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Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

Clover phyto-oestrogens in sheep in Western Australia

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Abstract: Oestrogenic isoflavone compounds in clover continue to cause reproductive losses in sheep in Western Australia. The phyto-oestrogens can cause two infertility syndromes in sheep, one of which resolves after the phyto-oestrogen is removed, while the other form of infertility is permanent and increases in severity with continued exposure to phyto-oestrogen. There is often a poor association between the extent of exposure to phyto-oestrogens and the severity of the infertility, but the reason for this is not fully known. The balance between epidemiological and laboratory-based research over the past 50 years has not always been optimal, delaying the application of measures to control the problem. To a large extent, this imbalance arose because numerous differences between species in their response to phyto-oestrogens were not appreciated.

INTRODUCTION

Oestrogenic infertility has been reported in domestic livestock on all continents, but the outbreak in Western Australia is the largest and most severe. Over the past 50 years, millions of ewes have been rendered infertile by phyto-oestrogens in Western Australia, a problem that still continues in a subclinical form today. The infertility is caused by oestrogenic isoflavone compounds found in certain strains of subterranean clover (*Trifolium subterraneum* L.).

The pathology of the oestrogenic infertility in sheep has been reviewed (1) and methods to recognise and diagnose the detrimental effects of phyto-oestrogens on ruminants have been summarised (2). However, there has been no overview of the historical context of the problem. This review examines the way in which researchers responded to the problems raised by the outbreak, and attempts to draw conclusions that might be useful in other situations where phyto-oestrogens are believed to be a problem.

WHY WESTERN AUSTRALIA?

Subterranean clover is grown across wide areas of southern Australia (Fig. 1). The initial development of subterranean clover pastures took place in South Australia, but clover oestrogens have not caused severe problems in that State, except on Kangaroo Island. Clinical problems in sheep caused by clover oestrogens have been reported in all the southern States of Australia, have been most severe and widespread in Western Australia.

The soils of the agricultural parts of Western Australia are old, infertile, and lacking in nutrients. Pastures are heavily dependent on the presence of leguminous plants to fix sufficient nitrogen to promote the growth of grasses. The Mediterranean type of climate in south Western Australia, with cool wet winters and hot dry summers, makes agriculture dependent on annual rather than perennial pastures. The optimal annual legume for this area has proven to be subterranean clover, which was imported

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accidentally from countries bordering the Mediterranean sea. When the subterranean clover was provided with a phosphate fertiliser such as superphosphate, abundant and productive pastures developed. The commercial exploitation of 'sub and super' in the 1930s led to rapid expansion of the Australian sheep industry, with large areas of land being cleared and sown to subterranean clover. These pastures were initially monocultures with little grass until the levels of nitrogen in the soil increased. The fact that most of the strains of clover sown were oestrogenic was not immediately apparent.

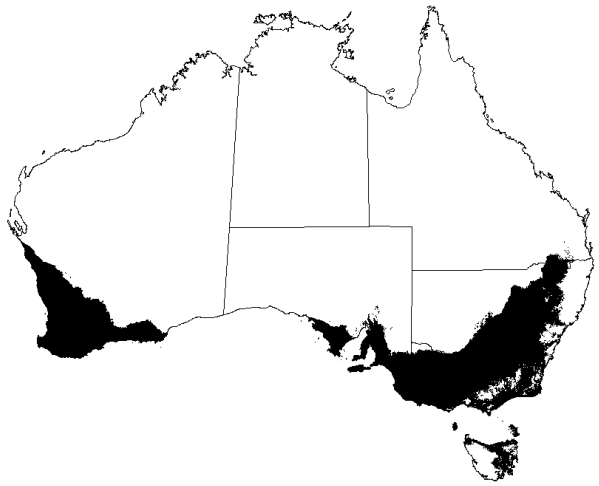

Fig. 1. Areas of Australia (shaded) suitable for growing subterranean clover

The Mediterranean-type climate is more extreme in Western Australia than in other parts of Australia. This favours the growth of subterranean clover and may be one reason why clover disease was more severe in this State. Furthermore, the first strains of clover commercialised in Western Australia (Dwalganup and Yarloop) were highly oestrogenic. None of the first 5 strains commercialised in the eastern States of Australia was oestrogenic, while 4 of the 5 strains in Western Australia contained high concentrations of oestrogenic isoflavones (3). It is not clear whether this is simply a coincidence. Other factors possibly responsible for the greater severity of the problem in Western Australia are discussed under 'Unresolved Issues'.

