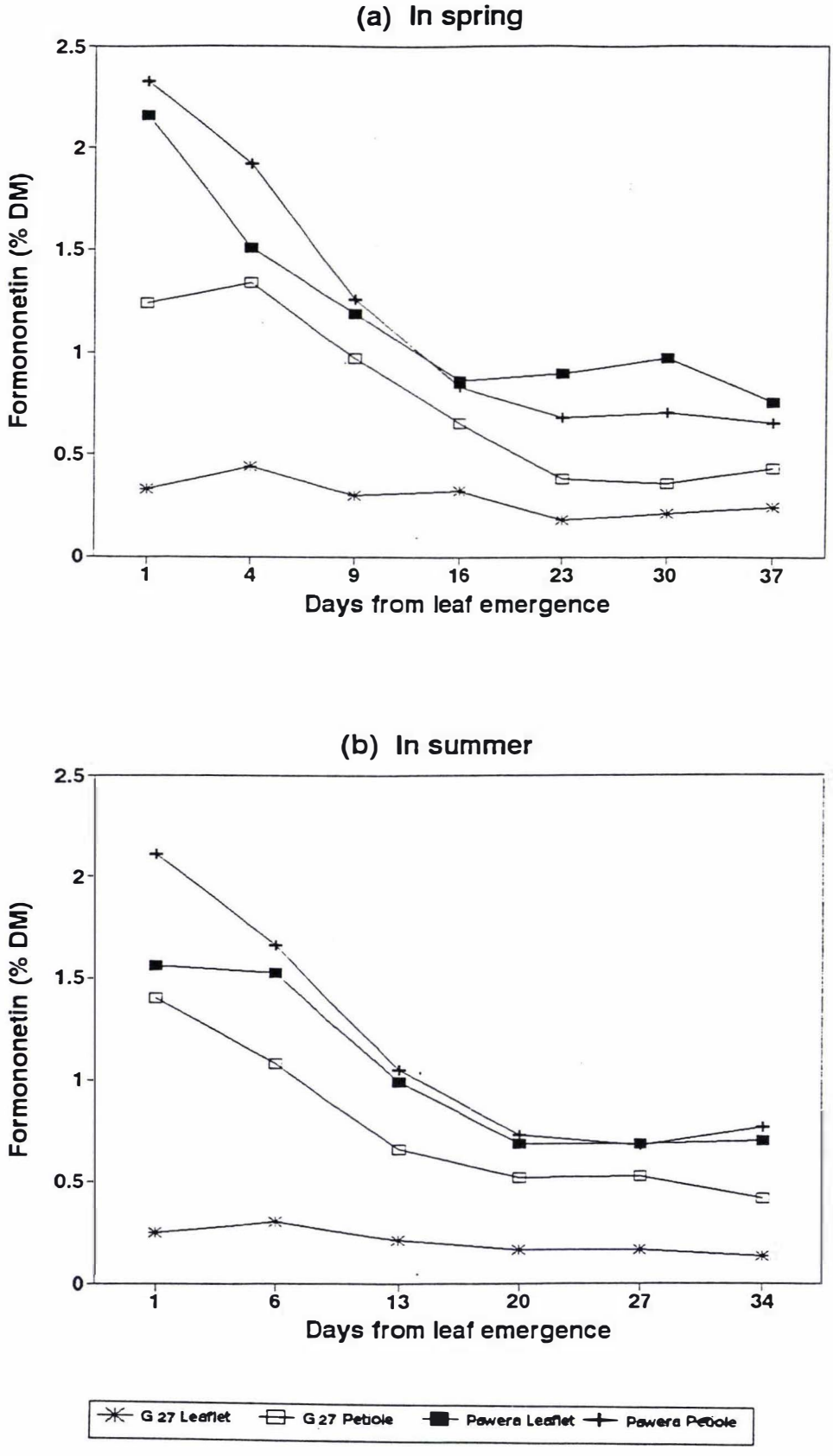


Figure 3.3. Mean formononetin concentrations in leaflets and petioles during vegetative leaf development in G27 and Pawera red clovers.

G27 leaflet concentrations (0.29 ± 0.02) changed little compared to Pawera leaflet concentrations which declined from >2% in very young leaflets to approximately one third of this concentration by the end of the leaf development. Within G27 the formononetin concentration was higher in petioles than leaflets throughout leaf development but was only significantly higher until day 16 ($P<0.05$). There was a closer similarity in leaflet and petiole formononetin concentrations for Pawera in contrast to that of G27.

Table 3.1. Mean formononetin concentrations in vegetative leaflets and petioles from leaf emergence to senescence in G27 and Pawera red clovers.

Age of leaf	Formononetin (% DM)			
	G27		Pawera	
(days)	Leaflet	Petiole	Leaflet	Petiole
1	0.33 ± 0.04^{ij}	1.24 ± 0.03^{d}	2.16 ± 0.10^{a}	2.33 ± 0.07^{a}
4	0.44 ± 0.02^{i}	1.34 ± 0.15^{cd}	1.51 ± 0.05^{c}	1.92 ± 0.19^{b}
9	0.30 ± 0.04^{ij}	0.97 ± 0.07^{e}	1.19 ± 0.03^{d}	1.26 ± 0.01^{d}
16	0.32 ± 0.05^{ij}	0.65 ± 0.03^{h}	0.86 ± 0.02^{efg}	0.83 ± 0.03^{efgh}
23	0.18 ± 0.01^{j}	0.38 ± 0.03^{ij}	0.90 ± 0.05^{ef}	0.68 ± 0.04^{gh}
30	0.21 ± 0.02^{j}	0.36 ± 0.03^{ij}	0.97 ± 0.06^{e}	0.70 ± 0.02^{gh}
37	0.24 ± 0.01^{ij}	0.43 ± 0.02^{i}	0.75 ± 0.08^{fgh}	0.65 ± 0.02^{h}

Means with different superscripts in the same row or same column differ significantly ($P<0.05$).

Formononetin concentration calculated for the whole leaf (leaflet + petiole) is shown in Table 3.2. The concentration was significantly lower in G27 leaves than Pawera leaves ($P<0.05$). Minimum values for formononetin concentration were reached sooner in Pawera (day 16) than those in G27 (day 23) in spring.

Formononetin concentration in the developing leaves was not significantly different from that in the corresponding leaflets ($P>0.05$) in both the clovers (Table 3.2). But the

Table 3.2. Mean formononetin concentrations in vegetative leaf (leaflet + petiole) during development.

Age of leaf (days)	Formononetin (% DM) G27	Pawera
1	0.42 ± 0.03^{fg}	2.17 ± 0.10^{a}
4	0.52 ± 0.03^{f}	1.55 ± 0.05^{b}
9	0.35 ± 0.04^{ghi}	1.20 ± 0.02^{c}
16	0.37 ± 0.04^{fgh}	0.86 ± 0.02^{de}
23	0.22 ± 0.01^{i}	0.86 ± 0.05^{de}
30	0.24 ± 0.02^{hi}	0.91 ± 0.05^{d}
37	0.28 ± 0.01^{ghi}	0.73 ± 0.06^{e}

Means with different superscript letters in the same row or same column differ significantly ($P<0.05$).

concentration was significantly lower than the corresponding petioles until day 16 of the leaf growth ($P<0.05$) in G27 red clover; in Pawera red clover it was not different from the corresponding petiole. Formononetin concentration in G27 leaves was 19-43% of that in Pawera leaves.

4.1.2. Dry weight per part and formononetin yield

Dry weights and formononetin yields of developing leaflets and petioles are shown in Table 3.3. G27 leaflets and petioles were significantly lighter ($P<0.05$) in weight compared to Pawera ones except in the younger parts (leaflet at day 1 and petiole at day 1 and 4) where differences were not significant.

Formononetin yield in G27 leaflets and petioles was significantly lower than in the Pawera leaflets and petioles respectively ($P<0.05$). In the youngest petioles, the concentration was not different between the two clovers.

Table 3.3. Dry weight and formononetin yield per leaflet or petiole from leaf emergence to senescence in G27 and Pawera red clovers.

Age	Dry Weight (mg)		Formononetin yield (mg)	
(days)	G27	Pawera	G27	Pawera
Leaflet				
1	8.98 ± 1.12^{f}	13.90 ± 0.80^{ef}	0.03 ± 0.01^{d}	0.30 ± 0.02^{a}
4	10.05 ± 1.56^{f}	16.95 ± 1.37^{de}	0.04 ± 0.01^{d}	0.26 ± 0.02^{ab}
9	12.30 ± 1.79^{ef}	24.20 ± 3.53^{bc}	0.04 ± 0.01^{d}	0.29 ± 0.05^{ab}
16	17.92 ± 1.75^{cde}	32.08 ± 3.62^{a}	0.06 ± 0.00^{d}	0.28 ± 0.03^{ab}
23	18.87 ± 1.05^{bcde}	34.92 ± 2.22^{a}	0.03 ± 0.00^{d}	0.31 ± 0.01^{a}
30	17.52 ± 1.73^{de}	24.82 ± 3.12^{b}	0.04 ± 0.00^{d}	0.24 ± 0.04^{b}
37	14.58 ± 1.10^{ef}	22.85 ± 0.99^{bcd}	0.03 ± 0.00^{d}	0.17 ± 0.02^{c}
Petiole				
1	0.86 ± 0.04^{h}	0.91 ± 0.10^{h}	0.01 ± 0.00^{f}	0.02 ± 0.00^{ef}
4	0.90 ± 0.06^{h}	1.42 ± 0.23^{gh}	0.01 ± 0.00^{f}	0.03 ± 0.01^{de}
9	1.03 ± 0.13^{h}	2.68 ± 0.19^{fg}	0.01 ± 0.00^{f}	0.03 ± 0.00^{cd}
16	3.25 ± 0.50^{ef}	5.45 ± 0.95^{cd}	0.02 ± 0.00^{ef}	0.04 ± 0.01^{bc}
23	4.80 ± 0.30^{d}	9.33 ± 0.86^{e}	0.02 ± 0.00^{ef}	0.06 ± 0.01^{a}
30	4.60 ± 0.77^{de}	7.00 ± 0.62^{b}	0.02 ± 0.00^{ef}	0.05 ± 0.01^{b}
37	4.08 ± 0.40^{def}	6.33 ± 0.39^{bc}	0.02 ± 0.00^{ef}	0.04 ± 0.00^{bc}

Means with different superscripts within same parameter and same part differ significantly ($P<0.05$).

Within G27, formononetin yield per leaflet or per petiole did not differ significantly at any stage.

Within Pawera, the formononetin yield was lowest ($P<0.05$) in the senesced leaflet but the yield was not significantly different in the leaflets from day 1 to day 30. In Pawera petioles, the yield increased with increasing age and was at a maximum on day 23.

4.2. Formononetin in leaves from the pre-flowering shoots

4.2.1. Formononetin concentration

Formononetin concentrations in leaflets and petioles of pre-flowering shoots are presented in Table 3.4. Mean formononetin concentration in G27 leaflets at different ages was less than 0.30% and was significantly lower than in Pawera leaflets ($P<0.05$). Mean formononetin concentration in various G27 petioles was lower than that in corresponding Pawera petioles but it did not differ significantly between the two strains except in the youngest petiole ($P<0.05$).

Table 3.4. Mean formononetin concentrations in leaflets and petioles on pre-flowering shoots of G27 and Pawera red clovers.

	Formononetin (% DM)	
Part	G27	Pawera
Leaflet1	0.29 ± 0.03^{g}	1.58 ± 0.09^{a}
Leaflet2	0.23 ± 0.01^{g}	0.93 ± 0.07^{b}
Leaflet3	0.22 ± 0.01^{g}	0.80 ± 0.03^{bcd}
Leaflet4	0.20 ± 0.01^{g}	0.84 ± 0.06^{bc}
Petiole1	0.97 ± 0.04^{b}	1.47 ± 0.18^{a}
Petiole2	0.56 ± 0.03^{ef}	0.68 ± 0.07^{cde}
Petiole3	0.53 ± 0.03^{ef}	0.59 ± 0.01^{ef}
Petiole4	0.47 ± 0.04^{f}	0.64 ± 0.04^{def}
All leaflets	0.23 ± 0.01	1.04 ± 0.09
All petioles	0.63 ± 0.05	0.85 ± 0.10
All leaves	0.35 ± 0.02	0.97 ± 0.09

Means with different superscripts in the same row or same column differ significantly ($P<0.05$).

Within G27 red clover the concentration did not differ between the four leaflets ($P>0.05$) but amongst petioles it was highest in the youngest one ($P<0.05$).

Within Pawera red clover, the youngest leaflet had the highest concentration ($P<0.05$), but the concentrations did not differ significantly in the three older leaflets. Concentration within Pawera petioles also was highest in the youngest petiole ($P<0.05$), but it did not differ between the older petioles.

The mean formononetin concentration in all the four leaves on pre-flowering shoots is shown in Table 3.4 (calculated from formononetin yields and dry weights of leaflets and petioles). The concentration in G27 leaves (leaflets+petioles) was 0.35% as compared to 0.97% in Pawera leaves ($P<0.05$).

4.2.2. Dry weight per part and formononetin yield

Formononetin yield and dry weight per part in the pre-flowering shoots is summarized in Table 3.5. G27 leaflets and petioles had lower ($P<0.05$) dry weights than the corresponding Pawera leaflets and petioles respectively except for the oldest leaflet, and the youngest and the oldest petioles.

Formononetin yield was significantly lower in G27 leaflets and petioles than the Pawera leaflets and petioles respectively ($P<0.05$).

The mean yield did not differ significantly ($P>0.05$) between G27 leaflets at various ages although it was lower in the oldest leaflet. The yield declined significantly ($P<0.05$) in Pawera leaflets with maturity.

Among petioles the yield was higher in second and third petioles in both the strains

G27 leaves weighed 69 percent of Pawera leaves but contained only 26 percent of the formononetin yield present in Pawera shoots (calculated from table 3.5).

Table 3.5. Average dry weight and formononetin yield per leaflet and per petiole of pre-flowering shoots.

	Dry Weight (mg)		Formononetin yield (mg)	
Part	G27	Pawera	G27	Pawera
Leaflets				
Leaflet1	44.56 ± 5.56^{cd}	67.25 ± 3.77^{b}	0.13 ± 0.02^{d}	1.06 ± 0.07^{a}
Leaflet2	72.31 ± 8.81^{b}	107.00 ± 5.29^{a}	0.16 ± 0.01^{d}	1.00 ± 0.11^{a}
Leaflet3	61.31 ± 6.24^{bc}	94.00 ± 5.28^{a}	0.13 ± 0.01^{d}	0.75 ± 0.06^{b}
Leaflet4	31.00 ± 8.88^{d}	42.50 ± 2.63^{cd}	0.06 ± 0.02^{d}	0.36 ± 0.04^{c}
Subtotal	209.18	310.75	0.48	3.17
Petioles				
Petiole1	13.63 ± 1.28^{e}	14.25 ± 0.25^{e}	0.13 ± 0.01^{cd}	0.21 ± 0.02^{b}
Petiole2	38.69 ± 3.18^{b}	51.75 ± 1.80^{a}	0.21 ± 0.00^{b}	0.35 ± 0.04^{a}
Petiole3	35.06 ± 5.59^{bc}	51.50 ± 2.90^{a}	0.18 ± 0.02^{bc}	0.30 ± 0.01^{a}
Petiole4	18.33 ± 5.94^{de}	26.00 ± 0.71^{cd}	0.08 ± 0.03^{d}	0.17 ± 0.01^{bc}
Subtotal	105.71	143.50	0.60	1.03
Overall Total	314.89	454.25	1.08	4.20

Means with different superscript letters within same parameter and same part differ significantly ($P<0.05$).

4.3. Formononetin at the early-flowering stage

4.3.1. Formononetin concentration

Formononetin concentration (% DM) in various parts of the plant at the early-flowering stage is given in Table 3.6. Mean formononetin concentrations were significantly lower in G27 red clover than those in Pawera red clover in all the upper

Table 3.6. Formononetin concentration in various parts of G27 and Pawera red clover shoots at early-flowering stage.

Part[1] of the shoot	Formononetin (% DM)	
	G27	Pawera
Flower and bract		
Flower	0.16 ± 0.02^{b}	0.23 ± 0.01^{b}
Fl.Bract	0.28 ± 0.04^{b}	1.22 ± 0.09^{a}
Flower+Bract	0.16	0.33
Leaves		
Leaf1	0.16 ± 0.01^{cd}	0.99 ± 0.07^{a}
Leaf2	0.15 ± 0.01^{cd}	0.95 ± 0.14^{a}
Leaf3	0.17 ± 0.01^{cd}	0.78 ± 0.11^{b}
Leaf4	0.16 ± 0.01^{cd}	0.34 ± 0.03^{c}
Leaf5	0.14 ± 0.03^{d}	0.20 ± 0.04^{cd}
R.Leaves	0.06 ± 0.01^{d}	0.18 ± 0.05^{cd}
All leaves	0.14	0.71
Axillary shoots		
Ax.sh.2	0.35 ± 0.04^{d}	1.52 ± 0.07^{a}
Ax.sh.3	0.37 ± 0.01^{d}	1.41 ± 0.13^{a}
Ax.sh.4	0.54 ± 0.03^{cd}	1.35 ± 0.08^{ab}
Ax.sh.5	0.46 ± 0.05^{d}	1.12 ± 0.17^{b}
R.Ax.sh.	0.43 ± 0.07^{d}	0.79 ± 0.11^{c}
All ax.sh.	0.43	1.28
Internodes		
IN1	0.35 ± 0.03^{e}	1.30 ± 0.12^{a}
IN2	0.49 ± 0.04^{de}	0.90 ± 0.11^{b}
IN3	0.39 ± 0.03^{de}	0.55 ± 0.07^{de}
IN4	0.35 ± 0.05^{e}	0.40 ± 0.06^{de}
IN5	0.32 ± 0.04^{e}	0.44 ± 0.07^{de}
INr	0.61 ± 0.05^{cd}	0.79 ± 0.11^{bc}
All INs	0.47	0.64
Overall	0.37	0.78

Means with different superscript letters within same group of parts differ significantly ($P<0.05$).

[1] Parts arranged from the youngest to the oldest (e.g. leaf 1 = youngest leaf on the shoot). R.Leaves = remainder leaves.

parts of the plant i.e. the flower bracts, three youngest leaves, all the axillary shoots and the two youngest internodes ($P<0.05$). In the plant parts where the differences between the two strains were not significant, G27 always had the lower formononetin concentration. The formononetin concentration calculated on the whole plant basis was 0.37% in G27 and 0.78% in Pawera red clover.

Within G27 red clover shoots, formononetin concentration was not different ($P>0.05$) among the leaves although the oldest leaf had the lowest concentration. The concentration also did not differ significantly between G27 axillary shoots. Amongst internodes, formononetin concentration was highest in the oldest but it did not differ significantly from the 2nd and 3rd youngest internodes.
In Pawera red clover, formononetin concentration was significantly higher in the two youngest leaves and declined with increasing age of the leaf ($P<0.05$). Similarly, in the axillary shoots of Pawera, the concentration declined in the older shoots ($P<0.05$). Among Pawera internodes, the youngest had the highest formononetin concentration ($P<0.05$); the concentration declined in the lower internodes but it increased again in the lowest internodes.

4.3.2. Dry weight per part and formononetin yield

Formononetin yield and dry weight per part of the early-flowering shoot are given in Table 3.7. Most parts of G27 red clover shoot had significantly lower weights than those of Pawera red clover. Total shoot weight was 1712 mg for G27 and 2038 mg for Pawera red clover. Overall formononetin yield from G27 and Pawera shoots was 6.37 and 15.96 mg respectively. Formononetin yield was significantly lower in most of the younger parts of G27 shoots (flower and bract, leaf 1-3, axillary shoot 1-3, internode 2-3) than those of Pawera shoots ($P<0.05$). Differences in yield were not significant between the other parts of the shoot.

Amongst the G27 leaves, axillary shoots, and internodes, formononetin yield did not change significantly with age. But within the parts of Pawera red clover shoot, formononetin yield increased with increasing age and then declined ($P<0.05$).

4.3.3. Distribution of formononetin in various parts of the shoot

The distribution (percent) of formononetin in different parts of the early-flowering

Table 3.7. Average dry weight and formononetin yield per part at early-flowering stage.

Part of	Dry Weight (mg)		Formononetin yield(mg)	
the shoot	G27	Pawera	G27	Pawera
Flower and bract				
Flower	52.50 ± 5.56^{b}	69.50 ± 2.50^{a}	0.08 ± 0.01^{b}	0.16 ± 0.01^{a}
Fl.Bract	5.00 ± 0.58^{c}	7.00 ± 0.58^{c}	0.01 ± 0.00^{c}	0.09 ± 0.01^{b}
Subtotal	57.50	76.50	0.09	0.25
Leaves				
Leaf1	32.50 ± 1.50^{e}	69.50 ± 5.19^{c}	0.05 ± 0.00^{d}	0.69 ± 0.07^{b}
Leaf2	75.50 ± 4.03^{c}	119.00 ± 6.24^{a}	0.11 ± 0.01^{cd}	1.12 ± 0.15^{a}
Leaf3	96.50 ± 6.95^{b}	125.00 ± 9.95^{a}	0.16 ± 0.01^{cd}	0.96 ± 0.13^{a}
Leaf4	81.00 ± 3.32^{bc}	80.00 ± 6.68^{bc}	0.13 ± 0.01^{cd}	0.27 ± 0.02^{c}
Leaf5	53.00 ± 5.26^{d}	25.50 ± 2.36^{e}	0.07 ± 0.01^{d}	0.05 ± 0.01^{d}
R.Leaves	71.00 ± 4.80^{c}	24.00 ± 5.35^{e}	0.05 ± 0.01^{d}	0.04 ± 0.01^{d}
Subtotal	409.50	443.00	0.57	3.13
Axillary shoots				
Ax.sh.2	14.00 ± 1.63^{d}	57.50 ± 8.38^{cd}	0.05 ± 0.01^{d}	0.89 ± 0.16^{c}
Ax.sh.3	73.50 ± 6.45^{bcd}	173.50 ± 25.93^{a}	0.27 ± 0.02^{cd}	2.37 ± 0.20^{a}
Ax.sh.4	63.50 ± 9.54^{cd}	123.50 ± 39.56^{abc}	0.34 ± 0.05^{cd}	1.62 ± 0.51^{b}
Ax.sh.5	139.50 ± 39.76^{ab}	40.50 ± 17.88^{d}	0.63 ± 0.16^{cd}	0.45 ± 0.21^{cd}
R.ax.sh.	147.00 ± 19.77^{a}	54.50 ± 24.86^{cd}	0.60 ± 0.06^{cd}	0.44 ± 0.22^{cd}
Subtotal	437.50	449.50	1.89	5.77
Internodes				
IN1	10.50 ± 2.50^{f}	31.50 ± 5.68^{f}	0.04 ± 0.01^{e}	0.39 ± 0.04^{de}
IN2	58.00 ± 4.69^{ef}	147.50 ± 15.02^{cd}	0.28 ± 0.02^{e}	1.34 ± 0.25^{bc}
IN3	117.00 ± 16.54^{cd}	275.00 ± 18.95^{b}	0.46 ± 0.08^{de}	1.48 ± 0.12^{b}
IN4	168.00 ± 9.49^{c}	233.50 ± 25.59^{b}	0.58 ± 0.08^{de}	0.91 ± 0.10^{cd}
IN5	106.50 ± 10.72^{de}	110.50 ± 18.36^{cd}	0.33 ± 0.05^{de}	0.47 ± 0.07^{de}
INr	347.50 ± 14.38^{a}	271.00 ± 43.75^{b}	2.13 ± 0.21^{a}	2.22 ± 0.53^{a}
Subtotal	807.50	1069.00	3.82	6.81
Overall total	1712.00	2038.00	6.37	15.96

Means with different superscripts letters within same parameter and same group of parts differ significantly ($P<0.05$).

