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Review

Fertility of Herbivores Consuming Phytoestrogen-Containing *Medicago* and *Trifolium* Species

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Abstract: Despite their unrivalled value in livestock systems, certain temperate, pasture, legume species and varieties may contain phytoestrogens which can lower flock/herd fertility. Such compounds, whose chemical structure and biological activity resembles that of estradiol-17α, include the isoflavones that have caused devastating effects (some of them permanent) on the fertility of many Australian sheep flocks. While the persistence of old 'oestrogenic' ecotypes of subterranean clover (*Trifolium subterraneum*) in pasture remains a risk, genetic improvement has been most effective in lowering isoflavone production in *Trifolium* species; infertility due to 'clover disease' has been greatly reduced. Coumestans, which can be produced in *Medicago* species responding to stress, remain a potential risk in cultivars susceptible to, for example, foliar diseases. In the field, coumestrol is often not detected in healthy vegetative *Medicago* species. Wide variation in its concentration is influenced by environmental factors and stage of growth. Biotic stress is the most studied environmental factor and, in lucerne/alfalfa (*Medicago sativa*), it is the major determinant of oestrogenicity. Concentrations up to 90 mg coumestrol/kg (all concentrations expressed as DM) have been recorded for lucerne damaged by aphids and up to 600 mg/kg for lucerne stressed by foliar disease(s). Other significant coumestans, e.g., 4'-methoxy-coumestrol, are usually present at the same time. Concentrations exceeding 2000 mg coumestrol/kg have been recorded in diseased, annual species of *Medicago*. Oestrogenicity of some *Medicago* species is also influenced by maturity and senescence. Studies in Israel, North America, Europe, New Zealand and Australia have confirmed that coumestans in lucerne, represent an acute or sub-acute loss of reproductive efficiency in herbivores, e.g., sheep, cattle, and possibly horses. When sufficiently exposed peri-conception, coumestrol, sometimes present in lucerne, be it as pasture, hay, silage, pellets, meal, and sprouts, is associated with what can be an insidious, asymptomatic, infertility syndrome. Most livestock research with oestrogenic lucerne has been conducted with sheep. Ewes may be at risk when the coumestrol concentration in their diet exceeds 25 mg/kg. In studies where lambing was compared for lucerne and a phytoestrogen-free treatment, the mean decrease in lambs born/ewe was 13%; ewes on lucerne, exhibited a lower frequency of multiple births.

Keywords: alfalfa; annual medics; clover disease; coumestans; coumestrol; fertility; isoflavones; lucerne

1. Introduction

The wool industry-supported, Australian Isoflavone Laboratory closed 20 years ago having assisted Australian plant breeders and research workers to alleviate the threat to the fertility of sheep posed by isoflavone-based *clover disease*. The laboratory also accorded considerable, if less, attention to the highly potent coumestans, phytoestrogens often found in *Medicago* species. In the 1960s and 1970s, a number of widespread research groups exposed the risk that coumestans pose, emphasising the significant increase in concentration associated with foliar diseases. In Australia, the surge in plant

breeding that followed the introduction of three new aphid pests of lucerne in 1977 [1], may have led to a perception that the steady and effective genetic improvement of *Medicago* species had improved resistance to diseases and that, as there are but few reports about infertility in *Medicago*-dependant livestock, coumestans need not concern producers.

In recent investigations into mare infertility (unpublished), we detected coumestrol in serum (0–5.7 μg/L). Coumestrol concentration was sometimes >100 mg/kg (all concentrations are expressed on a DM basis) in modern cultivars of well managed lucerne - both pasture and hay. Such samples included pasture where the proportion of foliage that appeared diseased was <15%. We reviewed the literature to identify pasture/fodder products containing phytoestrogens and understand their consequent effects. With coumestans in particular, we sought to understand the factors that stimulate their production, to estimate their likely impact on herbivores, to assess the usefulness of analytical chemistry for guiding plant breeders, clinicians, and producers, and to identify knowledge gaps for research and extension.

2. Phytoestrogens

2.1. Phytoestrogenic Compounds

Oestrogen is responsible for expressing mating behaviour in the female of many animal species. It controls the secretion of gonadotrophic hormones from the pituitary gland, especially luteinizing hormone, and causes hypertrophy and hyperplasia throughout the reproductive tract. Epithelial and stromal cells increase and expand in the uterus. The epithelial cells of the cervix make and secrete mucus; in the vagina they increase and become cornified. Blood flow increases. Effects on the ovary gland and mammary gland include the dysplasia of the ovarian granulosa cells, thus impeding the maturation of the ovarian follicles; the mammary gland secretes milk and teats expand [2].

Phytoestrogens are stable, non-steroidal, natural plant compounds of low molecular weight that are structurally or functionally similar to the endogenous oestrogens of mammals, particularly 17β-estradiol. When ingested, they can mimic the biological activity of oestrogens as they are able to pass through cell membranes and interact with the enzymes and receptors of cells. Their phenolic ring structure enables them to bind to oestrogen receptors of cells so that they can compete with endogenous steroid hormones/oestrogens such as estradiol-17β, and influence the oestrous cycle of many mammalian species [3].

Many phytoestrogenic compounds have been found in more than 300 plant species, including legumes, herbs, nuts, cereals, flax, sesame, and hops. Their function in plants is unclear; they may provide resistance to predators or fungi but are widely recognised for their association with anti-oestrogenic, oestrogenic, and genotoxic effects on herbivores [3,4]. Though common in many grasslands and pastures, oestrogenic plants must make up a major part of the diet before the effects on herbivores become obvious. The main groups of phytoestrogens are the isoflavones, flavones, stilbenes, lignans, and coumestans [3]:

- Isoflavones (e.g., genistein, daidzein, glycitein, formononetin, and puerarin) are primarily found in soybean (Glycine max), chickpeas (*Cicer arietinum*), and some clovers, most notably subterranean (sub.) clover (*Trifolium subterraneum*) and red clover (*T. pratense*) but also white clover (*Trifolium repens*) in which, more importantly, coumestans may also be present [5]. Amongst the *Trifolium* (clovers), 14 of 100 species examined by Francis et al. (1967) [6] were found to have contents comparable with sub. clover. Isoflavones in sub. clover are mainly found in the leaf tissue; the availability of suitable carbon substrate is the major determinant of isoflavone content [7,8]. Elevated isoflavone levels are observed in phosphorous deficient, but not potassium deficient, red clover [8] and in phosphorous deficient sub. clover, where leaf concentration of formononetin may quadruple [7,9]. Rossiter (1969) [10] also noted that isoflavone content may double in nitrogen deficient sub clover. Their concentration increases under other stresses such as water deficit, water-logging and disease. Fungal infection can increase isoflavones in

sub. clover [11]. While all varieties of isoflavone-containing clover species contain isoflavones, only some varieties have contents that result in economically significant oestrogenic potency. The variety Yarloop may contain an average of 4.8% dry weight as isoflavones [12]. The high potency of the Tallarook variety was noted early using a mice bioassay [13]. Lloyd Davies and Bennett (1962) [14] demonstrated the high potency of the cultivars Yarloop and Dwalganup by measuring the increased weight of the uterus and cervix of virgin and aged ewes. Isoflavones disappear when the clover wilts but are maintained by rapid drying and in well-made hay or silage [2,13,15]. Adams [2] summarised much of the research carried out on sub. clover-induced infertility of sheep in Western Australia.

- Flavones (e.g., luteolin, apigenin, querectin, chrysin, kaempherol, and wogonin). Numerous flavones are found in a diverse range of species including lucerne and white clover [16].
- Stilbenes (e.g., resveratrol) [16]
- Lignans (e.g., lariciresinol, matairesinol, and secoisolariciresinol can be metabolized by gastro-intestinal bacteria to the oestrogenic 'mammalian lignins' enterolactone and enterodiol) [16].
- Coumestans: At least 27 coumestans have been described [17,18], many of which have two or three synonyms. Coumestrol [syn 7'12' dihydroxy coumestan] has been found in 58 plants [2], especially legumes, (e.g., lucerne), other perennial *Medicago* (e.g., *M. falcata*), annual 'medics' (*Medicago* spp.), *peas (Pisum sativum*), soybean, limabeans (*Phaseolus lunatus*), pinto beans (*P. vulgaris*), and some clovers (e.g., white clover [5,19,20] and strawberry clover (*T. fragiferum*) [6]). The relative abundance of particular coumestans and flavones that white clover produces in the field varies considerably depending on which plant pathogen stimulates their production [21,22]. The coumestrol content in white clover was considered insufficient to explain the oestrogenic activity it was responsible for [5,20]; other phytoestrogens may be involved. Although it is the most commonly measured coumestan, coumestrol's concentration is likely to underestimate the oestrogenicity of the plant. Other coumestans include 4'-methoxy-coumestrol, 3'-methoxycoumestrol [syn 7,12-dihydroxy-11-methoxycoumestan], 11,12 dimethoxy-7-hydroxy coumestan, coumestrol dimethyl ether [syn 7'12' dimethoxy coumestans], aureol, lucernol, medicagol, repensol, sativol, trifoliol, wairol, wedelactone [syn 7-methoxy-5,11,12-trihydroxy-coumestan], and wedelolactone [17,18].

4'-methoxy-coumestrol is considered the second most important coumestan for oestrogenic activity. In annual medics it is usually detected in amounts somewhat greater than coumestrol, viz. a ratio of from 0.9–1.5 [23–25]. Some New Zealand studies with lucerne found coumestrol was the more dominant compound [26–28]. In their analysis of 5 coumestan-containing pastures and 7 coumestan-containing fodders/concentrates (lucerne - and some annual medics and clover, unnamed), Ferriera-Dias et al. (2013) [29] consistently found a much greater ratio of methoxy-coumestrol:coumestrol (mean for pasture 24.3, range 3.8–44.1; mean for fodder 3.6, range 2.9–6.8).

Most plants containing phytoestrogens include more than one of the above classes. Although the isoflavone content of sub. clover may account for >5% of dry matter [30] and greatly exceed the concentration of coumestrol observed in *Medicago* (Table 1), coumestrol is the phytoestrogen most able to bind to oestrogen receptors. Adams (1989) [2] found it bound with ten-fold the ability of the isoflavone-derived equol. Less than 10% of isoflavones in sheep plasma are present in free form whereas with coumestans, 20%–40% of them may be present in free form [25]. Phytoestrogens and the mycoestrogen zearalenone may be present together in pasture [31] and may have additive effects [2]. The significance of relatively low coumestan concentrations should not be overlooked if the diet contains other xenoestrogens (eg isoflavones and zearalenone). Synthetic xenoestrogens may also impair fertility but lie outside the scope of this review.

Factors controlling phytoestrogen content differ greatly with type; isoflavones are present in green material but disappear rapidly with senescence; coumestans are retained in senescent material [2]. While isoflavones are commonly found in sub. clover, at least at a very low level, coumestrol is often not detected in healthy, vegetative *Medicago* plants.

2.2. Genetic Influence on Phytoestrogen Production

Isoflavone concentration in subterranean clover was a significant characteristic in predicting the long term successful persistence amidst a wide range of diverse varieties [32]. Possibly, they lower its palatability. Although Yarloop was a vigorous winter grower and a relatively productive variety in terms of live weight gain and wool production of sheep (wethers) [33], Hume et al. (1968) [34] found that at maturity, the voluntary intake value of the low oestrogenic variety, Woogenellup, was 36% greater than that of mature Yarloop. Reduced palatability at maturity may reduce seed removal by sheep and help to explain the wide distribution of oestrogenic varieties. Such varieties/ecotypes need not have been deliberately sown; they may occur and spread by natural means such as the ecotypes, Cookardinia and Bookbook, found in Southern New South Wales [35]. They may also, unwittingly, be sown widely as contaminants (e.g., Dwalganup and Dinninup) present in commercial (uncertified) seedlots [36]. Old pastures can be expected to contain a significant proportion of varieties distinct from the commercial varieties that were sown; some out-crossing occurs [32]. Prediction of the oestrogenic potency of sub. clover in old pastures may therefore require analysis for phytoestrogens; reliance on variety identification by leaf and flower markings can be difficult and unreliable.

Plant selection/breeding has been effective for controlling isoflavone content in sub. clover [37] and red clover [38]. In *Medicago* species, genetic diversity for coumestan production has not been apparent. Early research workers advocated that plant breeders seeking to reduce coumestan shouldemphasise select for resistance to foliar pathogens and pests [18].

2.3. Anabolic Effects of Phytoestrogens

With forage legumes, observations need to be studied carefully to separate the phytoestrogenic effect on fertility from the beneficial effect on fertility due to the legumes' nutritive value, which is usually high relative to grass species. Reviews of feeding value have highlighted legume dominant pasture/fodder and its significant benefit relative to nitrogen-fertilised grass pasture/fodder. This benefit is associated with nutritive characteristics such as a high soluble/structural carbohydrate content that facilitates a fast rate of digestion and a high voluntary intake. Other important differences include the greater efficiency with which digested nutrients are utilised and a greater concentration of minerals in favour of legumes. A review of cattle production systems found that some legume dominant pastures significantly enhanced reproductive efficiency [39].

