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Red Clover Isoflavonoids in Feed, Plasma and Milk of Ruminants

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RED CLOVER ISOFLAVONOIDS IN FEED, PLASMA AND MILK OF RUMINANTS

Eeva Mustonen

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

Doctoral Programme in Clinical Veterinary Medicine

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To be presented, with permission of
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for public examination
in the Auditorium 1041, Viikinkaari 5, Helsinki
on 11th of December 2015, at 12 noon

HELSINKI 2015
I dedicate this work to my family, my daughters Hilma and Elli and husband Mikko
ABSTRACT

Forage legumes such as red clover (*Trifolium pratense* L.) are increasingly used in grassland-based production systems due to their ability to fix atmospheric nitrogen and reduce the need of fossil-fuel-based nitrogen fertilizers. Red clover is the principal forage legume available for silage production in northern Europe. It is an essential component of organic farming and has also become attractive in conventional agriculture.

Red clover is a valuable ruminant feed. It has a high crude protein content and sufficient digestible fibre. Red clover silage tends to increase dry matter intake and milk yield in dairy cows. The use of clover silage can lead to desirable changes in milk fatty acid composition, promote growth and increase live-weight gain in ewes and lambs. In addition, the antioxidant and antiviral capacity of isoflavonoids may be biologically significant.

The literature review for this thesis focuses on the principal isoflavones in red clover, their metabolism in the rumen and conjugation and excretion in ruminants. The role of the most important metabolite, equol, is reviewed regarding concentrations in plasma and milk. Furthermore, the biological effects, mainly for fertility, of isoflavones and the metabolite equol in ruminants are reviewed.

In Study I, all red clover varieties studied contained isoflavones; the main ones of which were formononetin (6.0–7.9 mg/g in dry matter DM) followed by biochanin-A (3.7–6.1 mg/g in DM). The concentrations of genistein (0.5–0.6 mg/g in DM) and daidzein (0.2–0.30 mg/g in DM) were considerably lower than formononetin concentration. There were statistically significant differences in isoflavone contents among the four clover varieties studied. The overall isoflavone content was highest in cultivar Ilte, which also had the highest concentration of formononetin. The effect of growing conditions (year), growth stage (harvest) and habitat (site) were significant for daidzein and formononetin concentrations. The total isoflavone and formononetin concentrations were highest in 2004, under poor weather conditions, at the northern location and with later harvest.

In the feeding experiment that represented the basis for Studies II and III, the ewes were fed red clover silage that contained on average 3.8 mg/g of biochanin-A, 0.7 mg/g of genistein, 0.6 mg/g of daidzein, and 6.8 mg/g of formononetin in DM. Timothy/meadow fescue (*Phleum pratense* L./*Festuca pratensis* Huds.) grass silage did not contain any isoflavones. There was no coumestrol in either silage. In the red clover group, estimated daily intake of isoflavones was 6 g of biochanin A, 1 g genistein, 1 g daidzein and for 10.5 g formononetin. In ewes fed red clover, the major part of formononetin ingested was metabolized, contributing to the increase of equol in the serum. The concentration of equol remained at a constant average level of 7.7 mg/l during the feeding period. In chiral (high performance liquid chromatography HPLC) analysis, the serum of ewes contained solely (S)-equol. The average concentration of formononetin was 0.073 mg/l. Furthermore, O-demethylangolensin was present in the serum of ewes fed red clover silage at an average concentration of 0.35 mg/l. No daidzein, genistein or biochanin A was found in serum samples of ewes. Daily weight gains of the ewes were 222 and 180 g/day in the red clover and control grass fed groups, respectively. Despite the planned isoenergetic diets, the red clover group ewes gained weight faster (*P<0.001*), and were significantly heavier (*P<0.01*) at the end of the experiment than were the control animals. A similar significant difference (*P<0.001*) was detected for carcass weights at slaughter, the weights being 30.5±1.5 and 25.6±2.1 kg in red clover and control grass fed groups, respectively.
All ewes in both groups became pregnant. There were no significant differences in the time of conception, numbers of foetuses per pregnancy or numbers of ovulations. Feeding red clover silage did not have any effect on mean or total weights of foetuses. However, the total mass of the uterus with its contents was significantly greater (P<0.01) in the red clover group compared with that in the control group. This difference was mainly explained by more foetal fluids in the red clover group (P<0.01).

In Study IV, the red clover silages contained 2.5–4.7 mg/g biochanin A, 0.5–0.7 mg/g genistein, 0.2–0.3 mg/g daidzein, and 3.0–6.5 mg/g formononetin in DM. Grass silage did not contain any isoflavones. The formononetin contents of the red clover silages were highest for the silage that had had the shortest growing time. The daily intake per cow of daidzein was 1.7–2.6 g and of formononetin 27–76 g. When red clover was fed to cows, low concentrations of formononetin, 0.004–0.035 mg/l, were recorded in the plasma. The equol concentration in plasma was 4.6–8.4 mg/l, but also 0.2–0.4 mg/l of O-DMA was recorded. Intake of formononetin (x) was strongly associated with the equol concentration in plasma (y=0.071x + 2.75, R² 0.71).

Equol contents in plasma were significantly higher (P<0.01) for the red clover than for the cows fed grass silage. Plasma equol contents were significantly higher (P<0.001) for cows fed early- than late-cut red clover silages, where the growth times were shortest. Equol concentrations in the milk of cows fed red clover were 458–643 μg/l and daily secretion of equol in milk was estimated to be 12–19 mg/day. Intake of formononetin (x) was only weakly associated with equol concentration in milk (y = 0.0035x + 0.358, R² 0.20). The equol contents in milk were significantly higher (P<0.01) for the red clover than for the grass silage fed cows. No O-DMA was found in milk.

Daidzein, genistein, formononetin, biochanin A, O-DMA, and equol concentrations were analysed from 12 organic commercial milk samples and from four conventionally produced control milk samples. Organic skimmed milk contained 411±65 μg/l of equol. There was some equol, 62±16 μg/l, in conventionally produced control milk samples. Furthermore, formononetin was detected in organic milk samples, but not in conventionally produced milk. Similarly, some daidzein was detected in organic milk samples, but due to the coelution of impurities it was not possible to quantify the amounts. There was no daidzein in conventionally produced milk samples. No genistein, biochanin A, or O-DMA was detected in either milk samples.

Combined fluorescence and ultraviolet detection using the HPLC method provided excellent accuracy and sensitivity for quantification of isoflavonoids from fodder, blood and milk samples. Using enantiopure equol as the authentic reference compound in the HPLC analysis of serum of ewes on long-term red clover feeding, (S)-equol and O-DMA were the main isoflavonoids detected. In addition, it is suggested that the estrogenic effects of metabolic equol in sheep reported earlier are solely or predominantly due to (S)-equol. In Finnish landrace sheep the fecundity of nulliparous ewes was not reduced by feeding red clover with high isoflavone concentrations for five months before, during, and after the breeding season. The volume of foetal fluids, however, increased, which could increase the risk of vaginal prolapse before term.

A strong association between formononetin intake and equol concentration in plasma was demonstrated. The equol content in cow’s milk can be as high as 600 μg/l with red clover silage feeding, even though only a small part of the formononetin is secreted into milk as the metabolite equol. The equol content in milk can be manipulated by varying the harvesting strategy of red clover. Shorter growing periods for red clover result in higher formononetin contents in the silage and equol content in the plasma and milk. Milk equol is derived from the formononetin of red clover silage and red clover-fed cows’ milk can be considered as a source of
equol in human nutrition. Finnish organic commercial milk can contain high levels of equol. This is due to the widespread use of red clover in organic farming.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four publications and one manuscript referred to in the text by their Roman numerals.

I Mustonen E, Tuori M, Kurki P, Isolahti M, Taponen J, Vanhatalo A. Cultivar, time of harvest and conditions during growing season have impact on red clover isoflavone content. Submitted to Journal of Agricultural and Food Chemistry.


### ABBREVIATIONS

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>BPD</td>
<td>biparietal diameter</td>
</tr>
<tr>
<td>BW</td>
<td>bodyweight</td>
</tr>
<tr>
<td>CRL</td>
<td>crown-rump length</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
</tr>
<tr>
<td>DM</td>
<td>dry matter</td>
</tr>
<tr>
<td>FLD</td>
<td>fluorescence detector</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>EDC</td>
<td>endocrine-disrupting chemicals</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>G</td>
<td>grass</td>
</tr>
<tr>
<td>H1</td>
<td>first harvest</td>
</tr>
<tr>
<td>H2</td>
<td>second harvest</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LOL</td>
<td>limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantification</td>
</tr>
<tr>
<td>NS</td>
<td>non-significant</td>
</tr>
<tr>
<td>O-DMA</td>
<td>O-demethylangolensin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>prostaglandin F₂α</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RC</td>
<td>red cover</td>
</tr>
<tr>
<td>RE</td>
<td>primary growth of early cut red clover</td>
</tr>
<tr>
<td>RL</td>
<td>primary growth of late cut red clover</td>
</tr>
<tr>
<td>RRE</td>
<td>re-growth of early cut red clover</td>
</tr>
<tr>
<td>RRL</td>
<td>re-growth of late cut red clover</td>
</tr>
<tr>
<td>RBA</td>
<td>relative binding affinity</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulators</td>
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1. INTRODUCTION

There is increasing interest in using forage legumes for ruminant feeding in grassland-based production systems. Legumes such as red clover (*Trifolium pratense L.*) can fix atmospheric nitrogen and thereby reduce the need for application of fossil-fuel-based nitrogen (N) fertilizers. Red clover is the major forage legume available for silage production in northern Europe. The use of forage legumes is essential in organic production and has become attractive in conventional agriculture (Halling et al. 2002, Wilkins et al. 2002, Halling et al. 2004, Vanhatalo et al. 2009).

Red clover is a valuable ruminant feed, having a high crude protein content and sufficient digestible fibre. Red clover silage increases dry matter intake and milk yield in dairy cows more than grass silages (Dewhurst et al. 2003, Bertilsson and Murphy 2003, Vanhatalo et al. 2006). In addition, replacing grass silage with red clover silage can lead to desirable changes in the fatty acid composition of milk (Dewhurst et al. 2006, Vanhatalo et al. 2007, Moorby et al. 2009). Red clover diets have been shown to promote growth and increase live-weight gain in ewes and in lambs (Fraser et al. 2004, Moorby et al. 2004, Speijers et al. 2005, Graves et al. 2012). The antioxidant and antiviral capacity of isoflavones could be biologically significant (Greiner et al. 2001, Hwang et al. 2003, Turner et al. 2004, Lecot et al. 2005, Martin et al. 2007, Akula et al. 2002).

Red clover, like other legumes, contains phytoestrogens. These are phenolic non-steroidal compounds with a similar steric structure as steroidal estrogens. This similarity gives them the ability to bind to estrogen receptors and exert estrogenic or anti-estrogenic effects. The most common phytoestrogens are isoflavones, lignans and coumestans. The four main isoflavones in red clover are formononetin, biochanin A, daidzein and genistein (Baber 2013).

Breeding problems affecting ewes grazing on subterranean clover (*Trifolium subterraneum L.*) pasture initiated isoflavone research in late 1940s (Bennetts et al. 1946). Isoflavonoids, and especially the metabolite equol, have been shown to cause fertility problems in sheep (Adams 1998). Despite the documented problems in sheep, only few case reports have been published concerning cattle (Thain 1965, Elghamry et al. 1969, Lookhart 1980, Kallela et al. 1984) even though the metabolism of isoflavones in sheep and cattle seems to be similar (Lundh 1990a). It has been shown that isoflavone metabolites derived from red clover are found in bovine milk and are thus part of the human diet (King et al. 1998).