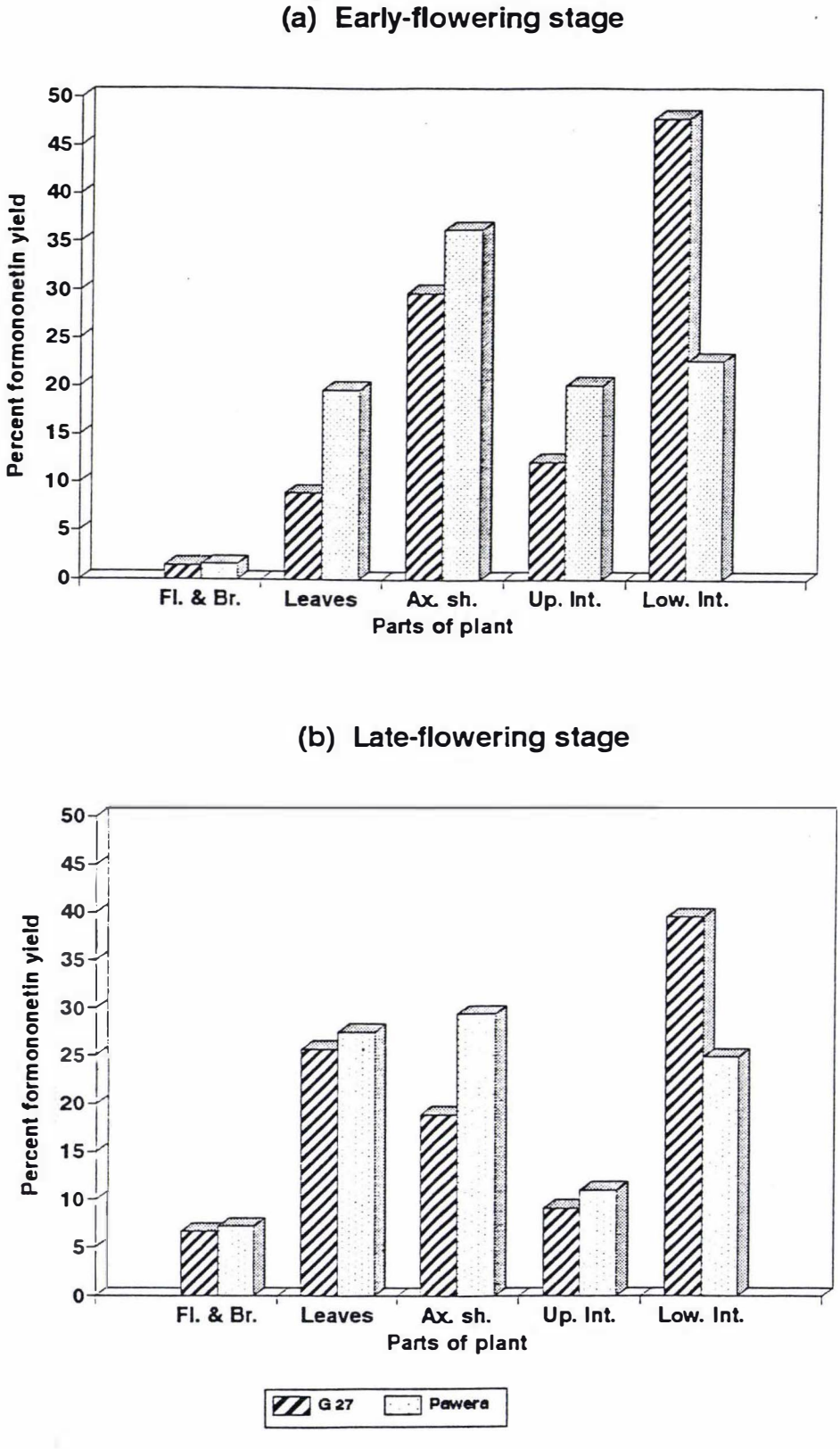


Figure 3.4. Distribution of formononetin in various parts of red clover shoots. G27 and Pawera

shoot is shown in Figure 3.4a (and Appendix Table 2.1). Leaves, axillary shoots and flower together contained 40% and 57% of the total formononetin yield of G27 and Pawera shoot respectively. Of the total formononetin yield from the shoots, 60% of G27 and 43% of Pawera resided in the stem portion (internodes). In G27 red clover, the lower internodes yielded the highest amount of formononetin (47.7%) at early-flowering stage. In Pawera red clover, the axillary shoots contained the highest formononetin yield (36%) at this stage.

4.4. Formononetin at the late-flowering stage

4.4.1. Formononetin concentration

Formononetin concentration in various parts of the plant at the late-flowering stage is presented in Table 3.8. Mean formononetin concentrations were significantly lower ($P<0.05$) in G27 than those in Pawera red clover in the younger parts of the plant i.e. flower bract, leaflets, two youngest petioles, all the axillary shoots and the youngest internode (IN1). In the flower, oldest leaflets and petioles, and all the internodes (except IN1), formononetin concentration was not significantly different between the two red clovers ($P>0.05$). The formononetin concentrations in the whole shoot (derived from total formononetin yield and total dry weight per shoot) (Table 3.9) were 0.22% and 0.44% in G27 and Pawera respectively.

Within G27, formononetin concentration did not differ between flower and bract. The concentration also did not differ between leaflets ($P>0.05$) although it was lower in the oldest ones. The oldest G27 petioles also had a lower formononetin concentration. Concentrations among G27 axillary shoots did not differ significantly ($P>0.05$). Among G27 internodes, the oldest one had the highest formononetin concentration but it did not differ significantly from other internodes except IN3.

Within Pawera, the bract had a significantly higher concentration than the flower. The concentration was higher in the youngest leaflet and petiole but it declined significantly in the older leaves ($P<0.05$). Formononetin concentration in the axillary shoots did not differ significantly. Among Pawera internodes the youngest had the highest formononetin concentration.

Table 3.8. Formononetin concentration in different parts of G27 and Pawera red clover shoots at late-flowering stage.

Part[1] of the shoot	Formononetin (% DM)	
	G27	Pawera
Flower and bract		
Flower	0.08 ± 0.01^{b}	0.09 ± 0.01^{b}
Fl.Bract	0.18 ± 0.02^{b}	0.76 ± 0.07^{a}
Flower+Bract	0.09	0.18
Leaflets and petioles		
Leaflet1	0.21 ± 0.00^{fg}	0.88 ± 0.06^{a}
Leaflet2	0.17 ± 0.01^{fg}	0.85 ± 0.10^{a}
Leaflet3	0.17 ± 0.00^{fg}	0.81 ± 0.09^{a}
Leaflet4	0.17 ± 0.02^{fg}	0.54 ± 0.15^{bcd}
R.Leaflets	0.12 ± 0.01^{g}	0.31 ± 0.13^{efg}
Petiole2	0.44 ± 0.02^{cde}	0.72 ± 0.03^{ab}
Petiole3	0.35 ± 0.02^{def}	0.59 ± 0.06^{bc}
Petiole4	0.36 ± 0.02^{def}	0.45 ± 0.07^{cde}
R. Pet.	0.18 ± 0.04^{fg}	0.18 ± 0.07^{fg}
All leaves	0.21	0.58
Axillary shoots		
Ax.sh.2	0.34 ± 0.02^{c}	1.14 ± 0.12^{ab}
Ax.sh.3	0.27 ± 0.01^{c}	0.95 ± 0.06^{b}
Ax.sh.4	0.30 ± 0.02^{c}	1.03 ± 0.07^{ab}
Ax.sh.5	0.42 ± 0.03^{c}	1.31 ± 0.08^{a}
R.Ax.sh.	0.51 ± 0.34^{c}	1.11 ± 0.16^{ab}
All ax.sh.	0.32	1.05
Internodes		
IN1	0.21 ± 0.01^{cd}	0.51 ± 0.09^{a}
IN2	0.19 ± 0.02^{cd}	0.32 ± 0.05^{bc}
IN3	0.14 ± 0.01^{d}	0.24 ± 0.03^{cd}
IN4	0.20 ± 0.02^{cd}	0.20 ± 0.03^{cd}
INr	0.31 ± 0.03^{bc}	0.37 ± 0.03^{b}
All INs	0.24	0.32
Overall	0.22	0.44

Means with different superscript letters within same group of parts differ significantly ($P<0.05$).

[1] Parts arranged with increasing age (e.g. leaf 1 = youngest leaf on the shoot). R.Leaflets = rest of the leaflets.

4.4.2. Dry weight per part and formononetin yield

Dry weight per part and formononetin yield per part in the late-flowering shoot are summarized in Table 3.9. Mean dry weight did not differ significantly between the majority of the shoot-parts of the two red clovers. Total shoot weight for G27 (1751 mg) and Pawera (1801 mg) was not different from each other but the total formononetin yield from G27 (3.85 mg) was almost half of that from Pawera (7.86 mg).

G27 red clover had significantly lower formononetin yield than Pawera in the flower bract, three youngest leaves, axillary shoots 2 and 3, and the oldest internodes ($P<0.05$). The yield was not significantly different between the two strains in other parts of the plant.

Within G27 the formononetin yield did not differ significantly between leaflets, or petioles, although there was a significant increase in the dry weight with increasing age. The same pattern occurred with axillary shoots and the younger internodes (IN1-IN4).

Within Pawera, the formononetin yield was not different in the three youngest leaflets but decreased significantly in the older leaflets ($P<0.05$). The yield was not different amongst various petioles or amongst younger internodes ($P>0.05$). Among axillary shoots, the formononetin yield increased significantly with increase in age and then declined ($P<0.05$) in the oldest ones.

4.4.3. Distribution of formononetin in various parts of the shoot

The distributions (percent) of formononetin in different parts of the late-flowering shoot are shown in Figure 3.4b (and in Appendix Table 2.2). Leaves, axillary shoots and flower together contained 51% and 64% of the total formononetin yield of G27 and Pawera shoot respectively. G27 and Pawera internodes contained 49% and 36% of the total formononetin yield from the shoots. In G27 red clover, the lower internodes yielded the highest amount (39.5%) of formononetin at the late-flowering stage. In Pawera red clover, the axillary shoots still yielded the highest amount of formononetin (29%)
with the leaves (27%) and lower internodes (25%) close to this level.

Table 3.9. Average dry weight and formononetin yield per part of late-flowering stage.

Part of	Dry Weight (mg)		Formononetin yield (mg)	
the shoot	G27	Pawera	G27	Pawera
Flower and bract				
Flower	255.50 ± 10.44^{a}	276.50 ± 7.50^{a}	0.20 ± 0.01^{b}	0.25 ± 0.01^{b}
Fl.Bract	31.50 ± 1.71^{b}	42.50 ± 4.35^{b}	0.06 ± 0.01^{c}	0.32 ± 0.03^{a}
Subtotal	287.00	319.00	0.26	0.57
Leaflets and petioles				
Leaflet1	42.50 ± 2.75^{def}	55.00 ± 3.70^{bcde}	0.09 ± 0.01^{bc}	0.48 ± 0.05^{a}
Leaflet2	49.50 ± 4.11^{cde}	58.50 ± 3.69^{bcde}	0.09 ± 0.01^{bc}	0.50 ± 0.07^{a}
Leaflet3	71.00 ± 3.70^{bc}	50.50 ± 1.50^{cde}	0.12 ± 0.01^{bc}	0.41 ± 0.04^{a}
Leaflet4	74.50 ± 2.99^{b}	36.00 ± 5.23^{efg}	0.13 ± 0.01^{bc}	0.19 ± 0.06^{b}
R.Leaflets	63.50 ± 16.46^{bcd}	54.00 ± 6.11^{bcde}	0.08 ± 0.02^{bc}	0.17 ± 0.08^{bc}
Petiole2	11.00 ± 1.29^{h}	13.50 ± 0.96^{h}	0.05 ± 0.00^{c}	0.10 ± 0.01^{bc}
Petiole3	22.50 ± 1.71^{fgh}	18.50 ± 2.22^{gh}	0.08 ± 0.01^{bc}	0.11 ± 0.00^{bc}
Petiole4	42.00 ± 2.71^{def}	19.50 ± 0.96^{gh}	0.15 ± 0.02^{bc}	0.09 ± 0.02^{bc}
R.Pet.	98.00 ± 18.81^{a}	67.00 ± 11.00^{bc}	0.20 ± 0.09^{b}	0.11 ± 0.03^{bc}
Subtotal	474.50	372.50	0.99	2.16
Axillary shoots				
Ax.sh.2	15.00 ± 5.07^{d}	21.50 ± 8.18^{d}	0.05 ± 0.02^{b}	0.23 ± 0.07^{b}
Ax.sh.3	75.50 ± 9.03^{ab}	84.00 ± 17.83^{a}	0.21 ± 0.03^{b}	0.79 ± 0.15^{a}
Ax.sh.4	86.00 ± 11.80^{a}	66.00 ± 12.46^{abc}	0.26 ± 0.03^{b}	0.67 ± 0.12^{a}
Ax.sh.5	39.50 ± 16.76^{bcd}	22.00 ± 12.17^{d}	0.16 ± 0.06^{b}	0.31 ± 0.18^{b}
R.Ax.sh.	9.00 ± 1.00^{d}	27.33 ± 11.10^{cd}	0.05 ± 0.04^{b}	0.31 ± 0.12^{b}
Subtotal	225.00	220.83	0.73	2.31
Internodes				
IN1	29.00 ± 1.73^{e}	40.50 ± 3.86^{de}	0.06 ± 0.00^{d}	0.20 ± 0.04^{cd}
IN2	61.00 ± 5.45^{de}	97.00 ± 7.55^{cd}	0.11 ± 0.00^{cd}	0.30 ± 0.04^{cd}
IN3	128.00 ± 3.56^{bc}	159.50 ± 16.40^{b}	0.18 ± 0.01^{cd}	0.37 ± 0.03^{c}
IN4	134.00 ± 13.95^{bc}	141.50 ± 12.34^{bc}	0.26 ± 0.02^{cd}	0.27 ± 0.02^{cd}
INr	412.50 ± 9.25^{a}	450.50 ± 53.90^{a}	1.26 ± 0.12^{b}	1.68 ± 0.26^{a}
Subtotal	764.50	889.00	1.87	2.82
Overall total	1751.00	1801.33	3.85	7.86

Means with different superscripts letters within same parameter and same group of parts differ significantly ($P<0.05$).

4.5. Formononetin intake

4.5.1. Early-flowering stage

When calculated only for the upper parts of the plant which are usually ingested by sheep (i.e. flower, flower bract, leaf 1-4, axillary shoots 2-4, and internodes 1-2), G27 and Pawera red clover contained 0.27% and 0.99% formononetin respectively (Figure 3.5a). The formononetin concentrations in the residual (ungrazed) parts of G27 and Pawera red clover were 0.42% and 0.59% respectively.

4.5.2. Late-flowering stage

In the upper parts of the plant ingested by sheep (i.e. flower, flower bract, leaf 1-4, axillary shoots 2-4, and internode 1), G27 and Pawera red clover contained 0.19% and 0.53 % formononetin respectively (Figure 3.5b). Formononetin concentrations in the residual (ungrazed) parts of G27 and Pawera red clover were 0.25 and 0.40% respectively.

5. Discussion

5.1. Formononetin in the plants

G27 red clover, a sixth generation selection within the cultivar 'Grasslands Pawera', was produced with an aim to lower formononetin concentration to levels where deleterious effects on sheep fertility were minimized if not eliminated. At the same time selection was directed to maintaining the high productivity of Pawera red clover. Results of the present study show that the overall formononetin level in the plant was reduced ≥50% at various stages of plant development. So the initial aim of the plant breeding programme was achieved in part. However, an unforeseen consequence of the selection programme was a differential distribution of formononetin within the red clover plant.

The selection of G27 red clover (for generations 1 to 6) was based on decreasing the formononetin concentration of plant leaflets. G27 leaflets had a formononetin concentration only 22% of Pawera leaflets (Table 3.4). The reduction in the formononetin concentration of G27 petioles (compared to Pawera) was not as great as

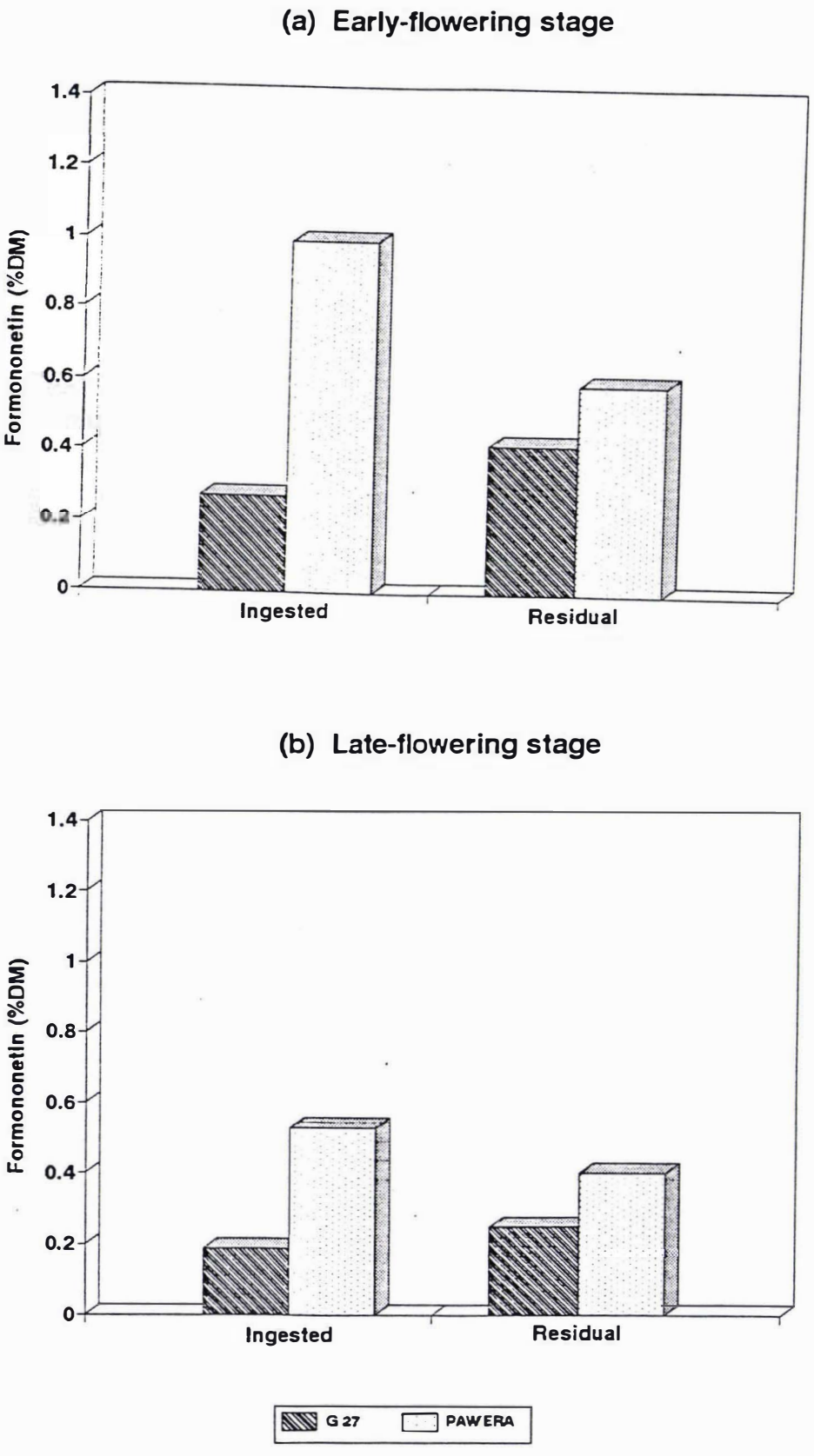


Figure 3.5. Formononetin concentration in the younger parts of G27 and Pawera red clover plants usually ingested by sheep, and in the residual parts left af er grazing.

that achieved in the leaflets. But, as petiole dry weights were much lower than leaflet dry weights (Tables 3.2, 3.5 and 3.8), the concentration of whole G27 leaf (leaflet + petiole) was closer to that of the leaflets than the petioles. In subterranean clover the green petioles (of Yarloop strain) have about nine times less oestrogenicity than the leaflets as measured by a teat length bioassay with wethers (Francis and Millington 1965a). The level of isoflavones in the petioles of different strains of subterranean clover was shown to be about 10% of the leaf blade. This is different from formononetin concentrations in leaflets and petioles in red clover which were similar to each other (as in Pawera) or even higher in petioles than leaflets (as in G27). The observation in Pawera red clover is in close agreement to that of McMurray *et al.*, (1986) who reported a formononetin concentration in red clover petioles almost similar to or slightly lower than that in the leaflets. The differential decrease in the formononetin concentration between G27 leaflets and petioles suggests that some of the processes involved in formononetin biosynthesis and degradation in the two components of the leaf may differ from each other.

A further difference was in the extent of the decrease seen in formononetin concentration which occurred as leaf growth and development proceeded. The apparent concentration decrease in G27 leaflets was only about half the decrease seen in Pawera leaflets and petioles and also in G27 petioles. It is not clear why this difference should have developed. Rossiter and Beck (1967) noted a progressive decline in the concentration of formononetin, genistein and biochanin A as the subterranean clover (Dwalganup cultivar) leaves became older. Along with a marked decline in formononetin in the leaves at early senescence, they observed a rise in another oestrogenic isoflavone, daidzein, which was not detected in younger leaves and which possibly originated from demethylation of formononetin, or alternatively from a failure of methylation of daidzein to formononetin. In the plants of the Yarloop cultivar, a decline in isoflavone concentration with advancing leaf age was also recorded. According to Stafford (1990), formononetin degradation can occur in a variety of reactions including glycoside hydrolysis, demethylation, hydration and oxidation. Breeding for a low formononetin content might have altered formononetin biosynthesis and degradation reactions in G27 leaflets.

The maximum concentrations of formononetin in Pawera and G27 leaves were present at the earliest stages of leaf growth, before unfolding of leaflets occurred. This result is similar to that recorded for subterranean clover (Rossiter and Beck, 1967). Rossiter

(1972) suggested that the isoflavones appeared to be synthesized mainly during a period of high metabolic activity in the leaf. Results obtained with G27 and Pawera red clover leaflets and petioles would support this suggestion.

In the flowering shoots, the reduction achieved in formononetin concentration in G27 stem was of the same order as that in the petioles, and much less than that in leaflets. Although the formononetin concentration in the youngest G27 internodes was lower than that in Pawera (at both early and late-flowering stages), it was not significantly lower in the older internodes. Formononetin concentration in Pawera red clover stem at early and late-flowering stage was comparable to that reported by McMurray *et al.*, (1986) who reported that red clover stem contained a formononetin concentration about one half to two thirds of that in leaflets. A differential decline in formononetin concentration of G27 stem gave rise to a different formononetin distribution between G27 and Pawera within the flowering shoot fractions. A much higher proportion of formononetin in G27 was in stem (49-63%) compared with that in Pawera stem (36-43%).

Formononetin concentrations in various parts of late-flowering shoots were usually lower than those in early-flowering shoots in both G27 and Pawera red clover, the overall decline being almost similar. McMurray *et al.*, (1986) also observed that formononetin concentration declined with age in various parts (laminae, petioles, and stem) of red clover plants harvested from vegetative to the dead inflorescence stage.

5.2. Dry weight

Apart from having a low formononetin concentration, G27 leaflets had a lower dry weight than the Pawera leaflets indicating that productivity of G27 red clover might be lower than Pawera red clover. But plant growth rates and yield per unit area of G27 and Pawera are very similar (R.G. Keogh, personal communication). Component composition is also similar at vegetative and flowering phases of growth. As G27 leaves are smaller than Pawera leaves, it follows that leaf (and shoot) densities will be higher for G27.

5.3. Plant-animal interaction

As oestrogenic infertility in sheep is related to the amount of phytoestrogens consumed, formononetin concentration *per se* is the important consideration rather than the amount of formononetin per plant (or part thereof). Moreover, it is the formononetin concentration of the components grazed rather than the mean concentration per plant which is the determinant of formononetin ingested.

High formononetin concentration has been the main factor limiting more extensive use of Pawera red clover in sheep pastures. It may cause temporary as well as permanent infertility in ewes (Kelly *et al.*, 1980; Shackell *et al.*, 1993). Pure swards of Pawera can not be safely grazed with ewes or ewe lambs for prolonged periods, and the most logical use for Pawera rich pastures is for fattening all classes of stock destined for slaughter, or for the production of high quality hay (Kelly *et al.*, 1979). A significantly lower formononetin concentration in G27 red clover at all stages of development makes it a better substitute for Pawera as well as for other late flowering, formononetin-rich red clovers. It would be safer to feed this clover to breeding ewes in order to limit fertility problems, and at the same time it could be used for fattening stock. Growth rates of sheep on G27 and Pawera red clover are similar which indicates that their feeding values and intakes are probably also very similar (R.G. Keogh, personal communication).

In Australia where some highly oestrogenic cultivars of subterranean clover have been the cause of permanent infertility in sheep due to the cumulative effects of formononetin, cultivars with a formononetin concentration of 0.3% or less (on a leaf dry weight basis) appear to be safe, and on this basis a maximum concentration of 0.2% (of the leaf dry weight) has been used as a guide to selection in breeding programmes (Collins and Cox, 1985). The mean formononetin concentration in G27 leaflets has been found to be less than 0.3% in the leaves on pre-flowering shoots but due to a higher formononetin concentration in petioles, the overall formononetin concentration in these leaves is 0.35% (which is about one third of that in Pawera). At early and late-flowering stages, the formononetin concentration of younger parts (including leaves and axillary shoots which usually make up sheep intake) of the G27 plant is less than 0.3%, so it may be safer for breeding ewes than pre-flowering growth. G27 is a late flowering red clover that would normally be at a reproductive stage of growth during late summer-early autumn when flushing and mating of ewes

occurs. So plants with a lower formononetin concentration at this stage might pose a lower risk to the fertility of ewes in addition to providing a larger amount of feed. Dedio and Clark (1968) also indicated that red clover after the flowering stage would be suitable for livestock in reducing reproductive difficulties. But according to Rossiter and Beck (1967), the oestrogenic activity of subterranean clover has not been shown to decline in potency during the later stages of clover growth. It might be due to an increased intake of clover leaf during the later stages of plant growth, thus compensating for the fall in leaf concentration of oestrogenic isoflavones. Alternatively, plant compounds other than those currently believed to be important might play some role.

In areas of New Zealand where red clover is grown for sheep grazing, it may not be a major part of the sward (Jagusch, 1983). So a slightly higher than the recommended safe concentration of formononetin in G27 is not expected to pose fertility problems in breeding ewes. The botanical composition of a pasture offered to grazing ewes may be associated with changes in fertility because grass may act as a diluent to the total intake of phytoestrogens (Davies and Maller, 1970), although it is not known whether the botanical composition on offer bears any relation to the relative amounts of grass and clover consumed by the sheep. Baxter *et al.*, (1993) observed no significant depression in fertility of sheep continually exposed to Pawera-perennial ryegrass pasture, when red clover contributed 20% and 30% of the annual and autumn herbage production. Due to its lower formononetin concentration, G27 may be safely integrated at higher proportions in the mixed pastures than Pawera. Study of the developing vegetative leaf suggests that G27 leaves attain the lowest formononetin concentration around 23 days of age. In a rotational grazing system, a lower formononetin intake should be expected if the sheep are grazed on pure stands of G27 or mixed pastures after a regrowth period which exceeds 23 days. The lowest formononetin level in developing leaf of Pawera red clover was noted at an earlier age (16 days) in spring, but it was similar to G27 (i.e. at 3 weeks of age) in summer.