In addition to such nutritive factors, oestrogenic materials, whether fed or implanted subcutaneously, have long been known to improve live weight gain and the efficiency of feed conversion by ruminants [40]. In trials where lambs were fed varying amounts of lucerne that contained coumestrol, progressing from lucerne meals through acetone extracts of different coumestrol potency to a final test of isolated coumestrol, a trend toward a positive growth response to elevated coumestrol levels was obtained with wether lambs, but not with ewes. Marked increases in teat length and in seminal vesicle and pituitary weights were obtained in animals fed the higher levels of coumestrol. Organoleptic tests consistently demonstrated improved tenderness and juiciness scores from lamb roasts for animals fed high-coumestrol diets [41]. Phytoestrogens stimulate protein deposition and live weight gain, both in monogastric and ruminant animals [42]. McClure et al. (1995) [43] found that the benefit, in live weight gain of sheep, when lucerne was compared with ryegrass, was more evident in wethers than in ewes. This effect may be associated with greater hormone production [44]. Pace et al. (2006) [45] fed lambs (27 kg) for a year on either Italian ryegrass (non-oestrogenic) or low oestrogen cultivars of subterranean clover. The clover contained isoflavones (797 mg/kg; formononetin content <80 mg/kg). Diets were supplemented to make them iso-proteic and iso-energetic. The reproductive efficiency of ewes was not affected; for both genders, significant benefits were observed in live weight gain for the clover diet. For the males only, significant benefits of clover included improved carcass and meat characteristics. The anabolic action of phytoestrogen was suggested as the cause for this difference between genders. From studies using young ovariectomised

rats, Nogowski (1999) [46] suggested that the effect of coumestrol is generally anabolic with regard to lipids and catabolic with carbohydrate metabolism and, in part, unrelated to its oestrogenic action.

3. Phytoestrogen Effects on Fertility

3.1. Oestrogenic Activity

Based on changes to the nucleic acid ratio in the uterine tissue of ewes, Little (1976) [47] found lucerne to have moderate oestrogenic activity. Boue et al. (2003) [48] evaluated the oestrogenic effects of seven legume extracts containing phytoestrogens. Methanol extracts were prepared from soybean, bean, lucerne sprout, mung bean sprout (*Vigna radiata*), kudzu root (*Pueraria lobata*), red clover (*Trifolium pratense*) blossom, and red clover sprout. All seven extracts exhibited preferential agonist activity toward oestrogen receptor beta (ERβ). As will be outlined below, there is overwhelming evidence that phytoestrogen exposure can have significant consequences for the reproductive health of a wide range of mammals. Phytoestrogens contained in soy-based proprietary feeds for the US laboratory rodent market were found sufficient to stimulate the uterus in rat and mice [49]. Animal models are significant in the study of phytoestrogens in the diet of humans [50].

Ewes grazing phytoestrogenic pasture exhibit a lowered frequency of multiple births; follicular development may cease [51]. Infertility in cattle is due to anovulation, or the development of cystic follicles. Phytoestrogenic effects depend on the dose and route of exposure, parameters which influence the concentration in serum. Timing of exposure is critical in determining phenotypic effects, different tissues have species-specific windows of sensitivity to morphological and functional disruption. These sensitive windows generally begin in the early prenatal period and extend in some cases through adulthood. Coumestans are produced by some plants as a response to stimuli. When present in forage legumes, their concentration is low relative to that of the isoflavones that are produced in some clovers, independently of stimuli. When coumestrol was given to ovarectomized ewes by intraruminal infusion, they displayed oestrogenic activity that was ~15 times as great as the isoflavones, genistein, biochanin A, and formononetin [52]. Thus, relatively low concentrations of coumestans in the diet are sufficient to influence fertility.

3.2. Mode of Action

The metabolism and physiological effects of phytoestrogens in livestock was reviewed by Cox and Braden (1974) [53]. Where their concentration is sufficient, both isoflavones and coumestans can lower reproduction by a number of means, including failure to ovulate, failure to conceive, and increased embryonic mortality. Phytoestrogens can act as both oestrogen agonists and antagonists thus causing either an oestrogenic or anti-oestrogenic effect. The levels of endogenous oestrogen may influence the actions of the weaker-binding phytoestrogens as the two forms compete [54]. In cows and ewes the activity of endogenous oestrogen is considered low and phytoestrogens have been reported to function primarily as oestrogen agonists, thereby causing an oestrogenic effect [40]. Estradiol-17-β regulates uterine prostaglandin production (mainly PGF2α luteolytic and PGE luteotropic), the fatty acids that regulate the oestrous cycle. As they alter oestrogenic feedback on the pituitary gland or the hypothalamus, phytoestrogens may affect gonadotrophins, stimulating both PGF2α and PGE2 in both cell types of bovine endometrium via an oestrogen receptor-dependent genomic pathway. As phytoestrogens preferentially stimulate PGF2α synthesis in epithelial cells of bovine endometrium, they may disrupt uterus function by altering the PGF2α to PGE2 ratio. This action of phytoestrogens on PGF2α may account, at least in part, for the reproductive disorders observed in ruminants [55]. Differences in their metabolic pathways may explain why coumestrol, which maybe conjugated to both sulphates and glucuronides [2], exhibits greater oestrogenic potency than the isoflavones [56].

In ruminants, phytoestrogens are absorbed from, or microbially degraded in, the rumen; little is excreted. The significance of various phytoestrogens varies with animal species. Rumen microbes, once they have adapted to the presence of genistein and biochanin A, will convert them to non-oestrogenic

phenols; that may take 7–12 days [2]. The significance of those isoflavones is therefore less for ruminant species than for monogastric species. Formononetin and daidzein are reduced and demethylated to a potent oestrogen, equol, and excreted in urine. The metabolism of individual sheep (or their rumen flora) may vary; some excrete the less oestrogenic metabolite 5'methoxy-equol [53]. Their breakdown metabolites are much more active than the phytoestrogens in increasing prostaglandin synthesis [57].

Intraruminally administered genistein, biochanin A, and formononetin may be detected in plasma within 2.5 h [58]. Genistein and daidzein were detected in blood and fat depots following intraruminal administration of biochanin A and formononetin. Such *O*-demethylation at the C4 position was also observed in sheep grazing sub. and red clover following intraruminal administration of biochanin A or formononetin. Plasma levels of free genistein, from 1 to 5 μg/100 ml, were associated with a graded uterine response. Free plasma formononetin and daidzein above 0.5 μg/100 ml seemed necessary for detectable uterotrophic action in a 5-day assay. For some clovers, isoflavone contents were poorly related to levels in plasma.

In the plasma of ewes, concentration of free coumestrol (Section 5) remained steady and appeared to be related to the amount of coumestans ingested when ewes were fed two diets differing in coumestan concentration, intakes of which were 514 and 952 mg/day, maintained over 16 days [25]. Production of cervical mucus remained basal suggesting that ewes became refractory with prolonged exposure to coumestans rather than deactivating them as occurs with the isoflavones genistein and biochanin A.

Phytoestrogens exert their oestrogenic effects primarily though binding to oestrogenic receptor α (in several organs of the female reproductive tract) and oestrogenic receptor β (in the prostate gland, testis, ovaries, lymph nodes, and brain), with a higher affinity for ERβ, and acting as agonists, partial agonists, and antagonists. Phytoestrogens may alter steroidogenesis though inhibition of 17β- and 3β-hydroxysteroid dehydrogenase, aromatase, and 5α-reductase and through stimulation of sex hormone-binding globulin. Cellular growth is inhibited by phytoestrogens' effects on protein tyrosine kinase, DNA topoisomerases, matrix metalloprotein, and vascular endothelial growth factor [4].

The peri-conception period is a time when reproductive performance and the quality of offspring, including foals [59], can be extremely sensitive to dietary factors. These can affect oocytes developing in the follicle as well as the young embryo. Alterations in the diet pre-mating affect oocyte maturity, blastocyst yield, prenatal survival, and the number of surviving offspring. Nutrition at this time can also affect the quality of embryos, resultant offspring, behaviour, and cardiovascular and reproductive function throughout post-natal life [60].

3.3. Clinical Signs

Phytoestrogens stimulate the reproductive tract of ewes and cows and enlarge mammary glands which may secrete a milk-like fluid. Hypertrophic effects are observed in the uterus, external genitalia appear swollen, and increased secretion of cervical mucus may be visible from the vulva. With cattle, oestrogenism signs, a swollen vulva, cervical mucus discharge, behavioural changes, and mammary development, first drew attention to the existence of phytoestrogens. In rodents, horses, cattle, sheep, and pigs, increased ingestion of phytoestrogens, such as genistein or coumestrol, induces marked, oestrogenic, clinical signs including edematous vaginal and cervical tissue, modified development of ovarian follicles, an increase in haemorrhagic follicles, abnormal follicular waves, ovarian dysfunction, early embryonic death, miscarriage, suppression of the hormone surge (viz. luteinizing and follicle stimulating hormones), repeat breeding, and oestrogenic syndrome (viz. mammary gland hypertrophy and milk-like secretions from elongated teats). In the male, alterations in testis development and a decrease in sperm count is induced [3].

Phytoestrogens came to prominence with the devastating effects of sub. clover observed in Australian sheep flocks [2]. The term *clover disease* is used to describe the effects observed in sheep grazing sub. clover in which the leaves of some varieties contain high concentrations of isoflavones (viz. 10–48 g/kg DM) [12,61]. Temporary and permanent infertility have been observed [62].

Ewes present with prolapsed uterus, dystocia, and an inability/unwillingness to deliver. Abnormalities include vaginal prolapse [63]. The main effects are reduced ovulation and delayed oestrus. Lamb marking rates, especially for maiden ewes, can fall to 20%–40% [64,65]. Adverse effects on the rate of egg transport and the number of sperm reaching the site of fertilisation has been suggested [66].

Sheep are considered more vulnerable to isoflavones than cattle [67,68]. Much higher plasma concentrations of equol in cattle compared with sheep when both species were fed red clover-grass silage, suggests that differences in their capacity to detoxify isoflavones does not explain the different vulnerability. The reason remains unclear [69].

Temporary infertility in ewes is associated with hormone disruption and subsequently less multiple ovulations; it resolves itself several weeks after ewes are removed from oestrogenic pasture. A swollen udder or reddened vulva may be noticed but the pathology is frequently subclinical [40]. With sheep, the genes controlling sexual differentiation are not fully deactivated at birth. Prolonged exposure to isoflavones causes trans-sexual re-differentiation in the adult ewe [62]. Permanent infertility results, mainly from disruption of the cervix which undergoes a uterus-like differentiation [70]. Diminished cervical folds hinder transportation of spermatozoa [71]. There is an increase in the cross-sectional area of lamina propria tissue lying underneath the cervical folds, mid-cervix. Histological examination of the cervix revealed that subclinical infertility was widespread in Western Australian flocks [72]. With isoflavone-containing red clover varieties, ewes exposed long-term had a high incidence of abnormalities of the external genitalia, decreased mating performance and fewer multiple births [73,74].

When the potential effect of phytoestrogen levels in the diet of ewes was studied with daily injections of 128 and 32 μg stilboestrol dipropionate prior to and during mating, the fertility of ewes fell almost to zero [75]. Subsequent work, with treatment groups of ~90 ewes, found that 8 μg was sufficient to reduce conception from 75% (nil dose) to 59% and increase the non-pregnant, non-return status of ewes from 4% to 25%. The number with twins declined from 12 to 5, the number with two *corpora lutea* from 19 to 5 and the ovulation rate from 1.29 to 1.09 [76].

Low conception in ewes may be observed at a formononetin intake of 40 mg/kg live weight [77]. The concentration of formononetin in sub clover is in the 0.1–1.5% range and from 0–1% in red clover. A level of at least 0.5% is usually associated with infertility [2]. If their oestrogen intake is 20–100 g/day, ewes frequently die before delivery. In a 4-year study involving two breeds of ewes and 5 cultivars of sub. clover, conception rates were significantly related to the concentration of formononetin in clover leaf [78]. With sub. clover, cultivar Dinninup, ewe cervical mucus bioassay indicated a potency equivalent to almost 40 μg stilboestrol [79]. Ewes artificially inseminated 17–21 days after grazing Dinninup (0.9% formononetin in dry petiole) had a lower proportion in oestrous and lower rates of ovulation ($p < 0.001$) and fertilisation ($p = 0.05$) compared with those on non-oestrogenic pasture. Ovulation rate, corpus luteum weight, and embryo mortality were affected.

4. Coumestans

In response to challenge, perhaps as a means of restricting infection, fungal, bacterial and viral pathogens lead to the formation of aromatic compounds, including coumarin derivatives, in affected plant species [80]. Coumestans are phenolic compounds (aromatic benzene ring compounds with one or more hydroxyl groups) that are produced by plants (Figure 1), mainly for protection against stress. Coumestans are not phenolic alexins, similar compounds which are secreted by perturbed plants and have anti-microbial properties [81]. Coumestan infertility has not attracted as much attention as isoflavone fertility which has been greatly reduced by genetic improvement and by the decommercialisation of 'highly oestrogenic' cultivars of *Trifolium* species. As long advocated [18], the coumestan risk is currently recognized in some plant breeding programs [82].

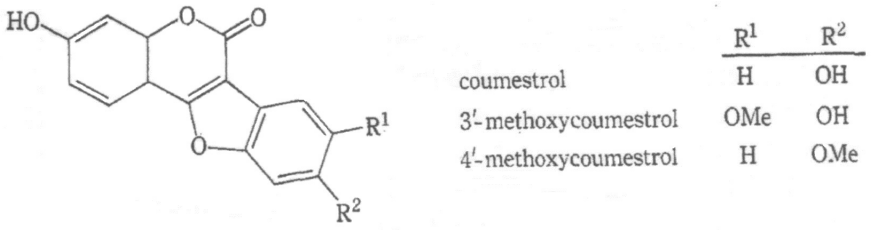

Figure 1. Coumestans common in *Medicago* species [72].