CLASSICAL 'CLOVER DISEASE'

Oestrogenic compounds were demonstrated in plants in 1928, a few years after the isolation of steroidal oestrogens from mammals (4), but they remained a scientific curiosity until the early 1940s, when severe outbreaks of oestrogenism were reported in sheep in Western Australia (5).

A number of different clinical manifestations were recorded, which later came to be grouped under the term 'clover disease'. These clinical conditions tended to occur relatively independently of one another, and included:

- maternal dystokia, often occurring in younger sheep. The problem can occur several months after the ewes are removed from oestrogenic pasture (6). The cervix failed to dilate properly and uterine contractions were very weak, so that the lamb was commonly not expelled and died *in utero*
- prolapse of the uterus in unmated ewes, particularly in spring when the clover grows rapidly and the amount of oestrogen in the pasture reaches its maximum
- mammary development and lactation in unmated ewes and wethers (castrate male sheep)
- enlargement of the bulbo-urethral glands in wethers, often accompanied by blockage of the urethra and death
- infertility in ewes, frequently accompanied by cystic endometrium. Lamb marking rates as low as 15% were recorded.

Most of these problems only occurred in sheep that had been exposed to large doses of phyto-oestrogen. Clinical outbreaks of clover disease continued until the early 1970s. Over time, the oestrogenicity of the pastures decreased, so that clinical clover disease largely disappeared. However, subclinical infertility still causes widespread economic loss.

THE INFERTILITY SYNDROME IN SHEEP

Surprisingly, there are two totally separate forms of infertility caused by phyto-oestrogens in sheep, that are called 'temporary' and 'permanent' infertility.

Temporary infertility results from the ingestion of phyto-oestrogens around the time of mating. Fertility normally recovers within 6 to 8 weeks of removal from the oestrogenic pasture (7). This infertility is common in other parts of the world where sheep or cattle are mated while grazed on pasture or forage containing phyto-oestrogens. The infertility results mainly from a reduced twinning rate, although failure to display oestrus and increased embryo mortality also occurs. In contrast to classical clover disease, clinical signs may be difficult to detect. Exposure to phyto-oestrogens may cause signs of oestrogenic stimulation in sheep or cattle, including mammary development or enlargement of the external genitalia, but short-term exposure to phyto-oestrogens can reduce the twinning rate in sheep in the absence of any external signs (8). Such infertility is difficult to detect, because the twinning rate of sheep is strongly influenced by the level of nutrition (the so-called 'flushing effect'; (9)) and the twinning rate depends on both the nutrient supply from the pasture and its content of phyto-oestrogen. Thus, widespread infertility may occur which would not be detected without measuring the oestrogenicity of the pasture at the time the sheep were mated (2). It is not yet clear whether ovarian function is affected directly, or indirectly through reduced stimulation by the gonadotrophic hormones luteinizing hormone and follicle stimulating hormone.

Subterranean clover contains oestrogenic isoflavone compounds only during its growing phase, when it is green. In Western Australia, sheep are normally mated in summer on dry, non-oestrogenic pasture, so that the lambs will be born 5 months later when the pasture is green. Thus, the ewes are mated at a time when they are not exposed to phyto-oestrogens, and the infertility that is expressed in this situation is both permanent and cumulative (10).

Permanent infertility is due mainly to low conception rates. The mechanism by which exposure to oestrogen can cause permanent infertility took a number of years to elucidate. The ewes ovulate normally and mate, but the cervix has a reduced ability to act as a reservoir for spermatozoa (11), so that sperm transport through the reproductive tract is impaired. This form of infertility has been observed only in sheep, cattle and horses being apparently unaffected (5). The fertility of rams was relatively unimpaired.