In addition to decreasing fertility problems in ewes, low formononetin concentration may improve palatability of clover. It has been shown that flavonoid glycosides might be unpalatable to sheep (Francis, 1973): taste testing by human subjects of purified isoflavone glycosides indicated a slight but distinctive astringent bitter taste. G27 red clover is more palatable to sheep than Pawera. It has been suggested that the intake of a forage is little affected by palatability *per se* when only one feed is available but

when there is a choice between forage components with equal accessibility, sheep will eat a greater proportion of the more palatable material (Black, 1990). It remains to be seen whether in a mixed pasture, a higher palatability of a low formononetin red clover like G27 may result in better utilization of the pasture. If so, the effective grazing of the red clover in the mixed pasture might also result in a better regrowth of the red clover plants.

The present study indicates that under field conditions, the newly selected G27 red clover contains significantly lower formononetin concentrations than the original Pawera red clover at different stages of plant growth and development. It has a proportionally lower plant formononetin yield, a major part of which is located in the stem. Thus G27 may be considered a safer feed for breeding ewes (and other stock) than Pawera red clover.

Figure 3.6 Sheep grazing Pawera and G27 red clover

CHAPTER IV

Follicular development, plasma progesterone, FSH, and equol concentration in ewes grazed on Pawera and G27 red clovers

1. Abstract

This study was conducted to investigate ovarian follicle development and plasma FSH concentrations in ewes grazing either a low formononetin selection G27 red clover, or high formononetin Pawera red clover, or non-oestrogenic (Control) pasture. Plasma progesterone levels and blood equol concentrations in ewes were also determined. The animals (n = 11 per treatment) grazed the respective treatment pastures for 15 days after oestrous synchronization. Ovarian surface follicles in the ewes were counted and measured by laparoscopy on day 13 followed by injection of a prostaglandin F2α analogue (PGF). Further observations on the ovaries were made 24 h and 72 h after PGF injection. The left ovary was taken from each ewe 72 h after PGF injection and was used for histological study. Blood sampling for FSH was done at 09.00 h, 12.00 h and 15.00 h daily for 5 consecutive days starting from day 12. Blood sampling for progesterone was done once daily on day 5, 8, and 12, and that for equol was done once on day 12. Blood equol concentration in ewes grazing G27 red clover (1.81 ± 0.28 μg/ml) was one fourth of that in ewes grazing Pawera red clover (7.25 ± 1.70 μg/ml) ($P<0.01$). Total number of surface follicles in the Pawera ewes (9.40 ± 1.13) was lower than that in G27 (15.36 ± 1.87) or Control ewes (16.18 ± 2.32) 24 h after PGF injection ($P<0.05$). Histological examination of the left ovaries showed that the number of healthy follicles with diameter (D) $1mm<D\leq2mm$ was lower in Pawera ewes (2.80 ± 0.66) than that in G27 (5.50 ± 1.04) or Control animals (5.18 ± 0.64) ($P<0.06$). Cellular atresia was observed in some of the large follicles ($D>4mm$) in the Pawera group but not in the other two treatments. No differences were observed in the mean plasma FSH concentrations between ewes from the three treatments at various sampling times. Mean progesterone concentrations were also not different between ewes in the different treatments. The results of this study suggest that grazing ewes on high formononetin clover might result in disturbances in follicle growth in the ovaries. Follicle growth in ewes on G27 red clover was not different from that in Control animals.

2. Introduction

Ewes grazed on oestrogenic clover before or during mating suffer from a temporary infertility characterized by lower ovulation rate and a reduced chance of conception. Fertility recovers within a few weeks of removal to non-oestrogenic pastures (Adams, 1990). The effects of grazing on oestrogenic clover are readily apparent as only 8 days of grazing before joining impaired the reproductive performance of ewes (Kelly *et al.*, 1980). The lower ovulation rate in ewes grazed on oestrogenic clover is due to fewer multiple ovulations and more ewes failing to ovulate (Lightfoot and Wroth, 1974; Kelly *et al.*, 1980).

Differences in follicular growth and atresia may be involved in the poor ovulation rate on oestrogenic pastures. Adams (1977) observed that ewes grazing oestrogenic subterranean clover developed excessive numbers of small and medium sized ovarian follicles, in many of which antrum formation was deficient. This abnormal development was accompanied by early etresia of the follicles, and this effect was visible by day 6 of grazing. Kelly *et al.*, (1976) also noted unusual signs of early atresia in ovarian follicles of the ewes given coumestans.

The poor reproductive performance of ewes grazing oestrogenic clover may be associated with disturbances in reproductive endocrine function. Changes have been observed in the pituitary gland of ewes grazing oestrogenic subterranean clover (Adams, 1977). They included degranulation of the δ basophils of the anterior lobe, vacuolation of cell nuclei, and increase in cell size suggesting that gonadotrophin metabolism might be altered in ewes on oestrogenic pasture. The depression in ovulation rate of ewes fed a high level of coumestans was overcome by treatment with PMSG suggesting interference exerted by coumestans on gonadotrophin release from the pituitary gland (Smith *et al.*, 1979). Nwannenna *et al.*, (1994) reported that red clover silage, fed for 14 days, appeared to reduce the magnitude and duration of the pituitary response to GnRH injections in ovariectomized heifers. There is also some evidence of disturbed luteal function in ewes ingesting phytoestrogens. In ewes failing to conceive on oestrogenic clover, progesterone concentrations began to fall on day 11-12, and reached oestrous levels on days 13-14, indicating a shortened period of corpus luteum function (Obst and Seamark, 1975). Lightfoot and Wroth (1974) reported that ewes grazing oestrogenic clover prior to and during joining had lighter corpora lutea compared to the control animals.

The oestrogenic compounds isolated from subterranean clover and red clover are isoflavones including formononetin, genistein, biochanin A and daidzein. However, evidence suggests that formononetin is mainly responsible for the oestrogenicity in sheep grazed on clovers (Millington *et al.*, 1964; Morley *et al.*, 1968; Davies *et al.*, 1970). It is now well established that in considering the biological effects of phytoestrogens, of particular importance are the metabolic changes they undergo in the rumen because formononetin has virtually no oestrogenic activity in laboratory animals nor in sheep when injected (Cox and Braden 1974a). It has been shown that equol is the major metabolite produced by degradation of formononetin by rumen microflora and it is oestrogenically active. Equol is probably the principal oestrogen responsible for reproductive disturbance in ewes grazed on oestrogenic clover (Shutt and Braden, 1968; Shutt *et al.*, 1970; Davies and Hill, 1989).

The present experiment was conducted to study follicular development and the levels of some reproductive hormones in ewes grazing on high formononetin Pawera red clover or low formononetin selection G27 red clover. The objective was not only to compare the effects of these two red clovers, but also to try to better understand the phenomenon of lower ovulation rate in ewes due to oestrogenic clover. In an attempt to establish causes for the difference in ovulation rate, specific objectives of the study were: (1) to compare follicular growth on the surface of ovaries of the ewes grazed either on Pawera red clover, G27 red clover, or Ryegrass-white clover (non-oestrogenic Control) pastures for two weeks, (2) compare follicle populations in the ovaries of these ewes around the time of ovulation, (3) compare blood equol concentrations in these ewes, (4) compare FSH concentrations in these ewes during the last four days of grazing in the follicular phase of the oestrous cycle, and (5) compare progesterone concentration in the ewes on three different days of the cycle.

3. Materials and methods

3.1. Animals and grazing treatments

Thirty-three 5 to 7-year old Border Liecester x Romney ewes with a mean body weight of 44.9 ± 0.5 kg were selected from a flock in April 1992. The ewes had a history of normal fertility and had no previous exposure to oestrogenic pastures. Oestrus was synchronized in the ewes by insertion of intravaginal sponges containing 40 mg

medroxy progesterone acetate for a period of 12 days. After sponge withdrawal, the ewes were joined with two harnessed teaser rams. Observations for oestrus were made at 08.00, 12.00 and 17.00 h daily. All the ewes were in heat within 48 h after sponge withdrawal. The ewes were weighed and allocated to the following three treatment groups (n = 11); (1) Pawera red clover, (2) G27 red clover, (3) Ryegrass-white clover (Control).

Animals were grazed on the treatment herbages from day 1-16 after oestrus was detected (day 0) except when removed from the treatment pastures for a 48 h period from 16.00 h on day 12 to 16.00 h on day 14 of the treatment to allow for laparoscopy (day 13) and laparotomy (day 14) to be conducted. They grazed a Ryegrass-white clover pasture for one hour only in the afternoon of day 13, during this 48 h off-treatment period. Animals were put back on respective treatments on the evening of day 14 with teaser rams. Ewes were slaughtered on day 16.

The clovers and Control pastures grazed in the trial were grown at Massey University's 'Sheep and Beef cattle Research Unit' in paddocks located next to each other. The Pawera and G27 paddocks contained at the start of grazing approximately 70% red clover. Herbage was sampled on day 1 and day 8 of the grazing period and formononetin concentration was measured by a fluorimetric method (Gosden and Jones, 1978).

In this and subsequent chapters, the groups of ewes grazed on Pawera red clover, G27 red clover, or Control pasture will be referred to as Pawera ewes, G27 ewes, and Control ewes respectively.

3.2. Observations on ovaries

On day 13 of the cycle, laparoscopy was performed on the ewes after tranquilizing with 0.5 ml of Acepromazine maliate (ACP) (C-Vet Ltd., Bury St. Edmunds, Suffolk, England). The ovaries of each ewe were examined and all ovarian surface follicles ≥1.5mm (measured with a calibrated manipulating probe) and ovulation points counted. After laparoscopy each ewe was injected intramuscularly with 125 μg cloprostenol (Estrumate, Coopers Animal Health, N.Z. Ltd., Upper Hutt, New Zealand), an analogue of prostaglandin F2α (PGF) to improve synchrony of corpus luteum regression. Further observations of the ovarian surface follicles were made 24 h

(by laparotomy) and 72 h (at slaughter) after PGF injection. Laparotomy was performed after tranquilizing each ewe with 0.5 ml ACP, and using an intravenous injection of 6 ml of 6% (w/v) pentobarbitone sodium (Nembutal, Boehringer Ingelheim, NSW, Australia) for general anaesthesia. The uterus and ovaries were exteriorized and all follicles ≥1.5mm in diameter were measured to the nearest 0.5 mm using a vernier caliper. Follicles were again measured in the same manner 72 h after PGF administration. Follicles were arbitrarily classified into three groups i.e. small (≤2.5 mm diameter), medium-sized (3 mm-4.5 mm) and large (≥5 mm). Ovaries were removed after slaughter for histological examination.

3.3. Histological procedure

The left ovary in each ewe was used for histological examination as it has been shown that the absolute number of normal and atretic tertiary (antral) follicles of the various size classes does not differ significantly between left and right ovaries (Brand and de Jong 1973). The histological procedure to prepare ovarian tissue, and classification of follicles was done as described by Turnbull *et al.*, (1977) and Xu *et al.*, (1989). Immediately after removal, the ovaries were cut in half and fixed in Bouin's solution for 24 h. Then they were moved to 70% ethanol. The ovaries were embedded with paraffin and serially sectioned at 10 μm. Every fifth section was mounted and stained with hematoxylin and eosin. The number of microscope slides each ovary required was 67.6 ± 1.9 containing 4 sections per slide. All follicles ≥0.2 mm were measured under a light microscope at 40x with the aid of an ocular micrometer. Follicle diameter was taken as the mean of two measurements at right angles to each other on the section where the area of the follicle was maximal. The basal membrane of the follicle was taken as its outer limit. These follicles were then observed at 400x for the assessment of health status. All follicles were arbitrarily classified into four groups according to diameter (D): D≤0.5, 0.5<D≤1, 1<D≤2, and D>2mm. Follicles were also divided into four health status classes of increasing degree of atresia. Healthy follicles were defined as follicles with no more than three degenerative cells with pycnotic nuclei. Early atretic follicles were defined as those with a small number of pycnotic nuclei or atretic bodies in the largest section. In addition, local destruction of the basal membrane and loss of orientation of the basal layer of granulosa cells were used as indications of early atresia. Advanced atretic follicles were identified by the presence of numerous atretic bodies. Late atretic follicles were defined as follicles with a large number of atretic bodies and widespread destruction of follicular structure. Presence of an antral cavity

in the follicles was also recorded and thus another classification was done on the basis of antrum formation. Classification of follicle health is given in Appendix 4.

3.4. Blood sampling

Plasma Progesterone. Ewes were blood sampled by venipuncture to measure peripheral plasma progesterone concentration once daily on day 5, 8, and 12 of the oestrous cycle.

Equol. Blood samples were collected from ewes on day 12 of the treatment within half an hour after removal from the grazing treatments.

Follicle stimulating hormone (FSH). Blood samples were taken from all the ewes for 5 consecutive days starting from day 12 of the oestrous cycle. Samples were collected at 09.00 h, 12.00 h and 15.00 h daily with only one sample collected on day 16 at 9.00 h prior to slaughter.

All blood samples were collected into heparinized vacutainers (Becton Dickinson and Company, USA) and centrifuged at 4°C for 15 minutes at 2700 g. The plasma was stored at -20°C until required for FSH and progesterone assays. Whole blood samples for equol determination were frozen until required.

3.5. Hormone assay

3.5.1. FSH

Plasma FSH levels were measured by a double antibody competitive binding radioimmunoassay using a kit and protocol supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Natonal Institute of Health, and the National Hormone and Pituitary Program, University of Maryland School of Medicine, Baltimore, U.S.A.). Lyophilized ovine FSH (NIAMDD-oFSH-I-1) was used for iodination by the chloramine-T method (Greenwood *et al.,* 1963) and ovine FSH (NIAMDD-oFSH-RP-1) as a reference standard. The antiserum (first antibody) was rabbit anti-ovine FSH (NIAMDD-anti-oFSH-1) used at a final tube dilution of 1:160,000. Information supplied by NIAMDD showed that the specificity of the FSH antiserum, in terms of its reactivity with highly purified oLH, oPRL, and oGH was

<0.2% for all the hormones tested.

Prior to assay, plasma samples were thawed overnight in a refrigerator at 4°C. All samples were analysed in two assays. Plasma samples (100 µl) (triplicates) or standards were pipetted into reaction tubes followed by 50 µl of the first antibody (anti-ovine FSH) and 50 µl of the trace (I^{125}-labelled FSH). The tubes were vortexed and allowed to incubate for 24 h at room temperature. The second antibody was donkey anti-rabbit gamma globulin (IDS, Tyne & Wear, England) in 6% PEG-PBS (polyethylene glycol in 0.01M phosphate buffered saline pH 7.5) which was used to agglutinate the first antibody-hormone complex and allow it to be centrifuged into a pellet. Second antibody (50 µl) was added to the mixture, incubated for 1 h and then centrifuged at 2400 g and 4°C for 30 minutes. The binding percentage of I^{125}-labelled FSH in the pellet was measured by an LKB-Wallac 1261 Multigamma counter (Wallace Oy, Finland). The assay had a linear range of 0.2-12.8 ng/ml and assay sensitivity of 0.16 ng/ml. The intra-assay coefficient of variation (c.v.) was 9.4% and inter-assay c.v. was 15.1% at mean FSH concentration of 1.5 ng/ml.

3.5.2. Progesterone

Plasma progesterone concentration was determined using the technique described by Kirkwood *et al.,* (1984). Samples (500 µl) were extracted with 5 ml toluene:hexane (1:2 v/v). The plasma was frozen overnight and solvent was then decanted into clean glass tubes, dried under air and redissolved in 500 µl ethanol. Duplicate 100 µl samples of ethanol extract were dispensed into plastic tubes and dried under air, as were duplicate 100 µl samples of standard ethanolic solutions of progesterone (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) with concentrations corresponding to plasma progesterone levels of 0.65 to 20 ng/ml.

A mixture containing antiserum at a final dilution of 1:18,000; [1,2,6,7-$^{3}H^{8}$]progesterone (Amersham, Bucks, U.K.) at 20 000 c.p.m./100µl; phosphate-buffered saline containing 0.02 M-EDTA and 0.1% gelatin (PBS-EG) in the ratio of 1:1:4 (by vol.) was added (600 µl) to each tube and vortexed. After overnight incubation at 4°C, 600 µl of 2.5% (w/v) charcoal (Norit A; A.H. Thomas Co., Philadelphia, U.S.A.) suspension in PBS-EG were added to the tubes, vortexed and then incubated at 4°C for 10 minutes. Tubes were then centrifuged at 3000 g for 10 minutes at 4°C. The supernatant was decanted into scintillation vials and 6 ml toluene-

triton scintillation fluid added before counting for 2 min in a Wallac 1409 liquid scintillation counter. Assay sensitivity was 0.56 ng/ml. Intra-assay c.v. was 11.98 (n = 17), and inter-assay c.v. was 13.45 (n = 71) for plasma pool containing mean progesterone concentration 7.0 ng/ml.

3.5.3. Equol

Equol assay was performed in a total of 14 randomly selected ewes in three treatments. The method used to determine equol concentration in whole blood samples is in Appendix 3.

3.6. Statistical analyses

Chi square test was applied to compare ovulation rate in the ewes after the first synchronized oestrus. Univariate procedure of the Statistical Analysis System computer package (SAS Institute Inc., 1988) was used to check that the follicle count data was a sample from a normal distribution. This procedure applies Shapiro-Wilk statistic (Shapiro and Wilk, 1965) for this purpose. Data in many of the size-classes were not normally distributed. The follicle numbers (x) on the ovarian surface as well as those from the histological study were analysed by analysis of variance (ANOVA) procedure after $\sqrt{x+½}$ transformation due to the presence of no follicles of a particular class in some animals. A comparison of the number of surface follicles between right and left ovaries was also made within each treatment after slaughter using paired t-test. Plasma progesterone levels were compared by analysis of variance for repeated measures. Effect of day of the cycle on progesterone concentration was analysed by ANOVA. FSH concentrations between treatments were compared by analysis of variance for repeated measures. For a comparison between days, FSH concentrations in blood samples taken from each animal on the same day were averaged and the effect of treatment on mean FSH concentration was analysed using analysis of variance for repeated measures. The differences between means were determined by t-test. Blood equol concentration between treatments was compared by ANOVA. The level of statistical significance was set at $P<0.05$, but closer probabilities (up to $P<0.10$) have been recorded in the text to show some marginal differences. All the data were analysed using the Statistical Analysis System computer package ((SAS Institute Inc., 1988). The data are presented as mean ± SEM.

4. Results

4.1. Herbage formononetin and uptake in the animal

The mean formononetin concentration (on a dry weight basis) of Pawera and G27 clover was 1.05 ± 0.12 and 0.34 ± 0.06 percent respectively.

The concentration of equol (free + conjugated) in blood on day 12 of treatment was significantly lower ($P<0.001$) in ewes grazing G27 red clover (1.81 ± 0.28 μg/ml) than that in ewes grazing Pawera red clover (7.25 ± 1.70 μg/ml) (Figure 4.1). No blood equol was detected in ewes grazing Ryegrass-white clover pasture.

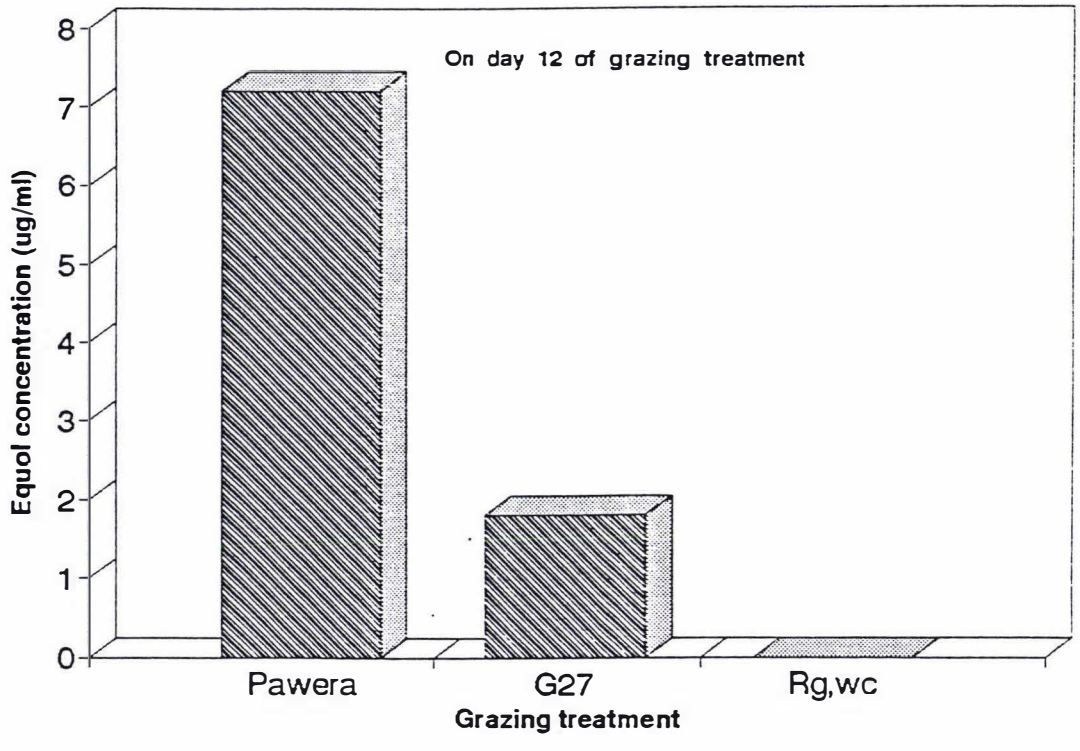


Figure 4.1. Blood equol concentration (μg/ml) in ewes grazing Pawera red clover, G27 red clover, or Ryegrass-white clover (Control) pastures.

4.2. Ovulation rate in ewes at start of the trial

Laparoscopic examination (on day 13 of the cycle) revealed that one of the Pawera ewes had failed to ovulate, although it was in heat at the start of the trial. Absence of a CL in this ewe was confirmed by the very low progesterone concentrations (≤0.09 ng/ml) and, therefore, data from this animal were excluded from all the analyses. One of the G27 ewes died soon after laparotomy and thus did not provide data on FSH concentrations and histological appearance of ovarian tissue.

Mean ovulation rates in Pawera ewes (1.40 ± 0.16), G27 ewes (1.36 ± 0.15), and Control ewes (1.64 ± 0.24) were not significantly different ($P>0.05$) from each other at the start of the trial after the first synchronized oestrus. Multiple ovulations (≥2) occurred in 40%, 36.4%, and 45.4% of ewes in Pawera, G27, and Control groups respectively.

4.3. Follicular development on the ovarian surface

All the ewes were in heat within 48-72 h after PGF injection. Five animals had already ovulated when slaughtered 72 h after PGF administration. They were one Control ewe (with 1 ovulation), two G27 ewes (with 2 ovulations each) and two Pawera ewes (with 2 and 1 ovulations). The ovulation points in these ewes have been added to the number of large follicles present at this stage, for the sake of comparison.

Table 4.1 summarizes the follicle development data. Mean numbers of follicles in various size-classes were not different between the three treatment groups on day 13 of the cycle although the Control animals had a higher number of medium-sized follicles ($P<0.06$) than that in Pawera or G27 group ewes. The total number of follicles on the ovarian surface was significantly lower ($P<0.05$) in Pawera ewes than that in G27 or Control animals 24 h after PGF injection. This difference was largely due to a larger number of small follicles in Control and in G27 ewes ($P<0.06$) than those in Pawera animals. On day 3 after PGF administration, the number of follicles in the various size-classes did not differ significantly between the three treatments.

Comparison of the number of follicles within each treatment is presented in Table 4.2. In the Pawera group, the mean number of large as well as small follicles increased

Table 4.1. The distribution of large, medium-sized, and small follicles measured on the ovarian surface in ewes grazed on either a high oestrogenic red clover (Pawera), a low oestrogenic red clover (G27), or Ryegrass-white clover (Control) pasture (mean ± SEM).

Observation time	Size class	Treatment		
		Pawera	G27	Control
Day 13 of cycle	Large	1.30 ± 0.45	1.00 ± 0.36	1.55 ± 0.25
	Medium	2.80 ± 0.77	2.27 ± 0.54	5.00 ± 0.97
	Small	2.90 ± 0.91	5.36 ± 1.11	3.18 ± 1.29
	Total	7.00 ± 0.80	8.64 ± 1.31	9.73 ± 1.75
Prostaglandin F2α (PGF) administered to ewes after laparoscopy				
24h post-PGF	Large	1.40 ± 0.37	1.45 ± 0.21	1.45 ± 0.28
	Medium	1.60 ± 0.50	2.36 ± 0.74	3.00 ± 0.66
	Small	6.40 ± 1.21	11.55 ± 1.60	11.73 ± 2.10
	Total	9.40 ± 1.13^{b}	15.36 ± 1.87^{a}	16.18 ± 2.32^{a}
72h post-PGF	Large	2.20 ± 0.29	2.00 ± 0.15	2.00 ± 0.23
	Medium	2.90 ± 0.59	4.50 ± 0.95	5.45 ± 1.18
	Small	9.30 ± 1.90	11.00 ± 2.20	11.45 ± 1.47
	Total	14.40 ± 1.94	17.50 ± 2.42	18.91 ± 1.62

Treatment means with different superscript letters in the same row differ significantly ($P<0.05$).

after PGF administration. The highest number of large ($P<0.09$) and small ($P<0.01$) follicles in these ewes was noted 72 h after PGF injection, but no difference was observed in the number of medium-sized follicles. Similar was the situation in the ewes grazing G27 red clover, in which the number of large follicles rose from 1.00 ± 0.36 before PGF to 2.00 ± 0.15 ($P<0.01$) 72 h after PGF injection, and the number of small follicles was also higher after PGF injection ($P<0.05$). In Control ewes, the mean number of large as well as medium-sized follicles present on the surface of ovaries was not different on the three days of observations. However the mean number of small follicles was significantly higher after PGF administration ($P<0.01$) compared to that

before PGF injection.