This thesis is focused on revealing the contents of the main isoflavones in red clover ruminant feeds in Finland, and the contents of isoflavone compounds in the blood of ruminants and in the milk of dairy cows. In addition, the possible fertility effects in ewes fed red clover silage were studied.
2. REVIEW OF THE LITERATURE

2.1. Red clover

Red clover is a perennial herb belonging to the pea family *Fabaceae*. The corolla is zygomorphic and the colour is typically violet-red. Red clover usually has many stems, which have an ascending to erect habit, and the height of the plant ranges from 15 to 50 cm. The leaves are alternate, stalked and stipulate. Red clover is widely distributed in Finland and has been found even in the most northern parts of Lapland (Nylén 1995, Lampinen et al. 2013).

2.2. Isoflavones

Plants can produce phenols or polyphenolic compounds. Isoflavones are subgroup of isoflavonoids; they are polyphenolic compounds found predominantly in legumes (Figure 1). Isoflavones occur as a variety of compounds that have a chromane skeleton with a phenyl substituent at the C3 position. Isoflavones are present as glucoside, malonylglucoside and aglycone forms in *Trifolium* species (Veitch 2009).

Nitrogen fixation of leguminous plants is based on *Rhizobium*-legume symbiosis in root nodules. It has been proven that isoflavones work in the nodulation process as signalling compounds and nod gene inducers (Bucar 2013). Isoflavones are stored in plant vacuoles either as glycoside or their 6''-O-malonylated form. These conjugated forms are more soluble than an aglycone form. Upon microbial infection the aglycones are generated from these precursors by hydrolysis of the stored form. The aglycones are precursors of pterocarps and isoflavans, produced to prevent plant pathogen infection. In this way isoflavones also work as phytoalexins and phytoanticipins (Bucar 2013, Edwards et al. 1997).

More than 30 isoflavone compounds have been isolated from red clover and related species as aglycones, glycosides or glycoside malonates (Wu et al. 2003). Qualitative analysis reveals that red clover isoflavones are mainly available as glycoside malonate conjugates, and the number of corresponding isoflavonoid aglycones is low (Edwards et al. 1997, de Rijke et al. 2001). In typical isoflavone studies, the biologically active aglycone forms are quantified after hydrolysis of the conjugated isoflavones with acids, bases or enzymes. This is done because the separation of malonyl and acetyl isoflavones from isoflavone glucosides and aglycones is challenging (Inbaraj and Chen 2012).

2.3. Isoflavones in red clover feed

In the Nordic countries, the total isoflavone content of red clover can range between 5–25 mg/g of dry matter (DM) (Pettersson et al. 1984, Kallela et al. 1988, Saloniemi et al. 1995, Sarelli et al. 2003, Andersen et al. 2009b, Saviranta et al. 2010a). For other grassland legumes, the amounts of isoflavones are quite small. For example, the concentrations of isoflavones, mainly formononetin, present in white clover (*Trifolium repens* L.) are 0.2–0.6 mg/g in DM. Small quantities of coumestrol have been found in lucerne (*Medicago sativa* L.) (0.033–0.063 mg/g in DM) and white clover (0.001–0.0089 mg/g in DM) (Saloniemi et al. 1995). In birdsfoot trefoil (*Lotus corniculatus* L.) there are only traces of isoflavones (0.004–0.012 mg/g in DM) (Sarelli et al.
Soybean (*Glycine max* (L.) Merr.) meal, used as a protein supplement for cattle, can contain 3 mg/g of isoflavones in DM (Cools et al. 2014).

Figure 1. Examples of isoflavonoids together with flavone and 17β-estradiol

Figure 1. Examples of isoflavonoids together with flavone and 17β-estradiol
Red clover cultivars differ significantly in isoflavone content which means that the genotype has significant impact on isoflavone concentration (Kallela et al. 1988, Sivesind and Seguin 2005, Tsao et al. 2006, Saviranta et al. 2008). Even if there are environmental factors affecting the concentration, such as soil, age and harvest of plant stand, the cultivar has the greatest influence on isoflavonoid concentration (Sivesind and Seguin 2005).

Formononetin and biochanin A are the predominant aglycone isoflavones in red clover (Wu et al. 2003, Sivesind and Seguin 2005, Tsao et al. 2006). Other isoflavones such as daidzein, genistein, glycitein, irilone, orobol, pratensein, pseudobaptigen, prunetin and calycosin have been found in smaller concentrations (He et al. 1996, Wu et al. 2003, Tsao et al. 2006). The amounts of isoflavones are highest in the leaves, intermediate in the stems and lowest in the flowers (Wu et al. 2003, Sivesind and Seguin 2005, Tsao et al. 2006). The highest formononetin and biochanin A concentrations are found in the leaves. In stems and flowers, formononetin predominates (Sivesind and Seguin 2005, Tsao et al. 2006, Saviranta et al. 2008).

Temperature affects isoflavone contents, lower temperatures increasing the formononetin concentration (McMurray et al. 1986). Distinctly higher isoflavone concentrations were detected in northern Finland compared with the the southern areas (Kallela et al. 1988). Nitrogen fertilizers reduced the isoflavone content in swards (Kallela et al. 1987), but a lack of phosphate increased the content (Butler et al. 1967), especially those of formononetin (McMurray et al. 1986). Elevated ozone levels as well as biotic and abiotic stress factors affect the roots and isoflavone concentrations can increase (Saviranta et al. 2010a, Saviranta et al. 2010b).

Harvest timing and season influence isoflavone content (McMurray et al. 1986, Kallela et al. 1988, Sivesind and Seguin 2005, Booth et al. 2006). There is abundant production of isoflavones during spring and early summer when the growth of plants is fastest. Isoflavone production declines by midsummer (Kallela 1980, McMurray et al. 1986), and increases in aftermath (Kallela et al. 1987). Formononetin diminishes in all parts of the plants as the plant gets older. The earlier the re-growth clover is harvested, the higher the isoflavone concentrations, particularly those of formononetin (McMurray et al. 1986). At the beginning of flowering, the isoflavone content of inflorescences is at its highest (Sivesind and Seguin. 2005), but after budding isoflavone contents decline (Sarelli et al. 2003). The highest concentrations of formononetin were detected at the early maturity stage of leaves and stems (Sivesind and Seguin 2005).

Preconservation of red clover affects isoflavone concentrations. Fresh herbage contains more formononetin than preconserved silage or hay. The quantity of formononetin was highest in fresh clover, intermediate in silage and lowest in dry hay (Sivesind and Seguin 2005). Longer wilting lowered isoflavone amounts in silage (Sarelli et al. 2003). A part of the estrogenic activity seems to disappear during drying; hay contains less phytoestrogens than silage (Kallela 1980, Saloniemi et al. 1995, Sarelli et al. 2003).

2.4. Metabolism in rumen

Isoflavones of red clover are mainly present in plant vacuoles as glycoside or 6''-O-malonate conjugates (Figure 2) (Beck 1964, Wu et al. 2003, Edwards et al. 1997). During mastication and rumination, ruminants chew plants leading to structural breaking of leaves and stems. The plant cuticle is removed, vascular bundles and other tissues are separated and the plant cell contents released (Kennedy 2005). Such crushing of the plant releases isoflavones (Beck 1964); plant enzymes or micro-organisms in the rumen hydrolyze
isoflavone glycosides to aglycones (Lundh 1995). Ruminal metabolism of isoflavones has been studied, mainly in sheep, and it is suggested to resemble qualitatively that of cattle (Lundh 1995). In the rumen, biochanin A is demethylated to genistein and formononetin to daidzein (Nilsson 1961, Nilsson 1962). Genistein is further degraded in the ruminal fluid and the major metabolite is para-ethylfenol. With regard to formononetin, the identified metabolites are daidzein and equol (Batterham et al. 1965). The presence of the formononetin–daidzein–equol pathway in rumen fluid was confirmed in sheep (Nilsson et al. 1967).

Figure 2. Examples of isoflavonoid glucosides

Formononetin, daidzein and equol are available for absorption from the rumen up to 24 h after ingestion (Dickinson et al. 1988). It has been shown that most aglycone isoflavones are metabolized further in the rumen and a fairly small proportion of isoflavones is absorbed and excreted as such (Shutt et al. 1970, Lloyd-Davies and Hill 1989). Even if the basic metabolism (Figure 3) of red clover isoflavones in the rumen has been clarified, alternative pathways may exist, and several metabolic conversions have not been completely defined (Lundh 1995, Cox and Davies 1988). For instance, it has been shown that bovine ruminal bacteria can convert daidzein and genistein to dihydrodaidzein and dihydrogenistein, and can produce equol from daidzein through dihydrodaidzein (Wang et al. 2007).

2.5. Isoflavone excretion

Isoflavones and their metabolites disappear very rapidly from the rumen and nearly completely from forestomachs and abomasum of ruminants (Lindner 1967, Shutt et al. 1970). When synthetic genistein, biochanin A and formononetin were given intraruminally to ewes, detectable amounts were measured in the lood within 2.5 h (Lindner 1967). When urinary phenols were measured before and after intraruminal
administration, biochanin A and genistein were mainly excreted as para-ethylphenol in urine, the major metabolite of formononetin being equol (Braden et al. 1967). Less than 1% of the isoflavones ingested were excreted in urine or faeces as such without further metabolism (Shutt et al. 1970). Experiments with radiolabelled formononetin confirmed that equol is the main metabolite in urine and plasma (Batterham et al. 1970). It was concluded that in relation to estrogenic responses, equol is of major importance in sheep eating clover that has a high formononetin content (Shutt and Braden 1968, Batterham et al. 1970).

Shutt et al. (1970) showed in a quantitative study in sheep that equol was the main metabolite in digesta and excreta after red clover feeding. The main excretion site was urine; it was equivalent to 70% of formononetin in the diet. About 86% of equol metabolized in the rumen was absorbed; the residence time for equol in the rumen was 1.7 h. Furthermore the predominant metabolite in plasma was equol (Shutt and Braden 1968, Shutt et al. 1970). It was concluded that formononetin – which itself has very little estrogenic activity – undergoes further metabolism in the rumen so that the end product is estrogenically active equol (Cox and Braden 1974, Cox and Davies 1988). Formononetin metabolism was studied in detail and confirmed using tritiated isoflavones (Lloyd-Davies and Hill 1989). They showed that formononetin was converted to equol within 48 h and 80% was excreted in urine. Equol disappeared from the rumen within 1 h, it was found in plasma within 2 h and peak concentration was detected in plasma within 24 hours (Lloyd-Davies and Hill 1989).

2.6. Conjugation

The majority of isoflavones and their metabolites are found in a conjugated form in plasma, the main conjugated form being glucuronides (Shutt et al. 1967) and 5 to 15% sulfconjugates (Cox and Davies 1988). Conjugation with glucuronic acid is believed to be the major detoxification mechanism (Shutt et al. 1967). The detoxification can take place in the liver (Shutt et al. 1967), but it was shown that glucuronidation starts already in the gastrointestinal epithelium because equol rarely exists in the free estrogenically active unconjugated form (Lundh 1990b). Lundh et al. (1988b) observed that the ruminant liver microsomes contribute very little to demethylation and the conjugative rate of formononetin and daidzein. Later Lundh (1990b) measured considerable glucuronidation activity toward formononetin, daidzein and equol in the gastrointestinal epithelium of rumen, reticulum, omasum and small intestine of sheep and cow. The overall activity was higher in sheep than in cattle, except in the intestinal mucosa, where conjugation capacity was higher in cattle (Lundh 1990b, Lundh 1995).

