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

A thesis presented in partial fulfilment of the
requirements for the degree of Doctor of
Philosophy in Animal Science at Massey University

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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 F2 α (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 ($1.81 \pm 0.28 \mu\text{g/ml}$) than on Pawera red clover ($7.25 \pm 1.70 \mu\text{g/ml}$) ($P < 0.01$). 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) $1\text{mm} < D \leq 2\text{mm}$ 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 ($D > 4\text{mm}$) 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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TABLE OF CONTENTS

Abstract	ii
Acknowledgement	v
List of tables	xiii
List of figures	xix
List of abbreviations	xx
Chapter I: Introduction	1
Chapter II: Review of literature	
1. Oestrogens and plant oestrogens	3
2. Oestrogenic effects in grazing animals	5
2.1. Sheep	5
2.2. Cattle	7
3. Mechanism of temporary infertility in sheep	7
3.1. Oestrous incidence	7
3.2. Ovulation rate	8
3.3. Ovum fertilization and gamete transport	9
3.4. Embryo mortality	9
3.5. Returns to service, and lambing performance	10
3.6. Corpus luteum function	10
3.7. Recovery period	10
4. Mechanism of permanent clover infertility in sheep	11
4.1. Oestrous incidence	11
4.2. Ovulation rate and corpus luteum function	11
4.3. Lambing rate	12
4.4. Sperm transport and cervical mucus	12
4.5. Embryo mortality	13
4.6. Changes in hypothalamus and pituitary gland	13
5. Metabolism of isoflavones in sheep	14
6. Factors affecting phytoestrogen concentration in legumes	17

6.1.	Genetic factor	17
6.2.	Plant growth stage	18
6.3.	Nutrients	19
6.4.	Other factors	19
7.	Conservation procedures and oestrogenicity of clover	19
8.	Performance of ewes on high and low formononetin cultivars	20
9.	Ewes immune to clover infertility	20
10.	Control of phytoestrogen induced infertility	21
10.1.	Breeding and use of low oestrogenic cultivars	21
10.2.	Other measures	22
11.	Oestrogenic clover and New Zealand	22
12.	Purpose and scope of the study	24

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	25
2.	Introduction	25
3.	Materials and methods	28
3.1.	Plant sampling	28
3.1.1.	Formononetin in vegetative (developing) leaves	29
3.1.2.	Formononetin in pre-flowering shoots	29
3.1.3.	Formononetin in plants at an early-flowering stage	29
3.1.4.	Formononetin in plants at a late-flowering stage	32
3.2.	Formononetin assay	32
3.3.	Statistical analyses	33
4.	Results	33
4.1.	Formononetin during leaf development	33
4.1.1.	Formononetin concentration	33
4.1.2.	Dry weight per part and formononetin yield	36
4.2.	Formononetin in leaves from the pre-flowering shoots	38
4.2.1.	Formononetin concentration	38
4.2.2.	Dry weight per part and formononetin yield	39
4.3.	Formononetin at the early-flowering stage	40
4.3.1.	Formononetin concentration	40

4.3.2.	Dry weight per part and formononetin yield	42
4.3.3.	Distribution of formononetin in various parts of the shoot	42
4.4.	Formononetin at the late-flowering stage	45
4.4.1.	Formononetin concentration	45
4.4.2.	Dry weight per part and formononetin yield	47
4.4.3.	Distribution of formononetin in various parts of the shoot	47
4.5.	Formononetin intake	49
4.5.1.	Early-flowering stage	49
4.5.2.	Late-flowering stage	49
5.	Discussion	49
5.1.	Formononetin in the plants	49
5.2.	Dry weight	52
5.3.	Plant-animal interaction	53

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

1.	Abstract	57
2.	Introduction	58
3.	Materials and methods	59
3.1.	Animals and grazing treatments	59
3.2.	Observations on ovaries	60
3.3.	Histological procedure	61
3.4.	Blood sampling	62
3.5.	Hormone assay	62
3.5.1.	FSH	62
3.5.2.	Progesterone	63
3.5.3.	Equol	64
3.6.	Statistical analyses	64
4.	Results	65
4.1.	Herbage formononetin and uptake in the animal	65
4.2.	Ovulation rate in ewes at start of the trial	66
4.3.	Follicular development on ovarian surface	66
4.4.	Ovarian follicular population	71
4.5.	Plasma FSH	73
4.6.	Plasma progesterone	73