Table 4.2. The distribution of large, medium-sized, and small follicles measured on the surface of ovaries in ewes, on three days; (comparison within each treatment group) (mean ± SEM).

Size class	Observation time		
	Day 13 of cycle	24h post-PGF	72h post-PGF
Pawera			
Large	1.30 ± 0.45	1.40 ± 0.37	2.20 ± 0.29
Medium	2.80 ± 0.77	1.60 ± 0.50	2.90 ± 0.59
Small	2.90 ± 0.91^{b}	6.40 ± 1.21^{a}	9.30 ± 1.90^{a}
Total	7.00 ± 0.80^{d}	9.40 ± 1.13^{d}	14.40 ± 1.94^{c}
G27			
Large	1.00 ± 0.36^{f}	1.45 ± 0.21^{ef}	2.00 ± 0.15^{e}
Medium	2.27 ± 0.54	2.36 ± 0.74	4.50 ± 0.95
Small	5.36 ± 1.11^{h}	11.55 ± 1.60^{g}	11.00 ± 2.20^{g}
Total	8.64 ± 1.31^{j}	15.36 ± 1.87^{i}	17.50 ± 2.42^{i}
Control			
Large	1.55 ± 0.25	1.45 ± 0.28	2.00 ± 0.23
Medium	5.00 ± 0.97	3.00 ± 0.66	5.45 ± 1.18
Small	3.18 ± 1.29^{l}	11.73 ± 2.10^{k}	11.45 ± 1.47^{k}
Total	9.73 ± 1.75^{o}	16.18 ± 2.32^{m}	18.91 ± 1.62^{m}

Treatment means with different superscript letters in the same row differ significantly ($P<0.05$).

On day 13 of the cycle and again on the following day (24 h after PGF injection), two animals from the Pawera group did not have any medium-sized or large follicles on the ovarian surface, although a few small follicles were present. All the other ewes had large and/or medium-sized follicles present on the ovarian surface throughout the observation period. By 72 h after PGF administration all ewes had a large follicle in the ovary.

Table 4.3. The distribution of large, medium-sized, and small follicles measured on the surface of right and left ovaries in ewes 72 hours after prostaglandin F2α injection (mean ± SEM).

Size class	Left ovary	Right ovary
Pawera		
Large	0.80 ± 0.33	1.40 ± 0.27
Medium	1.40 ± 0.43	1.50 ± 0.34
Small	4.60 ± 1.03	4.70 ± 1.18
Total	6.80 ± 1.02	7.60 ± 1.26
G27		
Large	0.70 ± 0.26	1.30 ± 0.26
Medium	2.00 ± 0.58	2.50 ± 0.82
Small	5.60 ± 1.08	5.40 ± 1.31
Total	8.30 ± 1.27	9.20 ± 1.24
Control		
Large	1.09 ± 0.25	0.91 ± 0.25
Medium	3.00 ± 0.76	2.45 ± 0.65
Small	4.55 ± 0.25^{b}	6.91 ± 0.86^{a}
Total	8.64 ± 1.05	10.27 ± 0.86

Treatment means with different superscript letters in the same row differ significantly ($P<0.05$).

Mean number of surface follicles between the right and left ovaries in the three treatments is presented in Table 4.3. The number of follicles in the various size classes, and the total number follicles on the surface did not differ between the right and left ovaries in the various treatments, except small sized follicles in the Control ewes which were significantly higher in the right ovary ($P<0.05$).

Table 4.4. The distribution of ovarian follicles classified for size and histological appearance, in ewes grazed on Pawera red clover, G27 red clover or Control pasture (mean ± SEM).

Size (mm)	Status[1]	Treatment		
		Pawera	G27	Control
≤0.5	H	47.10 ± 4.59	58.80 ± 9.59	68.00 ± 10.13
	E	0.10 ± 0.10	0.00 ± 0.00	0.18 ± 0.18
	A	0.30 ± 0.21	0.40 ± 0.31	0.27 ± 0.14
	L	1.20 ± 0.44	1.70 ± 0.56	1.18 ± 0.30
	Total	48.70 ± 4.60	60.90 ± 9.89	69.64 ± 10.35
0.5-1	H	4.80 ± 0.57	6.10 ± 1.03	6.55 ± 1.60
	E	1.20 ± 0.69	0.60 ± 0.27	0.91 ± 0.64
	A	1.10 ± 0.78	0.70 ± 0.26	1.36 ± 0.43
	L	2.30 ± 0.52	3.40 ± 1.08	3.18 ± 0.78
	Total	9.40 ± 1.51	10.80 ± 1.76	12.00 ± 2.12
1-2	H	2.80 ± 0.66	5.50 ± 1.04	5.18 ± 0.64
	E	2.40 ± 0.58	3.10 ± 0.53	2.55 ± 0.47
	A	2.20 ± 0.57	4.40 ± 1.11	2.36 ± 0.64
	L	1.10 ± 0.41	1.10 ± 0.46	2.09 ± 0.51
	Total	8.50 ± 1.51	14.10 ± 2.10	12.18 ± 1.41
>2	H	1.70 ± 0.54	2.10 ± 0.50	2.45 ± 0.55
	E	0.50 ± 0.31	0.20 ± 0.20	0.18 ± 0.12
	A	0.60 ± 0.27	1.60 ± 0.67	1.45 ± 0.55
	L	0.40 ± 0.16	0.40 ± 0.27	0.27 ± 0.14
	Total	3.20 ± 0.71	4.30 ± 0.75	4.36 ± 0.83
Total	H	56.40 ± 5.67	72.50 ± 11.36	82.18 ± 11.77
	E	4.20 ± 1.11	3.90 ± 0.67	3.82 ± 1.03
	A	4.10 ± 1.44	7.10 ± 1.66	5.45 ± 1.13
	L	5.10 ± 0.99	6.60 ± 1.54	6.73 ± 1.29
Overall Total		69.80 ± 6.96	90.10 ± 13.36	98.18 ± 13.68

[1] The symbols used for the status classs are; H = healthy; E = early atretic; A = advanced atretic; L = late atretic.

Differences between various treatments not significant ($P > 0.05$).

4.4. Ovarian follicular population

Follicle data from the histological study are presented in Table 4.4. Although the mean number of follicles in the various sizes was not significantly different between the three treatments, the number of healthy follicles with 1<D≤2 was found to be lower (P<0.06) in the Pawera ewes than that in G27 or Control animals. At this stage all the follicles had developed the antral cavity.

In the left ovary which was selected for histological examination, follicles >4mm in diameter were observed in 4 Pawera ewes (5 follicles), 4 G27 ewes (7 follicles), and in 7 Control ewes (10 follicles). Two out of five large follicles in Pawera ewes were in early stage of atresia. All the other large follicles were found to be healthy.

Table 4.5. The number of preantral and antral follicles and their appearance in the left ovaries of ewes grazed on various herbages (mean ± SEM).

Type of follicles	Status	Treatment		
		Pawera	G27	Control
Preantral		35.80 ± 3.65	37.40 ± 6.73	42.45 ± 5.28
Antral	Healthy	20.60 ± 3.20	35.10 ± 6.75	39.73 ± 10.29
	Atretic	13.40 ± 2.49	17.60 ± 2.95	16.00 ± 2.32
	Total	34.00 ± 4.94	52.70 ± 7.78	55.73 ± 11.68
Overall Total		69.80 ± 6.96	90.10 ± 13.36	98.18 ± 13.68

The diameter at which antrum formation in the follicle started, was not different between the treatments. Mean diameter at which the antral cavity was first observed in the ewes was 0.24 ± 0.01 mm. Almost all the follicles with a diameter larger than 0.33 ± 0.01 mm had developed an antral cavity. The follicle data classified on the basis of antrum formation are shown in Table 4.5. Atresia was not observed in the preantral follicles. The mean number of preantral, healthy and atretic antral follicles was not

Table 4.6. Mean plasma FSH concentration (ng/ml) in ewes grazing on either a high oestrogenic red clover (Pawera), or a low oestrogenic red clover (G27), or Ryegrass-white clover (Control) pasture.

Day of cycle	Sampling time (h)	Treatment		
		Pawera	G27	Control
12	0900	1.54 ± 0.24	1.79 ± 0.32	1.53 ± 0.27
	1200	1.57 ± 0.26	1.98 ± 0.40	1.50 ± 0.25
	1500	1.50 ± 0.27	2.07 ± 0.42	1.42 ± 0.29
13	0900	1.44 ± 0.21	2.19 ± 0.49	1.27 ± 0.26
		Prostaglandin F2α analogue (PGF) injected		
	1200	1.85 ± 0.28	2.14 ± 0.50	1.32 ± 0.28
	1500	1.72 ± 0.25	2.07 ± 0.45	1.43 ± 0.28
Day 1 post-PGF	0900	1.37 ± 0.23	1.31 ± 0.37	0.98 ± 0.13
	1200	1.18 ± 0.23	1.24 ± 0.31	0.94 ± 0.16
	1500	1.29 ± 0.26	1.41 ± 0.24	0.98 ± 0.13
Day 2 post-PGF	0900	0.97 ± 0.17[a]	1.45 ± 0.23[b]	0.86 ± 0.10[a]
	1200	1.30 ± 0.31	1.38 ± 0.19	1.13 ± 0.22
	1500	1.21 ± 0.27	1.36 ± 0.17	1.63 ± 0.25
Day 3 post-PGF	0900	1.40 ± 0.17	1.27 ± 0.11	1.41 ± 0.25

a b; Means with different superscripts differ significantly (P<0.05).

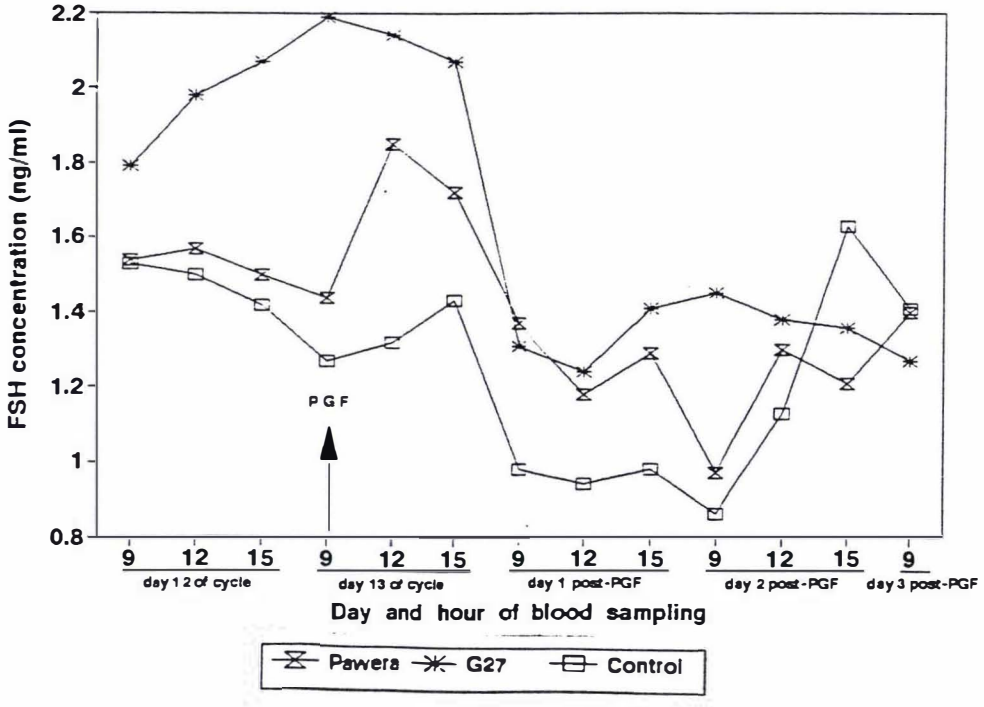


Figure 4.2. Plasma FSH concentrations in ewes grazing Pawera or G27 red clover, or Control pastures

significantly different between the three treatments.

4.5. Plasma FSH

Mean plasma FSH concentrations at different sampling times are shown in Table 4.6 and Figure 4.2. The mean FSH concentrations at different sampling times were not different statistically between the three treatments ($P>0.05$) except at one point i.e. 48 hours after PGF injection where mean FSH concentration in G27 ewes was higher ($P<0.05$) than the Control ewes. There was a marked decline in the mean FSH concentrations in each treatment within 24 h after PGF injection. When FSH concentrations from individual ewes were plotted against sampling time, all the G27 and Control ewes and 9 out of 10 Pawera ewes showed a decline in FSH within 24-30 h after PGF injection. In one of the Pawera animals, FSH concentration was still elevated 30 hours after PGF injection; this was one of the two Pawera ewes in which no large or medium sized follicles were present on the ovarian surface 24 h after PGF injection.

With this 'three blood samples per day' regimen (at 3 h interval), a preovulatory FSH rise was detected in 3 (30%) Pawera, 4 (40%) G27 and 5 (45.5%) Control ewes respectively. All the animals with corpora haemorrhagica at the time of slaughter (about 72 hours after PGF) had shown a rise in FSH concentration 51 h after PGF injection.

Means of the averaged FSH concentrations per day (Table 4.7) also did not differ significantly between the three treatment groups on any day of sampling.

4.6. Plasma progesterone

The mean progesterone concentrations in the peripheral plasma of ewes are presented in Table 4.8. Progesterone concentration did not differ significantly between the three treatment groups ($P>0.05$) on day 5 or 12 of the cycle. On day 8 of the cycle, progesterone concentration was significantly higher in Pawera ewes than that in G27 ewes ($P<0.01$). Within each treatment group, progesterone concentration was lowest on day 5. The hormone concentrations were not different ($P>0.05$) between day 8 and day 12 in each treatment.

Table 4.7. Plasma FSH concentration (ng/ml) in ewes grazed on various herbages; mean ± SEM of averages of the three daily samples.

Sampling day	Treatment		
	Pawera	G27	Control
Day 12 of cycle	1.54 ± 0.14	1.95 ± 0.21	1.48 ± 0.15
Day 13 of cycle	1.67 ± 0.14	2.13 ± 0.27	1.34 ± 0.15
	Prostaglandin F2α analogue (PGF) injected		
Day 1 post-PGF	1.28 ± 0.14	1.32 ± 0.17	0.97 ± 0.08
Day 2 post-PGF	1.16 ± 0.14	1.40 ± 0.11	1.21 ± 0.13
Day 3 post-PGF	1.40 ± 0.17	1.27 ± 0.11	1.41 ± 0.25

Differences between various treatments not significant (P>0.05).

Table 4.8. Plasma progesterone concentration (ng/ml) in ewes grazed on Pawera or G27 red clovers or Ryegrass-white clover (Control) pasture.

Day of cycle	Treatment		
	Pawera	G27	Control
5	1.00 ± 0.11^{b}	0.86 ± 0.11^{d}	0.93 ± 0.18^{f}
8	2.02 ± 0.27^{a}	1.16 ± 0.11^{cd}	1.71 ± 0.21^{e}
12	1.95 ± 0.29^{a}	1.51 ± 0.16^{c}	2.04 ± 0.28^{e}

Means with different superscript letters in the same column differ significantly (P<0.05).

There were a few animals (Pawera = 2 ewes, G27 = 2 ewes, Control = 1 ewe) in which progesterone concentration was lower on day 12 compared to that on day 8. Two of them (one Control and one Pawera ewe) had ovulated when slaughtered 72 h after PGF injection.

5. Discussion

A significantly lower blood equol concentration in ewes grazing G27 red clover compared to the Pawera group indicates that the formononetin content in the herbage is translated into a proportional concentration of equol in the blood. G27 red clover contained a formononetin content about one third of Pawera red clover, and the blood equol concentration in five randomly selected ewes grazing G27 was one fourth of that found in the ewes grazing Pawera. A plasma equol level of 4.09 μg/ml (4.04 μg/ml being conjugated) has been reported in adult Merino wethers after 0.5 h of red clover feeding (Shutt *et al.,* 1970). Equol, the major metabolite of formononetin, has been suggested to be the cause of infertility in ewes grazing oestrogenic red clover (Shutt and Braden, 1968). Equol is present in blood plasma predominantly (more than 90%) in a conjugated form, and only about 1% of it is present as unconjugated or free equol (Shutt *et al.,* 1970; Lundh *et al.,* 1990). The "free" fractions probably represent the biologically active form of the compound (Shutt, 1976), and a small proportion of sulphoconjugate is also biologically available (Cox *et al.,* 1984). Although proportions of biologically available equol in G27 and Pawera red clover were not determined in the present experiment, a significantly lower concentration of (conjugated + free) equol in G27 grazing ewes suggests that the new low formononetin selection should be safer than Pawera red clover.

This study provided some indication of decreased follicle development in ewes grazed on red clover. A relatively lower number of medium-sized follicles was found developing on the ovarian surface of ewes grazed either on Pawera or G27 red clover than in the Control ewes on day 13 of the oestrous cycle. By 24 hours after PGF injection, the total number of surface follicles was not different between Control and G27 red clover ewes, but it was significantly lower in the Pawera animals. In the latter group, 2 out of 10 ewes did not have any large or medium-sized surface follicles at this stage. McNatty *et al.,* (1982) showed that a large follicle appeared in the sheep ovary within 10 hours after the induction of luteolysis with a PGF analogue and that this presumptive preovulatory follicle emerged before the corpus luteum had ceased to function. Driancourt *et al.,* (1985) also suggested that in sheep, the recruitment of follicles which were candidates for ovulation occurred around 48 h before the LH peak and it probably coincided with luteolysis. The follicles capable of ovulating within the expected time from induced luteolysis to ovulation can come from a pool of antral follicles ≥2 mm in diameter suggesting that the ovary has a great flexibility in selecting

follicles for ovulation (Tsonis *et al.*, 1984). The observation in the present trial suggests that in ewes grazed on red clover, a lower number of medium sized follicles (3 mm-4.5 mm) was available for recruitment on day 13 of the oestrous cycle. By 24 h after PGF injection, the mean follicle number in red clover groups had improved, perhaps because the ewes had been away from red clover for 48 h at that time (discussed in detail in the following paragraph), but the total mean number was still lower in the Pawera ewes.

The number of large follicles did not differ in Control ewes between the three observation times, but there was an increase in the number of large follicles in Pawera as well as G27 group over time. A possible reason for this improvement might be because the ewes did not graze on treatment herbages for 48 hours in connection with laparotomy and laparoscopy conducted for follicular observation. This interval might be enough for ewes to show some recovery from the effect of grazing oestrogenic pasture on follicular development and ovulation/rate. It has been shown that isoflavones, in oestrogenic clover, ingested by sheep are metabolized in the rumen quite rapidly (Shutt *et al.*, 1970). About 90% of the formononetin ingested in red clover is metabolized in 1.5 h, predominantly to form equol in the sheep rumen which in turn is absorbed into the blood in a mean time of 1.7 h. Davies and Hill (1989) have shown that 52% of the equol produced in ewes given intrarumenal tritiated formononetin was excreted after 24 hours, and most of it (81%) was excreted in 48 hours. It may therefore be possible for some recovery in follicular development and ovulation rate to occur if ewes are kept away from oestrogenic pasture for 48 hours. Kelly *et al.*, (1980) observed that ovulation rates recovered within a cycle of removing ewes from Pawera red clover, whereas the incidence of return to service was still high.

The mean number of surface follicles in various size classes at slaughter was not different between the left and right ovaries of the Pawera, G27 or Control groups. However, the mean number of small follicles was higher in the right ovary than that in the left ovary in the Control ewes. As this was the only difference observed, any one ovary could be used for histological examination. Cahill *et al.*, (1979) did a microscopic study of three right and left ovaries from the same sheep and showed that the largest difference between the right and left ovary occurred for the smallest follicles in the growing phase and that the relative difference per class decreased as the size of the follicle increased.

Histological examination of left ovary 72 h after PGF injection showed a relatively lower number of healthy follicles in the Pawera ewes in the diameter range of 1mm<D≤2mm. Moreover, 2 of the 5 large follicles (>4mm in diameter) were found to be in early stages of atresia. All the >4mm follicles in the G27 and Control ewes were found to be healthy. Moor *et al.*, (1978) and Carson *et al.*, (1981) showed that the output of oestrogen is sharply reduced during atresia in all sizes of follicles, and indicated that the aromatizing capacity of follicles is a limiting factor at a very early stage in atresia and precedes most of the classical structural changes of cellular degeneration. Studies with fully grown preovulatory follicles of rats have shown that a decrease in ovulability precedes morphological evidence of atresia (Tsafriri and Braw, 1984). Therefore, the large follicles, although at an early stage of atresia by morphological standards, might have already lost the ability to ovulate. Abnormal follicular development accompanied by early atresia of the follicles also occurred in ewes grazing oestrogenic Dinninup cultivar of subterranean clover (Adams, 1977). Histological observation of the ovaries in the present experiment suggest that grazing the high formononetin clover may result in a low number of medium sized healthy follicles, and atresia of large follicles which may account for the lower ovulation rate (or ovulation failure) in the ewes.

Mean plasma FSH levels were not different in ewes grazing the various herbages. A marked decline in mean FSH concentration was observed in all three groups on the day following PGF injection which is in close agreement with Wallace *et al.*, (1988). These authors reported that overtime there was a decrease in the secretion of FSH during the first 18 hours after cloprostenol induced luteolysis. This marked, largely linear, depression in FSH during the follicular phase is followed by a peak of FSH secretion coincident with the LH surge (Campbell *et al.*, 1991). In the cloprostenol injected ewes, this preovulatory FSH peak has been reported to occur at various intervals after the injection (e.g. 61 ± 4 h, Baird *et al.*, 1981; 54.5 ± 3.4 h, Campbell *et al.*, 1991). In the present trial, a preovulatory rise in FSH was not found in a majority of the ewes, and it was especially so in Pawera animals where the lowest percentage of ewes showed a rise in FSH. This may in part be due to the fact that the blood sampling was done in an infrequent manner. In spite of this fact, it should be mentioned that a rise in FSH concentration was noted in all the ewes which had ovulated by 72 hours after PGF injection.

No decline in mean plasma progesterone levels was observed on day 12 of the oestrous

cycle in ewes grazed on various treatment pastures suggesting an undisturbed luteal function until that day. According to Goodman (1988) progesterone concentrations in peripheral blood begin to increase around day 3-4 of the oestrous cycle. Maximum concentrations are observed by day 10-12 and are maintained until luteolysis around day 14-15. Obst and Seamark (1975) reported that ewes failing to conceive on oestrogenic clover had progesterone concentrations that began to fall on day 11-12 and reached oestrous levels on days 13-14, indicating a shortened period of corpus luteum function. As the criterion of selection of ewes for progesterone assay was different in the latter study, it may explain the different results from those of the present trial.

It is concluded that ewes grazed on G27 red clover performed better than those on Pawera red clover in terms of blood equol levels, and follicle growth and development on the ovaries. Follicle development in G27 ewes was not different from that in Control ewes.

CHAPTER V

Sperm transport in ewes grazed on red clover

1. Abstract

Two experiments were conducted in a study to compare sperm transport in ewes which had either grazed on a high formononetin Pawera red clover, low formononetin G27 red clover, or Ryegrass-white clover (Control) pasture.

In the first experiment 84 Romney ewes were grazed for two oestrous cycles on various grazing treatments. The grazing treatments for cycle 1 and cycle 2 were: (1) Pawera / Pawera; (2) Control / Pawera; (3) Pawera / Control; (4) G27 / G27; (5) Control / G27; (6) G27 / Control; (7) Control / Control. The ewes were inseminated with 500 million fresh motile spermatozoa at the os cervix after 2 cycles of grazing, and killed either 2 or 24 h after insemination (n = 6 / slaughter time / treatment). Spermatozoa were flushed with normal saline from different parts of the reproductive tract and sperm numbers were determined. Mean number of spermatozoa in the cranial part of the cervix were not different between treatments 2 h after insemination. Spermatozoa were not recovered from the Fallopian tubes and uteri of many ewes, and thus no conclusion could be drawn from the experiment.

In the second experiment 30 Romney ewes were mated to rams after 28 days of grazing either on Pawera red clover, G27 red clover, or Control pasture (n = 10 per treatment). The ewes were killed 24 h after service and sperm were recovered from the tract and counted using an improved technique. The number of spermatozoa recovered from different parts of the tract did not differ significantly between treatments although there was a trend for the low formononetin (G27) group to have higher sperm numbers than Pawera group. Spermatozoa could not be recovered from the Fallopian tubes of 57% of Pawera ewes 33% of Control ewes, and 11% of G27 ewes.

2. Introduction

Permanent infertility in ewes caused by several seasons of grazing on oestrogenic clover has been shown to result from non fertilization of ova which is due to impaired sperm transport through the reproductive tract. Turnbull *et al.*, (1966) reported that

sperm were not found after mating in the Fallopian tubes of ewes which had grazed oestrogenic red clover for 6 years. The primary failure of sperm transport in the permanent clover-infertile ewes occurs when sperm do not enter the cervix in adequate numbers following service (Lightfoot *et al.*, 1967). The impaired sperm transport through the cervix is due to histological changes in the cervix (Adams, 1990), and to secretion of a thinner, more watery cervical mucus (Smith, 1971) with an abnormally low spinnbarkeit (or visco-elasticity) which has a decreased ability to orientate sperm migration (Adams, 1976a).

A temporary type of infertility in ewes joined on oestrogenic pastures may also involve an impaired sperm transport in the reproductive tract. Lightfoot and Wroth (1974) reported a decreased egg fertilization rate in ewes inseminated 17 to 21 days after placement on oestrogenic subterranean clover, compared to the ewes grazed on non-oestrogenic pasture. The difference between treatments in fertilization rate was associated with fewer spermatozoa on the zona pellucidae of the eggs recovered from the ewes grazed on oestrogenic clover. An adverse effect on the rate of egg transport in ewes grazing oestrogenic pasture may also affect fertilization by hastening the passage of the egg through the Fallopian tube (Holst and Braden 1972, Lightfoot and Wroth 1974). A daily dose of 25 μg of oestradiol-17β administered to ewes for 14 days preceding oestrus has also been shown to have a deleterious effect on the passage of spermatozoa through the cervix into the uterus within the first 2 h after insemination (Croker *et al.*, 1975).