In plasma, equol was mainly present in a conjugated form, but also small amounts of unconjugated free equol were detected (Shutt et al. 1967, Shutt and Braden 1968, Shutt et al. 1970). The concentration of free equol has been measured in several studies. In the plasma of ewes fed with red clover pellets, 1.5–6% of free equol was found (Shutt and Braden 1968). Later, in a quantitative study, 1–2% of total equol reported to be in the free form (Shutt et al. 1970). When fresh clover was fed to wethers and heifers, only 0.5% and 0.3%, respectively of equol was found in the unconjugated form (B Braden et al. 1971). In a more recent study, plasma concentrations for dairy cattle and wethers were compared. The free form constituted 5% of total equol in cows and 1% in sheep (Lundh et al. 1990). Plasma concentrations have been compared with different levels of formononetin fed. When ewes grazed red clover pastures either high 5.7 mg/g or low 2.5 mg/g in DM formononetin content, the concentration of conjugated equol in plasma rose during the first two days in both pastures. Thereafter, the values averaged from 0.24 to 0.08 mg/l. On low formononetin pasture free equol was undetectable for the first four days; later the average amount was 0.0036 mg/l. With high
formononetin pasture, the amount of free equol increased to an average level of 0.01 mg/l. When ewes were removed from the red clover pastures, equol was cleared from the blood the day following removal. In both cases the amount of free equol was approximately 4% of total equol (Kramer 1996).

Figure 3. Main metabolism of formononetin in sheep and cattle
2.7. Excretion to milk

Elimination of isoflavonoids in ruminants is not fully elucidated. It seems that most equol (70–80%) produced is excreted in urine and very little is found in faeces (Shutt et al. 1970, Lloyd-Davies and Hill 1989). Besides urine and faeces, isoflavonoids are excreted in milk. First identification of equol (81 μg/l) in cow milk was by Adlercreutz et al. (1986). Later, in measurements of isoflavonoids in Australian bovine milk, the highest concentrations found 293 μg/l were from Western Australian samples, where clover cultivars are common (King et al. 1998). Similar equol concentrations, 3–191 μg/l, were found in France in cow milk (Antignac et al. 2003, Antignac et al. 2004) and in the milk of goats fed clover 210–1120 μg/l (Sakakibara et al. 2004). Other isoflavone compounds, genistein, daidzein and para-ethylphenol were found in dairy milk, but concentrations were far smaller than those for equol (King et al. 1998, Antignac et al. 2003, Antignac et al. 2004, Sakakibara et al. 2004).

Research from the last decade on the isoflavonoid concentrations in dairy milk comes from Nordic countries. In Denmark, an equol concentration of 230 μg/l was reported for organic milk (Purup et al. 2005). The effect of feeding silage of white and red clover on isoflavone concentration of milk was studied in Norway; equol concentrations were particularly high, 272–364 μg/l, when red clover was fed (Steinshamn et al. 2008). In a Danish study, where silage of grass/red and white clover was fed, equol concentrations were low in milk, 186 μg/l. Grazing on red clover pastures increased equol concentration to 253–397 μg/l (Andersen et al. 2009a, Andersen et al. 2009b). In a Swedish study, silage mixtures containing red clover had the highest concentrations of formononetin and daidzein, and furthermore, very high equol concentrations in milk, 1297–1494 μg/kg, were found (Höjer et al. 2012).

2.8. Tissues concentrations

Traces of equol were found in meat (Moorby et al. 2004) and in other tissue samples (Urpi-Sarda et al. 2008). Urpi-Sarda et al. (2008) investigated tissue distribution of isoflavones, especially equol, in ewes fed red clover. There were 157.6 mg/kg bodyweight (BW) of total aglycone isoflavones in the daily diet of ewes. Ewes ingested 81.8 mg/kg BW formononetin, 64.8 mg/kg BW biochanin A, 7.6 mg/kg BW genistein and 2.9 mg/kg BW daidzein. The major isoflavones in silage, genistein, formononetin and biochanin A, were not recovered in plasma or in tissues. When analysed without hydrolysis, mono-glucuronides of daidzein and equol were the major forms in plasma and tissues. After hydrolysis by β-glucuronidase/sulfatase, the major forms found were equol and daidzein. Mean plasma levels of equol and daidzein were 18.20 and 8.55 μmol/l, respectively. The tissue distribution was unequal over the different tissues, but similar in both experimental animals. Tissue concentrations are presented in Table 1. Tissues were also analysed when non-hydrolysed to detect aglycone forms. Daidzein was not found as aglycones, but 8% of equol in ovaries, 1.9% in mammary glands and 2.6% in the aorta were non-hydrolysed (Urpi-Sarda et al. 2008).
Table 1. Tissue distribution of equol and daidzein in ewes fed red clover (Urpi-Sarda et al. 2008)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Equol nmol/g</th>
<th>Daidzein nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>kidney</td>
<td>181.06</td>
<td>71.23</td>
</tr>
<tr>
<td>liver</td>
<td>18.24</td>
<td>26.41</td>
</tr>
<tr>
<td>ovary</td>
<td>14.96</td>
<td>5.56</td>
</tr>
<tr>
<td>aorta</td>
<td>15.17</td>
<td>4.69</td>
</tr>
<tr>
<td>suprarenal glands</td>
<td>10.35</td>
<td>5.20</td>
</tr>
<tr>
<td>uterus</td>
<td>10.30</td>
<td>3.45</td>
</tr>
<tr>
<td>thyroid</td>
<td>8.55</td>
<td>1.84</td>
</tr>
<tr>
<td>brain hemisphere</td>
<td>0.73</td>
<td>0.36</td>
</tr>
<tr>
<td>brain cerebellum</td>
<td>1.70</td>
<td>0.34</td>
</tr>
<tr>
<td>brain olfactory lobe</td>
<td>2.69</td>
<td>0.69</td>
</tr>
<tr>
<td>mammary glands, pituitary gland, lungs, thymus, heart, muscle</td>
<td>less than 10 nmol/g total isoflavones</td>
<td></td>
</tr>
</tbody>
</table>

2.9. Mechanism of action

2.9.1. Background

Since Bennetts et al. (1946) reported on breeding problems of ewes that grazed on subterranean clover pasture, much research was done to reveal the cause. Initially, it was thought that genistein was responsible for the breeding problems (Curnow 1954) and that formononetin was inactive (Wong 1962). Later it was shown that the amount of formononetin in the subterranean clover strains was positively linked to estrogenic activity. By that time the estrogenic activity of fodder was measured using the wether teat length bioassay (Millington et al. 1964) or ewe uterine weight bioassay (Morley et al. 1964, Bennett and Dudzinski 1967).

2.9.2. Physiological effects and regulatory mechanisms of endogenous estrogens

Estradiol-17β is the primary estrogen, estrone and estriol representing other metabolically active estrogens. Estrogens have a wide range of physiological effects. They, mainly estradiol-17β, act on the central nervous system to induce behavioural estrus, and on the uterus to increase both the amplitude and frequency of contractions by amplifying the effects of oxytocin and prostaglandin F₂α. During the growth period of females, estrogens cause physical development of the female secondary sexual characteristics and stimulate the duct growth and development of the mammary gland. Estradiol-17β participates crucially in the regulatory mechanisms of secretion of gonadotrophic hormones. Estradiol has a negative feedback effect on
the secretion of follicle stimulating hormone (FSH) through the hypothalamus and the adenohypophysis. Estradiol-17β has a so-termed dualistic influence on the secretion of luteinizing hormone (LH). Normally the feedback effect is negative, keeping the amplitude of LH secretion low, but at the beginning of estrus, after reaching a certain threshold, the effect turns highly positive, inducing an LH peak that initiates the ovulatory process. In addition to the sexual and reproductive function, estrogens have biological effects on the cardiovascular, musculoskeletal, immune and central nervous systems (Hafez et al. 2000).

Cellular signalling of estrogens is mediated through two estrogen receptors (ER), ERα and ERβ, which belong to the nuclear receptor family. These subtypes mediate the biological effects of estrogens and antiestrogens. Different ligands of ERs produce different ER conformations. Estrogen receptors interact with different co-activators and co-repressors when they are ligand bound; this complex interaction regulates gene expression. Different mechanisms of target gene regulation affect the agonist/antagonist profile of the ligand. Selective Estrogen Receptor Modulators (SERMs) have a tissue and gene-specific mixed agonist/antagonist effect on ER (Nilsson and Gustafsson 2002). Heldring et al. (2007) described three pathways in the regulatory action of ER. The classical direct pathway consists of ligand activation of ER and a direct DNA-binding to the estrogen response element; this will lead to gene regulation. In a tied pathway; protein-protein interaction is needed after ligand activation. In this case gene regulation arises by indirect DNA-binding. A third, non-genomic pathway, has been observed in many tissues, but the mechanism is not fully known. In this pathway rapid physiological effects are seen without gene regulation.

ERs have special roles in the immune, skeletal, cardiovascular and central nervous systems in addition to their central role in reproductive development and physiology. ERα is possibly more involved in the feedback regulation of the hypothalamo-pituitary axis and levels of LH and FSH. ERβ is believed to have a special role in memory and learning. Both estrogen receptors are found on vascular endothelial cells, smooth muscle cells, myocardial cells and the mammary gland. ERα is important in bone maturation and homeostasis, but ERβ has a role also in female bone physiology. ERβ is expressed in the urogenital tract: bladder, urethra, testis, prostate, and ovary (Nilsson and Gustafsson 2002).

2.9.3. Equol – the most important metabolite

When equol was originally detected in the urine of mares in 1932 (Marrian and Haslewood 1932), it was believed to be estrogenically inactive. Nowadays equol is considered to be the most important compound in this respect due to its high estrogenic activity (Cox and Braden 1974, Setchell et al. 2002, Atkinson et al. 2005, Setchell and Clerici 2010). Equol occurs as two enantiomeric forms, (S)-equol and (R)-equol when chemically synthesized without enantioselective control. Human intestinal bacteria produce exclusively (S)-equol (Atkinson et al. 2005) as do bovine ruminal bacteria (Wang et al. 2007).

The relative binding activity of isoflavone compounds has been shown to be between 1/1000 and 1/10,000 of that of estradiol (Adams 1998). Shutt and Cox (1972) were the first to show this, when they compared the relative molar binding affinity of estradiol-17β to equol and other compounds. When the affinity of estradiol-17β was 100, the affinity for equol was 0.4. Isoflavonoids bind with a greater affinity to ERβ than to ERα (Kuiper et al. 1998). Pfitscher et al. (2008) tested receptor binding and transactivation of red clover isoflavones and their metabolites to ERα, androgen receptor (AR) and progesterone receptor (PR). Competitive binding assays with radiolabelled 17β-estradiol, 17α-methyltrienolone or progesterone studied binding to the respective ERα and ERβ, AR and PR. All tested compounds, except para-ethylphenol, bound to
ERα. Equol and a hydroxylated metabolite of genistein, 3'-OH-genistein, had the highest affinities for ERα, but their potency was a factor of about $1.6 \times 10^3$ lower than that of 17β-estradiol. The affinity of isoflavones and their metabolites for ERβ was higher than for ERα. Equol and genistein had the highest affinity for ERβ. Their potencies were about 24 times lower than those of 17β-estradiol. The response to AR and PR for all tested compounds was very low (Pfitscher et al. 2008). (S)-equol is largely ERβ selective (Kuiper et al. 1998, Kritchevsky et al. 2003). In a competitive binding assay, where the relative binding affinity (RBA) for estradiol-17β was 100%, the value for (S)-equol was 0.1% (Muthyala et al. 2004). Red clover isoflavones and their metabolites are able to bind to ERs and exert estrogenic effects, and are thus recognized as endocrine-disrupting chemicals (EDC) (Pfitscher et al. 2008).