The ewe develops permanent infertility because it remains slightly susceptible to the organisational activities of oestrogen after the normal period of organogenesis, so that oestrogen can continue to cause slow sexual differentiation during adult life. In mammals, the reproductive system in all individuals commences development during foetal life as female, but if the foetus has testes, these produce hormones that re-organise the reproductive system into a male phenotype. Oestrogen is one of the hormones produced by the foetal testis that plays a role in organising the defeminization and masculinization of the reproductive tract and the central nervous system during this period. In mammals other than the sheep, such organisational actions of oestrogen are restricted to the period of organogenesis during development. However, prolonged exposure of adult ewes to oestrogen can cause redifferentiation of the oestrogen target organs away from the adult female form to an undifferentiated or even a male form (1,12).

The resulting infertility is caused mainly by structural and functional changes in the cervix to become more like the uterus. The cervical folds are lost and the nature of the cervical mucus is altered, so that the cervix becomes more hostile for spermatozoa. Minor abnormalities can be detected in most of the oestrogen target organs; there is slight masculinization of the external genitalia, the secretion of luteinizing hormone becomes less sensitive to oestrogen, and sexual behaviour in response to oestrogens or androgens is defeminized and masculinized. However, these effects are slight and are counter-

balanced by altered secretion of endogenous oestradiol, so that it is the changes in the cervix that play the predominant role in the infertility (1).

These effects can be brought about in the ewe, not only by plant oestrogens, but also by prolonged treatment with oestradiol-17β (13). Thus, the sexual redifferentiation during adult life is not a specific effect of the phyto-oestrogen, but is an unusual response to oestrogen that is seen only in the sheep. The mechanism also explains why rams were not permanently affected, since they were already fully differentiated sexually.

A comparison of temporary and permanent infertility in Table 1 indicates that functionally, they can be distinguished in a number of ways.

Table 1. Comparison of temporary and permanent infertility arising from phyto-oestrogens

	Temporary infertility	Permanent infertility
Clinical effect	Low twinning	Poor conception
Organ affected	Ovary	Cervix
Exposure	Current, within past 8 weeks	Prolonged; 2 years
Dose required	Low	Moderate
Species	Most species	Sheep only

THE ISOFLAVONE PHYTO-OESTROGENS

The oestrogenic compounds in subterranean clover are the isoflavones genistein and biochanin A. Formononetin, which itself has low oestrogenicity, can be metabolised by gut microflora to the oestrogenic isoflavan, equol. Together, these compounds make up about 5% of the dry weight of green subterranean clover. The role of these compounds in plants is still unknown, but subterranean clover plants without any isoflavones (such as cv Uniwager) do not compete with other plants and survive in pasture.

The oestrogenic activity of the isoflavone compounds is low, with a relative binding affinity to the oestrogen receptor between 1/1000 and 1/10 000 that of oestradiol-17β. Their activity *in vivo* is further reduced by their rapid metabolism. However, the amounts of these compounds in the diet can be so high that they can result in oestrogenic stimulation of sheep greater than is ever achieved by endogenous steroidal oestrogen.

THE HISTORY OF CONTROL MEASURES

Bennetts *et al.* (5) recognised in 1946 that clover disease resulted from intake of an oestrogenic substance, or an oestrogenic precursor, from green subterranean clover. Initial research efforts focused on isolating and measuring the oestrogenic compound in the clover. Within 5 years, the isoflavone genistein, and later its methylated derivative biochanin A, were isolated and were shown to be oestrogenic in laboratory animals (4).

This triumph for the relatively primitive chemical methods then available led to a focus on laboratory-based chemical analyses and bioassays. The initial observations that the problem occurred only on the 'early' Dwalganup strain of subterranean clover (5), and that there were differences between strains of clover on their effects on sheep (14), were discounted. Bioassays of clover using guinea pigs indicated that the amount of oestrogenicity was similar in all strains of clover tested (15). Rapid chemical assays for genistein and biochanin A were developed and used to survey many strains of subterranean clover. Relatively high concentrations of genistein and biochanin A were found in all strains of clover tested, and it was suggested that breeding clover for low oestrogenicity was not a viable means of combatting the problem (16). The isoflavan formononetin was also isolated from clover, but found to be of low oestrogenicity when injected into mice and was discounted as a possible causative agent (4). No progress was made in combating the problem in the field, and research diverted to other topics.