Use of low oestrogenic cultivars of clover has been shown to decrease the infertility problem in sheep. Kaltenbach and Davies (1970) compared sperm transport in ewes which had grazed for five years on either high or low oestrogenic cultivars of subterranean clover. A larger proportion of ewes on high oestrogenic cultivars had low oviductal sperm counts and the fertilization rate was significantly lower than in ewes grazed on low oestrogenic cultivars.

This study was conducted to compare sperm transport in ewes which had either grazed on Pawera red clover, the low oestrogenic selection 'G27 red clover', or Ryegrass-white clover pasture (Control) before artificial insemination or natural mating. Two experiments were conducted for this purpose. In the first experiment, the ewes were grazed for 2 oestrous cycles on various grazing treatments, then inseminated and killed either 2 or 24 h after insemination to count sperm in the reproductive tract. In the

second experiment, the ewes were grazed for 4 weeks on red clover and Control pastures, mated to entire rams after removal from the treatment pastures and killed 24 h after mating to count sperm in the reproductive tract.

3. Experiment 1

3.1. Materials and methods

3.1.1. Animals and treatments

Mixed-age Romney ewes with a history of normal fertility and with an average body weight of 55.5 ± 0.7 kg were used from a Massey University sheep farm flock. For this purpose 92 animals were treated for 12 days with intravaginal sponges impregnated with 40 mg medroxyprogesterone acetate (MAP) and injected (i.m.) with 500 i.u. PMSG (Folligon, Intervet, Holland)intramuscularly at sponge removal. For convenience at artificial insemination, sponges were inserted at a weekly interval with 46 ewes treated at each sponge insertion. Four harnessed vasectomized rams were run with the flock for oestrous detection after sponge withdrawal. Eighty-four ewes which exhibited heat within 3 days of sponge withdrawal, were allocated to seven grazing treatments as outlined in Table 5.1.

Table 5.1. Trial design (sperm transport experiment 1)
(N = 84; n = 12)

Group	Grazing treatments	
no.	Cycle 1	Cycle 2
1	Pawera	Pawera
2	Control	Pawera
3	Pawera	Control
4	G27	G27
5	Control	G27
6	G27	Control
7	Control	Control

The treatment groups were balanced for live weight and age. Duration of the grazing treatment was equal to two oestrous cycles.
The grazing treatments started during the second week of February, 1991. Ewes in groups 2, 3, 5, and 6 were moved to cycle 2 pastures 18 days after the start of treatments. Red clover paddocks were break-grazed by use of flexinets (electric fencing) for better utilization of available herbage. The ewes which grazed Control pasture at any stage, were always grazed as one mob.

A harnessed vasectomized ram was placed with each group of ewes 4 days before the expected time of first oestrus on the treatment pastures. Teaser rams remained with the ewes throughout the second cycle. Ewes were inseminated at the end of the second oestrous cycle. The ewes in each treatment were further subdivided into two subgroups to be slaughtered either 2 or 24 hours after insemination (n = 6 per subgroup), to determine the number of spermatozoa recoverable from various parts of the reproductive tract. The 2 hour interval was chosen because it has been suggested that the numbers of spermatozoa recoverable from the cranial region of the cervix 2 h after insemination are closely related to the numbers in the oviducts 24 h later (Croker *et al.*, 1975). The 24 h interval was chosen because it would be a time closer to ovulation and sperm would be expected in Fallopian tubes about the time of ovulation.

At the start of the second oestrous cycle, ewes were weighed again. They were administered an anthelmintic drench, and given a foot bath with zinc sulphate solution as a few ewes were found limping. The animals were also ring crutched to minimize accumulation of faeces around the tail.

3.1.2. Artificial Insemination

Ewes detected in oestrus by 08.30 h were moved from treatment paddocks to the insemination shed located close to the slaughter house. All inseminations were conducted between 09.00 to 10.00 h.

Four Border Liecester rams of proven fertility were trained for semen collection using an artificial vagina. On day of insemination, semen was collected from at least three rams and each sample was examined for density and motility (each scored on a 0 to 5 scale). Only those semen samples which reached a minimum grading of 4 for both

density and motility, were used for insemination. The samples of high quality from these rams were pooled to minimize any effect of variation between rams. Motility of spermatozoa in the pooled ejaculate was determined by placing a small drop of semen on a warm slide and examining it microscopically. The number of spermatozoa per ml was estimated by haemocytometer counts and the volume required to provide 500 million motile sperm (dose per insemination) determined. During any one insemination period, a check was kept on the motility of the pooled ejaculate which was used undiluted. Procedures described by Evans and Maxwell (1987) were followed for semen collection and evaluation.

Cervical inseminations were done in the ewes by placing their hind quarters over a wooden rail. Semen was deposited in the external os cervix using 500 million spermatozoa, with the aid of a metallic speculum fitted with a light. Inseminations were completed within one hour of the semen collection. After insemination the ewes were left in a small yard with grass and were not disturbed until slaughter.

3.1.3. Recovery of spermatozoa

Ewes were slaughtered at 2 h or 24 h after insemination. The abdomen was opened by a ventral incision and the reproductive tract, minus vagina, was immediately removed. Ligatures were placed at each utero-tubal junction, at the base of the uterine body and at the posterior end of the cervix. Each tract was placed in a plastic bag and taken to the laboratory where it was divided into four segments; two Fallopian tubes, the uterus and the cervix in that order. The cervix was stored immediately at -20°C to be thawed and flushed at a later stage. Techniques used by Allison and Robinson (1972), Hawk and Conley (1975), and Pearce and Robinson (1985) were followed for recovery of spermatozoa. All the organs were flushed with sterile saline solution (0.9% w/v NaCl).

For each ewe, the Fallopian tubes were dissected from the broad ligament and flushed from the ovarian end, each with 3 ml of saline, into a single, labelled plastic vial. Each tube was stripped of saline solution three times during the flushing.

The uterine horns were dissected from the broad ligament and separated. Each horn was flushed with approximately 15 ml saline. It was clamped at both ends and injected with 8 ml of saline solution, manipulated vigorously, placed in a filter funnel and drained of its contents. The procedure was repeated with another 7 ml saline. Flushings

from the two horns were pooled in one vial.
Flushings from the Fallopian tubes and uteri were frozen at -20°C for subsequent counting of spermatozoa.

To recover the spermatozoa from the cervix, it was cut, while frozen into caudal, mid and cranial thirds and each third was divided into 6-8 longitudinal strips. The longitudinal strips were placed in a glass beaker containing 20 ml of saline and allowed to thaw for 30 minutes. Then the contents of the beaker were stirred vigorously for 30 seconds with a glass rod and left for at least one hour before sperm counting.
Instruments were cleaned and boiled for each section of the tract, and the hands of the operator were thoroughly washed before and after manipulation of each part.

The number of large follicles (≥5mm) and the corpora lutea on the ovaries was also recorded for each animal.

3.1.4. Estimation of number of spermatozoa

Uterus and Fallopian tube

The samples were thawed at room temperature. Two strips of vaseline, about 35 mm apart, were placed across the width of a microscope slide (using a syringe filled with vaseline), and 0.2 ml of the thoroughly mixed flushing solution was placed between them. A 20x40 mm cover slip was then placed over the vaseline strips and gently pushed down until the flushing solution occupied the area under the cover slip and all air bubbles were expressed. The slide was then allowed to settle for at least 20 minutes. The number of spermatozoa was counted in a single lengthwise traverse under x400 magnification. Each traverse represented 1/44.44 of the area of each slide. Six slides were prepared from each flushing. The volume of each flushing was measured and the total number of spermatozoa estimated.

Cervix

Following thorough mixing of the flushings, six haemocytometer chambers (0.1 mm deep) were filled with a pasteur pipette by the following method. The first chamber was filled from the pipette and the residue returned to the beaker. The contents of the

beaker were re-mixed and a second sample taken, and procedure repeated until all six chambers were filled. The slides were allowed to settle for at least 20 minutes. The number of spermatozoa in each chamber was counted using the standard technique for white blood cells (Cartwright, 1968), the mean was determined, and number of sperm present in the flushing was estimated.

3.1.5. Red clover sampling and formononetin assay.

Pawera red clover and G27 red clover grazed during the trial were at the flowering stage of growth. The paddocks were dominant in red clover and contained more than 70% Pawera or G27. Red clover samples from the plots grazed by sheep were regularly collected during the trial and were analysed for formononetin concentration as described in Chapter III.

3.1.6. Statistical analyses

Number of spermatozoa estimated in each segment was corrected by adding 10 to avoid zero values. Univariate procedure of the Statistical Analysis System computer package (SAS Institute Inc., 1988) was applied to check if the sperm count data in the various segments of the reproductive tract and various treatments were samples from normal distributions. Univariate procedure uses Shapiro-Wilk statistic (Shapiro and Wilk, 1965) for this purpose. Data in most of the cases were not normally distributed. A near normal distribution of the sperm data was obtained after $\log_{10}$ transformation. Analysis of variance procedure (ANOVA) was applied to the transformed data to compare the number of spermatozoa between the grazing treatments within each slaughter time. Live weight change in ewes after one cycle of grazing were also compared by ANOVA. Initial live weight was used as a covariate in this analysis. Formononetin concentrations in Pawera and G27 red clover were compared by t-test. Data are presented as mean ± SEM. All the analyses were performed using the Statistical Analysis System computer package (SAS Institute Inc., 1988).

3.2. Results

3.2.1. Formononetin concentration in red clover

Mean formononetin concentration in Pawera and G27 red clover during the grazing period is shown in Table 5.2. The concentration was significantly lower in G27 than that in Pawera red clover ($P<0.01$).

3.2.2. Live weight changes in ewes

Mean live weights of ewes at the start of the trial were not different between various groups (Table 5.3). All the groups of ewes gained weight during the first cycle except for one of the Control groups that grazed G27 in the second cycle (Control/G27 group).

3.2.3. Oestrous activity in ewes

All the ewes were marked by teaser rams after sponge withdrawal at the start of the trial (early February), but 25% of the animals did not show another heat until late March or early April. This might be due to the breeding season which starts in March in the region with highest oestrous activity towards end of March (Quinlivan and Martin, 1971). The inseminations of ewes started on March 11 and were completed on April 10. More than 70% of the ewes were inseminated within a 10 day period (i.e. 11-20 March). The data from four ewes which did not show heat until after 10th of April were excluded from the trial.

3.2.4. Ovarian activity

Ewes with fresh ovulation points were found at both 2 h and 24 h after insemination (10/40 vs 29/40). The number of ovulating ewes in each treatment was higher 24 h after insemination (Table 5.4). All the ewes without an ovulation point had large follicles present on the ovary except the ewes in Control/Pawera group where 3 out of 10 ewes had neither a large follicle nor a corpus luteum on the ovaries, although they had been marked by the teaser rams.

TABLE 5.2. Mean formononetin concentrations (% of herbage dry matter) in treatment pastures

Herbage	No. of samples	Formononetin (% DM)
Pawera	4	0.64 ± 0.10^{a}
G27	5	0.28 ± 0.04^{b}

Means with different superscript letters in the same column are significantly different ($P<0.01$).

TABLE 5.3. Live weight (kg) in ewes grazing various treatment herbages.

Grazing treatment cycle1/cycle2	No. of ewes	Observation time[1] relevant to treatment		
		Start	Middle	Weight gain
Pawera/Pawera	11	57.3 ± 2.3	60.1 ± 2.1^{a}	2.8 ± 0.7^{a}
Control/Pawera	10	55.2 ± 1.6	58.5 ± 2.2^{a}	3.3 ± 0.8^{a}
Pawera/Control	12	54.2 ± 1.6	57.1 ± 1.8^{a}	2.9 ± 1.5^{a}
G27/G27	12	56.2 ± 1.9	57.6 ± 2.3^{ab}	1.3 ± 0.7^{ab}
Control/G27	12	54.7 ± 1.7	53.9 ± 1.9^{b}	-0.8 ± 1.0^{b}
G27/Control	11	56.5 ± 1.9	58.6 ± 1.5^{a}	2.2 ± 0.8^{a}
Control/Control	12	54.7 ± 1.4	55.6 ± 1.9^{ab}	0.9 ± 0.4^{ab}

Means with different superscript letters in the same column are significantly different ($P<0.05$).

[1] Ewes were weighed at the start of the grazing treatment and then at the middle of the treatment that was the start of the second cycle.

3.2.5. Number of spermatozoa

Mean numbers of spermatozoa recovered from the reproductive tracts of ewes are shown in Table 5.4 and 5.5.

At 2 hours after insemination the mean numbers of spermatozoa recovered from various parts of the tract were not significantly different between ewes on the various treatments ($P>0.05$). Spermatozoa were always recovered from all three regions of the cervix although they decreased from the caudal to the cranial part in each treatment. The numbers of sperm cells recovered from the uterus and Fallopian tubes were very low compared to those in the cervix. No spermatozoa could be recovered from the Fallopian tubes of 13 ewes and uteri of 14 animals which were almost evenly distributed between treatment groups.

At 24 hours after insemination the mean numbers of spermatozoa recovered from the various parts were not significantly different between treatments ($P>0.05$). There were 17 (21%) ewes in which no spermatozoa were recovered from the cranial region of the cervix 24 hours after insemination. Similarly, no spermatozoa were recovered from uteri of 21% animals over all treatments. These ewes were almost evenly distributed among the treatment groups.

No spermatozoa were recovered from Fallopian tubes of 22 (27%) ewes. The distribution of these ewes was: 5 out of 6 (Pawera/Pawera); 2 out of 5 (Control/Pawera); 4 out of 6 (Pawera/Control); 3 out of 6 (G27/G27); 2 out of 6 (Control/G27); 2 out of 5 (G27/Control); 4 out of 6 (Control/Control).

Comparison of sperm numbers at the two intervals after insemination showed that in all the groups, the mean number of spermatozoa recovered from the caudal region of the cervix 2 h after insemination was significantly higher than that recovered at 24 h ($P<0.05$). Number of spermatozoa recovered from the middle region of the cervix was also higher 2 h after insemination than that 24 h after insemination, but significant differences were observed only in Control/G27, G27/G27, and Pawera/Control groups ($P<0.05$); the same groups showed a higher number of spermatozoa recovered from the cranial region of the cervix 2 h after insemination than 24 h. Number of spermatozoa recovered from the uterus did not differ at the two intervals after insemination. Number of spermatozoa recovered from the Fallopian tubes also did not differ between

TABLE 5.4. Number of spermatozoa recovered from reproductive tracts at 2 hours and 24 hours after cervical insemination of the ewes grazed on various pastures for 2 oestrous cycles.

Time interval and Grazing treatment	Number of ewes			Spermatozoa recovered			
	Examined	Ovulating	With large follicles	Fallopian tubes	Uterine horns	Cervix ($\times 10^3$)	Total ($\times 10^3$)
2 Hours							
Pawera/Pawera	5	3	2	1403 ± 1009	2483 ± 834	37538 ± 13934	37541 ± 13934
Control/Pawera	5	1	3	2450 ± 726	3521 ± 1950	31577 ± 15747	31583 ± 15749
Pawera/Control	6	2	4	3342 ± 2424	2209 ± 1703	12843 ± 3693	12848 ± 3697
G27/G27	6	1	5	386 ± 261	665 ± 372	36289 ± 15919	36290 ± 15919
Control/G27	6	1	5	7125 ± 6836	9803 ± 5376	43202 ± 20676	43219 ± 20675
G27/Control	6	1	5	557 ± 269	6148 ± 5017	34204 ± 24535	34211 ± 24534
Control/Control	6	1	5	102 ± 63	645 ± 202	78272 ± 52606	78272 ± 52606
24 Hours							
Pawera/Pawera	6	4	2	63 ± 53	19890 ± 18089	10100 ± 9318	10220 ± 9336
Control/Pawera	5	3	0	333 ± 171	4967 ± 3854	2383 ± 1348	2388 ± 1351
Pawera/Control	6	5	1	140 ± 93	1214 ± 866	231 ± 57	233 ± 57
G27/G27	6	5	1	244 ± 125	3120 ± 2937	2127 ± 1144	2130 ± 1147
Control/G27	6	4	2	2609 ± 1627	31089 ± 16021	2341 ± 1031	2374 ± 1046
G27/Control	5	4	1	18089 ± 17936	139996 ± 138322	2331 ± 1479	2489 ± 1621
Control/Control	6	4	2	104 ± 73	21621 ± 20882	3402 ± 2998	3424 ± 3018

TABLE 5.5. Number of spermatozoa recovered from various regions of cervix at 2 hours and 24 hours after cervical insemination of ewes grazed on various pastures for two oestrous cycles.

Time interval and Grazing treatment	No. of ewes	Spermatozoa recovered ($x10^3$) Region of the cervix		
		Cranial	Mid	Caudal
2 Hours				
Pawera/Pawera	5	590 ± 299	8098 ± 3826	28849 ± 10433
Control/Pawera	5	453 ± 313	15290 ± 9079	15833 ± 7322
Pawera/Control	6	234 ± 171	1622 ± 546	10987 ± 3657
G27/G27	6	539 ± 297	16263 ± 8516	19486 ± 9039
Control/G27	6	1792 ± 916	17284 ± 12435	24125 ± 10187
G27/Control	6	608 ± 435	1698 ± 903	31898 ± 24840
Control/Control	6	794 ± 381	10774 ± 5079	66703 ± 50000
24 Hours				
Pawera/Pawera	6	883 ± 830	2720 ± 2191	6498 ± 6301
Control/Pawera	5	748 ± 719	854 ± 467	781 ± 598
Pawera/Control	6	44 ± 32	98 ± 43	89 ± 42
G27/G27	6	173 ± 159	916 ± 877	1038 ± 559
Control/G27	6	885 ± 492	909 ± 430	547 ± 317
G27/Control	5	387 ± 362	1102 ± 880	841 ± 526
Control/Control	6	734 ± 608	2325 ± 2108	342 ± 284

2 and 24 h after insemination except in one group (Pawera/Control).

3.3. Discussion

There was an indication of "quiet ovaries" (with no ovulation or follicular activity) in Control/Pawera group (i.e. the ewes that grazed Pawera red clover for one cycle before insemination nd slaughter), but it was not observed in animals that grazed Pawera red clover for two cycles before slaughter. It has been observed that high oestrogenic

clover may cause oestrus without ovulation in ewes (Kelly *et al.,* 1980).

When the ewes were killed 2 h after insemination, sperm were found in all three regions of the cervix in all the ewes. The establishment of a sperm population of normal size in the anterior third of the cervix of the ewe soon after mating or insemination is essential for subsequent movement of normal numbers of sperm to the oviduct (Hawk, 1983). Croker *et al.,* (1975) suggested that data for the relative number of spermatozoa in the caudal, middle, and cranial cervix shortly after insemination could be interpreted as indicating relative ease of passage of spermatozoa through the cervix. They found that the number of spermatozoa recoverable from the cranial region of the cervix at 2 h after insemination is closely related to the number of spermatozoa present in the oviduct 24 h later. In the present experiment the mean sperm numbers in any part of the cervix were not different between various groups of ewes grazed either on the high or low oestrogenic red clovers or non-oestrogenic pasture using different grazing schedules. This indicated that about 34 days of grazing on high oestrogenic clover had not affected the ability of the cranial part of the cervix to act as a sperm reservoir. Lightfoot *et al.,* (1967) observed that motility of spermatozoa recovered from the cervix might be affected in ewes after exposure to phytoestrogens for several years. It is not known if sperm motility would be affected in ewes mated on oestrogenic clover.

Sperm could not be recovered from a substantial proportion of ewes from the upper portion of the tract (that is uterus and Fallopian tubes) in all the treatment groups 2 h or even 24 h after insemination. Similar observations have been recorded by Allison and Robinson (1970), and Croker *et al.,* (1975) who each used a dose of semen comparable to that used in the present experiment to inseminate the ewes. A higher number of spermatozoa might be more suitable for such studies.

A higher number of spermatozoa was recovered from the caudal region of the cervix 2 h after insemination than 24 h after insemination. The number recovered from the middle and the cranial regions of the cervix in 4 out of the 7 groups was not different at the two intervals after insemination. Hawk (1983) suggested that sperm numbers in the cervix of the ewe at 2 h after mating are probably higher than at any later time. That is, by 2 h, more sperm probably are moving from the cervix to the vagina and the uterus than are entering the cervix from the vagina. Declining sperm numbers in the posterior and middle segments of the cervix presumably reflect some movement of

sperm anteriorly into the uterus and oviducts, but most sperm lost from the cervix probably move into the vagina. Sperm numbers in the anterior third of the cervix have been shown to remain reasonably constant until at least 22 to 24 h after mating (Hawk, 1983), and the numbers in the uterus and oviduct of sheep increase gradually between 1 and about 24 hours after mating or insemination (Quinlivan and Robinson, 1969; Hawk, 1983; Lane *et al.*, 1993). But the sperm number recovered from the uterus and Fallopian tube in the present study was not different between 2 and 24 hours after insemination. Perhaps no differences could be observed due to many zero values and also to a low number of sheep per cell.

It is concluded that the numbers of spermatozoa recovered from the cranial part of the cervix 2 h after insemination were not different in ewes grazed on high or low oestrogenic red clover cultivars. No conclusions could be drawn from the uterine and oviductal sperm counts due to many zero counts of spermatozoa in these parts. These zero values could be due to techniques not being good enough to flush sperm from the reproductive tract, or to count the sperm in the flushed fluid. Moreover artificial insemination might not be as effective a method as natural mating may be, to study sperm transport after flushing the tract.

4. Experiment 2

In this experiment an effort was made to improve the transport in and recovery of sperm from the tract by using the following steps.

1- Natural mating was used to increase the number of spermatozoa inseminated.
2- Ewes were mated twice a day instead of once a day.
3- A larger volume of fluid was used (following Hawk and Conley, 1975) for a thorough flushing of various parts of the tract.
4- A modified method was used to count spermatozoa in the Fallopian tubes and uterine flushings. This involved concentrating of spermatozoa by allowing them to settle in 5 ml glass vials before counting in a haemocytometer chamber.

4.1. Materials and methods

This experiment was conducted during the breeding season of 1993 to study the

transport of spermatozoa in ewes mated after 28 days of grazing on Pawera, G27 red clover, or Ryegrass-white clover (Control) pasture..

4.1.1. Animals, treatments, and matings

Thirty mixed-age Romney ewes were weighed and allocated to three groups (n = 10) balanced for live weight and age. The groups were randomly assigned to one of the three grazing treatments which were (1) Pawera red clover, (2) G27 red clover, and (3) Ryegrass-white clover pasture (Control). Animals grazed the treatment pastures for 28 days from March 24 to April 21, 1993. On day 1 of the treatment, intravaginal sponges impregnated with 40 mg medroxyprogesterone acetate (MAP) were inserted in all the ewes for 12 days to synchronize oestrus. Harnessed vasectomized rams were run with the ewes for heat detection after sponge withdrawal. On day 14 of grazing treatments, blood sampling was done (from jugular vein) in animals to measure the concentration of equol (assay method described in Appendix II). The ewes were also weighed midway through the grazing treatment. After 28 days of grazing treatment, all the ewes were run as one group on Ryegrass-white clover pasture with two harnessed, vasectomized rams for heat detection.

Four entire Romney rams of proven fertility were used to pen mate the ewes. Mating marks by teaser rams were recorded twice a day at 08.00 h and 16.00 h. Ewes detected newly in heat were placed with another two or three ewes not in heat in a small yard for five minutes. After this settling down period, each ewe was mated once to each of two rams within a period of 10 minutes. Each of the mating rams was allowed only 2-3 services each day so that progressive diminution of sperm numbers with successive ejaculates which accompany frequent ejaculation would not occur (Salamon, 1962; Lightfoot *et al.*, 1967). Over the mating period the rams were used in such a way that successive mating ewes in each treatment were served by a different combination of rams. After mating, the ewes were again mixed with the other mob until slaughter 24 hours later for recovery of spermatozoa from the reproductive tract.

4.1.2. Recovery of spermatozoa

Ewes were slaughtered on the farm 24 h after mating to collect the reproductive tract as outlined in experiment 1. The cervix was stored immediately at -20°C to be thawed and flushed at a later stage. Spermatozoa from various parts of the tract were recovered

by a method similar to that used in experiment 1 with the following modifications.

Each Fallopian tube was flushed with 5-6 ml of saline, and each uterine horn was flushed with 50 ml of saline. Flushings from these parts were stored at -20 for subsequent sperm count.

The cervix was cut, while frozen, into caudal, mid and cranial segments of equal length. Each segment was divided into 6-8 longitudinal strips and stored overnight at 4°C in 50 ml of saline in a glass beaker.

4.1.3. Estimation of number of spermatozoa

4.1.3.1. Uterus and Fallopian tube.

To work out an efficient way of estimating the number of spermatozoa in the uterine and tubal flushings, where the number of spermatozoa are usually very low, three methods of recovering and counting spermatozoa were compared. Uterine flushing from one of the animals was used (referred to as 'test sample' in the following description) to compare these methods of sperm counting as follows.

i- Direct slide count.

The method is the same as that used to count sperm in tubal and uterine flushings in experiment 1. The number estimated in the test sample was 729 sperm/ml.

ii- Centrifugation of flushing and counting by haemocytometer chamber.