4.1. Coumestans in Annual Medics

Coumestans are usually insignificant in healthy, vegetative annual medics but at times, *Medicago truncatula* cultivars (viz. Jemalong and Cyprus) may contain substantial concentrations of coumestans [83]. Many workers have confirmed this for all the most commercially important species of *Medicago*: both annual (Table 1) and perennial (Table 2). Most workers have simply reported coumestrol; some have also analysed for other coumestans. Some workers have used mouse uterine weight or wether teat-length bioassays or fluorometry [5,84] while most have relied on either high performance liquid chromatography [18] or mass spectrometry to analyse for coumestans. Francis and Millington (1965 a, b) [37,85] found that coumestrol, in both annual *Medicago* spp. and lucerne, increased rapidly with the onset of flowering, especially during pod development (*no fungal pathogens were obvious*); it increased considerably in senescence. For *M. littoralis* (cv. Harbinger), coumestrol increased from 1 mg/kg in cotyledons to 18 (mid-winter), 40 (at first flower), 125 (full flower), 180 (obvious senescence), then at subsequent monthly samplings, the dry material tested 240, 256 and 335 mg/kg. Coumestrol was much greater in old leaves than in young leaves and much greater in old field-dried 'burr' (the prickly covered coiled pods) than in fresh pods. These workers did not measure flowering lucerne or lucerne seed pods, but coumestrol in mature, old leaves of lucerne did not exceed 45 mg/kg. They found that generally, the order of coumestrol concentration was *M. littoralis*>*M. truncatula*> *M. scutellata*=*M. polymorpha*. A survey of medic pastures in Western Australia indicated that levels of foliar disease were generally low and coumestrol concentration frequently exceeded 25 mg/kg (DM basis). On occasions, severe foliar disease and high coumestrol concentrations were observed. In two of the three years, significant positive correlations were obtained between the incidence/severity of spring black stem disease (*Phoma medicaginis*) and coumestrol content for both green and dry plant material [86]. Barbetti (1995) [87] studied four annual species of *Medicago* and also noted that the coumestrol concentration was correlated with incidence of disease. Depending on cultivar, coumestrol in diseased plants compared with fungicide treated plants increased between 230–500 mg/kg in stems and between 30–130 mg/kg in pods. Diseased *Medicago sphaerocarpos* (cv. Orion) produced up to 470 mg/kg in stems; diseased *Medicago truncatula* (cv. Caliph) produced 230 mg/kg coumestrol in stems. Francis and Millington (1971) [88] found that coumestan concentrations (viz. coumestrol + 4′ methoxy-coumestrol + 3′ methoxy-coumestrol) in *Medicago littoralis* of up to 737 and 332 mg/kg in stems and burr respectively; in *Medicago truncatula* stem, the level was 576 mg/kg (the disease status of these plants was not stated). In South Australian field studies conducted in 2013, *Medicago littoralis* (a mix of cvv. Angel, Herald and Jaguar) infected with both powdery mildew (*Erysiphe trifolii*) and spring black stem diseases contained 1050 mg/kg of coumestrol. In 2014, when only the former disease was present, the coumestrol concentration was 210 mg/kg [82]. Genetic diversity for resistance to powdery mildew has been found in this species [84] and the coumestrol concentration for soon to be released cultivars was compared with the commercial cultivars in the above mentioned field studies. In recent field experiments to compare cultivars, the coumestrol concentrations for new and older varieties averaged 37 and 383 mg/kg respectively [82].

Table 1. Coumestrol concentration in annual species of *Medicago.*

Cultivar, if Known	Stage of Growth	Plant Material Analysed	Disease [1]/Pest Status	Coumestrol (mg/kg, DM Basis) [3]	Reference
			Medicago truncatula		
Cyprus	fully podded	leaf	not stated	40–180	[89]
commercial	early podding	leaf	not stated	45–210	
Hannaford [2]	mature	stem	healthy	218 (576)	[88]
Cyprus [2]	mature	stem	healthy	232 (382)	
Jemalong [2]	mature	stem	healthy	132 (272)	
Caliph	mature	stem & pod (no seed)	*Phoma*, *Lepto.*, *Pseudo.*	230	[87]
Caliph	mature	stem & pod (no seed)	+fungicide	80	
Cyprus	mature	stem & pod (no seed)	*Phoma*, *Lepto.*, *Pseudo.*	350	
Cyprus	mature	stem & pod (no seed)	+fungicide	110	
not stated	green, late spring	tops	*Phoma* (low) rated <3 (0–10)	0–100	[86] (Western Australia survey)
not stated	dry, mature	tops	*Phoma* (low) rated 0 (0–10)	0–15	
			Medicago polymorpha		
var. denticulata	early burr	leaf	healthy	9 (16)	[88]
var. denticulata	early burr	leaf	*Uromyces*, low	24 (38)	
var. denticulata	early burr	leaf	*Uromyces*, medium	39 (50)	
var. denticulata	early burr	leaf	*Uromyces*, high	50 (80)	
Circle Valley	mature	stem	*Phoma*, *Lepto.*, *Pseudo.*	570	[87]
Circle Valley	mature	stem	+fungicide	470	
Santiago	mature	stem	*Phoma*, *Lepto.*, *Pseudo.*	470	
Santiago	mature	stem	+fungicide	290	
not stated	green, late spring	tops	*Phoma* (low) rated 0–10	0–250	[86] (Western Australia survey)
not stated	dry (mature)	tops	*Phoma* (low) rated 0–10	0–800	
not stated	dry (mature)	burr	*Phoma* (low) rated 0–10	0–200	
not stated	dry (mature)	burr	*Phoma* (high) rated 0–10	100–200	

Table 1. *Cont.*

Cultivar, if Known	Stage of Growth	Plant Material Analysed	Disease [1]/Pest Status	Coumestrol (mg/kg, DM Basis) [3]	Reference
			Medicago littoralis		
Harbinger	emergence	cotlydons	not stated	1	[83]
Harbinger	dry (mature)	senescent	not stated	335	
Harbinger	hay	tops	not stated	400 (810)	[23]
Harbinger [2]	mature	stem	healthy	528 (737)	[88]
	mature	leaf	spoted/physiogenic	2362	[90]
Angel, Herald, Jaguar	mature	top 5 nodes—including pods	*Phoma*, *Erysiphe*	1050	[82]
Experimental var.	mature	top 5 nodes—including pods	*Phoma*, *Erysiphe*	240	
Angel, Herald, Jaguar	mature	top 5 nodes—including pods	*Erysiphe*	383	
Experimental var.	mature	top 5 nodes—including pods	*Erysiphe*	37	
			Medicago murex		
Zodiac	mature	stem	*Phoma*, *Lepto.*, *Pseudo.*	880	[87]
Zodiac	mature	stem	+fungicide	270	
			Medicago scutellata		
wild type [2]	mature	stem	healthy	66 (122)	[88]
			Medicago sphaerocarpos		
Orion	mature	stem	*Phoma*, *Lepto.*, *Pseudo.*	470	[87]
Orion	mature	stem	+fungicide	170	

[1] *Phoma trifolii*, *Leptosphaerulina briosiana*, *Pseudopeziza medicaginis*, *Uromyces striatus*, *Erysiphe trifolii*, and *Stemphylium botryosum*; [2] Growing on the yellow sand soil type, coumestan concentrations were lower on a different (gravelly) soil type; [3] Figures in brackets represent total coumestans (if reported).

Table 2. Coumestrol concentration in perennial species of *Medicago.*

Cultivar, if Known	Stage of Growth	Plant Material Analysed	Disease [1]/Pest Status	Coumestrol (mg/kg, DM Basis)	Reference
		Medicago falcata			
Karlu	summer	tops	not studied	0–60	[91]
Karlu	summer	silage	not studied	26–44	
		Medicago sativa			
Ranger		leaves, 2 or >lesion/leaflet	*Pseudo.*	184	[92]
Ranger		leaves, 1 lesion/leaflet	*Pseudo*	40	
Ranger		leaves, 2 or >lesion/leaflet	*Lepto.*	72	
Ranger		leaves, 1 lesion/leaflet	*Lepto.*	29	
			Uromyces	400	[93]
Ranger and others	6 cuts/2 years	Tops	not stated	6–429	[94] (USA survey)
not stated	seed pod		not stated	340–560	EM Bickoff cited by [94]
Ranger	1st bud—bloom	leaf	diseased	62–92	[94]
Ranger	1st bud—bloom	leaf	+fungicide	29–35	
Ranger	1st bud—bloom	stem	diseased	32–112	
Ranger	1st bud—bloom	stem	+fungicide	30	
Buffalo	prebud	leaf and stem	*Phoma*	182–219	
Buffalo	prebud	leaf and stem	healthy	0–1	
Buffalo	$\frac{1}{4}$ bloom	leaf and stem	*Phoma*	60–74	
Vernal	prebud	leaf and stem	*Pseudopeziza*	33–48	
Vernal	prebud	leaf and stem	healthy	0	
Clone R-5	full bloom	leaf and stem	*Pseudopeziza*	9	
Clone R-5	full bloom	leaf and stem	healthy	3	

Table 2. *Cont.*

Cultivar, if Known	Stage of Growth	Plant Material Analysed	Disease [1]/Pest Status	Coumestrol (mg/kg, DM Basis)	Reference
Vernal	prebud	leaf and stem	*Leptosphaerulina*	0	[94]
Clone R-5	late bud	leaf and stem	*Leptosphaerulina*	31	
Clone R-5	late bud	leaf and stem	*Leptosphaerulina*	85	
Clone R-5	late bud	leaf and stem	healthy	0	
Buffalo	prebud	leaf and stem	*Stemphylium*	30–45	
Buffalo	prebud	leaf and stem	healthy	0	
Ranger	1/10 bloom	leaf and stem	Yellow Mosaic virus	30	
Ranger	1/10 bloom	leaf and stem	Yellow Mosaic virus	33	
Ranger	1/10 bloom	leaf and stem	healthy	19	
Clone R-5	$\frac{1}{2}$ bloom	leaf and stem	Yellow Mosaic virus	0	
not stated	hay stage	leaf and stem	Foggy—1524 m ASL	99	[18]
not stated	hay stage	leaf and stem	Clear—610 m ASL	32	
not stated	hay stage	commercial meal	not stated	Usu. <100	GO Kohler cited by [18]
Atlantic	vegetative	leaves	*Ascochyta imperfecta*	132–542	[95]
Atlantic	vegetative	leaves	*Colletotrichum trifolii*	76	
Atlantic	vegetative	leaves	*Uromyces*	115	
Atlantic	vegetative	Stem base	*Cylindrocladium scoparium*	88	
Atlantic	vegetative	roots	*Cylindrocladium scoparium*	247–362	
Atlantic	vegetative	leaves	*Cylindrocladium scoparium*	0	
Atlantic	vegetative	leaves	*Xanthomonas alalfae*	22–40	
not stated	sum.-autumn	tops	not stated	25–190	[26]
not stated	autumn grazed	tops	not stated	51–157	[27]

Table 2. *Cont.*

Cultivar, if Known	Stage of Growth	Plant Material Analysed	Disease [1]/Pest Status	Coumestrol (mg/kg, DM Basis)	Reference
Wairau	autumn grazed	tops	not stated	66–172	[28]
Wairau	basal bud	tops	Blue green aphid + fungicide	25	[96]
Wairau	basal bud	tops	Pea aphid + fungicide	13	
Wairau	basal bud	tops	+fungicide	2	
Wairau	prebud	tops	Aphid infestation	90	
Wairau	prebud	tops	+aphicide	23	
not stated	not stated	leaf and stem	diseased	0–159	[97] (NSW survey)
not stated	not stated	leaf and stem	healthy	0–19	
various	@ 60 d intervals	leaf	not stated; coumestrol high after humid weather	0–150	[98] (NSW survey)
various	@ 60 d intervals	stem	not stated; coumestrol high after humid weather	0–112	
CUF 101	@ 7 d intervals over spring, summer, aut.	leaf	rated 1–8; severity related to coumestrol	0–150	[98]
CUF 101		stem	rated 1–8; severity related to coumestrol	0–75	
not stated	summer/autumn		*Pseudo.*, *Lepto.*	100–350	[99] (France survey)
not stated	vege. to mature	whole tops	not stated	15–225	[100]
not stated	not stated	haylage	not stated	32	[29]

[1] *Phoma trifolii*, *Leptosphaerulina briosiana*, *Pseudopeziza medicaginis*, *Uromyces striatus*, *Erysiphe trifolii*, *Stemphylium botryosum*.