2.10. Effects in ruminants

2.10.1. Effects in sheep

The estrogenic potency of isoflavone compounds is fairly low, but the amounts in feed for sheep can be so high that estrogenic stimulation is possible (Adams 1998). Fertility problems in clover-fed ewes were discovered in Western Australia in the late 1940s. Bennetts et al. (1946) termed these problems ‘clover disease’. The disease has three manifestations: infertility, dystocia and prolapse of the uterus. Furthermore, maiden and non-bred ewes develop udders and secrete milk. Milk secretion has been seen in wethers, together with the enlargement of prostate and bulbo-urethral glands. It has been concluded that clinical manifestations of high isoflavone intake can vary (Adams 1998). Typically conception rate in flocks can be exceptional low, and endometrial cysts are detected as necropsy findings. During parturition, the opening of the cervix may be incomplete and uterine contractions weak, leading to stillbirths. In ewes, especially in young ones, dystocia due to maternal reasons can be detected. All these clinical signs can appear several months after exposure. In castrated rams, mammary glands may develop and they may even lactate. The bulbourethral gland may dilate leading to the obstruction of the urethra and death (Adams 1998).

Isoflavone intake can cause temporary subfertility or infertility. During the breeding season it may result in declining numbers of ovulations and/or conceptions. Fertility is restored in some weeks after changing the feeds. The disorder is insidious because the signs of hyperestrogenism usually do not appear, and the problem cannot be identified until lambing. There is less twinning and more barren ewes than normally. The pathological signs are due to estrogenic effects via adenohypophysis on ovaries and sperm transport (McDonald 1995, Adams 1998, Adams 1990).

Long duration exposure to isoflavone compounds can cause permanent, irreversible infertility in ewes without any clinical signs of classical clover disease. Several successive grazing periods on pastures rich in isoflavones impair fertility slowly but gradually and lead to infertility. The exposed ewes show estrous signs, are bred and ovulate normally, but pregnancy rates are poor. Affected ewes exhibit histological changes of the cervix. Folds of the mucous membrane disappear, the membrane itself thickens and glandular structures appear underneath the folds. The types of epithelial cells change so that the entire structure resembles more endometrium than cervix. These changes alter the secretory function of the cervix. Continuous exposure to isoflavone compounds reduces the normal response to endogenous estrogen leading to decreased viscosity of the cervical mucus. In ruminants, the cervix has an important role in sperm transport and as a sperm
reservoir. Hence, the changes can influence physiological functions and lead to subfertility and with time to permanent infertility (Adams 1990, Adams 1998). The level of nutrition, e.g. feeding under or above maintenance, together with insulin-like growth factor (IGF), have been suspected to modify the estrogenic effect that produces uterine-like histological changes in the cervix of ewes (Adams 1995b).

According to evaluations done in Australia, the content of estrogenic clover in the pasture determines the estrogenic potency; problems can be avoided by a strategic use of pastures. Pastures containing 3 mg/g or less formononetin in DM are believed to be safe for ewes. Formononetin concentrations above 8 mg/g in DM can lead to fertility problems (Marshall et al. 1976, Little 1996).

Red clover feeds exert a positive effect on growth. In a feeding trial, the performance of lambs fed with three different silages made of red clover, lucerne or perennial ryegrass (Lolium perenne L.) was evaluated. The lambs offered red clover silage had a higher voluntary silage DM intake, total DM intake and metabolisable energy (ME) intake than the lambs offered either lucerne or ryegrass silage. This resulted in a faster growth rate and increase in the condition score (Speijers et al. 2005). Lambs grazing the red clover pastures had a higher herbage intake and live-weight gain than those fed with lucerne (Fraser et al. 2004). Red clover sod-seeding in naturalized pastures promoted weight gain in lambs. Weight gain was higher with red clover than in control natural pastures or white clover sod-seeding pastures (Graves et al. 2012). In a study by Moorby et al. (2004), three pastures, high and low formononetin content red clover and perennial ryegrass were compared in a lamb feeding trial. Plasma concentrations of growth hormone and IGF-1 were highest in lambs grazing red clover with high formononetin content; they gained 40 g/day more live weight than the other lambs. There was a significant difference between clover fed and control animals also in mean teat length, the classical measure of estrogenic influence.

2.10.2. Effects in cattle

Despite the intensive research on clover disease in sheep, the effects of isoflavonoids on bovine reproduction are unclear, and there are very few case reports on this topic. In clinical studies using ovariectomized heifers, red clover isoflavones caused vulvar oedema, vaginal mucous discharge, milk-like secretion from the mammary gland, growth of teats and an increased weight of the uterus (Kallela 1968, Nwannenna et al. 1994). In single field cases, isoflavones from red clover or coumestans from lucerne were reported to cause fertility disorders in cows. In Germany, lucerne feeding induced the development of the mammary gland, vulvar oedema and dilatation of the uterus. Non-bred heifers began to lactate on clover pasture rich in formononetin (Elghamry et al. 1969). In a Finnish case report, cows fed with silage made of red clover had irregular estrous cycles with silent or absent estrous signs. In addition to this, vaginal discharge, early embryonic loss and premature deliveries were reported. Despite hormonal treatments the problems did not disappear until the forage was changed to grass silage (Kallela et al. 1984). Isoflavone compounds originated from red clover have been suspected to induce vaginal prolapses in cows in Finland (Sarelli et al. 2002). Subterranean clover was a possible cause of bovine infertility in Tasmania; in this case the development of ovarian cysts and anestrus were reported together with genital changes, udder enlargement and secretion of milk by maiden heifers (Thain 1965). In the United Kingdom, a group of heifers were either fed red clover containing 6.5 mg/g DM formononetin or grass silage prior to and during the time of insemination. In that study, no evidence of depressed fertility was seen; instead pregnancy rate to first service was significantly higher following feeding with red clover silage (Austin et al. 1982). It is noteworthy
that no significant effects on reproduction in cattle have ever been observed in Western Australia, where reproductive losses in sheep have been most severe (Adams 1998).

In several studies, red clover feeding had a positive effect on animal production. Inclusion of red clover in grass silage based diets of cows had a beneficial effect on production increasing the yields of milk, protein and lactose (Vanhatalo et al. 2006). With red clover based diets dry matter intake can increase by around 1 kg DM per day and energy corrected milk production on average by 0.9 kg per day (Bertilsson and Murphy 2003, Dewhurst et al. 2003, Vanhatalo et al. 2006). Red clover feeding increases the amount of polyunsaturated fatty acids in milk (Dewhurst et al. 2006, Vanhatalo et al. 2007, Moorby et al. 2009). When several datasets were reviewed, red clover feeding increased dry matter intake on average by 1.2 kg and milk yield by 1.5 kg per day when compared with grass silage. Milk fat content was lower with red clover feeding, but the proportion of the polyunsaturated fatty acids higher than with grass. Red clover based diets yielded higher concentrations of isoflavonoids, especially equol, in milk (Steinshamn 2010).

The metabolism of isoflavones in cattle is similar to that for sheep. Cattle are not, however, as sensitive to these compounds as sheep. The reason for this is unknown. The suggested differences in sensitivities have been proposed to arise from the differences in metabolism (Cox and Braden 1974) and especially in the detoxification capacity between the two species (Lundh et al. 1988b). However, according to Lundh (1995) factors other than differences in the conjugation rate between cattle and sheep explain the differences in sensitivity to isoflavones and their metabolites. Furthermore, Lundh (1995) suggested that these factors might operate at the receptor level since the concentration of ERs in the uterus is higher in ewes than in cattle (Koligian and Stormshak 1977, Henricks and Harris 1978). Due to the infertility problems encountered in sheep, isoflavonoids have received a negative reputation in animal production circles, even though infertility cases in cattle are uncommon (Adams 1995a).

Controversially, in recent studies of Woclawek-Potocka et al. (2006) the soy-derived isoflavone metabolites equol and para-ethylphenol were able to stimulate PGF$_2$α secretion in bovine corpus luteum. This stimulation takes place via an ER-dependent genomic pathway. Based on their earlier studies Woclawek-Potocka et al. (2006) concluded that these isoflavone metabolites disrupt reproductive efficiency and uterine function by modulating the ratio of PGF$_2$α and PGE$_2$. This leads to a high, non-physiological production of luteolytic PGF$_2$α in cattle during the estrous cycle and early pregnancy (Woclawek-Potocka et al. 2005, Pietrowska et al. 2006).
3. AIMS OF THE STUDY

The aims of the research included in this thesis were:

I: To use an expedient High Performance Liquid Chromatography (HPLC) method for the quantitative analysis of isoflavonoids, particularly for optically active equol, and to quantify red clover silage isoflavonoids by HPLC with ultraviolet-visible or fluorescence detectors using authentic reference compounds.

II: To study whether the variety, growing conditions, growing site and cutting time have an effect on the isoflavone content of red clover.

III: To determine whether formononetin in red clover silage fed to dairy cows is a source of equol in their plasma and milk. In addition, the influence of various harvesting strategies of red clover on the equol content of plasma and milk was examined.

IV: To study conception and early gestation of nulliparous Finnish landrace ewes fed red clover rich in formononetin. The hypothesis was that red clover feeding has negative effect on ovulation processes (number of ovulations and embryos) and reproductive organs.

V: To assess the amounts of isoflavonoids daidzein, genistein, formononetin, biochanin A, O-demethylangolensin, and especially equol, in both organic and conventionally produced commercial milk.
4. MATERIALS AND METHODS

4.1. Animals, study design and sampling

4.1.1. Animals

Articles II and III are based on one study comprising twenty nulliparous Finnish landrace ewes. The ewes were between 4.9 and 5.4 months old and weighed 30–38 kg at the beginning of the experiment. The experiment lasted for 23 weeks, from the 15th of August 2002 to the 22nd of January 2003, until the ewes were slaughtered. Ewes were fed either experimentally harvested red clover silage, or timothy/meadow fescue (*Phleum pratense* L./*Festuca pratensis* Huds.) grass silage, preserved in large wrapped bales.

Twelve Finnish Ayrshire cows in early lactation were used and six different diets investigated in Study IV. Two different grass silage batches were fed, which were prepared from the primary growth of early or late cut timothy-meadow fescue. The four silage batches made of red clover were prepared similarly from the primary growth of early or late cut red clover and their re-growth.

All animal studies were approved by the Ethics Committee of Animal Experiments, University of Helsinki.

4.1.2. Study design and feeding

In Study I four varieties of red clover (Betty, Saija, Ilte and Jokioinen) were cultivated in two different geographical locations (Mikkeli in Southeast and Ruukki in Northwest). During the growing season red clover fields for five cultivars were harvested twice, first in July and then in the autumn (August or September). Each variety was cultivated on four plots. The growing experiments were carried out during the 2003 growing season and repeated in summer 2004.