Two 5 ml aliquots from the test sample were centrifuged at 1850 g for 20 minutes, and another two for 10 minutes in siliconized vials. After centrifugation, 4.25 ml of the supernatant in each vial was decanted and the pellet resuspended in the left-over 0.75 ml saline. Both chambers of three haemocytometer (0.2 mm deep) were filled from each suspension and allowed to settle for 5 minutes. Spermatozoa were counted using the standard technique for white blood cells and the mean determined. Only the sperm heads were counted. Supernatants from these flushings were also examined for the presence of spermatozoa in six chambers each. Estimated sperm number per ml of the test sample (in the pellet and in the supernatant) is shown in Table 5.6.

Table 5.6. Counts of sperm in test samples of uterine flushing.

Vial no.	Centrifugation force and time	Sperm number estimated/ml	Sperm number in supernatant/ml
1	1850 g, 20 min	1256	0
2	1850 g, 20 min	1250	0
3	1850 g, 10 min	900	411
4	1850 g, 10 min	1148	600

iii- Settling of spermatozoa in glass tubes over time and counting by haemocytometer chamber.

Two aliquots of 5 ml each after thorough mixing of the test sample were put in siliconized-stoppered-glass vials and left undisturbed in a tube rack for 2-3 days to allow the particulate material to settle down. After this settling time 4.5 ml supernatant from each vial was decanted. Spermatozoa were counted after mixing the settled material by filling six haemocytometer chambers as described above. The number of spermatozoa estimated in the test sample by this method was 2437 and 2527 per ml in the two vials respectively.

Repeatability of the method was checked with two more flushings from different ewes, one from the uterus and the other from Fallopian tubes, and the method described above was found to be the most effective.

Comments.

The estimated number of spermatozoa in the same flushing was found to vary to a great extent when different methods for sperm counting were applied. The estimated number was higher after centrifugation at 1850 g for 20 minutes than that calculated after the slide method. Although no spermatozoa were found in the supernatant after centrifugation for 20 minutes, the estimated sperm number by this method was half of that calculated by allowing the sperm cells to settle in a similar volume for 2-3 days. It is possible that sperm cells are entangled in clumps of epithelial cells and other spermatozoa after centrifugation and thus are not evenly distributed in the pellet

suspension.

In the light of the results obtained from this small investigation, the method (iii) was used to count the number of spermatozoa in the uterine and Fallopian tube flushings in the present trial.

4.1.3.2. Cervix

Spermatozoa in the cervical flushings were counted using a haemocytometer chamber as done in the experiment 1.

4.1.1.3. Spermatozoa attached to ovum

When an ovary showed an ovulation had occurred, the fluid from the corresponding oviduct was examined for the egg under a stereo microscope. If recovered, a whole mount of the ovum was made (Chang, 1952). Two strips of melted vaseline were placed along the top and bottom of the slide. Each vaseline strip was lined with a small amount of medium (<5 µl) with micro pipette. The egg was placed in a small amount of saline between the vaseline lines. A coverslip was placed on, gently supported at two edges by vaseline. By gentle and careful pressing, the egg was anchored in the centre of the area between the coverslip and the slide. The coverslip was pressed down to obtain a good squash of the egg. This fresh preparation was examined at x400 to record the number of spermatozoa attached to the zona pellucida.

4.1.4. Red clover sampling and formononetin assay

Pawera red clover and G27 red clover grazed during the trial were at the vegetative stage of plant growth. Red clover sampling and formononetin assay were done as described in experiment 1.

4.1.5. Statistical analyses

Statistical analyses were performed as in experiment 1. Equol concentration between treatments was compared by using analysis of variance. Data are presented as mean ± SEM.

4.2. Results

4.2.1. Formononetin concentration in red clover

Mean formononetin concentration in Pawera and G27 red clover during the grazing period is shown in Table 5.7. The concentration was significantly lower in G27 than that in Pawera red clover (P<0.01).

TABLE 5.7. Mean formononetin concentrations (% of herbage dry matter) in treatment pastures

Herbage	No. of samples	Formononetin (% DM)
Pawera	3	0.65 ± 0.05^a
G27	3	0.32 ± 0.02^b

a b, significantly different (P<0.01).

TABLE 5.8. Equol concentrations (μg/ml) in peripheral blood of ewes grazing various treatment pastures

Grazing treatment	No. of ewes	Equol (μg/ml)
Pawera	5	6.25 ± 0.83^a
G27	5	3.23 ± 0.35^b
Contol	3	Undetectable

a b, significantly different (P<0.01).

4.2.2. Blood equol

Equol concentration in ewes was measured in five G27, five Pawera, and three Control ewes. Mean equol concentration on day 14 of treatment, was significantly lower ($P<0.01$) in ewes grazed on G27 red clover than that in ewes grazed on Pawera red clover (Table 5.8). No blood equol was detected in ewes grazed on Ryegrass-white clover pasture.

4.2.3. Live weight changes in ewes

Mean live weights of ewes at the start of the trial were not different between various groups (Table 5.9). Ewes in all the treatment groups gained weight during the first two weeks of grazing. The weight gain in various treatments was not different from each other ($P>0.05$).

TABLE 5.9: Live weight (kg) in ewes grazing various treatment herbages.

Treatment	No. of ewes	Observation time relevant to treatment		
		Start	Middle	Weight gain
Pawera	7	49.5 ± 3.3	53.6 ± 2.0	4.0 ± 1.6
G27	9	52.0 ± 2.4	55.5 ± 1.8	3.4 ± 0.8
Control	9	49.3 ± 3.1	52.3 ± 2.5	3.0 ± 1.5

4.2.4. Oestrous activity and matings

Due to loss of progestagen sponges from five ewes, the oestrous activity was spread over a longer period. 25 ewes in three treatments were mated within 13 days after removal from the treatment pastures. The remaining 5 ewes did not show heat and were found pregnant when observed by laparoscopy. They were probably served, only a few days before the trial started, due to entry of some rams in the flock from which the ewes were selected. Data from these ewes have not been included in any of the

TABLE 5.10. Number of spermatozoa ($x10^3$) recovered from reproductive tracts 24 hours after mating of the ewes, after 4 weeks of grazing red clover or Control pastures.

Grazing	Number of ewes			Spermatozoa recovered (10^3)			
treatment	Examined	Ovulating	With large follicles	Fallopian tube	Uterus	Cervix	Total
Pawera	7	4	3	30 ± 26	158 ± 96	2260 ± 582	2448 ± 671
G27	9	6	3	59 ± 28	535 ± 427	8060 ± 3016	8654 ± 3158
Control	9	8	1	30 ± 15	227 ± 87	4631 ± 1203	4888 ± 1256

analyses.

4.2.5. Ovarian activity

Of the total 25 ewes 18 (72%) had ovulated when killed about 24 hours after mating (Table 5.10). The rest of the ewes had large follicles present on the ovaries.

4.2.6. Number of spermatozoa

Sperm data are summarized in Tables 5.10 and 5.11. Mean numbers of spermatozoa recovered from the various parts of the reproductive tracts of ewes were not significantly different between the three treatments ($P>0.05$), although the means were always the lowest in Pawera.

TABLE 5.11. Number of spermatozoa ($x10^3$) recovered from various regions of cervix 24 hours after mating.

Grazing treatment	No. of ewes	Spermatozoa recovered from 3 regions of cervix		
		Cranial	Mid	Caudal
Pawera	7	690 ± 290	1371 ± 384	199 ± 90
G27	9	3083 ± 922	3831 ± 1734	1146 ± 588
Control	9	1644 ± 571	2154 ± 557	834 ± 462

Spermatozoa were always recovered in the three regions of cervix in the various treatments. No sperm cells could be recovered from the uteri of one Pawera and one G27 ewe. No spermatozoa were found in the Fallopian tubes of a total of 8 ewes in three treatment groups. The percentage of ewes in which no spermatozoa were recovered from the Fallopian tubes was the lowest (11%) in G27 group, and it was the highest (57%) in Pawera group. 33% of the Control ewes yielded no sperm from Fallopian tubes.

Details of the eggs recovered from the ewes in various treatments are given in Table

5.12. Spermatozoa were found attached to some of the eggs recovered in various treatments. In Pawera ewes sperm were also not recovered from the Fallopian tubes of two ewes in which sperm were not found attached to the eggs. Sperm were recovered from the Fallopian tubes of two G27 ewes which did not show sperm attached to the eggs recovered. In Control ewes sperm were recovered from the Fallopian tubes of two out of three ewes in which sperm were not found attached to the eggs.

TABLE 5.12. Distribution between treatments of ewes from which eggs were recovered 24 hours after mating.

Grazing treatment	Number of ewes			
	Examined	Ovulating	Yielding eggs	With sperm on egg(s)
Pawera	7	4	3	1
G27	9	6	4	2
Control	9	8	6	3

4.3. Discussion

The ewes grazing Pawera red clover had a mean blood equol concentration about twice that in ewes grazing G27 red clover. It is interesting to note that formononetin concentration in these two red clovers had a similar ratio. Equol which is a metabolite of formononetin, has been suggested to be the principal oestrogen responsible for reproductive disturbance in ewes grazed on oestrogenic clover (Shutt and Braden, 1968; Davies and Hill, 1989). A lower equol level in ewes grazing G27 indicates that this selection of red clover might be safer than Pawera red clover.

The number of spermatozoa recovered from various parts of the tract did not differ significantly between ewes that had either grazed high oestrogenic Pawera red clover or low oestrogenic G27 red clover or Control pastures, although the mean sperm numbers were always lowest in Pawera ewes, and highest in G27 animals. In spite of increasing the number of spermatozoa inseminated by mating to entire rams in the present trial, there were a few ewes in which no sperm were recovered from the

Fallopian tubes 24 hours after mating. Amongst these ewes the Pawera group had the highest percentage of animals with zero counts and the G27 group had the lowest percentage. If the presence of spermatozoa in the fallopian tube 24 h after mating is considered a criteria for fertility, G27 ewes would have the highest conception rate. In an earlier study, R. Keogh and M.F. McDonald (Personal communication) observed a markedly higher conception rate in ewes mated on G27 red clover than those mated on Pawera red clover or non-oestrogenic pastures.

It has been suggested that after traversing the cervix and uterus, biologically competent spermatozoa enter the oviducts within 6-8 h of mating early in oestrus and are then sequestered in the caudal isthmus for a further 17 or 18 h until release of the egg(s) is imminent (Hunter and Nichol, 1983). This population of spermatozoa, while found to be quite variable in number among experiments, is very small in relation to the sperm numbers inseminated (Saacke *et al.*, 1994). Lane *et al.*, (1993) proposed that low recovery rates of these sperm from the oviduct might be due to sperm binding to oviductal cells and also to the sperm recovery technique. According to them inclusion of a detergent in the flushing medium might improve the sperm recovery rate. A higher number of animals per treatment may be required to overcome the problem of low counts as well as high variability in the spermatozoa recovered from different parts of the tract to reach a certain conclusion.

Spermatozoa were found attached to the zona pellucidae of some of the eggs recovered, but not all of them. As the ovulations were quite fresh and the number of eggs recovered quite small, no inference could be drawn from these observations. Lightfoot and Wroth (1974) found fewer sperm on the zona pellucidae of the eggs recovered from the oestrogenic treatment ewes after 17-21 days of grazing.

The simple method to count spermatozoa in the uterine and oviductal flushings used in the present experiment seemed to be more efficient and accurate when compared with the other two methods. The repeatability of the method needs to be checked in future experiments.

It is concluded that no differences could be observed in sperm transport 24 h after mating in the reproductive tracts of ewes that had grazed high or low oestrogenic red clover for four weeks, although there was a trend for the low oestrogenic (G27) group ewes to have higher sperm counts in various parts of the tract.

CHAPTER VI

Ovarian activity and embryo survival in ewes grazing high and low oestrogenic red clovers

1. Abstract

In two trials, ewes (n = 16 per group) that were potential recipients for embryo transfer grazed on a high oestrogenic red clover (Pawera), a low oestrogenic red clover (G27), and Ryegrass-White clover (Control) pastures. The ewes grazed the respective pastures during 12 days of intravaginal sponge insertion, and for a further 23 days after induced oestrus. The interval from sponge removal to oestrus was shorter ($P<0.05$) in Control (42.0 ± 3.1 h) than Pawera (58.5 ± 4.4 h) and G27 (52.6 ± 3.2 h) groups in trial 1 and comparable intervals in trial 2 were 42.3 ± 1.7 h, 46.3 ± 4.2 h and 51.3 ± 4.9 h respectively. In both trials, the number of ovular ewes and ovulation rate were lower ($P<0.05$) in ewes grazing Pawera red clover. The ovulation rate in Pawera, G27 and Control ewes in trial 1 was 0.62 ± 0.15, 1.62 ± 0.18 and 1.93 ± 0.27; it was 0.31 ± 0.18, 1.17 ± 0.27 and 1.54 ± 0.14 for the three groups in trial 2. Following the transfer into suitable recipients of two embryos per ewe post mortem examination on day 35 showed a survival rate of 50%, 90% and 85% in Pawera, G27 and Control groups in trial 1 and 50%, 50% and 69% in trial 2. It was concluded that G27, a low oestrogenic red clover was safer than Pawera red clover in terms of ovulation rate when grazed around mating. Ovulation rate and embryo survival rate in ewes on G27 red clover was not different from that in Control ewes.

2. Introduction

Temporary infertility may occur in ewes grazing oestrogenically active clover prior to, and during the time of mating. The ewes exhibit an increase in return to service (Morley *et al.*, 1966; Kelly *et al.*, 1980; Shackell and Kelly, 1984), a reduced oestrous incidence (Lightfoot and Wroth, 1974; Kelly and Shackell, 1982), oestrus without ovulation (Ch'ang, 1958; Kelly *et al.*, 1980), and decreased ovulation rate (Lightfoot and Wroth, 1974; Kelly et al., 1980). Increase in returns to service on oestrogenic clover might be due to; (1) the return to service of ewes which show heat without ovulation, (2) a reduced fertilization rate via reduced sperm transport (Lightfoot and Wroth, 1974), and (3) disturbances in the normal ovum transport (Holst and Braden,

1972). Whether or not oestrogenic grazing induces additional embryonic mortality has not been determined (Lightfoot and Wroth, 1974).

The present study was conducted to compare ovarian activity in ewes grazing either Pawera red clover, G27 red clover, or Ryegrass-white clover pastures. Embryo transfers were done to overcome possible fertilization failures in ewes grazing red clover and also to examine embryo survival on oestrogenic pastures. Oestrous incidence in ewes after progestagen sponge removal was also recorded.

3. Materials and methods

This study comprised two trials with 48 ewes each. Trial 1 was started during the first week of May, 1991, and trial 2 during the last week of February, 1992. Animals used and the methods applied which were similar for both trials are described below.

3.1. Animals and management

Six to seven years old, non pregnant Romney ewes with an average body weight of 47.2 ± 1.0 kg (trial 1) and 47.0 ± 0.7 kg (trial 2) were used. The ewes had a history of normal fertility and had not grazed oestrogenic pasture before. Animals were given an anthelmintic drench, and foot bath with Zinc sulphate at start of the trials. Oestrus was synchronized in the ewes by insertion of intravaginal progestagen sponges (containing 40 mg medroxy progesterone acetate - MAP) for 12 days. Each animal received 300 i.u. PMSG (Folligon, Intervet, Holland) at the time of sponge withdrawal. Harnessed teaser rams were run with the ewes (one ram with each group) and marks were checked twice daily (0800 h and 1600 h) during the period 24-96 h after sponge removal. Ovulation rate was determined 5-6 days after heat by laparotomy or laparoscopy and ovular ewes received two good quality embryos from donor ewes grazing Ryegrass-white clover pasture. Recipient ewes were slaughtered 34-36 days after embryo transfer to check embryo survival. Viability of embryos was determined with reference to crown-rump length, the development of the chorion and allantois and the presence of blood in the circulatory system (Kelly and Allison, 1979).

3.2. Treatments

Ewes (n = 16 per treatment per trial) were either grazed on Pawera red clover, G27 red

clover, or Ryegrass-white clover pasture (Control). In each trial ewes were split into two blocks (for convenience) which started grazing treatment pastures two weeks apart. The ewes grazed on the various treatment pastures from the day of intravaginal sponge insertion until 6 days after induced oestrus, and for a further 17-18 days if they received embryos. After this the ewes from the red clover were moved to Ryegrass-white clover.

Pawera and G27 were sown as pure red clover plots and at the time of grazing contained 70 to 90% red clover. They were at a vegetative stage of growth when grazed in 1991, and at flowering stage in 1992. Samples of red clover leaves and upper parts of stem were collected at regular intervals during each trial and formononetin concentrations were determined.

3.3. Superovulation and embryo collection

In trial 1 (n = 20) and in trial 2 (n = 30) (Booroola x Romney) x Perendale ewes were used as embryo donors. Donors received intravaginal MAP sponges along with the recipients ewes. They were superovulated with a single intramuscular injection of 1200 i.u. PMSG (Folligon) 48 hours before sponge withdrawal. Sponges were removed from embryo donor ewes 24 h after the recipient ewes, and two harnessed entire rams were run with them. They also received intrauterine insemination 36 h after sponge removal. Embryos were recovered surgically as described by Tervit and Havic (1976), 5-6 days after insemination under general anaesthesia using Dulbecco's phosphate buffered saline containing 5% sheep serum. Embryos were evaluated for development stage and quality (Lindner and Wright, 1983). Two excellent or good quality embryos were transferred to each recipient ewe after laparotomy, in the horn ipsilateral to the corpus luteum.

3.4. Statistical analyses

Number of marked ewes, ovular ewes, ovulations per ewe, and embryos surviving per ewe were compared by Chi square method. Ovulation rate data with low frequency were combined and data re-analysed to confirm the statistical significance. Interval from sponge removal to oestrus was converted into frequency data by dividing into two classes i.e., number of ewes showing heat within 48 hours after sponge removal, and number of ewes showing heat after 48 hours; the data were then analysed by Chi

square method. Formononetin concentration in the two red clovers was compared by t-test. Data are expressed as mean ± SEM if not mentioned otherwise. All the analyses were performed using the Statistical Analysis System computer package (SAS Institute Inc., 1988).

4. Results

Mean formononetin levels for the treatment pastures are shown in Table 6.1. G27 red clover had formononetin concentrations less than half that of Pawera red clover in both the trials conducted during 1991 and 1992 ($P<0.05$).

Table 6.1. Mean formononetin concentrations (% of herbage dry matter) in treatment pastures.

Herbage	No. of samples	Formononetin mean ± SEM
Trial 1		
Pawera	5	1.31 ± 0.06^{a}
G27	5	0.59 ± 0.04^{b}
Control	3	Undetectable
Trial 2		
Pawera	3	1.09 ± 0.08^{c}
G27	3	0.34 ± 0.04^{d}
Control	2	Undetectable

Means with different superscript letters are significantly different ($P<0.05$).

4.1. Onset of oestrus

The occurrence of oestrus and ovarian activity in the three treatment groups are shown in Table 6.2. The percentage of marked ewes was lowest in the Pawera groups in both trials but the differences were not significant ($P>0.05$). In trial 1, mean interval from sponge removal to oestrus was not different in Pawera and G27 ewes but it was shorter

Table 6.2. Oestrus and ovarian activity in ewes grazed on Pawera or G27 red clover, or Ryegrass-white clover (Control) pasture.

Group	Number in treatment	Number marked(%)	Hours to oestrus mean±SEM	Ovular ewes %	No. of ewes with ovulations 0	1	≥2	Ovulation rate mean±SEM
Trial 1								
Pawera	16	14 (88)	58.5 ± 4.4[a]	56[a]	7	8	1	0.62 ± 0.15[a]
G27	16	16 (100)	52.6 ± 3.2[a]	94[b]	1	5	10	1.62 ± 0.18[b]
Control	14[1]	13 (93)	42.0 ± 3.1[b]	100[b]	0	4	10	1.93 ± 0.27[b]
Trial 2								
Pawera	16	12 (75)	46.3 ± 4.2	19[c]	13	1	2	0.31 ± 0.18[c]
G27	12[2]	12 (100)	51.3 ± 4.9	75[d]	3	5	4	1.17 ± 0.27[d]
Control	13[3]	13 (100)	42.3 ± 1.7	100[d]	0	6	7	1.54 ± 0.14[d]

Groups with different superscripts in the same column and in the same trial are different at the P<0.05 level.

[1] Two ewes were lost

[2] Four ewes lost sponges

[3] Three ewes lost sponges

(P<0.05) in the Control ewes. In the Control group, 73% of ewes were marked within 48 h after sponge removal, and all of them were marked within 56 h after sponge removal. In G27 31% and in Pawera 23% of ewes were marked within 48 h after sponge removal. In trial 2 the interval was again shortest for the Control ewes, but there was no significant difference between the three groups. All the Control ewes, 75% of the G27 ewes, and 67% of the Pawera ewes were marked within 48 hour after sponge withdrawal in trial 2.

4.2. Ovular ewes and ovulation rate

Percentage of ovular ewes, and mean ovulation rate were not different for ewes grazed on G27 red clover or Control pasture in the two trials (Table 6.2) but were significantly lower (P<0.05) in ewes grazed on Pawera red clover. Incidence of oestrus without

ovulation was quite high in ewes grazing Pawera red clover (36% and 75% in trial 1 and 2 respectively) but it was only 6% and 25 % on G27 red clover. The non ovulating ewes had some small to medium sized follicles (<4 mm), or they had flat ovaries as examined on day 5 after oestrus. In the second trial two unmarked Pawera ewes had mid cycle corpora lutea on the ovaries and one anovular G27 ewe had a follicle with a thick wall (probably luteinized).

All the Pawera group ewes had markedly enlarged udders and their uteri were tense and oedematous.

Table 6.3. Embryo survival and pregnancy status in ewes 35 days after transfer of good quality embryos (2 embryos/ewe).

Treatment group	No. of ewes implanted	Number of Embryos		Pregnancy status	
		Transferred	Surviving(%)	No.	Percentage
Trial 1					
Pawera	5	10	5 (50)	3	60
G27	11[1]	21	19 (90)	11	100
Control	10	20	17 (85)	10	100
Trial 2					
Pawera	2[2]	4	2 (50)	1	50
G27	9	18	9 (50)	5	56
Control	13	26	18 (69)	10	77

[1] One ewe received only one embryo.

[2] Three ewes were implanted with embryos but one died a week after embryo transfer.

4.3. Embryo survival

Embryo survival results for the two trials are presented in Table 6.3.

Owing to a low number of suitable embryos in Trial 1, only 25 ewes out of 38 that ovulated were transplanted with 2 embryos each. One of the G27 ewes received one embryo. In trial 2, One of the Pawera recipients died one week after embryo transfer, and is not included in the data.

Embryo survival and pregnancy rate (34-36 days after embryo transfer) were not different in ewes grazed on G27 red clover and Control pasture in trial 1, and trial 2, although in trial 2 survival rate was higher in the Control ewes. Pawera ewes were not included in the statistical analysis owing to the low number of embryos transferred into them.

5. Discussion

G27 red clover which had formononetin levels less than half of Pawera red clover was found safer in terms of number of ovular ewes and ovulation rate in the ewes grazed on this pasture around oestrus. A three week period of grazing by ewes on G27 red clover around oestrus resulted in an ovulation rate comparable to that on non-oestrogenic pasture. There were a few marked ewes (4 of 28 for both trials) that failed to ovulate on G27 red clover but the number of such ewes was significantly higher on Pawera red clover (14 of 26 marked ewes). Mean ovulation rate was also significantly lower in ewes grazing on Pawera red clover as compared to G27 and Control pastures. The results for high formononetin Pawera red clover were consistent with some earlier studies. Lightfoot and Wroth (1974) recorded a lower ovulation rate for ewes grazed on oestrogenic subterranean clover than those on non-oestrogenic pasture (1.13 vs 1.51). Kelly *et al.*, (1980) observed a mean ovulation rate of 0.79 in Romney ewes grazed on Pawera red clover vs 1.55 on Control pasture for 8 days before and during the first cycle of mating. Out of 24 anovular ewes in their study, 69% had been marked by the ram. Ch'ang (1958) also reported that some Romney ewe lambs showed oestrus without ovulation after grazing on a red clover pasture. It is not clear how the anovular ewes on high formononetin clover were able to show oestrous behaviour. It might have resulted from oestrogen secreted by some follicles that did not ovulate and became atretic, or the phytoestrogen might have played a role in it. There is some indication that grazing on Pawera red clover might disturb normal follicular development in the ewes, including low number of follicles in certain sizes, and possibly atresia at final stages (Chapter IV). This disturbance in follicular growth might also be the cause of ovulation failure in ewes grazed on the high formononetin clovers. The results of the present study show that oestrogenicity of G27 red clover fed to the ewes had been reduced to a level where at least three weeks of grazing around oestrus was far safer than grazing Pawera red clover.

The interval from sponge removal to heat was lowest in Control ewes in trial 1 but the differences were nonsignificant between the three groups in the second trial. In trials 1 and 2, 73% and 100% of Control group ewes were marked by the ram within 48 h after sponge removal. According to Lamond (1964), for an oestrous synchronization method to be considered satisfactory, 80-90% of the treated animals should show heat within 36-48 hours of device removal. So the method used in this study was efficient. Formononetin in red clover might have played a role in delaying occurrence of oestrus, but as large errors in the time of heat existed, no conclusion was made in regard to possible treatment effect.

Embryo survival rate after transfer of good quality fertilized eggs to the ewes (2 eggs per ewe) was not different between G27 and Control pastures as observed at slaughter 35 days after transfer. Ewes had grazed respective treatment pastures 3 weeks before and 17-18 days after embryo transfer. It has been observed that embryonic mortality later than the 18th day of pregnancy is negligible (Moore *et al.,* 1960; Quinlivan *et al,* 1966), so the live embryos at the time of slaughter in the present study had very low chances of being lost at a later stage. It has been shown that the procedures of collection and transfer of embryos have no marked adverse effect on viability of embryos, and that the maternal capacity to support embryos, rather than intrinsic viability of embryos, is the major factor determining the success or failure of transfer (Moore, 1985). Because only a few ewes grazing Pawera received embryos, the effect on embryo survival could not be determined. In the present study the method used to check embryo survival was not successful for the Pawera group due to the low number of ovular ewes. Variability in superovulatory response in donors also played some role, as insufficient good quality embryos were available in one of the blocks in the first trial, but this was not a hindrance in trial 2 or in block 1 of trial 1. Had ovulation failure not been a problem, 'the method of fertilized egg transfer' might have produced a clearer picture regarding the effect of high formononetin consumption on embryo survival in ewes.