5. Discussion	75
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CHAPTER V: Sperm transport in ewes grazed on red clover

1. Abstract	79
2. Introduction	79
3. Experiment 1	81
3.1. Materials and methods	81
3.1.1. Animals and treatments	81
3.1.2. Artificial Insemination	82
3.1.3. Recovery of spermatozoa	83
3.1.4. Estimation of number of spermatozoa	84
3.1.5. Red clover sampling and formononetin assay	85
3.1.6. Statistical analyses	85
3.2. Results	86
3.2.1. Formononetin concentration in red clover	86
3.2.2. Live weight changes in ewes	86
3.2.3. Oestrous activity in ewes	86
3.2.4. Ovarian activity	86
3.2.5. Number of spermatozoa	88
3.3. Discussion	90
4. Experiment 2	92
4.1. Materials and methods	92
4.1.1. Animals, treatments, and matings	93
4.1.2. Recovery of spermatozoa	93
4.1.3. Estimation of number of spermatozoa	94
4.1.3.1. Uterus and Fallopian tube	94
4.1.3.2. Cervix	96
4.1.3.3. Spermatozoa attached to ovum	96
4.1.4. Red clover sampling and formononetin assay	96
4.1.5. Statistical analyses	96
4.2. Results	97
4.2.1. Formononetin concentration in red clover	97
4.2.2. Blood equol	98
4.2.3. Live weight changes in ewes	98
4.2.4. Oestrous activity and matings	98

4.2.5.	Ovarian activity	100
4.2.6.	Number of spermatozoa	100
4.3.	Discussion	101

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

1.	Abstract	103
2.	Introduction	103
3.	Materials and methods	104
3.1.	Animals and management	104
3.2.	Treatments	104
3.3.	Superovulation and embryo collection	105
3.4.	Statistical analyses	105
4.	Results	106
4.1.	Onset of oestrus	106
4.2.	Ovular ewes and ovulation rate	107
4.3.	Embryo survival	108
5.	Discussion	109

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

1.	Abstract	111
2.	Experiment 1	112
2.1.	Introduction	112
2.2.	Materials and methods	113
2.2.1.	Animals	113
2.2.2.	Treatments	113
2.2.3.	Matings	114
2.2.4.	Statistical analyses	115
2.3.	Results	115
2.3.1.	Formononetin concentration in red clover	115
2.3.2.	Ovulation rate	116
2.3.3.	Returns to service and conception pattern	116
2.3.4.	Litter size	117

2.4.	Discussion	118
3.	Experiment 2	120
3.1.	Introduction	120
3.2.	Materials and methods	121
3.2.1.	Animals and grazing treatments	121
3.2.2.	Ovulation rate and mating of ewes	121
3.2.3.	Litter size	122
3.2.4.	Statistical analyses	122
3.3.	Results	122
3.3.1.	Live weights	124
3.3.2.	Ovulation rate	124
3.3.3.	Conception rate	124
3.3.4.	Litter size	124
3.4.	Discussion	126
CHAPTER VIII: General discussion and conclusions		129
Appendices		135
References		140

LIST OF TABLES

Table	Page
3.1. Mean formononetin concentrations in vegetative leaflets and petioles from leaf emergence to senescence in G27 and Pawera red clovers.....	35
3.2. Mean formononetin concentrations in vegetative leaf (leaflet + petiole) during development	36
3.3. Dry weight and formononetin yield per leaflet or petiole from leaf emergence to senescence in G27 and Pawera red clovers.....	37
3.4. Mean formononetin concentrations in leaflets and petioles on pre-flowering shoots of G27 and Pawera red clovers	38
3.5. Average dry weight and formononetin yield per leaflet and per petiole of pre-flowering shoots	40
3.6. Mean formononetin concentrations in various parts of G27 and Pawera red clover shoots at early-flowering stage	41
3.7. Average dry weight and formononetin yield per part at early-flowering stage.....	43
3.8. Mean formononetin concentrations in different parts of G27 and Pawera red clover shoots at late-flowering stage	46
3.9. Average dry weight and formononetin per part of late-flowering stage	48
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	67
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)	68
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 F2 α injection.....	69
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	70
4.5.	The number of preantral and antral follicles and their appearance in the left ovaries of ewes grazed on various herbage	71
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.....	72
4.7.	Plasma FSH concentration (ng/ml) in ewes grazed on various herbage; mean \pm SEM of averages of the three daily samples	74
4.8.	Plasma progesterone concentration (ng/ml) in ewes grazed on Pawera or G27 red clovers or Ryegrass-white clover (Control) pasture	74
5.1	Trial design (sperm transport experiment 1)	81
5.2.	Mean formononetin concentrations in treatment pastures.....	87