Twenty nulliparous ewes used in Study II and III were allocated to two groups. One group of ten animals was fed an estrogenic diet consisting experimentally harvested red clover silage, while the other ten ewes served as controls and were fed non-estrogenic timothy/meadow fescue grass silage. Silages were analysed for energy and protein content and chemical composition. Fresh water, mineral and vitamin supplements were available *ad libitum*. The groups had continuous access to silage and special attention was paid to ensure *ad libitum* feeding. Fresh silage was delivered twice a day, in the morning and afternoon, and the daily remainder of silage was always at least 5%. The ewes were weighed weekly to evaluate nourishment supply. The live weight and growth rate of the ewes were later used to evaluate metabolized energy supply, dry matter intake and intake of isoflavones. The breeding period lasted eight weeks. At the beginning of the breeding period, the rams were introduced into the flocks, one ram to each group. To minimize the ram effect, the rams were changed daily among flocks. Six weeks after the end of the breeding period, the ewes were slaughtered and their reproductive organs collected for analysis. During the experiment blood samples were taken every 4th week for isoflavone and urea analysis. Samples for progesterone analysis were collected once a week.

In Study IV, a cyclic change-over design experiment with twelve Finnish Ayrshire cows and six dietary treatments were used. Dietary treatments consisted of six different silages offered *ad libitum* and supplemented with 9.5 kg/d of a concentrate, consisting of a mixture of barley-oats and rapeseed expellers.
The two grass silages were prepared from primary growth of early or late cut timothy-meadow fescue. The four red clover silages were prepared similarly from a primary growth of early (RE, July 1) or late (RL, July 14) cut red clover and their re-growth (RRE and RRL, August 24, respectively). The concentrate mixture was distributed to the cows in six equal doses during the day. In addition, the cows were given mineral supplements daily and they had free access to water. The study consisted of four 21-d experimental periods, with an adaptation period from day 1 to day 13 followed by a sampling period from day 14 to day 21. All feeds given and the leftovers were weighed daily. The milk yields were recorded daily.

4.1.3. Sampling, samples and measurements

In Study I, four red clover varieties were cultivated in four different plots, and each plot was harvested twice during the growing seasons of 2003 and 2004. Two samples were taken from each plot, from both harvests and the two sites used.

In the experiment that produced material for Studies II and III, silage samples for isoflavone determinations were collected from every bale fed. Blood samples for urea and isoflavone determinations were collected four times, every fourth week from the beginning of the breeding season until culling. For the urea analysis, double samples were collected at the same time in the morning on two consecutive days. Blood plasma samples for progesterone determinations were collected once a week in the morning; this was done seven times altogether, after the breeding season until slaughter. To monitor rams' fertility, semen was collected using an artificial vagina at the beginning and at the end of the breeding period.

The reproductive organs of the ewes were collected for analysis. Total weight of the organs, cleaned from adipose and vulvar tissue, was recorded. The total length of cervix was measured. The ovaries were removed and the number of corpora lutea counted. The entire uterus (without cervix), with contents, was weighed. The number of foetuses was counted, the gender determined, and the foetuses were individually weighed. Biparietal diameters (BPD) and crown-rump lengths (CRL) were measured. Foetal fluids, as well as uterine tissue with foetal membranes, were weighed. Histological specimens were taken from udder, teat, vagina, three samples from the cervix (caudal, middle and cranial parts), both horns of the uterus, both ovaries, and cotyledons from the gravid uterus. One ewe with a mummified foetus did not have cotyledons and therefore those specimens were not available.

In Study IV twelve Finnish Ayrshire cows and six dietary treatments were used. All feeds given and the leftovers were weighed daily. The milk yields were recorded daily. During the last week of each period, milk samples were taken from four consecutive milkings and pooled to form one sample per cow and period. Samples from the silages were collected daily during the last week of each period and pooled to form one sample per period. On the last day of each period, blood samples were collected twice, by vacuum puncture of a jugular vein, before morning feeding and 3 h thereafter.

In Study V, twelve organic and four conventionally produced cartons of milk were purchased in random order from ordinary grocery stores. All the cartoons had been processed by Valio, the main dairy company in Finland that collects milk nationwide.
4.2. Analytical methods

4.2.1. Isoflavone analysis

High performance liquid chromatography (HPLC) was used to analyse isoflavonoids in the silage, serum, plasma and milk. Analysis methods were modified from Franke et al. (1998), Lundh et al. (1988a) Antignac et al. (2003) and Sarelli et al. (2003).

The basic instrumentation used was Liquid Chromatograph 1100 (Degasser, Japan); Binary Pump, fluorescence detector (FLD), diode array detector (DAD), analytical column Zorbax Eclipse XDB-C18 (Agilent Technologies, Germany) and ChemStation data system (Agilent Technologies, Germany).

4.2.1.1. Silage samples

Hydrolysis
The silage samples, 5 g, were first chopped and crushed, and then shaken in 25 ml water. After incubation for 60 min at ambient temperature, 10 ml of 3.5 M hydrogen chloride (HCl) and 80 ml ethanol were added to hydrolyse the isoflavone compounds. The mixture was shaken and heated to boiling and cooled and stored in a refrigerator at +6 °C.

Extraction, filtration and dilution
Silage sample extraction with 50 ml ethanol was repeated three times by shaking for 1 min. The silage sample, container and funnel were rinsed with 100 ml ethanol and the combined ethanol extracts filtered through a Buechner funnel (filter paper Whatman 40). The extract was evaporated into a 20 ml volume and transferred into a 50 ml volumetric flask and filled to mark with ethanol. After 48–72-h storage in a refrigerator, the ethanolic silage extract was filtered (0.45 μm GHP Acrodisc GF-filter) and dilutions were prepared into 10 ml volumetric flasks.

External standards
Concentrations of external standards (genistein, daidzein, biochanin A, coumestrol and formononetin in ethanol) of 0.4–12.5 μg/ml were used. Each calibration curve used for quantification was characterised by a coefficient of determination ($R^2$) better than 0.999. The stock solution concentration used was 25 μg/ml.

Analysis
Samples were analysed using two different HPLC detectors, ultraviolet and/or fluorescence. Authentic reference compounds were used to identify isoflavones and metabolites. The eluents were methanol and water adjusted with trifluoroacetic acid (TFA) to pH3. Ultraviolet detections (UV) at 262 nm and fluorescence excitation at 254 nm and emission 465 nm were used. Daidzein, genistein and biochanin A were analysed with the UV detector, coumestrol and formononetin with the fluorescence detector.
Analytical conditions
Two solvents were used during HPLC analysis: TFA adjusted water (A) and methanol (B). The gradient used was as follows: 0 mins, 46% B in A; 0-23 mins, 46%-100% B in A (linear gradient); 23-25 mins, 100% B; 25-32 mins, 46% B in A (isocratic). Flow was 1 ml/min. Injection volume was 10 μl and column oven temperature was +40°C.

4.2.1.2. Serum and plasma samples

Hydrolysis
Flavone 200 μl (12.5 μl/ml in ethanol) was added to centrifuge tubes and evaporated to dryness under a stream of nitrogen. One ml of serum or plasma sample, 100 μl of β-glucuronidase, 80 μl sulfatase and 820 μl of sodium acetate buffer (0.2 M, pH 5.0) were added. The mixture was shaken and tubes incubated in a mixer for 18 h at +37 °C.

Sample clean-up and extraction in ether
After incubation, 100 μl of glacial acetic acid and 5 ml of diethyl ether were added. The mixture was shaken and centrifuged, refrigerated and centrifuged again. The organic phase was transferred into centrifuge tubes. Extraction procedure was repeated twice. Finally combined extracts were evaporated to dryness under a stream of nitrogen.

Dilution
After evaporation, 150 μl methanol (MeOH) and 50 μl of sodium acetate buffer (0.2 M, pH 5.0) were added. The mixture was shaken and centrifuged. The samples were filtered into a HPLC microvial. Mixture of standards (biochanin A, daidzein, formononetin, genistein, O-DMA, equol and flavone) was added to a centrifuge tube, evaporated to dryness under a stream of nitrogen and 150 μl methanol and 50 μl of sodium acetate buffer were added and vortexed. Samples were split into two equal portions and stored in ampoules at +6 °C. Calibration was performed daily. Each calibration curve used for quantification was characterized by a coefficient of determination (R²) better than 0.999. When higher concentrations than those in calibration curve were present, the sample volume was diluted twofold.

Analysis
For analysis, MeOH/sodium phosphate buffer eluent (pH 6) was used. Ultraviolet detection was done at 281 nm, fluorescence excitation at 254 nm and emission at 465 nm. Flavone was added to samples and standards to calculate the recoveries.

Analytical conditions
Two solvents were used during HPLC analysis: 10 mM disodium hydrogen phosphate buffer (A) and methanol (B). The gradient used was as follows: 0 mins, 46% B in A; 0-23 mins, 46%-100% B in A (linear gradient); 23-25 mins, 100% B; 25-32 mins, 46% B in A (isocratic). Flow was 1 ml/min. Injection volume was 10 μl and column oven temperature was +40°C.
4.2.1.3. Chiral HPLC analysis

Chiral HPLC analysis of serum sample is based on the synthesis of enantiopure isoflavans and was performed at The Laboratory of Organic Chemistry (Jokela 2011).

4.2.1.4. Milk samples

Hydrolysis
Flavone 200 μl (12.5 μg/ml in ethanol) was added into a 50 ml vial for recovery assessment. The solvent was evaporated under nitrogen and 5 ml of fat free milk was added together with 100 μl of β-glucuronidase (500 units) and 80 μl of sulfatase (40 units). The sample was incubated at 37°C for 2 h.

Sample clean up and elution
After the addition of 1 ml of ammonium acetate buffer (1.5 M) and 7.5 ml of diethyl ether, samples were vortexed for 3 min, centrifuged, chilled at -80 °C, and re-centrifuged at +4 °C. The ether extract was removed and evaporated under a stream of nitrogen at +40°C. This procedure was repeated three times. Sodium acetate buffer (2 ml, pH 5.0) containing 20% methanol was added and samples were centrifuged at 4752 x g for 5 min at +48 °C. The supernatant was then applied onto SPE cartridges that had been activated with 2 ml of methanol and 2 ml of water. After rinsing the cartridges with 5 ml of 5% methanol, samples were eluted with 10 ml of acetonitrile/MeOH (9:1). Samples were evaporated under nitrogen at +60 °C and reconstituted in 150 μl of methanol and 50 μl of 0.2 M sodium acetate buffer (pH 5.0). Samples were then centrifuged and the supernatant was filtered into a vial.

Analysis
Detection was done by both ultraviolet diode (UV) diode array detector (DAD) at 262 nm for flavone, genistein, O-DMA and biochanin A and fluorescence detector (FLD) excitation 254 nm, emission 465 nm for daidzein and formononetin and emission 310 nm for equol.

Analytical conditions
Two solvents were used during HPLC analysis: 10 mM disodium hydrogen phosphate buffer (A) and methanol (B). The gradient used was as follows: 0 mins, 46% B in A; 0-23 mins, 46%-100% B in A (linear gradient); 23-25 mins, 100% B; 25-32 mins, 46% B in A (isocratic). Flow was 1 ml/min. Injection volume was 10 μl and column oven temperature was +40°C.