The results of this study suggest that G27 red clover, a low formononetin selection within cultivar Pawera, is safer than Pawera red clover when grazed for three weeks around oestrus. This short period of grazing G27 red clover resulted in an ovulation rate in ewes that was not different from the ewes grazed on non-oestrogenic pasture, and was significantly better than that on Pawera red clover. Embryo survival after transfer of fertilized eggs was also not different in G27 and Control ewes.

CHAPTER VII

Reproductive performance of ewes after grazing on G27 or Pawera red clovers

1. Abstract

In the first experiment, 150 ewes grazed either on a high oestrogenic red clover (Pawera), or a new, low oestrogenic selection of red clover (G27), or Control pastures before mating. The treatment groups (n = 25) and grazing lengths prior to mating were (1) Pawera, 6 weeks; (2) G27, 6 weeks; (3) G27, 12 weeks; (4) G27 / Ryegrass-white clover (Rg-wc), 6 weeks / 6 weeks; (5) Rg-wc (Control 1), 6 weeks, and (6) White clover (Control 2), 6 weeks. The ewes were mated as one group on Rg-wc pasture immediately after the grazing treatments. Formononetin concentration was significantly lower in G27 (0.26%) than in Pawera red clover (0.55%) ($P<0.05$). Ovulation rates in ewes after the first service were similar for all treatment groups ($P>0.05$). The incidence of return to service was significantly higher in Pawera ewes (72.7%) than in any of the other groups ($P<0.01$). The return rates for the other groups were 33.3% (G27/6 weeks), 25.0% (G27/12 weeks), 4.8% (G27/Rg-wc), 9.5% (Rg-wc) and 14.3% (White clover). Most ewes which were mated at the next two cycles became pregnant. The litter size was higher for Pawera group ewes, as a high percentage of returning ewes delivered multiple lambs, but the differences were not significant between different groups. It was concluded that the fertility in the ewes grazing G27 red clover was better than that in ewes grazing Pawera red clover after the first cycle of mating close to feeding treatments, however, there may have been some impairment in contrast to animals grazing Control pasture.

A second experiment was conducted to study if the temporary infertility in ewes induced by oestrogenic clover, would recover accompanied by a concomitant increase in ovulation rate and/or litter size following their removal to a 'safe' pasture. Romney ewes (N = 155) either grazed on Pawera red clover or Ryegrass-white clover (Control) pasture for four weeks. Afterwards all ewes grazed Ryegrass-white clover pasture and were mated in 3 groups either at the first, third or sixth week post-treatment. Mean ovulation rate was significantly lower ($P<0.05$) in the Pawera ewes (1.22 ± 0.06) than in the Controls (1.49 ± 0.07) after two weeks of grazing on respective treatments. Ovulation rates in ewes after the 1st, 2nd and 3rd mating cycle were 1.60 ± 0.12, 1.41 ± 0.10, 1.27 ± 0.09 (red clover), and 1.64 ± 0.10, 1.67 ± 0.12, 1.30 ± 0.09 (Control)

respectively. Ovulation rate and litter size were similar for the two treatment groups after joining in week 1, 3 or 6 post-treatment (P>0.05). The results showed that temporary infertility in ewes, induced by oestrogenic clover, did not recover accompanied by a concomitant increase in ovulation rate and/or litter size following their removal to a 'safe' pasture.

2. Experiment 1

2.1. Introduction

Plant oestrogens can cause a temporary or permanent infertility in sheep (Lightfoot, 1974). Ewes mated on, or mated after a short term grazing on an oestrogenic clover suffer from an impairment to fertility which resolves within a few weeks following removal to non- oestrogenic pasture (Morley *et al.*, 1966; Kelly *et al.*, 1980). Ewes grazed for several seasons on oestrogenic clover may suffer cumulative and therefore permanent infertility characterized by reduced fertilization but relatively normal ovulation rate (Adams 1990).

Temporary infertility is characterized by a lower ovulation rate, an increase in return to service, and a lower lambing rate (Morley *et al.*, 1966; Lightfoot and Wroth 1974; Kelly *et al.*, 1980; Shackell and Kelly, 1984). After removal from oestrogenic clover, ovulation rate appears to recover earlier whereas return to service takes longer to return to normal (Morley *et al.*, 1966; Kelly *et al.*, 1980).

Results of the study reported in Chapter VI showed that ovulation rate in ewes on low formononetin G27 red clover was significantly higher than that on Pawera red clover during three weeks of grazing around oestrus. A high proportion of ewes grazed on Pawera red clover failed to ovulate, but the number of anovular ewes was much lower in the G27 group. The objective of the present trial was to study the reproductive performance of ewes mated after 6 or 12 weeks grazing on G27 red clover, or 6 week grazing on Pawera red clover, or Control pastures. The specific objectives were to compare, (1) ovulation rates in ewes after removal from G27 or Pawera red clover, (2) returns to service in ewes mated after removal from the low and high formononetin red clover, (3) the recovery of ewe fertility from the deleterious effects (if any) of grazing on red clover, and (4) lambing performance of ewes after completion of the matings (for three heats).

2.2. Materials and methods

2.2.1. Animals

Mixed age Romney ewes, with a history of normal fertility, from a flock at Massey University were allocated to one of six treatment groups (n = 25) balanced for age and live weight. One half of the ewes were 2.5 years old while the remainder ranged from 4-6 years in age. Average live weight of the ewes at the start of the trial was 57.4 ± 0.6 kg. The ewes had no previous exposure to oestrogenic pasture.

2.2.2. Treatments

The trial was conducted in the breeding season of 1990. Treatment pastures and grazing periods before mating for various groups are shown in Table 7.1.

Table 7.1. Design of the trial to determine reproductive performance of ewes after various grazing treatments.

(N = 150; n = 25)

Treatment Group		Grazing period (weeks)
1	Pawera red clover	6
2	G27 red clover	6
3	G27 red clover	12
4	G27 / Ryegrass-white clover	6 / 6
5	Ryegrass-white clover (Rg-wc) (Control 1)	6
6	White clover (Control 2)	6

Ewes in groups 3 and 4 were put on G27 red clover in the first week of January. All the other ewes were kept on Ryegrass-white clover pasture during this time as one

mob. After 6 weeks of grazing on G27, group 4 ewes were moved to Ryegrass-white clover pasture. In the second week of February, the remainder of the ewes were also put on the respective grazing treatments and all ewes were drenched for parasite control. All the animals were shorn on 21st of February. Grazing finished on 23rd of March.

Live weight was monitored at weekly intervals during grazing treatments. An effort was made to keep the weight gain similar in all groups by restricting feed available to the heavier group/s, to minimize the effects of the weight change on ovulation rate.

Red clover samples were collected at regular intervals during the trial and were chemically assayed to determine formononetin concentration (Gosden and Jones, 1978). Pawera and G27 plots grazed in the trial contained 70-80% red clover as estimated by visual assessment. The plants were at the flowering stage at the time of grazing.

2.2.3. Matings

All ewes received intravaginal sponges impregnated with 40 mg medroxy progesterone acetate (MAP) for a period of two weeks from February 21, to synchronize oestrus. Harnessed teaser rams were used for heat detection which occurred about 9th March. Two days before the following natural heat, the ewes were run as one mob on Ryegrass-white clover pasture along with four harnessed, entire rams of proven fertility. Ovulation rate was recorded at laparoscopy 5 days after the first mating which occurred during the last week of March and the first week of April. Return to service was checked by regular change of crayons fixed to entire rams in the following two cycles. Pregnancy and the number of lambs per ewe (litter size) were determined by using real-time ultrasound seven weeks after the third time of mating (mating period = 44 days).

Due to the likelihood of facial eczema outbreak during early autumn which is common in the area, the ewes were drenched with zinc salts at biweekly intervals as a prophylactic measure (Smith *et al.*, 1977). The ewes were blood sampled at the start of the trial, at mating time and at the end of the trial. Serum gamma-glutamyltransferase (GGT) was measured to estimate any impairment of liver function (Towers and Stratton, 1978). There was an attack of the disease during the first mating cycle and

affected ewes were excluded from the trial.

2.2.4. Statistical analyses

Ovulation rate, return to service, and litter size data were analysed by the Chi square method. Formononetin concentrations in the two types of red clover were compared by t-test. Data are presented as mean ± SEM if not mentioned otherwise. All statistical analyses were performed using the Statistical Analysis System computer package (SAS Institute Inc., 1988).

2.3. RESULTS

2.3.1. Formononetin concentration in red clover

Mean formononetin concentration in Pawera (4 samples) and G27 red clover (10 samples) was 0.55 ± 0.08 and 0.26 ± 0.01 % (of dry matter) respectively. The concentration was significantly higher in Pawera than that in G27 red clover ($P<0.05$).

Table 7.2. Ovulation rate in ewes after grazing different red clover and Control pastures.

Treatment Group	Grazing period (weeks)	No. of ewes	Ovulation rate[1] (mean ± SEM)	No. (%) of ewes with ≥2 ovulations
Pawera	6	22	1.50 ± 0.13	10 (45.5)
G27	6	24	1.46 ± 0.10	11 (45.8)
G27	12	16	1.50 ± 0.16	7 (43.8)
G27 / Rg-wc	6 / 6	21	1.48 ± 0.13	9 (42.9)
Rg-wc (C1)[2]	6	21	1.43 ± 0.11	9 (42.9)
Wc (C2)[3]	6	21	1.62 ± 0.11	13 (61.9)

[1] Group means not significantly different ($P>0.05$).

[2] Ryegrass-white clover (control 1)

[3] White clover (control 2)

2.3.2. Ovulation rate

Details of ovulation rate in the ewes during the first cycle of mating are in Table 7.2. All the ewes included in the trial ovulated. Ewes in the White clover group had a higher mean ovulation rate due to slightly more ewes with multiple ovulations than those in the other groups, but the mean ovulation rates were not significantly different between the various treatment groups (P>0.05).

Table 7.3. Returns to service and conception pattern in the ewes a ter grazing different red clover and Control pastures.

Treatment Group	Grazing period (weeks)	No. of ewes	No. (and %) of ewes returning to 1st service	Number (and %) of ewes conceiving in		
				cycle 1	cycle 2	cycle 3
Pawera	6	22	16 (72.7)	6 (27.3)	14 (63.6)	1 (4.5)
G27	6	24	8 (33.3)	16 (66.7)	6 (25.0)	2 (8.3)
G27	12	16	4 (25.0)	11 (68.8)	3 (18.8)	1 (6.2)
G27 / Rg-wc	6 / 6	21	1 (4.8)	20 (95.2)	0 (0.0)	1 (4.8)
Rg-wc (C1)[1]	6	21	2 (9.5)	18 (85.7)	1 (4.8)	1 (4.8)
Wc (C2)[2]	6	21	3 (14.3)	18 (85.7)	2 (9.5)	1 (4.8)

[1] Ryegrass-white clover (control 1).

[2] White clover (control 2).

2.3.3. Returns to service and conception pattern

Return to service and conception pattern of ewes are presented in Table 7.3. A significantly higher percentage of ewes grazed on Pawera red clover returned to first service than did ewes in other groups (P<0.01). There was no difference in the fertility due to grazing G27 for 6 or 12 weeks. Return rates were greater in the ewes which grazed G27 until the end of treatment than in the animals which grazed Rg-wc or White clover (group 2 and 3, 12 of 40 ewes vs Controls, 5 of 42 ewes; P<0.05). A

majority of the ewes that returned to service after the first cycle, conceived in the following mating cycle (i.e. 14 out of 16 Pawera ewes, and 26 out of 34 ewes over all treatments). Three ewes were not pregnant after a third cycle (one each from the Pawera, G27/12 weeks, and Rg-wc group).

2.3.4. Litter size

Litter size (Table 7.4) after 44 days of matings was not different between various treatment groups (P>0.05). The ewes which grazed on G27 and then Ryegrass-white clover did not have many multiple births, somewhat different to that found in the Control groups or to those grazing G27 alone. The Pawera ewes had the highest mean litter size due to a higher percentage of ≥2 lambs (13 out of 22) compared to that in Controls (21 out of 42), and G27 groups (group 2 & 3, 19 out of 40). Triplet birth was the maximum number observed in two ewes both of them in the Pawera group.

Table 7.4. Litter size in ewes after completion of three mating cycles.

Treatment Group	Grazing period (weeks)	No. of ewes	Litter Size[1] (mean ± SEM)	Number (and %) of ewes with 0 lambs	1 lambs	≥2 lambs
Pawera	6	22	1.64 ± 0.15	1 (4.5)	8 (36.4)	13 (59.1)
G27	6	24	1.54 ± 0.10	0 (0.0)	11 (45.8)	13 (54.2)
G27	12	16	1.31 ± 0.15	1 (6.2)	9 (56.3)	6 (37.5)
G27 / Rg-wc	6 / 6	21	1.19 ± 0.09	0 (0.0)	17 (80.9)	4 (19.1)
Rg-wc (C1)[2]	6	21	1.38 ± 0.13	1 (4.8)	11 (52.4)	9 (42.8)
Wc (C2)[3]	6	21	1.57 ± 0.11	0 (0.0)	9 (42.9)	12 (57.1)

[1] Between treatment differences in litter size not significant (P>0.05).

[2] Ryegrass-white clover (control 1)

[3] White clover (control 2)

2.4. DISCUSSION

An important way to minimize phytoestrogen-related fertility problems in clovers is through the selection and breeding of cultivars low in formononetin. Monitoring the reproductive performance of ewes on them is necessary to establish their safety before they are released for commercial use. Pawera red clover has been shown to contain high concentrations of formononetin throughout the year, and this drastically reduced ovulation and conception rate in ewes joined while grazing it (Kelly *et al.*, 1979; Kelly *et al.*, 1980). G27 red clover contained less than half the amount of formononetin in Pawera. In the present trial the ewes that grazed G27 red clover showed a significantly lower rate of return to service than the Pawera ewes. There were more returns to service in the G27 groups compared to those in the Controls but the size of the difference was less than that after grazing on Pawera clover. More returns to service on G27 than the Controls indicate that the low formononetin concentration of 0.26%, as noted in G27 red clover in the present trial, may not be completely safe for fertility if the ewes are mated on or shortly after grazing the G27 red clover-dominant pasture. In a comparison of the effect of selected red clover cultivars, Moseley *et al.*, (1984) noted that even a low formononetin concentration of 0.21% was able to cause oestrogenic effects on uterine tissue, and pituitary release of LH in ewes. They concluded that, in order to avoid these effects through breeding new varieties of red clover, the formononetin concentration must be below 0.2%. Data from long term grazing with three strains of subterranean clover also suggested that the lowest possible levels of formononetin should be sought in clover breeding programmes (Adams 1987a). As red clover is usually not a major part of the sward in the areas of New Zealand where it is grown for sheep grazing (Jagusch, 1983), the formononetin concentration in this new cultivar is expected to be further lowered after dilution with non-oestrogenic pasture. Combinations of G27 red clover with non-oestrogenic pasture in various ratios for sheep grazing need to be studied to determine effective and safe grazing management.

Although the deleterious effects of six weeks of grazing on Pawera red clover apparently disappeared by the second cycle in this trial, as most of the returning ewes conceived in the very next cycle, a significantly higher percentage of Pawera group ewes potentially had later lambing dates and consequently the group had a greater spread of lambing compared to the ewes that had grazed G27 red clover for 6 or even 12 weeks. Increased returns to service in ewes grazed on oestrogenic clover might be due to a decreased egg fertilization rate as observed by Lightfoot and Wroth (1974).

They noted that the difference between oestrogenic clover and non-oestrogenic Control treatments in fertilization rate were associated with fewer spermatozoa on the zona pellucida of the eggs recovered from the ewes grazed on oestrogenic clover. In the sperm transport trial described in Chapter V (experiment 2) where ewes were mated after 4 weeks of grazing on red clover or Control pasture, no spermatozoa were recovered from the Fallopian tubes of a higher percentage of Pawera ewes 24 h after mating than of G27 or Control animals, although due to a high variability in the number of spermatozoa recovered, no statistical differences could be found in mean values.

The litter size was higher for the Pawera ewes, as a high percentage of returning ewes delivered multiple lambs, but the differences were not significant between the different groups. The higher litter size in Pawera ewes might be due to seasonal variation as a majority of the Pawera ewes conceived in a different cycle. Within season variation in ovulation rate has been shown in several studies (e.g. McDonald and Ch'ang, 1966). Another possibility is that it was an after-effect of the short term grazing on oestrogenic pasture. An elevated ovulation rate has been reported in ewes after removal from oestrogenic pasture which they had grazed for three years, and were permanently infertile (Adams *et al.*, 1979). Is the gain in fecundity after a short term grazing on Pawera repeatable? This question was addressed in another experiment. The results of the investigation into the residual effect of grazing Pawera red clover on reproductive performance of ewes are presented later in this Chapter.

The mean ovulation rates (checked after the grazing treatments finished) were not different in various red clover and Control groups. Although ovulation rates in the present trial were not checked while the ewes were grazing on the different treatment pastures, results of another study (Chapter VI) showed a significantly higher ovulation rate in the ewes grazing on G27 red clover than those grazing Pawera red clover. It has been noted that ovulation rates in ewes recover shortly after removal from oestrogenic clover (Morley *et al.*, 1966; Kelly *et al.*, 1980).

In the present experiment the reproductive performance of ewes that had grazed the low formononetin G27 red clover, was better than that of the ewes that grazed the high formononetin Pawera red clover because of fewer returns to service and thus earlier mean lambing date.

3. Experiment 2

Residual effect of a four weeks grazing of Pawera red clover on reproductive performance of ewes in the following six weeks

3.1. Introduction

In the previous trial where the ewes were mated on non-oestrogenic pasture immediately after six weeks of grazing Pawera, G27 red clover, or Control pastures, a significantly higher percentage of the Pawera ewes returned to service. Fertility in the Pawera ewes recovered after one cycle. The litter size after 34 days of mating, although not significantly different, was found to be higher in the Pawera group compared to the G27 or Ryegrass-white clover Control ewes (Table 7.4). It was speculated that the higher litter size might be a part of the phenomenon of recovery from phytoestrogen induced infertility, and that a continuous presence of high levels of phytoestrogenic substance in the blood for a few weeks might interfere with the control mechanism of oestradiol over the hypothalamus and in turn give rise to a higher ovulation rate. In that trial a higher number of ewes from the Control and G27 groups conceived during the first cycle compared to a low percentage of ewes from the Pawera group. It is also possible that the variation in the conception time might have contributed to the higher ovulation and lambing rate in Pawera ewes (McDonald and Ch'ang, 1966).

In permanent phytoestrogenic infertility, where ovulation rate remains relatively normal, there is some evidence of an increased ovulation rate in Merino ewes which had grazed oestrogenic pasture for three years (Adams *et al.*, 1979). The difference was reported to be statistically significant at the peak of the breeding season.

The present trial was conducted to determine whether the ewes recover with a concomitant increase in ovulation and lambing rate after short term grazing on oestrogenic pasture. The residual effect on reproductive performance of ewes following grazing highly oestrogenic Pawera red clover (for four weeks) was studied in the subsequent 3 cycles and compared with ewes that had grazed non-oestrogenic pasture.

3.2. Materials and methods

3.2.1. Animals and grazing treatments

Non-pregnant Romney ewes (N = 155) drawn from the same flock and with a history of normal fertility were used during the 1992 mating season (March - June). The ewes were 6-7 years old and had no previous exposure to oestrogenic clover. They were divided into two groups with similar average weight during the third week of March and then grazed either Pawera red clover (R) or Ryegrass-white clover (Control-C) pastures. After 28 days of this grazing treatment, the ewes on red clover were transferred to the Ryegrass-white clover pasture. At this stage, the ewes in each group were divided into three subgroups, identified as R1, R2 and R3, or C1, C2 and C3 in the red clover and Control treatments respectively. The subgroups were paired between treatments and grazed on non-oestrogenic pasture in three mobs. Ewes were weighed at start of the trial, at the end of 28 days grazing, and three weeks after the end of the treatment period.

The Pawera red clover was a fresh regrowth containing more than 70% red clover. Herbage was sampled at the start and middle of the grazing period for formononetin assay.

3.2.2. Ovulation rate and mating of ewes

Intravaginal progestagen sponges (each containing 40 mg medroxy progesterone acetate) were placed in the ewes at the start of the grazing treatments for a period of 12 days. Ovulation rate in all the ewes was recorded by laparoscopy 6 days after induced heat. The subgroups within each treatment were balanced for ovulation rate as determined at laparoscopy. The first post-treatment oestrus in ewes occurred within one week after removal from treatment. Mobs 1, 2 and 3 were joined with three entire rams in weeks 1, 3 and 6 post-treatment respectively. Two-tooth Romney rams were used for the first mating period but they were replaced by three mature Border Leicester rams for the second and third mating periods. Ovulation rate in each mob was checked 6 days after mating. Mean ovulation rates were compared between the two treatment groups after induced heat and then between the treatment subgroups in the same mating mob.

3.2.3. Litter size

Ewes in each subgroup were slaughtered 27 to 36 days after mating. Reproductive organs were collected, tagged and taken to the laboratory where pregnancy status of each ewe and number of developing embryos (expressed as litter size) were determined (as described in Chapter VI). Conception rate and litter size were compared between the treatment subgroups that were mated as one mob.

3.2.4. Statistical analyses

Ovulation rate, conception rate and litter size between treatment groups were compared by the Chi square method. Differences in live weight gain between treatments were compared by t-test. The data are presented as mean ± SEM. All statistical analyses were performed using the Statistical Analysis System computer package (SAS Institute Inc., 1988).

3.3. Results

The mean formononetin concentration present in Pawera clover was 1.00 ± 0.11 percent on a dry weight basis.

TABLE 7.5. Live weight change (kg) in ewes grazing Pawera red clover or Control pastures.

Observation time relevant to treatment	Red clover ewes (n=78)	Control ewes (n=77)	Significance
Start	53.3 ± 0.5	53.1 ± 0.6	NS
At end	59.9 ± 0.6	55.2 ± 0.6	P<0.01
3 weeks post-treatment	61.1 ± 0.6	56.5 ± 0.7	P<0.01

TABLE 7.6. Effect of grazing Pawera red clover or Control (ryegrass-white clover) pastures, on ovulation rate (OR) in ewes.

Time of observation	Treatment group					
	Red clover			Control		
	No. of ewes	OR mean ± SEM	Ewes with ≥2 CLs (%)	No. of ewes	OR mean ± SEM	Ewes with ≥2 CLs (%)
3rd week of treatment	73[1]	1.22 ± 0.06[a]	19 (26)	74[2]	1.49 ± 0.07[b]	37 (50)
1st week post-treatment	25	1.60 ± 0.12[a]	14 (56)	25	1.64 ± 0.10[a]	16 (64)
3rd week post-treatment	27	1.41 ± 0.10[a]	11 (41)	24[3]	1.67 ± 0.12[a]	15 (63)
6th week post-treatment	26	1.27 ± 0.09[a]	7 (27)	27	1.30 ± 0.09[a]	8 (30)

Means in same row with different superscript letters are significantly different ($P<0.05$).

[1] 5 ewes lost sponges.

[2] 3 ewes lost sponges.

[3] 1 ewe died.

3.3.1. Live weights

Mean live weights of ewes in the two treatment groups were not different at the start of the trial (Table 7.5). Both the groups gained weight during 28 days of grazing treatment. Ewes that grazed Pawera red clover gained significantly more weight than the ewes that grazed Control pasture ($P<0.01$). Both the groups continued to gain weight until three weeks after treatment.

3.3.2. Ovulation rate

Ovulation rate in the ewes is shown in Table 7.6. Mean ovulation rate was significantly higher in Control ewes (due to a higher percentage of multiple ovulating ewes) than that in Pawera ewes after the induced heat ($P<0.05$). Ewes at this time were grazing the two different pastures. Mean ovulation rates were not different in the two treatment subgroups at the first post-treatment heat. The differences in mean ovulation rate remained non significant between the two treatments at week three (second cycle) and week six (third cycle) after treatment ($P>0.05$).

3.3.3. Conception rate

Conception rate and litter size data are summarized in Table 7.7. The conception rate was lower in Pawera ewes (48% - subgroup R1) than that in Control ewes (64% - subgroup C1) after joining in week one, but the difference was not significant ($P>0.05$). Conception rates were not different between the two treatment sub-groups after joining in week three and week six after treatment.

3.3.4. Litter size

Mean litter sizes after joining in weeks 1, 3, and 6 post-treatment were consistently lower in Pawera than in Control ewes but the differences were not significant ($P>0.05$) (Table 7.7). Lower litter sizes in Pawera ewes were due to a lower percentage of ewes with multiple (≥ 2) lambs. Almost a similar rise and fall in mean litter size was observed in ewes from the two treatments during the second and third mating periods.

TABLE 7.7. Residual effect of grazing Pawera red clover or Control pastures on conception rate (CR) and litter size in ewes mated on non-oestrogenic pasture.

	Treatment group*							
	Red clover				Control			
Joining time after treatment	n	CR (%)	Litter Size mean ± SEM	Ewes with ≥2 lambs(%)	n	CR (%)	Litter Size mean ± SEM	Ewes with ≥2 lambs(%)
1st week	25	48	0.68 ± 0.16	5 (20)	25	64	0.92 ± 0.16	7 (28)
3rd week	26	88	1.15 ± 0.12	7 (27)	24	88	1.38 ± 0.16	11 (46)
6th week	26	85	0.92 ± 0.09	2 (8)	27	89	1.11 ± 0.11	6 (22)

* All between treatment differences are not significant.