Ten years later, and almost 20 years after the outbreak was first reported, bioassays of different strains of subterranean clover were carried out in sheep, using the change in weight of the uterus of ovariectomised ewes. This work demonstrated conclusively that clover strains did differ in their oestrogenic effect on sheep (17). Within 2 years, plant breeders using another sheep bioassay (increase in the teat length of wethers) showed that the oestrogenicity of clover strains for sheep was not related to their content of genistein, but it was related to the amount of formononetin present (18). The apparent importance of this compound led to the hypothesis that the metabolism of the isoflavones in the ruminant might be different from that in monogastric animals. A series of studies on the metabolism of isoflavone compounds in the rumen showed that genistein and biochanin A were rapidly broken down to non-oestrogenic para-ethyl phenol, but formononetin is demethylated to the more highly oestrogenic isoflavan equol (19). Equol is rapidly absorbed from the rumen and excreted in the urine, in contrast to the steroidal oestrogens, which are excreted predominantly in the faeces of sheep.

These observations were followed by the rapid implementation of plant breeding programs to reduce the oestrogenicity of pastures. However, the delay in research meant that the effective oestrogenicity of pasture remained higher than necessary for many years, because the relative abundance of strains of subterranean clover in pastures today is a function of the duration of use (3). Strains of clover with low concentrations of formononetin were developed and promoted. Initially, a limit of less than 0.5% formononetin was established because clinical clover disease was not observed in sheep grazed on clovers with less than this amount. Later studies indicated that even this concentration of formononetin could cause subclinical infertility, and plant breeders aimed to reduce the concentration of formononetin as much as possible. The release of low-oestrogen cultivars, together with the maturation of pastures, led to the virtual disappearance of clinical clover disease.

Subsequent research was carried out to develop diagnostic tests to measure the presence of the subclinical infertility. Several methods were investigated, and quantitative histology of the extent of the uterine-like change in the cervix was found to be the most reliable estimate of the severity of permanent oestrogenic infertility in the field (20). Use of these techniques showed that low level losses from ewes failing to conceive are widespread in Western Australia. However, farmers compensated for this by changing their management; for example, using the ‘flushing’ effect by feeding the sheep better (9) so that their twinning rate increased to achieve an acceptable overall lambing rate (20). Other research examined animal based preventative measures, including immunization against phyto-oestrogens, breeding sheep resistant to the effects of phyto-oestrogens, and treatment with progesterone implants. These techniques are all potential means to combat the problem, but none of these methods is economically viable at the present level of reproductive loss.

UNRESOLVED ISSUES

The most important issue still outstanding is our inability to predict the effective oestrogenicity of pastures. Sheep have frequently been observed to graze highly oestrogenic pastures without developing clinical signs of clover disease. Much research has been expended to define factors that apparently protect sheep in some circumstances, but no fully satisfying explanation has yet emerged. This issue was raised at the time of the initial outbreak, when it was noted that sheep had apparently grazed on clover pastures for 15 years without obvious problems before clover disease emerged (5), and has continued to stimulate research (21).

It was suggested initially that war-time limitations on the importation of phosphate fertiliser caused the clovers to become oestrogenic and it was shown that the concentration of formononetin increased when the available phosphate levels are low (22). However, limited phosphate fertilizer results in restricted growth of pasture, so that although the concentration increases, the total amount of phyto-oestrogen in the feed available to the sheep is not changed. It is now believed that phosphate fertiliser had little impact in the epidemiology of the disease (22). Following this conclusion, it was suggested that a change in the source of fertilizer may have affected the oestrogenicity of the clover through an unrecognized trace component; much of Western Australia is deficient in trace elements as well as the macro-elements of nitrogen and phosphate. Experiments were carried out to examine whether variations in the availability of cobalt and selenium explained the erratic nature of the problem. Large dietary

supplements of cobalt increased the severity of clover disease, presumably through increasing ruminal demethylation of formononetin, but no convincing role for selenium was detected (23). It was concluded that this line of research was also not productive.

Clover disease was most severe on newly sown pastures which were pure clover, and the clinical problem waned as the pastures matured, even though sheep continued to consume large quantities of phyto-oestrogen (23). The decrease in the problem occurred partly because the intake of clover became diluted with grasses as the level of nitrogen in the soil built up. However, this did not fully explain the decline in clover disease, because the relationship between the severity of the infertility and clover content was linear over a large range (24), while clinical problems appeared and disappeared in a sporadic manner that could not be related simply to the amount of oestrogenic clover in the pasture.