4.2.1.3. Identification and recovery

Isoflavones and metabolites were identified using authentic reference compounds as external standards in all studies. In Study V, calibration curves were established in the concentration range of 1.172–37.5 μg/ml for equol and 0.391–12.5 μg/ml for the other compounds with a correlation over 0.999. In Study IV, calibration curves were established in the concentration range of 1.172–75 μg/ml for equol and 0.195–12.5 μg/ml for the other compounds with a coefficient of determination (R²) better than 0.999. The limit of detection (LOD) and limit of quantitation (LOQ) were determined at signal-to-noise ratios of over 3 and 10, respectively. Identification was confirmed by comparing the UV-spectra of the eluting peaks with reference
compounds. Flavone was used as a standard to calculate the recovery in serum, plasma and milk analysis. The recovery level for flavone was 93 ± 6% in Study II, 80 ± 6% in Study V, and 93 ± 11% in Study IV.

4.2.2. Plasma analyses (Study III)

4.2.2.1. Urea analysis

Urea was measured with an automatic analyser (KONE Pro Selective Chemistry Analyser, Thermo Fisher Scientific, Vantaa, Finland). An enzymatic spectrophotometric method (Gutmann and Bergmayer 1974) was used to determine urea concentration in plasma.

4.2.2.2. Progesterone analysis

The concentration of progesterone was measured using a radioimmunoassay commercial kit (Coat-A-CountR Progesterone, Diagnostic Products Corporation, Los Angeles, USA). All samples were analysed in a single run. The intra-assay coefficients of variation were 5.9, 6.8, and 3.7% at the levels 5.5, 9.6, and 42.9 nmol/l, respectively. The detection limit of the assay was 0.3 nmol/l.

4.2.3. Examination of animals (Study III)

4.2.3.1. Estimation of the duration of pregnancy

The estimation of the duration of pregnancy on ewes was based on foetometry. The age of each foetus was estimated in two ways based on biparietal diameter (BPD) and crown-rump length (CRL). The calculations based on BPD were performed according to the formula “age = 21.4+1.85*(BPD cm)” presented by Haibel and Perkins (1989), where the BPDs were obtained from ultrasonographic measurements in Finnsheep. The foetal age based on CRL was calculated using the formula “age (in days) = 69.88*ln(CRL cm)-130.39”. If the age was less than 75 days, the formula “age (in days) = 43.08*ln(CRL cm)-47.68” was used (Joubert 1956). A mean estimated age for each foetus was then calculated from these two approximations. The difference between these two calculations (age based on CRL and BPD measurements) was 2.3±1.4 days, with a maximum difference of 5 days. The age/duration of the pregnancy was arbitrarily decided to be the age of the largest foetus. In pregnancies with more than one foetus, the difference in the estimated age between the largest and smallest foetus was 3.0±1.7 days, with a maximum difference of 6 days. After evaluating the stage of pregnancy, as described earlier, the progesterone results for the ewes were adjusted to correspond to the stage of pregnancy. For the statistical analysis, a period was selected during which progesterone results were available from at least six ewes in both groups in each week. The differences in progesterone concentrations between the groups could be analysed from the eighth to thirteenth week of pregnancy.

4.2.3.2. Semen and histopathological examination

In the Study III semen samples from both rams were analysed to ensure their fertility at the rams at the
beginning of the breeding period. In one ejaculate from each ram, the sperm motility was 60% and 75%, total number of sperm $3.2 \times 10^9$ and $3.0 \times 10^9$, and sperm concentration $4.2 \times 10^9$/ml and $4.0 \times 10^9$/ml. Both rams were considered to be of normal fertility.

The Pathology Unit of the Faculty of Veterinary Medicine, University of Helsinki performed the histopathological examinations (cervix, mammary gland, vaginal epithelium, uterine tissue, cotyledons, ovaries) using standard processing methods and haematoxylin-eosin staining.

4.3. Statistical analyses

In Study I, experimental design was a completely randomized block design with four blocks (replicates) at both experimental sites. The herbage crop was harvested twice during summer and the trial was repeated the following year using the same blocks. Data collected were subjected to mixed model variance analysis, using the MIXED procedure of Statistical Analysis System (SAS Institute Inc, Cary, NC, USA) to identify significant treatment effects and interactions. Arithmetical mean values for isoflavones were calculated for both growing season (the years 2003 and 2004), for sites (Mikkeli and Ruukki), harvests (July and September) and cultivars (Betty, Saija, Ilte and Jokioinen). The statistical model included year, experimental site, harvests and cultivars and all their interactions. Replicate (year * experimental site) was a random factor in the model. Residuals for the entire data set were checked for normality and outliers using the UNIVARIATE procedure of SAS. The data comprised 128 observations for daidzein and genistein, but as for formononetin, biochanin A and total isoflavones two observations were considered outliers and excluded from the analysis. In the variance analysis, natural logarithmic data transformations were used. Post-anova comparisons between the treatments were made using a Tukey's test. Significant differences among treatment were considered at $P < 0.05$.

In Study III, data were analysed using SPSS software, version 11.0 for Windows. Differences between the groups in carcass weight, corrected live weight gains (the effect of weight of the uterus with its contents removed), time of conception, number of ovulations, number of foetuses, length and width of the cervix, and length of teats were tested using a Mann-Whitney test. Differences in live weight gains and urea concentrations were analysed by repeated measures analysis of variance, with feeding group as the between-subject factor and time as the within-subject factors. Differences in progesterone concentrations were analysed by repeated measures analysis of variance, with feeding group as the between-subject factor and stage of pregnancy as the within-subject factors. Significances of time/stage effects and time/stage by feeding group interaction effects were evaluated using Greenhouse-Geisser-adjusted $P$-values. The effect of feeding group on weight of foetuses (mean and total), uterus with its contents, foetal fluids and uterine tissue, including placetas and foetal membranes, were studied using covariance analysis with stage of pregnancy, number of foetuses and corrected daily weight gain as covariates. The differences were considered significant at $P<0.05$.

In Study IV, the daily intake of isoflavonoids, concentrations of isoflavonoid compounds in the plasma and milk samples, as well as their daily secretion into the milk, were calculated and subjected to ANOVA, using the mixed procedure of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA). Intake of isoflavonoid compounds and secretion into the milk were based on the mean values from the last 6 d of the experimental period. The statistical model used included block, period, block x period interaction and treatment as fixed factors, with the cow within the block as the random factor. The sums of squares of the treatment effects
were further divided into the following pre-planned single degree-of-freedom comparisons: (1) effect of cut number of red clover silage diets; (2) effect of growth stage of red clover silage diets; (3) interaction between the cut number and growth stage of red clover diets; (4) effect of plant species, i.e. red clover vs. grass silage diets; (5) interaction between the plant species and growth stage of the silage diets.
5. RESULTS

5.1. Isoflavone concentrations and intake (Studies I, II, III and IV)

The main isoflavones and their concentrations in four red clover cultivars analysed in Study I were formononetin 6.0–7.9 mg/g in DM and biochanin-A 3.7–6.1 mg/g in DM. The concentrations of genistein, 0.5–0.6 mg/g in DM and daidzein 0.2–0.3 mg/g in DM, were considerably lower than formononetin concentrations. The total isoflavone and formononetin concentrations were highest in the variety Ilte, under poor weather conditions (2004), at the more northerly location and for autumn harvest (Tables 2 and 3). The total and formononetin concentrations (mg/g DM) for different red clover cultivars on first (H1) and second (H2) harvest in years 2003 and 2004 are presented in Figures 4 and 5. Concentrations from cultivar Bjursele, grown on the exclusion areas of the trial fields, are also presented in the figures. All five cultivars are used in Finland.

Table 2. Isoflavone concentrations of red clover, mg/g dry matter. Natural logarithmic transformed means are presented in parenthesis.

<table>
<thead>
<tr>
<th>Year</th>
<th>Experimental site</th>
<th>Harvest number</th>
<th>Cultivar†</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>2004</td>
<td>South-Finland</td>
<td>North-Finland</td>
<td>H1</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.31 (−1.20)</td>
<td>0.23 (−1.51)</td>
<td>0.25 (−1.44)</td>
<td>0.30 (−1.27)</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.51 (−0.70)</td>
<td>0.53 (−0.65)</td>
<td>0.52 (−0.67)</td>
<td>0.52 (−0.67)</td>
</tr>
<tr>
<td>Formononetin</td>
<td>6.26 (1.82)</td>
<td>7.40 (1.98)</td>
<td>6.60 (1.86)</td>
<td>7.06 (1.93)</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>4.13 (1.38)</td>
<td>5.22 (1.61)</td>
<td>4.39 (1.44)</td>
<td>4.95 (1.55)</td>
</tr>
<tr>
<td>Total</td>
<td>11.2 (2.40)</td>
<td>13.4 (2.57)</td>
<td>11.7 (2.44)</td>
<td>12.8 (2.53)</td>
</tr>
</tbody>
</table>

†Superscripts indicate differences (a,b P=0.05; c,d,e P<0.001) between the four red clover varieties. SE=standard error.
Table 3. P-values from the analysis of variance of the isoflavone concentration of four red clover cultivars grown for two years at two experimental sites and harvested twice a year.

<table>
<thead>
<tr>
<th></th>
<th>Daidzein</th>
<th>Genistein</th>
<th>Formononetin</th>
<th>Biochanin A</th>
<th>Isoflavone total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>0.0017</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Experimental site</td>
<td>0.0495</td>
<td>NS</td>
<td>0.0020</td>
<td>0.0060</td>
<td>0.0013</td>
</tr>
<tr>
<td>Year x Site</td>
<td>NS</td>
<td>0.0181</td>
<td>NS</td>
<td>0.0196</td>
<td>NS</td>
</tr>
<tr>
<td>Cultivar</td>
<td>&lt;0.0001</td>
<td>0.0227</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Harvest</td>
<td>0.0002</td>
<td>0.0033</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Year x Harvest</td>
<td>&lt;0.0001</td>
<td>0.0031</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Site x Harvest</td>
<td>0.0020</td>
<td>NS</td>
<td>NS</td>
<td>0.0011</td>
<td>0.0268</td>
</tr>
<tr>
<td>Cultivar x Harvest</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>0.0074</td>
<td>0.0048</td>
</tr>
<tr>
<td>Year x Cultivar x Harvest</td>
<td>NS</td>
<td>NS</td>
<td>0.0160</td>
<td>NS</td>
<td>0.0419</td>
</tr>
<tr>
<td>Site x Cultivar x Harvest</td>
<td>NS</td>
<td>0.0269</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, non significant (P >0.05). Interactions for Year x Cultivar, Site x Cultivar, Year x Site x Cultivar, Year x Site x Harvest and Year x Site x Cultivar x Harvest were all non significant.

Figure 4. Total isoflavone concentrations (mg/g dry matter) for different red clover cultivars at first (H1) and second (H2) harvest in 2003 and 2004.