3.4. Discussion

Mean ovulation rate in the ewes grazing oestrogenic Pawera red clover was significantly lower than in those grazing non-oestrogenic Control pasture due to differences in multiple ovulations. A significantly lower ovulation rate was observed in ewes grazed on Pawera red clover in another experiment due to a large number of anovular ewes (Chapter VI). It shows that oestrogenic Pawera red clover can decrease ovulation rate in ewes by completely blocking the ovulation and also by a decrease in multiple ovulations. This finding is in agreement with Kelly *et al.,* (1980).

Ovulation rates were not different between Pawera and Control groups within one week of ewes grazing red clover being moved to non-oestrogenic pasture, and were not different at 3 and 6 weeks post-treatment. In addition, the first experiment reported in this Chapter showed no differences in ovulation rates of ewes one week after grazing on Pawera, G27 red clover, or Control pastures. This indicates that ovulation (and ovulation rate) is probably influenced only by the high phytoestrogen level in circulation, and that it may recover within one week after 4-6 weeks of grazing on highly oestrogenic Pawera red clover. Morley *et al.,* (1966) also suggested that the deleterious effects of red clover grazing on multiple ovulation might be due to the action of oestrogens directly on the ovary. It has been shown that isoflavones in oestrogenic clover ingested by sheep are metabolized in the rumen quite rapidly (Shutt *et al.,* 1970). About 90% of the formononetin ingested is metabolized in 1.5 h predominantly to form equol in the sheep rumen which in turn is absorbed into blood in a mean time of 1.7 h. Equol is considered to be the oestrogen responsible for infertility in ewes (Shutt *et al.,* 1968). Conjugation detoxifies plant oestrogens, and only a very small percentage of equol is present in a biologically active form in sheep grazing on high formononetin clover (Shutt *et al.,* 1970; Lundh *et al.,* 1990). Davies and Hill (1989) have shown that 52% of the equol produced in ewes given intrarumenal tritiated formononetin was excreted after 24 hours, and most of it (81%) was excreted in 48 hours. So conjugation plus a rapid excretion of equol may relieve the animal body from the deleterious effect being exerted on ovulation/rate control mechanism within a couple of days after removal from oestrogenic clover resulting in a rapid recovery of ovulation/rate.

Ewes grazed on Pawera red clover did not have an ovulation rate higher than that in the Control ewes at any stage of the trial, despite a higher live weight gain during

treatment. Rather the mean ovulation rate in the red clover ewes was consistently lower than that in the Control ewes during the three mating cycles. Similarly, mean litter size was consistently lower in the red clover group than that in the Control group although the differences were not statistically significant. A rise in the mean litter size was observed in both treatments for ewes mated during the second cycle post-treatment. This may be due to different rams (possibly with better fertility) used in the second mating period.

The assumption that recovery from temporary phytoestrogenic infertility might accompany a rise in ovulation rate and/or litter size was not proved. There is evidence of a higher ovulation rate in ewes suffering from permanent infertility due to a long term grazing (for several seasons) on oestrogenic clover (Adams *et al.*, 1979). It has been shown that ewes suffering from permanent infertility are less able to elicit an increase in LH after treatment with oestrogen due to long term interference with the hypothalamic control mechanism (Findlay *et al.*, 1973; Adams and Martin 1983), and it may account for a higher number of ovarian follicles developing to meet the increased threshold for oestrogen in the hypothalamus (Adams *et al.*, 1979). In a normal oestrous cycle, at luteolysis, the secretion of oestradiol begins to increase in response to an increasing frequency of LH pulses, culminating in a peak of oestradiol secretion that triggers the preovulatory surge of GnRH and the gonadotrophins (Scaramuzzi *et al.*, (1993). Endogenous oestrogens largely come from the one or two biggest follicles on the ovaries. Following administration of exogenous oestrogen a large release of LH has been shown to occur in normal ewes (Goding *et al.*, 1969; Scaramuzzi *et al.*, 1971). Bolt (1981) showed that exogenous oestradiol tended to suppress the concentrations of both FSH and LH followed by an oestrous-like peak in both gonadotrophins. Hearnshaw *et al.*, (1977) examined the hypothesis that the ingestion of oestrogenic clover for a few days by ovariectomized ewes might result in a release of LH, and further that this release might interfere with the large release of LH normally observed when ovariectomized ewes were treated with oestradiol-17β. They found that the ingestion of oestrogenic clover can evoke increase in LH secretion in ovariectomized ewes. Moreover, a normal surge of LH was observed following oestradiol administration indicating that the phytoestrogens did not interfere with the mechanism responsible for this LH release after 7 days of grazing and therefore probably did not cause any saturation or refractoriness of hypothalamic receptor sites. However, the hypothalamo-pituitary axis might have become refractory if the ewes had continued to ingest the oestrogenic clover for a greater length of time before being

sampled for LH. The hypothalamic response of ewes that grazed oestrogenic red clover for 28 days in the present trial, may fall into the same category as described in the latter study. Thus any residual oestrogenic effect in terms of ovulation rate may be lacking and the hypothalamus may maintain a normal threshold for oestrogen after such a short exposure (28 days) to the clover.

However, other possibilities to explain the higher lambing rate observed in Pawera red clover ewes in experiment 1 of this Chapter could include the later time when mating and conception occurred. Ovulation rate and lambing rate can vary with successive heat periods (McDonald and Ch'ang, 1966; Quinlivan and Martin, 1971). It is concluded that recovery of fertility after a short term grazing on oestrogenic clover might not result in an above normal ovulation/litter size in ewes.

CHAPTER VIII

General discussion and conclusions

The main objective of the present study was to determine the reduction in the oestrogenic effects brought about by lowering the formononetin content in 'G27' red clover, a 6th generation selection within the highly productive and persistent, but highly oestrogenic 'Pawera' red clover. The effect of the selection was measured, both, in various parts of the plant at different maturity stages when grown under field conditions, and in ewes grazed on the clover around mating time. Comparison was made not only of the reproductive performance of the ewes grazed on the two types of red clover, but also of some of the basic mechanisms controlling fertility.

In 1974 Lightfoot reviewed the recommendations proposed for the control of infertility problems in ewes due to grazing on oestrogenic clover. Among these recommendations, which are as appropriate today as when promulgated (Davies, 1987, Croker *et al.*, 1989), the methods involving changes in pasture are the more important, and they are relevant to both permanent and temporary types of infertility. They include (i) the use of clovers low in phytoestrogens; (ii) dilution of oestrogenic cultivars in the pastures; and (iii) optimum fertilizer use, especially adequate phosphate applications to the pasture. These methods are aimed at reducing the quantity of phytoestrogens produced and, therefore, ingested by the animals. The use of low oestrogenic cultivars is the most important recommendation to follow. Formononetin has been found to be the major isoflavone present in subterranean clover and red clover with regard to oestrogenic effects and infertility in ewes (Millington *et al.*, 1964; Morley *et al.*, 1968; Davies *et al.*, 1970). Development of techniques for rapid determination of formononetin made it easier to carry out programmes for breeding of low formononetin strains of clover where the desired types were not available. Breeding for low formononetin may bring about a permanent change in the cultivar produced and make it safe for extensive grazing by all stock.

A formononetin concentration lower than 0.3% of the dry matter was achieved in the leaflets of G27 which is almost 25% of the concentration in the original material. The formononetin level of <0.3% in grazing material was suggested to be safe when ewes were grazed for several years on oestrogenic clover (Marshall, 1973). The reductions achieved in petioles and other parts of the G27 plant were not proportional to that

noted in the leaflets. So the overall formononetin concentration of the G27 plants at the flowering stage was 50% of the concentration in Pawera plants. Knowledge of the relative potency of the botanical fraction is important in the determination of optimum use of oestrogenically active forages (Francis and Millington, 1965); observation has shown that sheep consume, initially at least, mainly leaf blade. Since the formononetin concentration in petioles is higher than the concentration in the leaflets of G27 (a result of the selection programme), the best animal management on G27 clover may be light to moderate grazing (Williams, 1988) if pure swards of red clover are used for feeding to sheep.

Formononetin is not oestrogenic itself to sheep, but it is metabolized, mainly, to equol in the rumen, and equol is oestrogenic (Shutt and Braden, 1968). Blood equol concentration in sheep grazing G27 red clover-dominant paddocks was two to four times lower than that in sheep grazed on Pawera red clover in trials conducted in two different years. It shows that formononetin in the high and low oestrogenic red clovers is metabolized to equol in almost similar proportions as formononetin is present in the plant. While the determination of formononetin in the plants is easier and more practical, blood equol level is a measure of the formononetin intake by the sheep. In addition to equol another oestrogenic metabolite of formononetin, 4'-methyl equol may be present in appreciable amounts in some conditions (Cox and Braden, 1974b; Nottle and Beck, 1974). The equol level measured in the present study included both conjugated plus free fraction. Only the free fraction of equol is biologically active (Shutt *et al.,* 1970). It remains to be determined if sheep have a better ability to conjugate more of a lower level of equol produced while grazing on G27 red clover.

The main aim of breeding the low formononetin G27 red clover, of course, was to minimize the reproductive disturbances in ewes grazed on this cultivar. One of the major effects occurring in ewes grazed on oestrogenic clover around oestrus is a low ovulation rate (Lightfoot and Wroth, 1974; Kelly *et al.,* 1980). The mean ovulation rate in ewes grazed on G27 red clover for three weeks near to oestrus was not different from that in Control ewes, and it was significantly higher than that in ewes grazed on Pawera red clover (Chapter VI). The pattern of follicle growth in the ovaries in ewes grazed on G27 also showed that the low formononetin red clover selection resulted in a higher follicle number than in ewes grazed on high formononetin Pawera red clover. While follicular atresia was observed in some of the large follicles in Pawera ewes, all the large follicles in G27 ewes were healthy like those in Control animals. The number

of follicles increased in ewes grazed on G27 or Pawera red clover during a 48 h period of removal from red clover but the follicle number remained uniform in Control animals. This suggested that the phytoestrogens in circulation might have suppressed follicle development in red clover ewes (Chapter IV). Study of ovarian activity (Chapter VI) showed that a significantly higher percentage of Pawera ewes failed to ovulate compared to the G27 and Control ewes.

One of the most prominent features of temporary infertility that occurs in ewes grazing oestrogenic clover around mating, is an increased incidence of returns to service (Kelly *et al.*, 1980). A significantly lower percentage of ewes in the G27 group returned to service than that in the Pawera group when mated after 6 or 12 weeks grazing on red clover (Chapter VII, experiment 1). Although the return rate in the G27 ewes was higher than that in Control ewes, a previous study showed no differences between performance of ewes when mated on G27 or non-oestrogenic pasture (Williams, 1988). Another study conducted in 1989 showed a markedly higher conception rate in ewes mated on G27 red clover than the control ewes (R.G. Keogh and M.F. McDonald, personal communication). The present study showed that although formononetin concentration in G27 red clover has been decreased significantly (Chapter III), it may affect reproductive performance of ewes compared with Control animals but these oestrogenic effects are not consistent between years. Other subtle attributes associated with G27 in comparison to Ryegrass-white clover may account for some of the variation.

Disturbed sperm transport through the reproductive tract of ewes might be a cause of low fertility in ewes mated on oestrogenic clover. In the present study no statistical differences occurred in the mean number of spermatozoa recovered from the Fallopian tubes and other parts of the tract 24 h after natural service, but the ewes on G27 red clover showed higher sperm numbers than those in Pawera or Control groups (Chapter V, experiment 2). Lack of spermatozoa in a higher percentage of ewes grazed on Pawera red clover suggested that high formononetin clover might impair sperm transport in the reproductive tract as noted by Lightfoot and Wroth (1974). Although differences were not statistically significant, the tendency was for a greater number of spermatozoa to be recovered from G27 than from Control or Pawera animals. The reasons for the apparent inconsistency in results obtained from year to year on G27 are not known. Possible factors which may exaggerate the oestrogenic effects may include the levels of 'free' equol, or the presence of the fungal oestrogen, zearalenone.

Although zearalenone was not measured in the red clovers in this study, levels of 1-4 ppm are to be found in G27 in the autumn (R.G. Keogh, personal communication). Levels in excess of 1 ppm are known to affect fertility (Smith *et al,* 1987).

A clear picture of sperm transport could not be obtained in the present study due to great variation in the number of sperm recovered from individual ewes (Chapter V, particularly experiment 1). More reliable results from such a study may be obtained by mating/inseminating ewes at a uniform interval from the start of oestrus, use of a uniform quality of semen, development of improved techniques for recovery and counting of spermatozoa in different parts of the reproductive tract, and use of a larger number of animals, provided appropriate sperm counting techniques can be utilized. A simple technique was tried in the present investigation (Chapter V, experiment 2) to concentrate the spermatozoa in the uterine and oviductal flushings before counting. The technique allowed the spermatozoa in a 5 ml aliquot of fluid to settle down in a glass tube. The supernatant was discarded and the spermatozoa were counted in the 0.5 ml concentrate. The method was found to be more effective than counting after centrifugation, or counting the fluid without concentration.

Embryo survival in ewes on G27 red clover was not different from that in ewes on Control pasture after transfer of eggs on day 5-6 of the oestrous cycle. It has been observed that most of the embryonic mortality in sheep occurs in the period immediately preceding the 18th day after mating (Quinlivan *et al.,* 1966) during either blastulation or extension of embryonic membranes (Wathes, 1992). Due to a very low number of ovulatory ewes in the Pawera group, it could not be determined if a high formononetin clover would induce additional embryo loss (Chapter VI). The ovulation rate recovers in ewes within a few days after removal from oestrogenic pasture, but fertility takes a few weeks to become normal (Chapter VIII). So the egg transfer technique may be utilized to study embryo survival in ewes after removing them from oestrogenic clover. The technique of egg transfer used to study embryo survival in the present study did overcome the potential fertilization problem and it gave control over the number of embryos present, but it would not take into account the quality of ova released by ewes which grazed on oestrogenic clover, and also egg survival before the day of transfer (usually day 5 or later). Separate studies may be needed to get this information. The present study at least showed that grazing on a low formononetin clover, like G27, did not increase the embryonic loss in ewes.

In some cases the performance of ewes grazed on G27-dominant paddocks around oestrus was not comparable to that of ewes on Control pasture. This suggests that pure swards of G27 might not be completely safe for sheep if grazed around mating. As red clover in New Zealand is usually grown in association with non-oestrogenic grasses for animal grazing, a slightly higher than the recommended safe level of formononetin concentration in the plants is not expected to pose fertility problems, if any, in breeding ewes. In future studies, the reproductive performance of ewes on G27 red clover should be investigated using larger numbers of ewes grazed on different combinations of G27 red clover and non-oestrogenic grass. Studies should also be conducted to monitor the effects of long term grazing of G27 red clover on ewe reproduction as small effects may become apparent after prolonged grazing or exposure to low levels of plant oestrogen.

The major control programme for clover infertility has been to produce and disseminate cultivars of clover with a low content of formononetin. In New Zealand the selection of G27 red clover used in these experiments was based on lowering the formononetin content of leaflets and is a step in this direction. It is interesting to speculate on what outcome may have been achieved if different selection criteria had been used from the outset. Would there have been differential reduction in formononetin concentration between leaflets and other parts of the red clover plant? or would the selection process have been shortened? Would greater progress towards the aim of reducing formononetin levels have been made? The scope to further reduce formononetin levels in leaflets is limited and it would seem desirable that whole plant analysis of formononetin would be a more appropriate selection criterion. It is noteworthy that this approach has been used in subsequent selections (W. Rumball, personal communication).

A further point of importance regarding the selection of plants on the basis of lowering the concentration of individual isoflavones relates to the ecological stability of plants. Is red clover which has a low formononetin concentration more susceptible to pests and diseases? and if so what consequences are likely to occur to the productivity and persistence of such red clovers? If palatability is increased what will this mean in terms of the intensity of defoliation by stock and the rate of regrowth of the clover, particularly in mixed pasture associations. Perhaps future selections should take account not just of a narrow outcome in terms of lowering the formononetin content to reduce oestrogenicity, but also, concomitantly, to increase levels of other secondary

plant compounds to improve the ecological stability.

The follicle growth and ovulation rate in ewes on G27 red clover were not different from the Control ewes, and were better than those on Pawera red clover. The performance of ewes after grazing the low formononetin G27 red clover, was better than that of the ewes that grazed the high formononetin Pawera red clover because of fewer returns to service and thus an earlier mean lambing date. Sperm transport in the reproductive tract, and embryo survival in ewes after transfer of fertilized eggs was also not different in G27 and Control treatments. A lower formononetin concentration, and a related, significantly improved reproductive performance of ewes shows that the oestrogenicity of this version of G27 red clover has been reduced to a great extent compared to the Pawera red clover from which the selection was made. Developments in plant selection methods should lead to even greater improvements in reproductive performance.

Appendix 1

Formononetin content of developing leaves of G27 and Pawera red clovers during summer

Appendix Table 1.1. Mean formononetin concentrations in vegetative leaflets and petioles from leaf emergence to senescence in G27 and Pawera red clovers during summer.

Age of leaf (days)	Formononetin (% DM)			
	G27		Pawera	
	Leaflet	Petiole	Leaflet	Petiole
1	0.25 ± 0.01^{hi}	1.40 ± 0.12^{c}	1.56 ± 0.09^{bc}	2.11 ± 0.05^{a}
6	0.30 ± 0.02^{hi}	1.08 ± 0.04^{d}	1.53 ± 0.08^{bc}	1.66 ± 0.14^{b}
13	0.21 ± 0.04^{i}	0.66 ± 0.04^{ef}	0.99 ± 0.05^{d}	1.05 ± 0.05^{d}
20	0.16 ± 0.01^{i}	0.52 ± 0.03^{fg}	0.69 ± 0.04^{ef}	0.73 ± 0.06^{e}
27	0.16 ± 0.01^{i}	0.53 ± 0.04^{fg}	0.69 ± 0.05^{ef}	0.68 ± 0.05^{ef}
34	0.13 ± 0.02^{i}	0.42 ± 0.04^{gh}	0.70 ± 0.05^{ef}	0.77 ± 0.03^{e}

Means with different superscripts differ significantly ($P<0.05$).

Appendix Table 1.2. Mean formononetin concentrations in whole leaf (leaflet + petiole) during development.

Age of leaf (days)	Formononetin (% DM)	
	G27	Pawera
1	0.49 ± 0.02^{d}	1.67 ± 0.08^{a}
6	0.52 ± 0.03^{d}	1.57 ± 0.04^{a}
13	0.35 ± 0.03^{e}	1.01 ± 0.03^{b}
20	0.29 ± 0.01^{e}	0.70 ± 0.04^{c}
27	0.32 ± 0.02^{e}	0.68 ± 0.05^{c}
34	0.25 ± 0.02^{e}	0.73 ± 0.03^{c}

Means with different superscript letters differ significantly ($P<0.05$).

Appendix Table 1.3. Dry matter weight and formononetin yield per leaflet and per petiole from leaf emergence to senescence during summer.

Age	Dry Weight (mg)		Formononetin yield (mg)	
(days)	G27	Pawera	G27	Pawera
Leaflet				
1	12.63 ± 0.47^{f}	19.12 ± 1.20^{f}	0.03 ± 0.00^{c}	0.30 ± 0.04^{b}
6	20.87 ± 1.12^{ef}	27.87 ± 0.94^{de}	0.06 ± 0.00^{c}	0.42 ± 0.01^{a}
13	28.50 ± 1.19^{de}	42.00 ± 2.48^{bc}	0.06 ± 0.01^{c}	0.42 ± 0.04^{a}
20	35.75 ± 2.95^{cd}	51.00 ± 3.00^{a}	0.06 ± 0.00^{c}	0.35 ± 0.02^{b}
27	31.00 ± 5.29^{d}	50.25 ± 4.64^{ab}	0.05 ± 0.01^{c}	0.34 ± 0.03^{b}
34	29.00 ± 2.04^{de}	42.00 ± 3.76^{bc}	0.04 ± 0.01^{c}	0.30 ± 0.04^{b}
Petiole				
1	3.37 ± 0.13^{g}	4.87 ± 0.43^{fg}	0.05 ± 0.01^{d}	0.10 ± 0.01^{cd}
6	8.50 ± 0.46^{efg}	9.63 ± 0.52^{ef}	0.09 ± 0.01^{cd}	0.16 ± 0.02^{b}
13	13.50 ± 0.29^{e}	21.25 ± 2.56^{d}	0.09 ± 0.01^{cd}	0.22 ± 0.02^{a}
20	19.50 ± 1.32^{d}	33.00 ± 1.08^{ab}	0.10 ± 0.01^{cd}	0.24 ± 0.02^{a}
27	24.75 ± 5.02^{cd}	37.25 ± 2.21^{a}	0.14 ± 0.03^{bc}	0.25 ± 0.02^{a}
34	21.25 ± 1.25^{d}	29.25 ± 2.46^{bc}	0.09 ± 0.01^{cd}	0.23 ± 0.03^{a}

Means with different superscripts within same parameter and same part differ significantly ($P<0.05$).

Appendix 2

Distribution of formononetin in red clover shoots at flowering stage

Appendix Table 2.1. Distributions (percent) of formononetin per component in red clover shoots at early flowering stage.

Part of the plant	% Formononetin yield	
	G27	Pawera
Flower and bract		
Flower	1.26	1.00
Fl.Bract	0.16	0.56
Subtotal	1.42	1.56
Leaves		
Leaf1	0.78	4.32
Leaf2	1.73	7.02
Leaf3	2.51	6.02
Leaf4	2.04	1.69
Leaf5	1.10	0.31
R.Leaves	0.78	0.25
Subtotal	8.94	19.61
Axillary shoots		
Ax.sh.2	0.78	5.58
Ax.sh.3	4.24	14.85
Ax.sh.4	5.34	10.15
Ax.sh.5	9.89	2.82
R.Ax.sh.	9.42	2.76
Subtotal	29.67	36.16
Internodes		
IN1	0.63	2.44
IN2	4.39	8.40
IN3	7.22	9.27
IN4	9.11	5.70
IN5	5.18	2.96
INr	33.44	13.91
Subtotal	59.97	42.68

Appendix Table 2.2. Distributions (percent) of formononetin per component in red clover shoots at late flowering stage.

Part of the plan	% Formononetin yield	
	G27	Pawera
Flower and bract		
Flower	5.19	3.18
Fl.Bract	1.56	4.07
Subtotal	6.75	7.25
Leaves		
Leaflet1	2.34	6.11
Leaflet2	2.34	6.36
Leaflet3	3.12	5.22
Leaflet4	3.38	2.42
R.Leaflets	2.08	2.16
Petiole2	1.30	1.27
Petiole3	2.08	1.40
Petiole4	3.90	1.15
R. Pet.	5.19	1.40
Subtotal	25.73	27.49
Axillary shoots		
Ax.sh.2	1.30	2.93
Ax.sh.3	5.45	10.05
Ax.sh.4	6.75	8.52
Ax.sh.5	4.15	3.94
R.Ax.sh.	1.30	3.94
Subtotal	18.95	29.38
Internodes		
IN1	1.56	2.54
IN2	2.86	3.82
IN3	4.67	4.71
IN4	6.75	3.44
INr	32.73	21.37
Subtotal	48.57	35.88

Appendix 3

Equol Assay

The equol assay was done by Dr. Anton Erasmuson, National Chemical residue Analytical Laboratory, Upper Hutt, New Zealand. Method of equol assay as supplied by Dr. Erasmuson is detailed below.

The samples were thawed in air. Tube was mixed and 5 ml of sample transferred to a 50 ml Falcon tube.

1. *Control recoveries.* These were determined for each batch of samples using blood previously found to be blank. Zero, 5, 5, 15, and 50 µl of the 1 ppm standards mix was added to 5 ml of blood to obtain spikes at 0, 1, 1, 3, and 10 ng/ml.

2. *Internal standard.* 20 µl of diethylstilbestrol at 1 µg/ml was added to all samples as an internal standard.

3. *Enzyme hydrolysis.* Glucuronidase buffer (2.5 ml) was added to tubes for hydrolysis. The caps were screwed on and the tubes vortexed briefly and then hydrolyzed overnight at 37°C.

4. *Extraction.* Extraction was carried out with 30 ml of extractant (30% t-butyl-methyl-ether in n-hexane). Strong shaking was avoided to minimize formation of emulsions. Tubes were sonicated for about 30 seconds and centrifuged at 1000 g for 5 minutes at room temperature. The mixture was sucked off keeping the top layer; extraction was repeated to get a second extract to add to the first. Combined extracts were rotary evaporated until barely or nearly dry. They were transferred using extractant (1 ml) through a Whatman 1 paper filter to a tapered glass tube. The flask was rinsed out twice more with extractant (1 ml) and filtered/combining all three into the same tube. The material was blown down with dry nitrogen and removed when just dry. Flask contents were transferred to a HPLC vial using 200 µl HPLC phase.

5. *Chromatography.* 150 µl was injected onto HPLC column (Whatman PAC Partisphere 12.5 cm length, 5 µm particle size). Appropriate fraction containing equol (predetermined) was collected from Pharmacia Frac 300 fraction collector.

6. Collected samples were derivatised using system developed for equol.

7. *GC/MS analysis.* Derivatised sample was analysed by GC/MS and because of high abundance, equol was monitored at 194 and 288 m/z rather than the isotopically more abundant 192 and 286 ions.

Equol concentration was determined by regressing sample value against equol standards.

Appendix 4

Classification of follicle health

Description	Pycnotic nucelei	Atretic bodies	Structural break down
Healthy	≥ 3	Nil	Nil
Early atretic	≥ 10	Nil	Nil
Advanced atretic	> 20	Present	Partial
Late atretic	Present	Present	Widespread

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