Clover monocultures may be a particularly potent for sheep. Fig. 2 shows the fertility of a flock on a farm that had not experienced clinical problems previously. The farm experienced a rapid end to spring for two years in succession (1981 and 1982), and as a result the annual grasses in the pasture were unable to set seed. The loss of grasses resulted in the pastures becoming clover monocultures in subsequent years. The rapid appearance of clover disease infertility led to the sheep being sold, and the farmer sowed grasses back into his pastures.

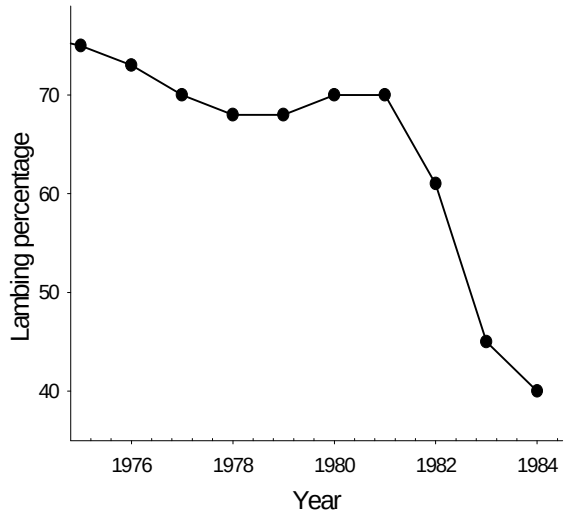

Fig. 2. Decline in fertility after grasses were removed from pasture.

If clover monocultures are particularly dangerous for sheep, it is probably not simply because the sheep have no choice of diet. The nutritional state may affect the severity of infertility that develops. Nutritional restrictions on the sheep were probably exacerbated in the 1940s by the demand for maximum production for the war effort and the shortage of phosphate fertilisers, both of which would lead to a lower nutrient supply to the sheep and loss of grasses from the pastures. Pure clover pastures provide limited pasture growth after germination at the end of autumn, so that the nutrient supply for sheep is limited during their initial exposure to phyto-oestrogen. The nutritional state of the sheep may determine whether or not the animal demonstrates the organisational effect of oestrogen (12). Diet may also affect metabolism of isoflavone phyto-oestrogens in the rumen, and so the effective oestrogenicity (25). These two observations are intriguing possibilities that have broader implications than the disappearance of clover disease.

Another feature that still defies explanation is the failure of oestrogenic isoflavones to affect the fertility of cattle. The dairy industry in Western Australia depended on the oestrogenic Yarloop strain of subterranean clover for many years, but no significant effects on reproduction in cattle were ever observed. Despite this, the metabolism of isoflavones is similar in both species, and concentrations of oestrogenic isoflavones circulating in the plasma of animals fed oestrogenic forage are comparable in sheep and cattle (26). Most outbreaks of oestrogenism in cattle have been reported as due to coumestans

or fungal oestrogens, and reports of problems from isoflavone phyto-oestrogens are rare (27). The factor that protects cattle from oestrogenic isoflavones is still unknown.

CONCLUSIONS

The history of our approach to clover disease illustrates a number of factors that may provide some useful guidelines to current researchers and research funders.

1 The failure to undertake thorough epidemiological studies before resorting to laboratory analysis was very costly. Reliance on laboratory-based work, rather than pursuing expensive and difficult field studies to confirm the early observations of differences between clover strains, delayed an effective response to the problem by more than a decade.

2 The research process was impeded by unrecognised differences between species in their responsiveness to phyto-oestrogens. Species differences acted in 3 different ways:

- differences in metabolism, both between species and possibly among individuals within a species, both in sheep (19) and in humans (28). Such differences are probably magnified by diet effects (25).
- differences in response to oestrogen. For example, oestrogen administered during adult life has not been reported to cause permanent infertility in any mammalian species other than sheep.
- differences in the background of endogenous oestrogen concentrations in which the phyto-oestrogen acts. A seminal study (29) showed that phyto-oestrogens may have either oestrogenic or anti-oestrogenic effects, depending on the concentration of endogenous steroid in the animal. For example, the same dose of genistein may have either oestrogenic or anti-oestrogenic effects, depending on whether it supplements low endogenous concentrations of oestradiol, or antagonises high concentrations of endogenous oestradiol (29).