4.2. Coumestans in Lucerne

Lucerne (syn. alfalfa; *Medicago sativa* L.), a productive and nutritious perennial pasture legume and a major fodder crop, has been cultivated for over 2500 years. Like many legumes, lucerne contains phytoestrogens and, from time to time, ingestion of lucerne has been linked to hyper-oestrogenism and reduced fertility in domestic animals. Research on lucerne has usually revealed wide variation in the concentration of coumestrol. Its production is poorly understood but the variation appears to be more to do with environmental conditions (e.g., factors that suit the activity of foliar diseases caused by fungal pathogens) than with the genetics of the host plant. The greatest coumestrol contents are usually observed after budding [95] when the crop is likely to be most vulnerable to stress. Cheng et al. (1953) [101] found that coumestrol concentration in lucerne increased with the number of cuts taken within the one season. Although Loper and Hanson (1964) [92] and Adams (1989) [2] noted that coumestan concentration in lucerne appeared unaffected by temperature extremes, stage of growth, or available phosphorus; others have elaborated and shown the main factors influencing coumestrol production in lucerne are stage of growth and physiogenic effects [18], foliar diseases, and insect predators. Environmental stresses such as temporary inundation/water-logging, frost, and radiation cannot yet be dismissed. Jansen et al. (1998) [102] found that the accumulation of plant secondary metabolites was influenced by UV-B (short wave) radiation; in red clover the concentration of isoflavones has been shown to increase with ambient UV-B radiation [103]. In Hawaii, lucerne grown at 1500 m above sea level in moist, foggy weather contained coumestrol (99 mg/kg). This was higher than the concentration in lucerne from 600 m ASL (32 mg/kg) where it received greater solar radiation [18].

Work in Canada found coumestrol in lucerne was higher in year 2 than in year 1 stands and that the concentrations of coumestrol and some of the oestrogenic flavones (luteolin, apigenin, and querectin) varied considerably with stage of maturity, sites, and harvest dates. Concentrations were low for all these at early flowering. Luteolin, apigenin, and querectin concentrations then increased and in the flower, compared with stem/leaf material, were 225%, 690% and 410% greater respectively. Concentration of coumestrol was similar between plant parts and in the whole plant reached 225 mg/kg at maturity. In flower and stem, the concentration was greatest for apigenin, then querectin then coumestrol and then luteolin [100,104].

Using young lamb bioassays, McLean (1967) [105] found that the oestrogenic potency of lucerne was greater in autumn than in spring but rose considerably towards the end of spring. Coumestrol is claimed to be relatively stable over several years and may persist in mature/senesced material, hay, silage, and dehydrated pellets [30,100]. Swinney and Ryan (2005) [103] found that freeze-drying herbage samples for analysis inhibited coumestrol expression; vacuum drying enhanced it.

4.3. Coumestrol Production Stimuli

With white clover, coumestrol can increase in autumn without obvious disease signs [5] but infection by foliar pathogens increases the concentration of coumestans [21] and increases the plant's oestrogenic activity in mice, rat and sheep [5,22]. Similar effects occur with lucerne. In the US, Loper and Hanson (1964) [92] found that coumestrol concentration was low in lucerne except when infected with leaf-spot pathogens. Sherwood et al. (1970) [95] did not detect coumestrol in the roots, leaves, and stems of uninfected plants grown in the glasshouse. They found it accumulated quickly at the infection site following infection with all of the five pathogens they studied (Table 1). Accumulation paralleled the development of infection; coumestrol was not translocated to uninfected parts of the plant.

In the field, Hanson (1965) [106] measured low coumestrol contents in the semi-arid states of California and Utah (~12 mg/kg being the mean from six cuttings taken over a two-year period). Highest mean values were obtained in Iowa (125 mg/kg), Pennsylvania (88 mg/kg), Kansas (71 mg/kg), North Carolina (52 mg/kg), and Nebraska (49 mg/kg). Coumestrol contents in respective states were *roughly proportional* to the expected frequency of disease occurrence. In France, coumestrol concentrations of 350 mg/kg have been recorded in lucerne affected by foliar disease in late summer

and autumn [99]. Similarly, in US studies, concentrations of coumestrol of up to 600 mg/kg have been found in diseased lucerne [106]. Bickoff et al. (1960) [107] compared virus-infected white clover with virus-free clones of white clover. The respective concentrations of coumestrol (mouse uterine weight bioassay) were 105 and 13 mg/kg.

Lucerne cultivars may vary in coumestrol concentration [106,108–110], but the variation attributable to genetic diversity has generally proved to be minor. In US studies, differences in coumestrol content of lucerne varieties were comparatively small and were in approximate order of susceptibility to foliar diseases. Lahontan had the highest coumestrol content, Du Puits and Vernal, the lowest [106]. Coumestrol concentration in lucerne increases when the plant is stressed, especially by foliar disease, insect predation, and nutrient deficiencies [64,111,112]. In studies where lucerne was infected separately with the pathogens *Pseudopeziza medicaginis* and *Leptosphaerulina briosiana*, coumestrol concentrations were 184 and 72 mg/kg respectively [93]. Hall (1984) [97] tested 68 samples of lucerne from the coastal region of New South Wales. Samples were assessed as healthy (n = 6) or diseased (n = 62). The mean coumestrol concentration for these two groups was 1.6 (range 0–9.5) and 37.4 (range 0–159) mg/kg respectively. For samples from inland regions, the corresponding levels were healthy (n = 30) and diseased (n = 26). The mean coumestrol concentration was 0.6 (range 0–19) and 10.5 (range 0–57) mg/kg respectively. Hall and Waterhouse (1985) [98] subsequently sampled 30 lucerne crops over 12 months at 2-monthly intervals. Some samples contained in excess of 25 mg/kg for each date of sampling except that of 9 May. The highest concentrations were found in February after a wet, humid summer when 45% of samples exceeded 25 mg/kg (mean concentration in leaf was 44 mg/kg); autumn concentrations were commonly high. Concentration was significantly related to severity of leaf disease. High levels were often noted in inland irrigated stands. In a simultaneous, serial-sampling conducted in the Hunter Valley, 30% of 22 samples taken each week exceeded 25 mg/kg (46% for autumn samples; range 0–150 mg/kg). They concluded that caution should be exercised with regard to animal breeding on lucerne.

Coumestrol in lucerne is increased following infestation by aphids [94,113]. Under field conditions, where the yield of lucerne was ~1.2 t/ha (DM), $\log_e$ coumestrol concentration over the summer was linearly related to aphid numbers [96]. The coumestrol concentration in severely aphid-damaged lucerne was sufficient to impair ewe fecundity. Subsequent controlled studies in the glasshouse showed that similar levels of coumestrol were found in leaf and stem tissue of aphid-damaged plants and, at ~23 aphids per stem, blue-green aphid infestations caused higher coumestrol (25 mg/kg) than similar population levels of pea aphid (13 mg/kg); uninfested plants contained 2 mg/kg. The coumestrol content of lucerne and annual medics can be decreased significantly when foliar diseases [18,87,94,110] and aphids [96] are controlled with herbicide and aphicide. Aphicide use in the field increased yield nearly 3-fold in the latter study.

5. Coumestan Infertility

A daily coumestrol intake of 4 mg/kg live weight stimulated uterine growth in rats [49]. While lucerne in the diet has been associated with lowered reproductive performance in sheep, cattle, chinchillas, chicken, Guinea pig, pigs, goats, rabbits, and rice rats [18], no accounts of devastating reproductive problems arising from coumestans of lucerne were found in contrast to the effects of high formononetin-containing varieties of subterranean clover (*Trifolium subterraneum* L.) [114]. Some workers have concluded that phytoestrogens in lucerne had only minimal effects on the fertility of sheep [91,115].

In contrast to isoflavones, coumestans are relatively resistant to microbial degradation. Coumestans increase uterine weight. Morgan and Parberry (1980) [116] found that the uterine weight of mice increased 120% when infected lucerne (viz. 15% of leaf area covered with *Pseudopeziza medicaginis* leaf spot) was fed compared with mice fed healthy lucerne. The probability of individuals within the treatment group having a much greater response increased 2–4-fold. Feeding white clover to rats, Nykänen-Kurki et al. [5] recorded a positive increase in uterine weight despite a low coumestrol

concentration in clover (<9 mg/kg). A low level of isoflavones was also present in the clover. Whitten et al. (1992) [49] also reported that coumestrol produced true uterine growth in rats—and appeared to have cumulative effects. Coumestrol reduced ovarian weight and increased apoptotic cell death in the ovaries of adult rats exposed to it during lactation [117,118].

When 28 ewes were fed on lucerne containing coumestrol and compared with 28 ewes fed a coumestrol-free diet in Spain (Table 3), 43% of the former ewes displayed macroscopic changes within the genital tract. Alterations were especially noted in the uterus (greater than normal development of the cervical folds); prolonged exposure to lucerne led to permanent effects on the reproductive organs [119]. Coumestans can produce an oestrogenic syndrome; lambs fed lucerne containing coumestrol (119 cf. 22 mg/kg) for three weeks had greatly increased mammary and vulval development; some expressed milk [120]. Ovary development in ewe lambs may be reduced [121].

5.1. Sheep

5.1.1. Suppression of Oestrus

Unlike isoflavones which do not inhibit oestrous [122], coumestans have a great ability to suppress oestrus by interfering with the ovarian secretion of oestrogen. Newsome and Kitts (1977) [123] showed that ewes fed on lucerne had higher levels of phytoestrogen in their plasma and lower levels of endogenous oestrogens than ewes fed grass (*Dactylis glomerata*), suggesting that gonadotropin stimulation of the ovary was reduced by the presence of phytoestrogen in the plasma. With coumestrol in white clover, Sanger and Bell (1959) [19] found it affected fertilisation but not ovulation. The chemical shape of coumestrol orients its two hydroxy groups in the same position as the two hydroxy groups in estradiol, allowing it to inhibit the activity of aromatase and hydroxysteroid dehydrogenase [124]. These enzymes are involved in the biosynthesis of steroid hormones, the inhibition of which modulates hormone production [125]. Kelly et al. (1976) [24] fed ewes on pelleted *Medicago littoralis* (cv. Harbinger) containing ~504 mg/kg of coumestrol and ~614 mg/kg of 4'-methoxy-coumestrol. For these and a similar flock fed on a phytoestrogen-free diet, 17 and 92% of ewes came into oestrous, respectively. Of those on the coumestans-containing diet that came into oestrus, 58% did not have a recently formed *corpus luteum*. These workers suggested that the inhibition of oestrus associated with coumestans is the result of their impeding the pituitary's production of endogenous oestrogen. Subsequently, Smith et al. (1979) [51] suggested that coumestans interfered with the release of follicle stimulating hormone from the pituitary. Hettle and Kitts (1983) [126] found that the peak concentration of luteinising hormone was elevated and delayed further into the oestrous period in ewes fed phytoestrognic lucerne relative to those fed grass hay.

For ewes fed medic (*Medicago littoralis*) hay supplying approximately 146 mg coumestrol and 124 mg 4'-methoxy-coumestrol per day, Shutt et al. (1969) [23] observed plasma levels of 0.5–0.7 µg 'free' coumestrol and 1.2–4.0 µg conjugated coumestrol per 100 ml. These levels were associated with oestro-genomimetic changes in the composition of the cervical mucus. 4'-methoxy-coumestrol was not detected in the plasma, which suggested that it had been converted to coumestrol by O-demethylation.

5.1.2. Ovulation

The impact of coumestans on fertility can be masked by a reduced incidence of multiple ovulations. After noting a three-fold lower rate of twinning on lucerne compared with grass, several further detailed studies were carried out involving over 1700 ewes. Coumestrol was only detected where plants were affected by disease. Embryo loss was up to 43% on some lucerne treatments compared with 19% on grass. Ovulation rate was linearly related to dietary coumestrol content over the range 0 to 100 mg/kg; the number of ovulations fell from 1.44 to 0.98; ewes with high ovulation rates were more sensitive to coumestans [51].

Table 3. Observations on ewes grazing on lucerne or white clover peri-conception.

Pasture/Feed	Coumestan Concentration	Significant Results	Reference
Lambing studies			
white clover vs. grass (Columbia premiparous and 2YO ewes over 3 year)	Clover positive to mouse uterine weight assay	3% less lambs/ewe on clover cf. grass. Oestrous delayed; 41% conceived at 1st service cf. 66% for ewes on grass	[127]
lucerne vs. grass, white clover (3128 ewes over 3 year)	not assessed	11% less lambs/ewe on lucerne due mainly to less multiple births	[128]
lucerne vs. grass, white clover (900 adult Border Leicester x Corriedale ewes over 10 weeks)	not assessed	11% less lambs/ewe, and 2.65 cf. 0.3% barren, lucerne v grass	[26]
lucerne vs. grass, white clover (800 adult Border Leicester x Corriedale ewes over 7 weeks)	60–150 mg/kg coumestrol (+0–40 mg/kg 4′-methyl–coumestrol)	12% less lambs/ewe, and 3.0 cf. 1.0% barren, lucerne v grass	
lucerne vs. grass, white clover (Coopworth adult ewes, 2 year)	51–104 mg/kg coumestrol (+9–91 mg/kg 4′-methyl–coumestrol)	32% less lambs, lucerne v grass clover; 28% decrease in multiple births	[27]
lucerne vs. grass, white clover (Romney Marsh adult ewes, 2 year)	82–157 mg/kg coumestrol (+41–154 mg/kg 4′-methyl–coumestrol)	19% less lambs, lucernce v grass clover; 17% decrease in multiple births	
lucerne vs. grass, sub. clover (1800 Merino and crossbred ewes, mixed ages over 3 year)	not assessed	8.5% less lambs, lucerne v grass clover. Fertile ewe % nsd.	[129]
M. falcata grazed then fed as silage vs. grass silage (34 ewes, 14 weeks)	0—60 mg/kg coumestrol	conception and lambing both nsd; ewes conceived 5 days later on lucerne.	[91]
Case studies, uterus and ovulation observations			
lucerne vs. grass, white clover	66–172 mg/kg coumestrol (+33–145 mg/kg 4′-methyl–coumestrol)	ovulation depressed 29% following consumption during last half of oestrous cycle	[28]
varied lucerne treatments (1750 ewes over 2 year)	up to 600 mg/kg coumestrol in leaf. Fed coumestrol doses of 0–100 mg/kg	ovulation depressed 34%; lambing 14.6 %. dose linearly related to no. of ovulations from 1.44 to 0.98	[51]
primiparous ewes	up to 350 mg/kg coumestrol. Summary of case studies, all involving diseased stands.	60% barren at 45 days post insemination	[99]
primiparous ewes		>10% aborted 5 months into pregnancy	
adult ewes		5% aborted 5 months into pregnancy	
lucerne vs. coumestrol-free diet (56 ewes over 10 months)	25–30 mg/kg coumestrol	43% lucerne ewes had macroscopic changes in the cervix and uterus	[119]
lucerne ad lib, day -7 to day 17 vs. maintenance diet of faba, oat hull pellet. 70 AI'd ewes	not assessed	21% less foetuses/ewe, lucerne v pellets—nsd. lucerne ewes had less multiple ovulations (0.15 cf. 0.26)s	[130]
'flushing' studies, viz. lucerne compared with low quality feed			
lucerne vs. senescent grass, clover pasture	not assessed	lucerne ewes had greater multiple ovulations (0.36 cf. 0.27)	[131]
Lucerne vs. senescent pasture (300 ewes over 2 months)	not assessed	19% more lambs/ewe, lucerne v senescent pasture. Barren ewes %—nsd	[132]