In the feeding experiment in Studies II and III, the ewes were fed red clover silage which containing on average 3.8 mg/g of biochanin A, 0.7 mg/g of genistein, 0.6 mg/g of daidzein, and 6.8 mg/g of formononetin in DM. Timothy/meadow fescue grass silage did not contain any isoflavones. There was no coumestrol in either silage. In the group fed red clover, estimated daily intake of isoflavones was 6 g biochanin A, 1 g genistein, 1 g daidzein and 10.5 g formononetin.
5.2. Isoflavones and their metabolites in the blood of ruminants (Studies III and IV)

With ewes fed red clover, a major part of the formononetin ingested was metabolized, contributing to the increase of equol in serum. The concentration of equol remained at a constant average level of 7.7 mg/l during the feeding period. In chiral HPLC analysis, the serum of ewes contained solely (S)-equol. Formononetin was found at an average concentration 0.073 mg/l. Furthermore, O-DMA was present in the serum of ewes fed red clover silage, with an average concentration of 0.35 mg/l. No daidzein, genistein or biochanin A were found from serum samples of red clover fed ewes (Table 5).
Table 5. Mean amounts mg/l (± s.d.) of isoflavones and their metabolites in serum samples of ewes

<table>
<thead>
<tr>
<th>Date</th>
<th>Biochanin A</th>
<th>Genistein</th>
<th>Daidzein</th>
<th>Formononetin</th>
<th>O-DMA</th>
<th>Equol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group E = red clover diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.10.2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.078 (0.046)</td>
<td>0.32 (0.12)</td>
<td>8.6 (1.6)</td>
</tr>
<tr>
<td>14.11.2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.071 (0.036)</td>
<td>0.42 (0.14)</td>
<td>9.0 (1.2)</td>
</tr>
<tr>
<td>12.12.2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.067 (0.041)</td>
<td>0.11 (0.074)</td>
<td>5.6 (2.6)</td>
</tr>
<tr>
<td>14.01.2003</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.076 (0.043)</td>
<td>0.55 (0.22)</td>
<td>7.9 (1.3)</td>
</tr>
<tr>
<td>Average</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.073 (0.041)</td>
<td>0.35 (0.22)</td>
<td>7.7 (2.2)</td>
</tr>
<tr>
<td></td>
<td>Group C = timothy/meadow fescue diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.10.2002</td>
<td>0</td>
<td>0</td>
<td>0.13(0.15)</td>
<td>0</td>
<td>0.59 (0.30)</td>
<td>0.86 (0.44)</td>
</tr>
<tr>
<td>14.11.2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12.12.2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14.10.2003</td>
<td>0</td>
<td>0</td>
<td>0.032 (0.091)</td>
<td>0</td>
<td>0.15 (0.30)</td>
<td>0.29 (0.49)</td>
</tr>
</tbody>
</table>

When red clover was fed to cows, low concentrations of formononetin, 0.004–0.035 mg/l, were found in the plasma. The equol concentration in plasma was 4.6-8.4 mg/L, but also 0.2–0.4 mg/l of O-DMA was found.

The intake of formononetin (x) was strongly associated with the equol concentration in plasma ($y=0.071x + 2.75, R^2 0.71$). The equol contents in plasma were significantly higher (P<0.01) for the cows fed red clover than for those fed grass silage. The equol contents in plasma were significantly higher (P<0.001) for cows fed early cut red clover silages with the shortest growth time as compared with those fed with late cut red clover (Table 6).

Table 6. Isoflavones and their metabolites in plasma and milk of cows fed primary or regrowth of grass or red clover silage harvested early or late.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Grass</th>
<th>Red clover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest</td>
<td>Primary growth</td>
<td>Primary growth</td>
</tr>
<tr>
<td>Silage diet</td>
<td>GE</td>
<td>GL</td>
</tr>
<tr>
<td>Intake of isoflavonoid phyto-oestrogens (g/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Formononetin</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total†</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Concentration in plasma (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equol</td>
<td>0.84</td>
<td>1.50</td>
</tr>
<tr>
<td>Equol 4th period</td>
<td>0†</td>
<td>0†</td>
</tr>
<tr>
<td>O-DMA</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Formononetin</td>
<td>0.00</td>
<td>0.002</td>
</tr>
<tr>
<td>Total</td>
<td>0.86</td>
<td>1.52</td>
</tr>
<tr>
<td>Concentration in milk (μg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equol</td>
<td>171</td>
<td>287</td>
</tr>
<tr>
<td>Equol 4th period</td>
<td>0†</td>
<td>0†</td>
</tr>
<tr>
<td>Secretion into milk (mg/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equol</td>
<td>4.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

GE, grass early; GL, grass late; RE, red clover early; RL, red clover late; RRL, regrowth of RL; RRE, regrowth of RE; O-DMA, demethylangolensin.

* Contracts: C1, Cut number of red clover; C2, Growth stage of red clover; C3, C1 x C2 interaction; C4, Plant species i.e. red clover v. grass; C5, Plant species x growth stage interaction.

† Genistein and biochanin A are included in total intake of phyto-oestrogens.

‡ Possibility for stealing of red clover silage was totally prevented during the fourth experimental period.
5.3. Examinations of the ewes (Study III)

The ewes fed red clover had significantly (P<0.001) higher concentrations of urea in their blood compared with control ewes during the entire follow-up period. In the red clover group, the concentration of urea was highest, and the difference between the groups was greatest during the breeding period (Figure 6).

Figure 6. The mean (+ s.d.) serum urea concentrations in ewes red clover (RC) or grass silage (G) during the experiment. The difference is statistically significant at every time point (p<0.001).

Figure 7. The mean (+ s.d.) plasma progesterone concentrations in red clover (RC) and grass silage (G) fed ewes. The difference is statistically significant at every time point (P < 01).

Progesterone concentrations in the red clover group were significantly (P<0.01) lower during the entire period analysed (Figure 7). No significant differences were detected in histopathological samples from cervix, mammary gland, vaginal epithelium, and uterus when the red clover group ewes were compared with grass-fed control ewes.
5.4. Effect of red clover feeding on the performance of ewes (Study III)

Daily weight gains of the ewes were 222 and 180 g/day in the red clover and grass fed control groups, respectively. Despite the planned isoenergetic diets, the ewes fed red clover gained weight faster (P<0.001), and were significantly heavier (P<0.01) at the end of the experiment. A similar significant difference (P<0.001) was detected in the carcass weights at slaughter, the weights being 30.5±1.5 and 25.6±2.1 kg in red clover and grass fed control group, respectively.

All ewes in both groups became pregnant. There were no significant differences in the time of conception, numbers of foetuses per pregnancy or numbers of ovulations. Feeding red clover silage did not have any effect on the mean or total weights of foetuses. However, the total mass of the uterus with its contents was significantly greater (P<0.01) in the red clover group compared with that in control group. This difference was mainly explained by the greater amount of foetal fluids in the red clover group (P<0.01).

5.5. Equol in milk (Study IV and V)

The equol concentrations in the milk of cows fed red clover were 458–643 μg/L and daily secretion of equol in milk was estimated to be 12–19 mg/day. The intake of formononetin (x) was only weakly associated with equol concentration in milk (y = 0.0035x + 0.358, R² 0.20). The equol contents in milk were significantly higher (P<0.01) for the red clover than for the cows fed grass silage. No O-DMA was found in milk (Table 6).

Daidzein, genistein, formononetin, biochanin A, O-DMA, and equol concentrations were analysed from 12 organic commercial milk samples and from four conventionally produced control milk samples. Organic skimmed milk contained 411±65 μg/l of equol, some equol, 62 ±16 μg/l, was present in conventionally produced control milk samples. Furthermore, formononetin was detected in organic milk samples but not in conventionally produced milk. Similarly, some daidzein was detected in organic milk samples, but due to the coelution of impurities it was not possible to quantify the amounts. There was no daidzein in conventionally produced milk samples. No genistein, biochanin A, or O-DMA was detected in either milk samples.
6. DISCUSSION

6.1. Analysis of isoflavonoids

Combined fluorescence and UV detection using the HPLC method provide excellent accuracy and sensitivity for the quantification of isoflavonoids from fodder, blood and milk samples. Isoflavones and metabolites were identified using authentic reference compounds as external standards in all studies. Some reference compounds are commercially available, but the most important ones, enantiopure (S)-equol and (R)-equol, racemic equol and O-DMA, were synthesized in the Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, Finland. Authentic reference compounds are essential in qualitative and quantitative analysis of isoflavonoids in silage, serum, plasma and milk. With chiral HPLC analysis developed by the Laboratory of Organic Chemistry (Jokela 2011) it was possible to study equol present in the serum of ewes. With enantiopure equol as an authentic reference compound in the HPLC analysis of serum of ewes, it is concluded that for long term red clover feeding, (S)-equol and O-DMA are the main isoflavonoids found in the ovine serum. Furthermore, is it reasonable to assume that equol in the milk of ruminants is the (S) enantiomer.

6.2. Isoflavones in red cover

Isoflavone contents of red clover were 6–12 mg/g in DM. The formononetin content of red clover ranged between 3–8 mg/g in DM. Variation depended on the red clover cultivar used (I) and short duration of growing time, e.g. time of harvest (I,IV). Poor growing season conditions such as rain or coldness in northern Finland had a minor effect on isoflavone contents (I). Previously in Finnish red clover silages, isoflavone concentrations were 10–25 mg/g in DM and formononetin content 7–10 mg/g in DM (Kallela et al. 1988, Saloniemi et al. 1995, Sarelli et al. 2003). The present thesis shows that the total isoflavone and formononetin concentrations of red clover in Finland are approximately at the same level as previously reported (Kallela et al. 1988, Saloniemi et al. 1995, Sarelli et al. 2003). Red clover is the major forage legume available for silage production in northern Europe. Since use of red clover is essential in organic production and increasing in conventional farming, it is important to know the levels of isoflavones in red clover feeds for ruminants. All five cultivars studied are in use in Finnish farms. According our results, considerable changes in red clover isoflavone concentrations can be achieved by choosing a suitable cultivar of red clover and with planning the harvest time. By varying the harvesting time, the formononetin content of silage can be doubled from 3 to 6.5 mg/g in DM (Study IV). It is also important to remember that rainy and cold weather during the growing season increase concentrations of isoflavones in red clover. Thus it is suggested that by altering the harvesting strategy red clover based feeds can be manipulated to contain less or more formononetin. These methods can be used to lower the plasma concentration of equol in ruminants especially sheep. The methods can also be used when producing milk with desirable amounts of equol.

6.3. Intake and blood concentrations of isoflavonoids in ruminants

In this thesis, the intake of formononetin (x) was shown to be strongly associated with the equol concentration in plasma of cows \( y = 0.071x + 2.75, R^2 0.71 \). When forage solely consisted of red clover (IV), daily intake of formononetin in dairy cows ranged between 27 and 76 g per day. Similarly, the amount of
Equol in plasma ranged between 4.6 and 8.4 mg/l, respectively. Equol contents in plasma were significantly higher (P<0.001) in cows fed early as compared with late cut red clover silages and significantly higher (P<0.01) for the red clover than for cows fed the grass silage. The equol concentrations in plasma were well in line with earlier studies. Braden et al. (1971) fed freshly cut red clover forage to heifers and reported plasma equol concentrations of between 1 and 6 mg/l. In the studies of Lundh et al. (1990) the daily intake of formononetin of dairy cows was 13–14 g, the equol concentration was 2 mg/l in plasma. In an earlier study with an intake of 3.5 g formononetin, the maximum equol level of 0.27 mg/l in plasma was detected (Lundh et al. 1988a). It is obvious that even small amounts of red clover can produce measurable amounts of equol in plasma of cows. This was detected by chance in Study IV. Slight intake of red clover was difficult to prevent totally in the feeding experiment and as a consequence equol was found in small quantities in the plasma of grass-fed cows as well, despite the lack of isoflavones in the grass silage.