3 Once the epidemiological features were clear, development of accurate chemical assays and their application to plant breeding rapidly provided a solution to the problem.

4 Phyto-oestrogens cause widespread subclinical infertility that is mostly unrecognised, because the reduced level of fertility are accepted as normal (20).

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REFERENCES

1 N.R. Adams. *Aust. Vet. J*, 1990, **67**, 197.
2 N.R. Adams. *J. Anim. Sci.* 1995, **73**, 1509.
3 P.S. Cocks, J.R. Phillips. *Aust. J. Agric. Res.* 1979, **30**, 1035.
4 R.B. Bradbury, D.E. White. *Vitamins Horm.* 1954, **12**, 207.
5 H.W. Bennetts, E.J. Underwood, F.L. Shier. *Aust. Vet. J.* 1946, **22**, 2.
6 N.R. Adams, M.E. Nairn. *Aust. Vet. J.* 1983, **60**, 124.
7 F.H.W. Morley, A. Axelsen, D. Bennett. *Aust. Vet. J.* 1966, **42**, 204.
8 J.F. Smith, K.T. Jagusch, L.F.C. Brunswick, R.W. Kelly. *NZ. J. Agric. Res.* 1979, **22**, 411.
9 I.E. Coop. *J. Agric. Sci., Camb.* 1966, **67**, 305.
10 P.G. Schinckel. *Aust. Vet. J.* 1948, **24**, 289.
11 R.J. Lightfoot, K.P. Croker, H.G. Neil. *Aust. J. Agric. Res.* 1967, **18**, 755.
12 N.R. Adams. *Proc. Soc. Expl Biol. Med.* 1995, **208**, 87.
13 N.R. Adams. *J. Reprod. Fert.* 1986, **76**, 727.

14 Anon. *A study of the reaction in wethers grazed on subterranean clover pasture dominated by (a) Bacchus Marsh strain, and (b) Dwalganup strain, respectively*. (East Melbourne, CSIR, 1948).

15 G. Alexander, R.H. Watson. *Aust. J. Agric. Res.* 1951, **2**, 480.

16 D.H. Curnow, R.C. Rossiter. *Aust. J. Expl Biol. Med. Sci.* 1955, **33**, 243.

17 H. Lloyd Davies, D. Bennett. *Aust. J. Agric. Res.* 1962, **13**, 1030.

18 A.J. Millington, C.M. Francis, N.R. McKeown. *Aust. J. Agric. Res.* 1964, **15**, 527.

19 R.I. Cox, A.W. Braden. *Proc. Aust. Soc. Anim. Prod.* 1974, **10**, 122.

20 N.R. Adams, M.R. Sanders, A.J. Ritar. *Aust. J. Agric. Res* 1988, **39**, 71.

21 Anon. *Infertility disease in sheep grazing subterranean clover*. (Melbourne, Commonwealth Scientific and Industrial Research Organization, 1952).

22 R.C. Rossiter. *Aust. Vet. J.* 1970, **46**, 141.

23 M.R. Gardiner, M.E. Nairn. *Aust. Vet. J.* 1969, **45**, 215.

24 H. Lloyd Davies, R.A. Maller. *Proc. Aust. Soc. Anim. Prod.* 1970, **8**, 394.

25 H. Lloyd Davies, J.L. Hill. *Aust. J. Agric. Res.* 1989, **40**, 157.

26 T.J.O. Lundh, H.I. Pettersson, K.A. Martinsson. *J. Agric. Fd Chem.* 1990, **38**, 1530.

27 K. Kallela, K. Heinonen, H. Saloniemi. *Nord. VetMed.* 1984, **36**, 124.

28 S.C. Karr, J.W. Lampe, A.M. Hutchins, J.L. Slavin. *Am. J. Clin. Nutr.* 1997, **66**, 46.

29 Y. Folman, G.S. Pope. *J. Endocr.* 1966, **34**, 215.