Coop and Clark (1960) [128] reported that ewes mated on lucerne recorded a 10–12% decrease in lambing, mainly due to a reduced number of twins. Coop (1977) [26] suggested the decrease was likely to be greater in high fecundity breeds (Table 3). In extensive, long-term studies, Donnelly, Morley, and McKinney (1982) [129] found that ewes grazing on lucerne were heavier at joining and gained more weight in the weeks before joining than did ewes on phalaris, subterranean clover pasture. Ewes were joined in autumn, some in mid March and some in early April. The proportion of fertile ewes was similar on both pastures, but ewes grazing lucerne had fewer multiple births; this resulted in 8% fewer lambs per ewe joined in crossbred flocks and 9% fewer in Merino flocks. In a pen-feeding study where fresh lucerne was fed *ad libitum* to oestrous-synchronised and artificially inseminated ewes for 17 days after insemination, the proportion with multiple births relative to ewes fed a maintenance ration of pellets based on faba bean and oat hulls, was lowered from 0.34 to 0.18, but not when ewes were fed for only 7 days after insemination [133]. The reduction in multiple foetuses was attributed to the lucerne-treated ewes' greater intake of feed; coumestan content was not reported. In French studies, several flocks on lucerne suffered, with 60% of primiparous young ewes failing to conceive (as determined by echography 45 days post insemination). Five months into pregnancy, the verified abortion rate was >10% in premiparous ewes and 5% in adult ewes [99]. In the seven studies where lambing was observed for lucerne compared with an oestrogen-free treatment [26,27,91,128,129] (Table 3), 13.4% less lambs were born to ewes on lucerne (range 0% to 32%).

5.1.3. Advice to Industry

In his review, Cox (1978) [134] found no evidence of permanent infertility and did not consider that coumestans represented too serious a problem for Australian livestock. Other reviewers concluded that the potential problem of lucerne-linked infertility in livestock is probably more serious than is generally recognized; they warn that moderate or transient cases probably escape detection or are simply not recognized as being linked to lucerne [2,18,105].

Nutritional supplements that are sometimes provided a few weeks before mating (flushing) may result in higher ovulation rates in ewes that are not in good body condition. Ventner and Greyling (1994) [135] found that flushing ewes on lucerne could increase ewe live weight and lift lambing percentage by 5%–10%. During risk-prone periods for plant pathogens, research workers in New South Wales advised that, for breeding stock, managers should consider carefully how to graze lucerne. They suggested that it might be better to continuously graze lucerne at a moderate rate of stocking rather than to spell it [98]. Robertson et al. (2015 b) [132] recommend grazing ewes on lucerne prior to and during joining in autumn as a means of increasing the number of lambs born relative to those grazed on low quality feed, viz. senescent annual grasses (*Hordeum leporinum*, *Bromus* spp.).

The diet of breeding cows [136] and ewes should be balanced for protein and have an adequate energy value. High levels of soluble protein can cause early embryonic death through increased levels of urea nitrogen in the blood [137]. A most nutritious feed, lucerne can improve live weight gain and reproduction and such benefits may mask the role that coumestans can have (e.g., less multiple ovulations). As a result of a detailed study in New Zealand in which conception rates fell by 22%–29% on oestrogenic lucerne, McLeod (1978) [28] emphasized the risk of leaving ewes on lucerne in the week prior to joining. Current recommendations to animal breeders are to delete or dilute the oestrogenic component of the diet. In Western Australia, the Department of Agriculture advised animal breeders to inspect lucerne, white clover, and annual medic pastures for foliar diseases prior to joining breeding stock. Paddocks with heavily diseased plants should be avoided until after mating. They recommended producers avoid closing up pasture for extended periods in spring in order to discourage fungal diseases taking hold [64]. Primiparous and young ewes appear to be the most vulnerable to the effects of coumestans [99].

5.1.4. Tolerance Levels

Coumestans in herbage appear rapidly in the plasma following ingestion by herbivores. Their concentration reflects that in the herbage and, as that increases, so too do the clinical signs associated with infertility. Differing tolerance levels for coumestrol in the diet of sheep have been reported. Some workers found that 'moderate amounts' of coumestrol in lucerne (*Medicago sativa/M. falcata*) (viz. up to 60 mg/kg) had no effect on ewe fertility [91,115]. New Zealand workers have attributed significant depression in ewe fertility to the presence of coumestrol in lucerne at mating [26,27,138,139]. Pasture containing ~1000 mg/kg will inhibit oestrus and ovulation [24] but pasture containing ~200–400 mg/kg will only depress ovulation [27]. Smith et al. (1980) [140] reported that a coumestrol concentration of 25 mg/kg DM was sufficient to suppress the ovulation rate of ewes, a claim supported by earlier work [22,141].

For ewes fed medic (*Medicago littoralis*) hay supplying approximately 146 mg coumestrol and 124 mg 4'-methoxy-coumestrol per day, Shutt et al. (1969) [23] observed plasma levels of 5–7 μg 'free' coumestrol and 12–40 μg conjugated coumestrol per litre. These levels were associated with oestrogenomimetic changes in the composition of the cervical mucus. 4'-methoxy-coumestrol was not detected in the plasma, which suggested that it had been converted to coumestrol by *O*-demethylation. Other workers have confirmed the greater concentration as conjugates relative to free coumestrol (Table 4).

Table 4. Coumestrol and methoxy-coumestrol levels in the plasma of ewes, goats and mares fed or administered varying levels of coumestans.

Species	Diet and Intake	Plasma Coumestrol		Plasma Methoxy-Coumestrol		Reference
		Form	Concentration (μg/L)	Form	Concentration (μg/L)	
Ewes (n = 5)	medic hay (*M. littoralis*) containing 300 mg/kg coumestrol and 340 mg/kg methoxy-coumestrol. Intake: 146 mg coumestrol and 124 mg 4′-methoxy-coumestrol/d	free	5–7	free	not detected	[23]
		conjugate	12–40	conjugate	not detected	
Ewes	not stated	free	1.0–3.1			[134]
		sulphate conjugate	1.8–5.0			
		glucuronide conjugate	6.1–7.0			
Ewes	fed 514 mg coumestrol/d for 16 d	free	3.7			[25]
		conjugate	14.5			
	fed 952 mg coumestrol/d for 16 d	free	8.1			
		conjugate	28.1			
Goats	lucerne hay. 12 mg coumestrol/head/d	free	2–3.9			[142]
Mares—(Thoroughbred & Holstein, n = 16, 6–11YO, 540kg LW)	lucerne clover haylage (5–8 kg/d), concentrate + pasture and hay. Haylage contained 3 mg/kg coumestrol and 10 mg/kg methoxy-coumestrol	free	0.03–0.24 [1]	free	0.06–0.18 [1]	[29]
		conjugate	0.32–1.07 [1]	conjugate	0.45–1.04 [1]	

[1] Data converted from published units (viz. n mol/L).

5.2. Cattle

Coumestans suppress oestrus and genital development in heifers, and coumestrol in lucerne usually induces temporary infertility in cattle as well as sheep. In Israel, Foltin (1959) [143] suspected that seasonal infertility in dairy cows was associated with a diet dominated by lucerne. Adler and Trainin (1959, 1960, 1961) [144–146] found that the reduced fertility in dairy cows and precocious mammary and genital development in heifers, was associated with lucerne containing the equivalent of 52 mg/kg estradiol. Lotan and Adler (1966) [147] similarly confirmed that increased lucerne feeding led to irregular oestrous cycling and lowered bovine conception. Bickoff et al. (1969) [18] cite observations from New England, USA, where 19 of 32 dairy cows fed lucerne hay were treated for cystic ovaries in one year; 6 were treated on 4–6 occasions. The oestrous cycle was disrupted. Adler and Trainin (1960) [145] associated coumestrol in lucerne with irregular oestrous cycles, cystic ovaries, and lowered fertility in cattle. Adams (1995) [77] also described the effects of phytoestrogens in cattle and referred to signs resembling those associated with cystic ovaries. When Romero et al. (1997) [148] fed 608 dairy cows on lucerne silage containing coumestrol (67 mg/kg), 1264 inseminations resulted in 376 gestations. Lookhart (1980) related oestrogenic effects in cattle with a coumestrol content in lucerne haylages > 37 mg/kg [149]. Mostrom and Evans (2011) [3] advised that the critical range of coumestrol in cattle feed was 18–180 mg/kg.

5.3. Horses

White clover has been suspected of causing resorption of equine embryos in Kentucky [150]. In three controlled experiments conducted in Portugal and Poland, groups of mares were grazed on or fed coumestrol-containing diets. Coumestrol and 'methoxy-coumestrol' (possibly 4'-methoxy-coumestrol [151]) increased after ingestion of oestrogenic plant material [29,151]. In Experiment I, coumestans in a legume-grass pasture declined during winter but were always present in the plasma of the grazing mares (Table 4). This was despite the low concentration of coumestans in ingested feed of 0–7 μg/kg coumestrol [reported as 0.25-26.6 nM/kg] and 3–77 μg/kg methoxy-coumestrol [associated with the sparse presence of *Medicago polymorpha* and *M. truncatula;* Dr Maria Joao Fradinho, Institute of Animal Reproduction and Food Research, Polish Academy of Science, pers. comm.]. In Experiment 2 (n = 6), a ration of lucerne pellets (1–3 μg/kg coumestrol; 7–18 μg/kg methoxy-coumestrol) was increased from 0.25 to 1 kg/mare/day. The coumestan concentration in plasma was markedly higher on days 13 and 14 than on day 0. Plasma coumestrol peaked at 3.5 h post-distribution of the pellets; methoxy-coumestrol peaked at 1.5 h. By Day 13, free coumestrol in plasma was fluctuating in the range of 0.1–0.9 nM/L; conjugated forms of coumestrol were present in the range of 2.5–4.5 nM/L. Similarly for methoxy-coumestrol, free form was in the range of 0.2–0.6 nM/L and the conjugated form was in the range of 1.5–3.5 nM/L. In their third experiment, lucerne and clover haylage was fed to mares for five months. The haylage contained coumestrol (~3 mg/kg) and 3'-methoxy-coumestrol (~10 mg/kg). This apparently low intake of coumestrol was associated with lack of ovulation, uterine edema and accumulation of uterine fluid.

Anovulatory haemorrhagic follicles in mares may correlate with follicular cysts observed in cows [152] and may reflect the disruption to normal endocrine function caused by coumestans as discussed above (5.1.1). Three possible causes of anovulatory haemorrhagic follicles have been suggested [153]: viz. low follicular oestrogen levels [154], insufficient gonadotropin stimulation (which can result in low levels of follicle stimulating hormone and/or luteinizing hormone) [155], or haemorrhage into the interior of the follicle [156].

6. Conclusions

While plant breeding has succeeded in reducing the isoflavone content of modern cultivars of *Trifolium* species, the difficulty of removing old sub. clover ecotypes and their hybrids from the seedbank remains a significant challenge for sheep breeding in some regions, for example, Kangaroo

Island, South Australia, where plant analysis remains a valuable tool for identifying problematic pasture [65].

The concentration of coumestrol and other coumestans in *Medicago* species and their products can sometimes exceed that at which consequent oestrogenic activity lowers fertility in various herbivore species. Some variation in methodologies sometimes limited our ability to compare the coumestan results of different workers. Diverse chemical and biological assays have been employed over time and among laboratories. Numerous synonyms appear to have been used for some coumestans while some workers have not identified coumestans other than coumestrol. Clarity in referring to other coumestans and metabolised conjugates has sometimes been lacking.

The most likely danger from coumestans appears to be when plants are infected with foliar diseases and/or when the plant is in an advanced stage of maturity. Fungal spores commonly build up with humid conditions in summer and autumn. The influence of root diseases and others stresses on the accumulation of coumestans in lucerne (e.g., including grazing management, plant nutrition, drought, frost/low temperature, and temporary inundation) warrants investigation.