5.3.	Live weight (kg) in ewes grazing various treatment herbage	87
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	89
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	90
5.6	Counts of sperm in test samples of uterine flushing	95
5.7.	Mean formononetin concentrations in treatment pastures	97
5.8.	Equol concentrations ($\mu\text{g}/\text{ml}$) in peripheral blood of ewes grazing various treatment pastures	97
5.9.	Live weight (kg) in ewes grazing various treatment herbage	98
5.10.	Number of spermatozoa recovered from reproductive tracts 24 hours after mating of the ewes, after 4 weeks of grazing red clover or Control pastures	99
5.11.	Number of spermatozoa recovered from various regions of cervix 24 hours after mating	100
5.12.	Distribution between treatments of ewes from which eggs were recovered 24 hours after mating	101
6.1.	Mean formononetin concentrations in treatment pastures	106
6.2.	Oestrus and ovarian activity in ewes grazed on Pawera or G27 red clover, or Ryegrass-white clover (Control) pasture	107
6.3.	Embryo survival and pregnancy status in ewes 35 days after transfer of good quality embryos	108

7.1	Design of the trial to determine reproductive performance of ewes after various grazing treatments.....	113
7.2.	Ovulation rate in ewes after grazing different red clover and Control pastures	115
7.3.	Returns to service and conception pattern in the ewes after grazing different red clover and Control pastures	116
7.4.	Litter size in ewes after completion of three mating cycles	117
7.5.	Live weight change (kg) in ewes grazing Pawera red clover or Control pastures	122
7.6.	Effect of grazing Pawera red clover or Control (Ryegrass-white clover) pastures, on ovulation rate (OR) in ewes	123
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	125

Appendix Tables

1.1.	Mean formononetin concentrations in vegetative leaflets and petioles from leaf emergence to senescence in G27 and Pawera red clover during summer	135
1.2.	Mean formononetin concentrations in whole leaf (leaflet + petiole) during development	135
1.3.	Dry weight and formononetin yield per leaflet and per petiole from leaf emergence to senescence during summer	136
2.1.	Distributions of formononetin per component in red clover shoots at early-flowering stage	137

2.2. Distributions of formononetin per component in
red clover shoots at late-flowering stage 138

LIST OF FIGURES

Figure	Page
2.1.	Metabolic conversions of isoflavones in the sheep 15
3.1.	Pawera and G27 red clover plants 27
3.2	Parts of the red clover plant 30
a.	Red clover leaf 30
b.	Red clover pre-flowering reproductive shoot 30
c.	Flowering shoot 31
3.3.	Mean formononetin concentrations in leaflets and petioles during vegetative leaf development in G27 and Pawera red clovers 34
3.4.	Distribution of formononetin in various parts of G27 and Pawera red clover shoots 44
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 after grazing 50
3.6.	Sheep grazing Pawera and G27 red clovers 56
4.1.	Blood equol concentration in ewes grazing Pawera, G27, or Control pastures 65
4.2.	Plasma FSH concentrations in ewes grazing Pawera, G27, or Control pastures 72

LIST OF APPENDICES

Appendix		Page
I	Formononetin content of developing leaves of G27 and Pawera red clovers during summer	135
II	Distribution of formononetin in red clover shoots at flowering stage	137
III	Equol assay	139
IV	Classification of follicle health	139a

LIST OF ABBREVIATIONS

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