In the ad libitum red clover feeding, the average formononetin intake of ewes (II) was around 10 g per day when red clover contained 6.8 mg/g formononetin in DM. Shutt et al. (1970) concluded that equol accounts for most of the phytoestrogenic activity in sheep fed on clover containing high levels of formononetin. In that study, a high intake of isoflavones, 9 g per day, of which 60% was formononetin, increased the concentration of equol conjugates in plasma to 3–4.4 mg/l. Later Lundh et al. (1990) fed 2.7–3.5 g of formononetin in red clover to wethers and found approximately 1–2 mg/l of equol in plasma. In New Zealand red clover varieties with high formononetin (high F) 9.9 mg/g in DM and low formononetin (low F) 2.7 mg/g were compared. The blood equol concentrations for sheep caused by low F and high F varieties were 1.81 mg/l and 7.25 mg/l, respectively (Anwar 1993). In our study with the daily intake of 10 g, we found 7.7 mg/l of equol in hydrolysed serum samples. This is in good agreement with the earlier results. The same two varieties from New Zealand were compared in the study of Kramer et al. (1996), and the cultivars had 5.7 mg/g formononetin in DM (high F) and 2.5 mg/g in DM (low F). During the grazing experiment conjugated equol concentrations were 0.24 mg/l in high formononetin pasture and 0.08 mg/l in low formononetin pasture. Also Moorby et al. (2004) reported low concentrations of equol, 0.09 mg/l, in their study where lambs grazed on red clover pasture having a mean formononetin concentration of 4.7 mg/g in DM. Explanation for these differences must be grazing, where a ewe can choose what to eat whereas in feeding trials by Shutt et al. (1970) and Lundh et al. (1990) sheep were fed indoors and feed intake more closely monitored.

It can be concluded that formononetin intake is strongly associated with the equol concentration in plasma and the total equol values in plasma of ruminants can range between 4-8 mg/l in Finland when pure red clover is fed. This thesis provides basic knowledge on formononetin and equol levels, if a further evaluation on injurious or positive effects of red clover feeding in ruminants is needed. Side effects of red clover feeding arise in discussion periodically, since the isoflavone content of red clover is well known. The rarity of documented isoflavonoids-related fertility problems in cattle is not known.

6.4. Effects of red clover feeding on the performance of ewes

The red clover silage contained abundant amounts of isoflavones, but feeding did not affect the fertility of ewes. There were no differences in the numbers of foetuses per pregnancy. The mean plasma progesterone concentration was significantly lower in ewes fed red clover than those fed grass during the entire follow-up period. The total uterine weight including contents was significantly heavier in the ewes fed red clover than for those fed grass, this difference in weight being mainly due to the greater volume of foetal fluids.
Other studies, mainly from Australia, show that estrogenic pastures significantly reduce the number of ewes in estrus, the ovulation rate and the fertilization rate (Marshall 1973, Lightfoot and Wroth 1974, Obst and Seamark 1975, Anwar 1993). In New Zealand grazing a pure red clover sward (formononetin between 8-12 mg/g in DM) before and during mating reduced the incidence of estrus and ovulation rates, and resulted in more returns to service when compared with ewes grazing white clover-grass pastures (Kelly et al. 1980). Later Kelly et al. (1982) studied estrous response of pasture isoflavones in high and low fecundity ewes grazing red clover with high formononetin (7 mg/g in DM) or ryegrass-white clover (control). In their study mean ovulation rates were consistently, but not significantly, lower in the red clover group compared with the control ewes. The Finnish landrace breed produces 1.8 and 2.5 lambs on average at first and later pregnancies, respectively (Parikka 2010). Thus, the fecundity of ewes was not compromised by the relatively high formononetin intake. On the contrary, the fertility, when evaluated as lambs per pregnancy, of ad libitum fed ewes was numerically slightly higher than the mean national figures for this breed. In our study the effect of equol was tested with a small number of ewes from high fecundity breed. Even if the small number of ewes clearly limits the value of this feeding trial, it can be concluded that landrace ewe fertility is not easily weakened with high plane of feeding.

The red clover silage appeared to be tastier than the grass silage used as the control diet and the sheep ate it readily. Red clover diets with high crude protein promote growth and increase live weight gain (Fraser et al. 2004). It has also been suggested that elevated formononetin content in red clover can increase the growth of lambs (Moorby et al. 2004). Red clover pastures promote weight gain in ewes and lambs compared with control feeds (Speijers et al. 2005,Graves et al. 2012). High crude protein intake in red clover leads to significantly higher plasma urea concentrations. It has been speculated that excess dietary urea could negatively affect viability of embryos (Mcevoy et al. 1997), but in our study there were no differences in fecundity between the feeding groups.

Interestingly, the mean plasma progesterone concentration was significantly lower in ewes fed red clover than in control ewes during the entire follow-up period. A similar low progesterone level was detected with estrogenic Yarloop clover feeding, but from the 90th day of pregnancy onward. The resulting abnormally high plasma estrogen:progesterone ratio was the suspected cause of lambing difficulties, such as incomplete dilatation of the cervix (Obst et al. 1971, Obst et al. 1972, Obst and Seamark 1975). Plasma progesterone concentrations at different levels in feeding regimes have been studied intensively. Increasing dietary intake has been shown to reduce peripheral progesterone concentrations. It has been concluded that high feed intake increases liver blood flow and metabolic clearance, which leads to lowered plasma progesterone concentration (Parr et al. 1993a, Parr et al. 1993b). These diet-induced alterations are mediated by insulin signalling (Smith et al. 2006). A high level of nutrition during mating or pregnancy leads to lower progesterone concentration (Boone et al. 1975, Smith et al. 2006) and low feed intake can increase plasma progesterone concentration (Shevah et al. 1975). In some studies, overfeeding during mating led to lower pregnancy rates (Parr et al. 1987). It remains somewhat open as to whether the decreased progesterone concentration (Study III) induced by feeding red clover was due to increased feed intake only or also due to isoflavones.

The total uterine weight including contents was significantly heavier in the ewes fed red clover than in those fed grass. The difference in weight was mainly due to the greater volume of foetal fluids. Although red clover ewes gained weight more rapidly and were heavier at the end of the experiment than control ewes, this is probably not the explanation for the finding, because in this case the foetuses would have been expected to be heavier also. The classical way of measuring the estrogenic activity of fodder has been the uterine weight bioassay, which is based on the uterine weight responses to synthetic estrogens and estrogenic pastures.
(Morley et al. 1964, Bennett and Dudzinski 1967). In a more recent study, uteri of diethylstilbestrol-exposed lambs were heavier than those of a control group. Uteri showed more oedema and cellular proliferation (Morrison 2003). Estrogenic stimuli can clearly increase uterine weight, but knowledge is lacking about its effect on volume of foetal fluids, although it is hypothesised that the estrogen/progesterone ratio could affect the amount of foetal fluids (Wintour et al. 1986). A more obvious explanation for greater volume of foetal fluids might be excess urea in the maternal and foetal blood due to high plane of nutrition. This might lead to excess of foetal urine and greater volume of foetal fluids.

6.5. Equol in milk

The intake of formononetin was strongly associated with the equol concentration in plasma. Milk equol is clearly derived from the formononetin in red clover silage. Timothy–meadow fescue silages used as controls did not contain isoflavonoids or coumestans. The intake of formononetin and equol concentration in milk are not so strongly associated, suggesting that a relatively small amount of formononetin is secreted into milk as the metabolite equol. In addition, the transfer rate of isoflavonoids from feed to milk may be higher at low intake than at high intake. This could explain the reasonable amounts of equol found in Study IV in the milk of grass silage-fed cows, which were able to snatch small amounts of red clover. Equol concentrations in our studies ranged from 65 to 643 μg/l which is well in line with other studies. King et al. (King et al. 1998) obtained bovine milk samples from different farms in Australia, and the mean equol concentration ranged from 45 to 293 μg/l. Steinshamn et al. (2008) fed clover silage to dairy cows with or without concentrate supplementation. With red clover feeding, daily formononetin intake ranged from 47 to 34 g/d and milk equol content from 364 to 273 μg/l, respectively. In study V Finnish commercial organic skimmed milk contained equol averaging 411 μg/l, whereas that for conventionally produced milk averaged 62 μg/l. The occurrence of isoflavonoids in commercial milk samples was investigated in France and equol concentrations of 14–293 μg/l were recorded. Organic milk samples contained equol averaging 191 μg/l, whereas conventional milk samples averaged only 36 μg/l (Antignac et al. 2003, Antignac et al. 2004, Antignac et al. 2009). Purup et al. (2005) presented similar figures for Danish bulk milk samples (230 and 41 μg/l, respectively). Recently milk equol concentrations were evaluated in several new studies. When different legume-grass silages were compared for milk isoflavone concentration, the silage mixtures with red clover had the highest concentrations of formononetin and daidzein and produced high average amounts of equol, 1297-1494 μg/kg, in milk. Furthermore, large variations of 500–2600 μg/kg in equol concentration of milk between cows were observed (Höjer et al. 2012). In a recent Norwegian study, the concentrations of equol were 1199 and 86 μg/kg, respectively (Adler et al. 2014).

The results presented in this thesis and earlier observations demonstrate that animal feeding does explain differences in the isoflavonoid contents of milk, red clover forage being the main source of equol. Due to its nitrogen-fixing ability, red clover is widely used in organic agriculture, which illustrates why the isoflavonoid concentrations in the organic milk samples are higher than in conventionally produced milk. These results show that cows fed red clover produce more equol in their milk, which may spark further interest in organically produced milk or milk specially produced for high equol concentration. The milk equol may contribute to human equol supply. In the human equol is predominantly a product of intestinal bacterial metabolism from soy foods containing daidzein (Satchell et al. 2005) and cannot presumably be found as such in any food other than milk or blood products of ruminants. The possible health benefits and risks associated with (S)-equol are being intensively studied since equol is classified as a natural selective estrogen.
receptor modulator (Jackson 2011, Mattison 2014). It is suggested that cow milk or blood products, with their unique nutrient contents and with (S)-equol represent an important research theme and this thesis contributes basic knowledge in this field of research.
7. CONCLUSIONS

- Combined fluorescence and UV detection using the HPLC method provide excellent accuracy and sensitivity for the quantitation of isoflavonoids from fodder, blood and milk samples. With enantiopure equol as an authentic reference compound in the HPLC analysis of serum of ewes, it is concluded that during long term red clover feeding, (S)-equol and O-DMA are the main isoflavonoids present in ovine serum. In addition, it is suggested that the estrogenic effects of metabolic equol in sheep reported earlier in the literature are solely or predominantly due to (S)-equol.

- The cultivar choice and time of the harvest affect the formononetin content of fodder. Weather conditions during the growing season as well as growing habitat have an effect on isoflavone concentrations. After a poor growing season and in a cold climate, more isoflavones are detected in the red clover fodder.

- A strong association between formononetin intake and equol concentration in plasma was shown. The equol content in milk can be manipulated by varying the harvesting strategy of red clover. Shorter growing periods for red clover resulted in higher formononetin content in the silage and equol content in the plasma and milk.

- The fecundity of nulliparous Finnish landrace sheep was not reduced by feeding red clover with high isoflavone concentrations for five months before, during and after the breeding season. The amount of foetal fluids was, however, increased, which may increase the risk of vaginal prolapse before term.

- The equol content in cow milk can be as high as 600 μg/l with red clover silage feeding, even though only a small part of the formononetin is secreted into milk as the equol metabolite. Milk equol is derived from the formononetin of red clover silage and milk from cows fed red clover can be considered as a source of equol in human nutrition. Finnish organic commercial milk can contain high levels of equol due to the widespread use of red clover in organic farming.
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