The work described merely shows that, in some situations, lucerne or annual medics grazed immediately before and after breeding can lower reproductive performance. This review should not be misinterpreted and divert producers from the greater use of *Medicago* and *Trifolium* species which are the basis of productive pasture in many regions and whose wider use offers great potential for improving productivity. Breeders should, however, be aware of the possible risk, especially for young or high value animals and for those drafted for artificial insemination.

Research on knowledge gaps, including better prediction of coumestan production, utilisation of coumestan-containing herbage around the time of mating, and tolerance levels of coumestans in the diet of various classes of vulnerable livestock, would establish guidelines for predicting the risk from phytoestrogenic *Medicago* and identifying and extending appropriate management to ensure that the fertility of livestock is not compromised. Coordinated interdisciplinary research and extension is needed to better define the problem, quantify the risk, and improve diagnosis. Research is needed on how various phytoestrogens are metabolized by gastro-intestinal microorganisms and to what extent their diversity may alter the bioactivity of phytoestrogens.

In the meantime, flock managers should consider selecting *Medicago* cultivars that exhibit high resistance to foliar diseases and pests, controlling pests, and, in the critical weeks around the time of mating, avoid grazing *Medicago* with leaf damage or disease. Caution is desirable if grazing *Medicago* in full flower; consider removing ewes 2–3 weeks prior to joining. While flushing ewes on healthy and vegetative regrowth may not affect ovulation rates, the risk will be heightened if ewes are flushed on advanced flowering or mature *Medicago.* Analysis for phytoestrogens should be considered, especially for breeders relying on purchased lucerne and medic hay or pellets.

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References

1. Irwin, J.A.G.; Lloyd, D.L.; Lowe, K.F. Lucerne biology and genetic improvement—an analysis of past activities and future goals in Australia. *Aust. J. Agric. Res.* **2001**, *52*, 699–712. [CrossRef]
2. Adams, N.R. Phytoestrogens. In *Toxicants of Plant Origin*; Cheeke, P.R., Ed.; CRC Press: Boca Raton, FL, USA, 1989; Volume 4, pp. 25–32.
3. Mostrom, M.; Evans, T.J. Phytoestrogens. In *Reproductive and Developmental Toxicology*; Gupta, R.C., Ed.; Academic Press—Medical: Amsterdam, The Netherlands, 2011; pp. 707–722.
4. Clarke, D.; Wiseman, H. Phytoestrogens. In *Bioactive Compounds in Foods*; Gilbert, J., Senyuva, H., Eds.; John Wiley and Sons: Oxford, UK, 2009; pp. 173–198.

5. Nykänen-Kurki, P.; Saloniemi, H.; Kallela, K.; Saastamoinen, I. Phyto-oestrogen content and oestrogenic effect of white clover. In *White clover in Europe: State of the art*; Frame, J., Ed.; Food and Agriculture Organisation of the United Nations: Rome, Italy, 1993.
6. Francis, C.M.; Millington, A.J.; Bailey, E.T. The distribution of oestrogenic isoflavones in the genus *Trifolium*. *Aust. J. Agric. Res.* **1967**, *18*, 47–54. [CrossRef]
7. Rossiter, R.C.; Beck, A.B. Physiological and ecological studies on the oestrogenic isoflavones in subterranean clover (*T. subterraneum* L.). II. Effects of phosphate supply. *Aust. J. Agric. Res.* **1966**, *17*, 447–456. [CrossRef]
8. Butler, G.W.; Steemers, M.A.T.; Wong, E. The effect of nitrogen, phosphorus and potassium supply on the isoflavone content of the leaves of red clover. *N. Z. J. Agric. Res.* **1967**, *10*, 312–315. [CrossRef]
9. Alexander, G.; Rossiter, R.C. The effects of fertilizer treatments on the oestrogenic potency of Trifolium subterraneum. *Aust. J. Agric. Res.* **1951**, *3*, 24–28. [CrossRef]
10. Rossiter, R.C. Physiological and ecological studies on the oestrogenic isoflavones in subterranean clover (*T. subterraneum* L.) VII. Effects of nitrogen supply. *Aust. J. Agric. Res.* **1969**, *20*, 1043–1051. [CrossRef]
11. Parbery, D.G.; Gardner, W.K.; Golebiowski, T. Stimulation of isoflavonoid content in subterranean clover by infection with a fungus. *J. Aust. Inst. Agric. Sci.* **1984**, *50*, 113–116.
12. Rossiter, R.C. Evaluation of genotypes of subterranean clover in a Mediterranean environment; a personal and historical account. In Proceedings of the 5th Australian Agronomy Conference, Perth, Australia, 25–30 September 1989; pp. 13–23. Available online: http://www.regional.org.au/au/asa/1989/donald-oration/p.htm (accessed on 24 May 2016).
13. Bickoff, E.M.; Booth, A.N.; Livingston, A.L.; Hendrikson, A.P. Estrogenic activity of fresh and dried red and subterranean clovers. *J. Anim. Sci.* **1961**, *20*, 133–136.
14. Lloyd Davies, H.; Bennett, D. Studies on the oestrogenic activity of subterranean clover on sheep reproduction in south Western Australia. *Aust. J. Agric. Res.* **1962**, *13*, 1030–1040. [CrossRef]
15. Shehata, M.N.; Hassan, A.B.; El-Shazly, K. Identification of the Oestrogenic Isoflavones in Fresh and Fermented Berseem Clover (*Trifolium alexandrinum*). *Aust. J. Agric. Res.* **1982**, *33*, 951–956. [CrossRef]
16. Moutsatsou, P. The spectrum of phytoestrogens in nature: Our knowledge is expanding. *Hormones* **2007**, *6*, 173–193. [PubMed]
17. The Netherlands Metabolomics Institute. 2015. Available online: http://www.metabolomicscentre.nl/ (accessed on 24 May 2016).
18. Bickoff, E.M.; Spencer, R.R.; Witt, S.C.; Knuckles, B.E. *Studies on the Chemical and Biological Properties of Coumestrol and Related Compounds*; Technical Bulletin No. 1408; US Department of Agriculture - Agricultural Research Service: Washington, DC, USA, 1969; p. 95.
19. Sanger, V.L.; Bell, D.S. Estrogenic activity in green forage crops and its effects on breeding ewes. *J. Am. Vet. Med. Assoc.* **1959**, *134*, 237–239. [PubMed]
20. Saba, N.; Drane, H.M.; Herbert, C.N.; Newton, J.E.; Betts, J.E. Effect of disease on the oestrogenic activity and coumestrol content of white clover. *J. Agric. Sci.* **1972**, *78*, 471–475. [CrossRef]
21. Wong, E.; Latch, G.C.M. Effect of fungal diseases on the phenolic contents of white clover. *N. Z. J. Agric. Res.* **1971**, *14*, 633–638. [CrossRef]
22. Wong, E.; Flux, D.S.; Latch, G.C.M. The oestrogenic activity of white clover. *N. Z. J. Agric. Res.* **1971**, *14*, 639–645. [CrossRef]
23. Shutt, D.A.; Braden, A.W.; Lindner, H.R. Plasma coumestrol levels in sheep following administration of synthetic coumestrol or ingestion of Medic hay (*Medicago littoralis*). *Aust. J. Agric. Res.* **1969**, *20*, 65–69. [CrossRef]
24. Kelly, R.W.; Adams, N.R.; Lindsay, D.R. Effect of coumestans on reproduction in the ewe. *Aust. J. Agric. Res.* **1976**, *27*, 253–259. [CrossRef]
25. Kelly, R.W.; Lindsay, D.R. Plasma coumestrol levels and cervical mucus responses in ewes ingesting coumestan-rich feeds. *Aust. J. Agric. Res.* **1978**, *29*, 115–121. [CrossRef]
26. Coop, I.E. Depression of lambing performance from mating on lucerne. *Proc. N. Z. Soc. Anim. Prod.* **1977**, *37*, 149–151.
27. Scales, G.H.; Moss, R.A.; Kelly, R.W. Reproductive performance of ewes mated on lucerne. *Proc. N. Z. Soc. Anim. Prod.* **1977**, *37*, 152–157.

28. McLeod, B.J. An Investigation into the Mechanisms Involved in the Depression of Ovulation Rates in Ewes Grazing Oestrogenic Lucerne. Master's Thesis, Massey University, Palmerston North, New Zealand, 1978. p. 103. Available online: http://mro.massey.ac.nz/bitstream/handle/10179/5010/02_whole.pdf?sequence=2 (accessed on 24 May 2016).
29. Ferreira-Dias, G.; Botelho, M.; Zagrajczuk, A.; Rebordao, M.R.; Galvao, A.M.; Pinto Bravo, P.; Piotrowski-Tomala, K.; Szostek, A.Z.; Wiczkowski, W.; Piskul, M.; et al. Coumestrol and its metabolite in mares' plasma after ingestion of phytoestrogen-rich plants: Potent endocrine disruptors inducing infertility. *Theriogenology* **2013**, *80*, 684–692. [CrossRef] [PubMed]
30. Morley, F.H.W. A New Look at Phytoestrogens. In *Proceedings New South Wales Divion, Australian Veterinary Association Annual General Meeting*; Australian Veterinary Association: Sydney, Australia, 1966; p. 4.
31. Reed, K.F.M.; Moore, D.D. A preliminary survey of zearalenone and other mycotoxins in Australian silage and pasture. *Anim. Prod. Sci.* **2009**, *49*, 696–703. [CrossRef]
32. Cocks, P.S.; Craig, A.D.; Kenyon, R.V. Evolution of subterranean clover in South Australia. II. Change in genetic composition of a mixed population after 19 years grazing on a commercial farm. *Aust. J. Agric. Res.* **1982**, *33*, 679–695. [CrossRef]
33. Day, H.R. *A Comparative Production Study of Three Strains of Sub Clover*; 1965–66 Report of the Kangaroo Island Research Centre. South Australian Department of Agriculture: Adelaide, Australia, 1966.
34. Hume, I.D.; Somers, M.; McKeown, N.R. Nutritive evaluation of two strains of mature subterranean clover. *Aust. J. Exp. Agric. Anim. Husb.* **1968**, *8*, 295–300. [CrossRef]
35. Stewart, G. *Variation in Sub Clover in Southern New South Wales*; Small Seeds Bulletin, New South Wales Department of Agriculture: Sydney, Australia, 1983.
36. Dear, B.S.; Sandral, G.A. *Subterranean Clover in NSW—Identification and Use*; Agfact P2.5.16; New South Wales Agriculture: Dubbo, Australia, 1997; p. 36.
37. Francis, C.M.; Millington, A.J. Varietal variation in the isoflavone content of subterranean clover: Its estimation by a microtechnique. *Aust. J. Agric. Res.* **1965**, *16*, 557–564. [CrossRef]
38. Rumball, W.; Keogh, G.; Miller, J.E.; Claydon, R.B. Grasslands G27 red clover (*Trifolium pratense* L.). *N. Z. J. Agric. Res.* **1997**, *40*, 369–372. [CrossRef]
39. Reed, K.F.M. A review of legume-based vs. nitrogen-fertilized pasture systems for sheep and beef cattle. In *Forage Evaluation: Concepts and Techniques*; Wheeler, J.L., Mochrie, R.D., Eds.; CSIRO and American Forage and Grassland Council: Melbourne, Australia, 1981; pp. 401–417.
40. National Research Council. *Hormonal Relationships and Applications in the Production of Meats, Milk and Eggs*; National Research Council: Washington, DC, USA, 1959.
41. Oldfield, J.E.; Fox, C.W.; Bahn, A.V.; Bickoff, E.M.; Kohler, G.O. Coumestrol in alfalfa as a factor in growth and carcass quality in lambs. *J. Anim. Sci.* **1966**, *25*, 167–174. [PubMed]
42. Trenkle, A.; Burroughs, W. Physiological effects of estrogens in animal feeds with emphasis on growth in ruminants. In *Nutrition and Drug Interrelationships*; Hathcock, J.N., Coon, J., Eds.; Academic Press: New York, NY, USA, 1978; pp. 577–611.
43. McClure, K.E.; Solomon, M.B.; Parrett, N.A.; Van Keuren, R.W. Growth and tissue accretion in lambs fed concentrate in drylot, grazed on alfalfa or ryegrass at weaning or after backgrounding on ryegrass. *J. Anim. Sci.* **1995**, *73*, 3437–3444. [PubMed]
44. Moorby, J.M.; Fraser, M.D.; Theobald, V.J.; Wood, J.D.; Haresign, W. The effect of red clover formononetin content on liveweight gain, carcass characteristics and muscle equol content of finishing lambs. *Anim. Sci.* **2004**, *79*, 303–313.
45. Pace, V.; Carbone, K.; Spirito, F.; Iacurto, M.; Terzano, M.G.; Verna, M. The effects of subterranean clover phytoestrogens on sheep growth, reproduction and carcass characteristics. *Meat Sci.* **2006**, *74*, 616–622. [CrossRef] [PubMed]
46. Nogowski, L. Effects of phytoestrogen-coumestrol on lipid and carbohydrate metabolism in young ovariectomised rats may be independent of its estrogenicity. *J. Nut. Biochem.* **1999**, *10*, 664–669. [CrossRef]
47. Little, D.A. Assessment of several pasture species, particularly tropical legumes, for oestrogenic activity. *Aust. J. Agric. Res.* **1976**, *27*, 681–686. [CrossRef]
48. Boué, S.M.; Wiese, T.E.; Nehls, S.; Burow, M.E.; Elliott, S.; Carter-Wientjes, C.H.; Shih, B.Y.; McLachlan, J.A.; Cleveland, T.E. Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. *J. Agric. Food Chem.* **2003**, *51*, 2193–2199. [CrossRef] [PubMed]

49. Whitten, P.L.; Russell, E.; Naftolin, F. Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth. *Steroids* **1992**, *57*, 98–106. [CrossRef]
50. Jefferson, W.N.; Patisaul, H.B.; Williams, C.J. Reproductive Consequences of Developmental Phytoestrogen Exposure. *Reproduction* **2012**, *143*, 247–260. [CrossRef] [PubMed]
51. Smith, J.F.; Jagusch, K.T.; Brunswick, L.F.C.; Kelly, R.W. Coumestans in lucerne and ovulation in ewes. *N. Z. J. Agric. Res.* **1979**, *22*, 411–416. [CrossRef]
52. Braden, A.W.H.; Hart, N.K.; Lamberton, J.A. The oestrogenic activity and metabolism of certain isoflavones in sheep. *Aust. J. Agric. Res.* **1967**, *18*, 335–348. [CrossRef]
53. Cox, R.I.; Braden, A.W. The metabolism and physiological effects of phytoestrogens in Livestock. *Proc. Aust. Soc. Anim. Prod.* **1974**, *10*, 122–129.
54. Folman, Y.; Pope, G.S. The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vagino trophic compounds of low potency. *J. Endocrinol.* **1966**, *34*, 215–225. [CrossRef] [PubMed]
55. Woclawek-Potocka, I.; Acosta, T.J.; Korzekwa, A.; Bah, M.M.; Shibaya, M.; Okuda, K.; Skarzynski, D.J. Phytoestrogens modulate prostaglandin production in bovine endometrium: Cell type specificity and intracellular mechanisms. *Exp. Biol. Med.* **2005**, *230*, 326–333.
56. Bickoff, E.M.; Livingston, A.L.; Hendrikson, A.P. and Booth, A.N. Relative potencies of several estrogen-like compounds found in forages. *J. Agric. Food Chem.* **1962**, *10*, 410–412. [CrossRef]
57. Woclawek-Potocka, Y.; Okuda, K.; Acosta, T.J.; Korzekwa, A.; Pilawski, W.; Skarzynskia, D.J. Phytoestrogen metabolites are much more active than phytoestrogens themselves in increasing prostaglandin F2α synthesis via prostaglandin F2α synthase-like stimulation in bovine endometrium. *Prostagladins Other Lipid Mediat.* **2005**, *78*, 202–217. [CrossRef] [PubMed]
58. Lindner, H.R. Study of the fate of phyto-oestrogens in the sheep by determination of isoflavones and coumestrol in the plasma and adipose tissue. *Aust. J. Agric. Res.* **1967**, *18*, 305–333. [CrossRef]
59. Morley, S.A.; Murray, J.A. Effects of Body Condition Score on the Reproductive Physiology of the Broodmare: A Review. *J. Eq. Vet. Sci.* **2014**, *34*, 842–853. [CrossRef]
60. Ashworth, C.J.; Toma, L.M.; Hunter, M.G. Nutritional effects on oocyte and embryo development in mammals: Implications for reproductive efficiency and environmental sustainability. *Phil. Trans. R. Soc. London B Biol. Sci.* **2009**, *364*, 3351–3361. [CrossRef] [PubMed]
61. Bennetts, H.W.; Underwood, E.J.; Shier, F.L. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust. Vet. J.* **1946**, *22*, 2–12. [CrossRef] [PubMed]
62. Adams, N.R. Permanent infertility in ewes exposed to plant oestrogens. *Aust. Vet. J.* **1990**, *67*, 197–201. [CrossRef] [PubMed]
63. Pugh, D.G. Theriogenology of Sheep and Goats. In *Sheep and Goat Medicine*; Schrefer, J.A., Ed.; Saunders: Philadelphia, PA, USA, 2002; p. 173.
64. Croker, K.P.; Nichols, P.G.H.; Barbetti, M.J.; Adams, N.R. *Sheep Infertility from Pasture Legumes*; Farmnote. 41/2005; Western Australian Department of Agriculture: Perth, Australia, 2007.
65. Dohle, L. Sheep in Clover May Give You Less Than You Bargained. Natural Resources, South Australia. 2015. Availlable online: http://www.naturalresources.sa.gov.au/kangarooisland/news/150928-sheep-in-clover (accessed on 6 November 2015).
66. Lightfoot, R.J.; Wroth, R.H. The mechanism of temporary infertility in ewes grazing oestrogenic subterranean clover prior to and during joining. *Proc. Aust. Soc. Anim. Prod.* **1974**, *10*, 130–134.
67. Lightfoot, R.J. A look at recommendations for the control of infertility due to clover disease in sheep. *Proc. Aust. Soc. Anim. Prod.* **1974**, *10*, 113–121.
68. Austin, A.R.; Aston, K.; Drane, H.M.; Saba, N. The fertility of heifers consuming red clover silage. *Grass Forage Sci.* **1982**, *37*, 101–106. [CrossRef]
69. Lundh, T.J.-O.; Pettersson, H.I.; Martinsson, K.A. Comparative levels of free and conjugated plant oestrogens in blood plasma of sheep and cattle fed oestrogenic silage. *J. Agric. Food Chem.* **1990**, *38*, 1530–1534. [CrossRef]
70. Adams, N.R.; Sanders, M.R. Development of uterus-like redifferentiation in the cervix of the ewe after exposure to estradiol-17 beta. *Biol. Reprod.* **1993**, *48*, 357–362. [CrossRef] [PubMed]
71. Lightfoot, R.J.; Croker, K.P.; Neil, H.G. Failure of sperm transport in relation to ewe infertility following prolonged grazing on oestrogenic pastures. *Aust. J. Agric. Res.* **1967**, *18*, 755–765. [CrossRef]

72. Adams, N.R.; Sanders, M.R.; Ritar, A.J. Oestrogenic damage and reduced fertility in ewe flocks in south Western Australia. *Aust. J. Agric. Res.* **1988**, *39*, 71. [CrossRef]
73. Newton, J.E.; Betts, J.E. The effects of red clover (*Trifolium pratense* var. Redhead), white clover (*Trifolium repens* var. S 100) or perennial ryegrass (*Lolium perenne* var. S 23) on the reproductive performance of sheep. *J. Agric. Sci.* **1973**, *80*, 323–327. [CrossRef]
74. Shackell, G.H.; Wylie, J.G.; Kelly, R.W. Effects of prolonged exposure of ewes to oestrogenic pasture. 2. Occurrence of abnormalities of the external genitalia and altered mating performance. *N. Z. J. Agric. Res.* **1993**, *36*, 459–464. [CrossRef]
75. Morley, F.H.W.; Bennett, D.; Axelsen, A. The effect of stilboestrol administered during an autumn mating on reproduction in Merino sheep. *Aust. J. Agric. Res.* **1963**, *14*, 660–669. [CrossRef]
76. Morley, F.H.W.; Axelsen, A.; Bennett, D. Effects of grazing red clover during the mating season on ewe fertility. *Proc. Aust. Soc. Anim. Prod.* **1964**, *5*, 58–61.
77. Adams, N.R. Detection of the effects of phytoestrogens on sheep and cattle. *J. Anim. Sci.* **1995**, *73*, 1509–1515. [PubMed]
78. Lloyd Davies, H.; Rossiter, R.C.; Maller, R. The effects of different cultivars of subterranean clover (*T. subterraneum* L.) on sheep reproduction in the South-west of Western Australia. *Aust. J. Agric. Res.* **1970**, *21*, 359–369. [CrossRef]
79. Lindsay, D.R.; Francis, C.M. Cervical mucus measurement in ovariectomised ewes as a bioassay of synthetic and phyto-oestrogens. *Aust. J. Agric. Res.* **1968**, *19*, 1069–1076. [CrossRef]
80. Farkas, G.L.; Kiraly, Z. Role of phenolic compounds in the physiology of plant diseases and disease resistance. *J. Phytopathol.* **1962**, *44*, 105–150. [CrossRef]
81. Bhattacharya, A.; Sood, P.; Citovsky, V. The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. *Mol. Plant Pathol.* **2010**, *11*, 705–719. [CrossRef] [PubMed]
82. Howie, J.; Ballard, R.; Peck, D.; Hill, J. *A Strand Medic Resistant to Powdery Mildew for the Eyre Peninsula and Mallee*; Minnepa Field Report. South Australia Research and Development Institute: Urrbrae, Australia, 2015; p. 4.
83. Reid, R.L. *Manual of Australian Agriculture*, 5th ed.; Australian Institute of Agricultural Science: Sydney, Australia, 1990.
84. Nair, R.M.; Howie, J.; Ballard, R.; Hutton, R.; Charman, N.; Preston, C. Genetic improvement of strand medic (*Medicago littoralis* Rohde ex Lois.) for Australian farming systems. In Proceedings of the 12th Australian Agronomy Conference, Brisbane, Australia, 26–30 September 2004; Available online: http://www.regional.org.au/au/asa/2004/symposia/6/3/716_nairrm.htm#TopOfPage (accessed on 26 July 2016).
85. Francis, C.M.; Millington, A.J. Wether bioassay of annual pasture legumes IV. The oestrogenic activity of annual medic pastures. *Aust. J. Agric. Res.* **1965**, *16*, 927–935. [CrossRef]
86. Croker, K.P.; Barbetti, M.J.; Nichols, P.G.H. Incidence of coumestrol in medic pastures in Western Australia. *Proc. Aust. Soc. Anim. Prod.* **1994**, *20*, 416.
87. Barbetti, M.J. Relative resistance, associated yield losses and phyto-oestrogen production from fungal foliar diseases in new and old annual *Medicago* cultivars. *Aust. J. Agric. Res.* **1995**, *46*, 441–450. [CrossRef]
88. Francis, C.M.; Millington, A.J. The presence of methylated coumestans in annual *Medicago* species: Response to a fungal pathogen. *Aust. J. Agric. Res.* **1971**, *22*, 75–80. [CrossRef]
89. Millington, A.J.; Francis, C.M.; McKeown, N.R. Wether bioassay of annual pasture legumes I. Oestrogenic activity in *Medicago tribuloides* var. Cyprus, relative to 4 strains of *Trifolium subterraneum*. *Aust. J. Agric. Res.* **1964**, *15*, 520–526. [CrossRef]
90. Loper, G.M. Accumulation of coumestrol in barrel medic (*Medicago littoralis*) [sic]. *Crop Sci.* **1968**, *8*, 317–319. [CrossRef]
91. Sormunen-Cristian, R.; Taponen, S.; Saastamoien, I.; Mela, T.; Saloniemi, H. Yellow-flowered lucerne: Properties and influence on performance and reproduction of ewes. *Agric. Food Sci. Fin.* **1998**, *7*, 437–446.
92. Loper, G.M.; Hanson, C.H. Influence of controlled environmental factors and two foliar pathogens on coumestrol content of alfalfa. *Crop Sci.* **1964**, *4*, 480–482. [CrossRef]
93. Loper, G.M.; Hanson, C.H.; Graham, J.H. Coumestrol content of alfalfa as affected by selection for resistance to foliar diseases. *Crop Sci.* **1967**, *7*, 189–192. [CrossRef]

94. Hanson, C.H.; Loper, G.M.; Kohler, G.O.; Bickoff, E.M.; Taylor, K.W.; Kehr, W.R.; Stanford, E.H.; Dudley, J.W.; Pedersen, M.W.; Sorensen, E.L.; et al. *Variation in Coumestrol Content of Alfalfa as Related to Location, Variety, Cutting, Year, Stage of Growth and Disease*; Agricultural Technical Bulletin 1333; US Department of Agriculture: Washington, DC, USA, 1965; p. 72.
95. Sherwood, R.T.; Olah, A.F.; Oleson, W.H.; Jones, E.E. Effect of disease and injury on accumulation of a flavonoid estrogen, coumestrol, in Alfalfa. *J. Phytopathnol.* **1970**, *60*, 684–688. [CrossRef]
96. Kain, W.M.; Biggs, D.R. Effect of pea aphid and blue-green lucerne aphid (*Acyrthosiphon* spp.) on coumestrol levels in herbage of Lucerne (*Medicago sativa*). *N. Z. J. Agric. Res.* **1980**, *23*, 563–568. [CrossRef]
97. Hall, D.G. Coumestrol content of lucerne in NSW. *Proc. Aust. Soc. Anim. Prod.* **1984**, *15*, 689.
98. Hall, D.G.; Waterhouse, D.B. Coumestrol content of lucerne in the central west and Hunter valley of NSW. In Proceedings of the 3rd Australian Agronomy Conference, Hobart, Australia, January–February 1985; Available online: http://www.regional.org.au/au/asa/1985/concurrent/sub-clover-pasture-legumes/p-11.htm (accessed on 28 May 2016).
99. Le Bars, J.; Le Bars, P. Recent acute and sub-acute mycotoxicoses recognized in France. *Vet. Res.* **1996**, *27*, 383–394. [PubMed]
100. Seguin, P.; Zheng, W.; Souleimanov, A. Alfalfa Phytoestrogen Content: Impact of Plant Maturity and Herbage Components. *J. Agron. Crop Sci.* **2004**, *190*, 211–217. [CrossRef]
101. Cheng, E.; Story, C.D.; Payne, L.C.; Yoder, L.; Burroughs, W. Detection of oestrogenic substances in alfalfa and clover hays fed to fattening lambs. *J. Anim. Sci.* **1953**, *12*, 507–514.
102. Jansen, M.A.; Gaba, V.; Greenberg, B.M. Higher plants and UV-B radiation: Balancing damage, repair and acclimation. *Trends Plant Sci.* **1998**, *3*, 131–135. [CrossRef]
103. Swinny, E.E.; Ryan, K.G. Red Clover Phytoestrogens: UV-B Radiation Increases Isoflavone Yield, and Postharvest Drying Methods Change the Glucoside Conjugate Profiles. *J. Agric. Food Chem.* **2005**, *53*, 8273–8278. [CrossRef] [PubMed]
104. Seguin, P.; Wenju, Z. Phytoestrogen content of alfalfa cultivars grown in eastern Canada. *J. Sci. Food Agric.* **2006**, *86*, 765–771. [CrossRef]
105. McLean, J.W. Lucerne and phytoestrogens. In *The Lucerne Crop*; Langer, R.H.M., Ed.; Reed: Wellington, New Zealand, 1967; pp. 276–282.
106. Hanson, C.H. Varietal Improvement of Alfalfa as related to Product Quality and Utility. In Proceedings of 9th Technical Alfalfa Conference, Lincoln, NE, USA, 17 November 1965; pp. 5–9.
107. Bickoff, E.M.; Livingston, A.L.; Booth, A.N.; Thomson, C.R.; Hollowell, E.A.; Beinhart, E.G. Some variation in estrogenic activity in fresh and dried white clover clones and the Ladino variety. *J. Anim. Sci.* **1960**, *19*, 1143–1149.
108. Stob, M.; Davis, R.L.; Andrews, F.N. Strain differences in the oestrogenicity of alfalfa varieties. *J. Anim. Sci.* **1957**, *16*, 850–853.
109. Weitzkin, G.; Marinov, U.; Roderig, H. Coumestrol in some alfalfa varieties in Israel. *Refuah Vet.* **1968**, *25*, 112–116.
110. Purves, R.G.; Hood, N.D.; Dunbier, M.W. The effect of cutting management and fungicide application on coumestans levels in three lucerne cultivars. In *Proceedings of 34th New Zealand Weed Pest Control Conference*; New Zealand Weed Pest Control Society: Blenheim, New Zealand, 1981; pp. 25–28.
111. Bickoff, E.M. *Oestrogenic Constituents of Forage Plants*; Commonwealth Bureau of Pastures and Field Crops: Hurley, Berkshire, UK, 1968; Review Series 1; p. 39.
112. Shemesh, M.; Ayalon, N.; Lindner, H.R. Coumestrol and 4′-*O*-methyl coumestrol in alfalfa grown in Northern Israel. Possible effects of a foliar pathogen (*Pseudopeziza medicaginis*). *Refuah Vet.* **1969**, *26*, 1–7.
113. Loper, G.M. Effect of aphid infestation on the coumestrol content of alfalfa varieties. *Crop Sci.* **1968**, *8*, 104–106. [CrossRef]
114. Lloyd Davies, H. Limitations to livestock production associated with phytoestrogens and bloat. In *Temperate Pastures, Their Production, Use and Management*; Wheeler, J.L., Pearson, C.J., Robards, G.E., Eds.; CSIRO: Melbourne, Australia, 1987; pp. 446–456.
115. Ruttle, J.L.; Goret, E.A. Effect of alfalfa on ewe fertility. *J. Anim. Sci.* **1968**, *27*, 1104.
116. Morgan, W.C.; Parbery, D.G. Depressed Fodder quality and increased oestrogenic activity of lucerne infected with *Pseudopeziza medicaginis*. *Aust. J. Agric. Res.* **1980**, *31*, 1103–1110. [CrossRef]

117. Saloniemi, H.; Wahala, K.; Nykanen-Kurki, P.; Kallela, K.; Saastamoinen, I. Phytoestrogen Content and Estrogenic Effect of Legume Fodder. *Exp. Biol. Med.* **1995**, *208*, 13–17. [CrossRef]
118. Kara, E.; Tsanakalis, F.; Papaioannidou, P. Is coumestrol a natural beneficial compound or a hazardous agent? Available online: http://www.frontiersin.org/10.3389/conf.fphar.2010.60.00136/event_abstract (accessed on 24 May 2016).
119. Cantero, A.; Sancha, J.L.; Flores, J.M.; Rodriguez, A.; Gonzalez, T. Histopathological changes in the reproductive organs of Manchego ewes grazing on lucerne. *J. Vet. Med. Ser. A-Zentralblatt.* **1996**, *43*, 325–330. [CrossRef]
120. Oldfield, J.E.; Fox, C.W.; Bickoff, E.M. Effects of estrogenic activity in alfalfa on growing lambs. *J. Anim. Sci.* **1960**, *19*, 1281.
121. Valderrábanoa, J.; Ramóna, J.P.; Barberána, M. Morphological alterations in the reproductive organs of ewe lambs reared on lucerne. *Anim. Prod.* **1988**, *47*, 271–274. [CrossRef]
122. Morley, F.H.W.; Axelsen, A.; Bennett, D. Recovery of normal fertility after grazing on red clover. *Aust. Vet. J.* **1966**, *42*, 278. [CrossRef]
123. Newsome, F.E.; Kitts, W.D. Effects of alfalfa consumption on estrogen levels in ewes. *Can. J. Anim. Sci.* **1977**, *57*, 531–535. [CrossRef]
124. Amin, A.; Buratovich, M. The Anti-Cancer Charm of Flavonoids: A Cup-of-Tea Will Do! *Recent Patents Anti-Cancer Drug Discov.* **2007**, *2*. [CrossRef]
125. Blomquist, C.H.; Lima, P.H.; Hotchkiss, J.R. Inhibition of 3α-hydroxysteoid dehydogenase (3α-HSD) activity of human lung microsomes by genistein, daidzein, coumestrol and C18-, C19- and C21 hydroxysteroids and ketosteroids. *Steroids* **2005**, *70*, 507–514. [CrossRef] [PubMed]
126. Hettle, J.A.; Kitts, W.D. Effects of phytoestrogenic alfalfa consumption on plasma LH levels in cycling ewes. *Anim. Reprod. Sci.* **1983**, *6*, 233–238. [CrossRef]
127. Engle, P.H.; Bell, D.S.; Davis, R.R. The effect of Ladino clover, birdsfoot trefoil and bluegrass pasture on the rate of conception among ewes. *J. Anim. Sci.* **1957**, *16*, 703–710.
128. Coop, I.E.; Clark, V.R. The Reproductive Performance of Ewes mated on Lucerne. *N. Z. J. Agric. Res.* **1960**, *3*, 922–933. [CrossRef]
129. Donnelly, J.R.; Morley, F.H.W.; McKinney, G.T. The productivity of breeding ewes grazing on lucerne or grass and clover pastures on the tablelands of Southern Australia. 1. Reproduction. *Aust. J. Agric. Res.* **1982**, *33*, 1085–1097. [CrossRef]
130. Robertson, S.M.; Clayton, E.H.; King, B.J.; Knott, S.; Morgan, B.; Friend, M.A. Lucerne pasture *ad libitum*, after day 7 post insemination, may increase embryo mortality in ewes. *Proc. Aust. Soc. Anim. Prod.* **2014**, *30*, 66.
131. King, B.J.; Robertson, S.M.; Wilkins, J.F.; Friend, M.A. Short-term grazing of lucerne and chicory increases ovulation rate in synchronised Merino ewes. *J. Reprod. Sci.* **2010**, *121*, 242–248. [CrossRef] [PubMed]
132. Robertson, S.M.; Clayton, E.H.; Friend, M.A. Reproductive performance of ewes grazing lucerne during different periods around mating. *Anim. Reprod. Sci.* **2015**, *162*, 62–72. [CrossRef] [PubMed]
133. Robertson, S.M.; Clayton, E.H.; Morgan, B.; Friend, M.A. Reproductive performance in ewes fed varying levels of cut lucerne pasture around conception. *Anim. Reprod. Sci.* **2015**, *158*, 75–85. [CrossRef] [PubMed]
134. Cox, R.I. Plant oestrogens affecting livestock in Australia. In *Effects of Poisonous Plants on Livestock*; Keeler, R., Van Kempen, K.R., James, L.F., Eds.; Academic Press: New York, NY, USA, 1978; pp. 451–464.
135. Venter, J.L.; Greyling, J.P.C. Effect of different periods of flushing and synchronized mating on body weight, blood glucose and reproductive performance in spring-mated ewes. *Small Ruminant Res.* **1994**, *13*, 257–261. [CrossRef]
136. Ferguson, J.D.; Chalupa, W. Impact of Protein Nutrition on Reproduction in Dairy Cows. *J. Dairy Sci.* **1989**, *72*, 746–766. [CrossRef]
137. Berardinelli, J.G.; Weng, J.; Burfening, P.J.; Adair, R. Effect of excess degradable intake protein on early embryonic development, ovarian steroids, and blood urea nitrogen on days 2, 3, 4, and 5 of the estrous cycle in mature ewes. *J. Anim. Sci.* **2001**, *79*, 193–199. [PubMed]
138. Thomson, N.; Jagusch, K.T. Effect of lambing date on the utilisation of grass/clover and lucerne pastures during mating. *Proc. N. Z. Soc. Anim. Prod.* **1976**, *36*, 184–189.
139. Jagusch, K.T.; Smith, J.F.; Kelly, R.W. Effect of feeding lucerne during mating on the fertility of ewes. *Proc. N. Z. Nutr. Soc.* **1977**, *2*, 161.

140. Smith, J.F.; Jagusch, K.T.; Brunswick, L.E.C.; McGowan, L.T. The effect of lucerne feeding on the ovulation rate in ewes. *Proc. N. Z. Soc. Anim. Prod.* **1980**, *40*, 44–49.
141. Bennett, D.; Morley, F.H.W.; Axelsen, A. *Bioassay* responses of ewes to legume swards. II Uterine weight results from swards. *Aust. J. Agric. Res.* **1967**, *18*, 495–500. [CrossRef]
142. Shemesh, M.; Lindner, H.R.; Ayalon, N. Affinity of rabbit uterine oestradiol receptor for phyto-oestrogens and its use in a competitive binding radioassay for plasma coumestrol. *J. Reprod. Fert.* **1972**, *29*, 1–9. [CrossRef]
143. Foltin, E. Observations on bovine infertility in the Jordan Valley. *Refuah Vet.* **1959**, *16*, 193.
144. Adler, D.; Trainin, J.H. Apparent hyper-oestrogenism in dairy cows. *Refuah Vet.* **1959**, *16*, 40.
145. Adler, D.; Trainin, J.H. Diet and bovine fertility. *Vet. Rec.* **1960**, *72*, 1171–1172.
146. Adler, D.; Trainin, J.H. The apparent effect of alfalfa on the reproductive performance of dairy cattle. In Proceedings of the 4th International Congress of Animal Reproduction, The Hague, The Netherlands, 5–9 June 1961; pp. 451–456.
147. Lotan, E.; Adler, J.H. Early effects of excessive alfalfa feeding on bovine fertility. *Refuah Vet.* **1966**, *23*, 110–112.
148. Romero, R.C.M.; Tarrago, C.M.R.; Munoz, M.R.; Arista, R.R.; Rosado, G.R. Oestrogenic syndrome in dairy cows by alfalfa consumption with large amounts of coumestrol. *Veterinaria Mexico.* **1997**, *28*, 25–30.
149. Lookhart, G.L. Analysis of coumestrol, a plant estrogen, in animal feeds by HPLC. *J. Agric. Food Chem.* **1980**, *28*, 666–667. [CrossRef] [PubMed]
150. Shemesh, M.; Shore, L.S. Effects of Environmental Estrogens on Reproductive Parameters in Domestic Animals. *Israel J. Vet. Med.* **2012**, *67*, 6–10.
151. Botelho, M.; Rebordao, M.R.; Balvao, A.M.; Pinto Bravo, P.; Piotrowska-Tomala, K.; Szostek, A.Z.; Wiczkowski, W.; Piscula, M.; Skarzynski, D.J.; Fradinho, M.J.; et al. Phytoestrogen coumestrol and its metabolites in mares' plasma after clover mixed pasture and alfalfa pellets ingestion. In *Forages and Grazing in Horse Nutrition*; Saastamoinen, M., Fradinho, M.N.J., Santos, A.S., Miraglia, N., Eds.; European Federation of Animal Science: Wageningen, The Netherlands, 2012; Scientific Series 132; pp. 49–53.
152. McCue, P.M.; Squires, E.L. Persistent anovulatory follicles in the mare. *Theriogenology* **2002**, *58*, 541–543.
153. Mottershead, J. Anovulatory Hemorrhagic Follicles. 2007. Available online: http://www.equine-reproduction.com/articles/AHF.shtml (accessed on 24 May 2016).
154. Pierson, R.A. Folliculogenesis and ovulation. In *Equine Reproduction*; McKinnon, A.O., Voss, J.L., Eds.; Lea and Febiger: Philadelphia, PA, USA, 1993; pp. 161–171.
155. McKinnon, A.O. Ovarian abnormalities. In *Equine Diagnostic Ultrasonography*; Ranaten, N.W., McKinnon, A.O., Eds.; Williams and Wilkins: Baltimore, MD, USA, 1997; pp. 233–251.
156. Ginther, O.J. *Reproductive Biology of the Mare: Basic and Applied Aspects*; Equiservices: Cross Plains, WI, USA, 1992.