Final report

Phase 2- Maximising the reproductive potential of the meat sheep industry by eliminating high-oestrogen clovers, more live lambs on the ground

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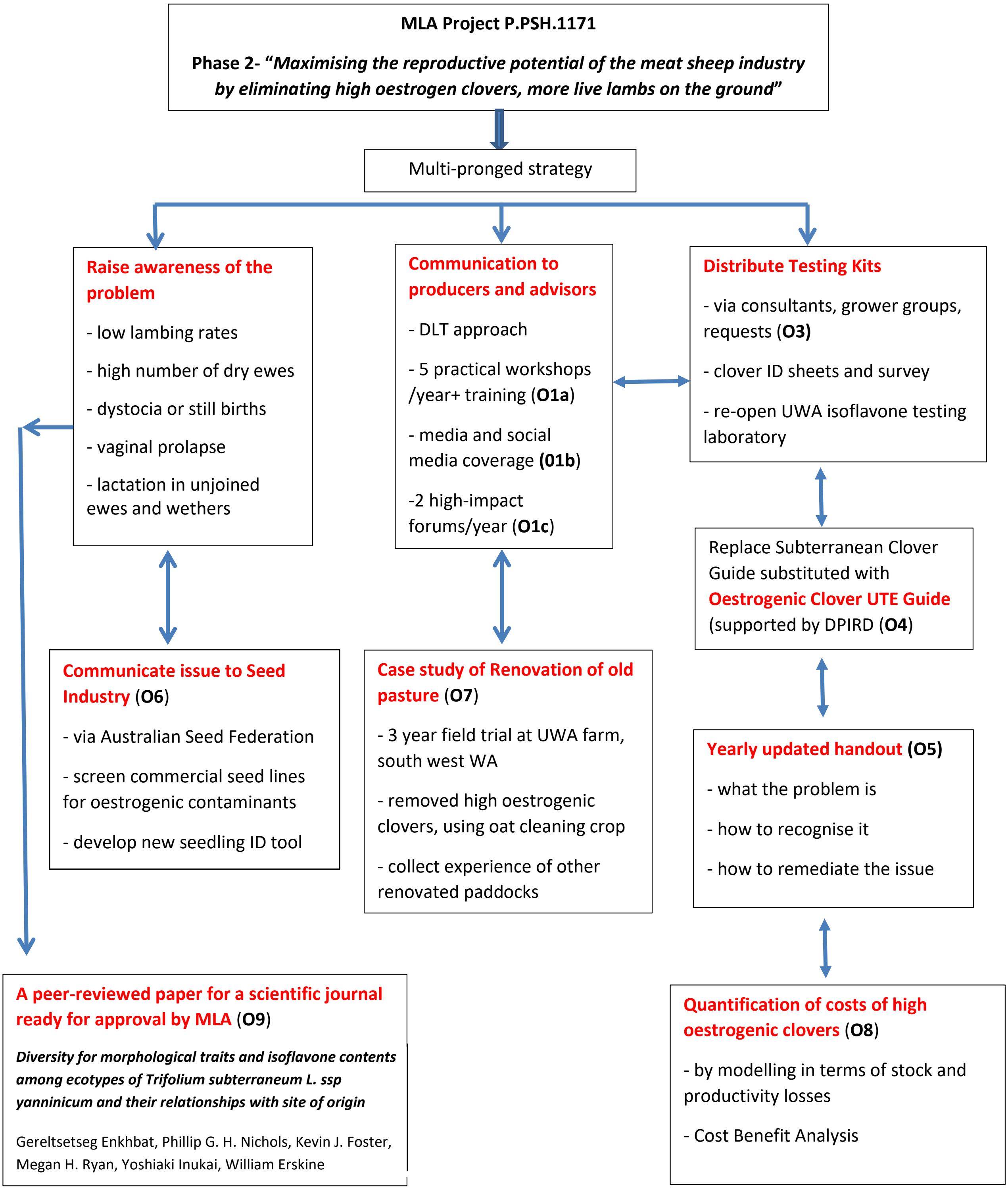
Abstract

Reproductive difficulties in sheep grazing highly oestrogenic subterranean clover (subclover) were described as 'clover disease' in the 1940s. By the 1990s, this issue was thought resolved through release of new cultivars. However, there are ongoing reports of poor lambing percentages in Merinos ewes and these can be explained by the persistence of highly oestrogenic cultivars that contain unsafe levels of formononetin in their green leaves.

This project has substantially raised awareness of the continuing issue of highly oestrogenic subclover pastures. This included training to identify oestrogenic cultivars, and on how to remediate the issue in the short and long term, for 975 producers, consultants, veterinarians and students. We also developed an oestrogenic pastures fact sheet and a Ute Guide. Around 600 leaf samples submitted by producers were tested in the re-opened diagnostic centre at the University of Western Australia. Of these, 60% contained higher formononetin than the safe threshold. Dinninup was the most common oestrogenic subclover. Several cultivars released as safe, had a substantial number of samples over the safe limit. Large variation was present in the level of formononetin for individual cultivars, especially Dinninup, the most common oestrogenic subclover identified. It seems that unknown factors are impacting formononetin levels in field samples. We also demonstrated through a field experiment that the soil seedbank of oestrogenic clovers can be very greatly reduced by three years of cropping with in-crop herbicide control of oestrogenic subclover seedlings. We also developed new subclover identification tools to aid the quick identification of oestrogenic subclover contaminants in seed lots.

Overall, the project has increased awareness of the ongoing issue of 'clover disease' and demonstrated that the occurrence oestrogenic subclover pastures is substantially larger than previously considered. A number of recommendations are made for future research and extension to further address this issue.

Executive summary



Subterranean clover (subclover) is the main annual pasture legume in southern Australia, with 42 million sheep grazing 30 million ha of subclover-based pasture. High levels of the phytoestrogen formononetin in some older highly-oestrogenic subclover cultivars—notably Dinninup, Dwalganup, Geraldton and Yarloop—can cause temporary and permanent infertility and dystocia in ewes, as well as lamb and ewe mortality. By the 1990s, it was thought that 'clover disease' had been banished to history, mainly because low-formononetin cultivars were developed and were widely adopted. Consequently, extension activities diminished, and there was a widespread loss of corporate memory among producers, agronomists, consultants, veterinarians and government agencies. Due to oestrogenic subclover being a forgotten problem, producers tended to attribute poor ewe productivity and reproductive health issues to other causes. Therefore, there was an urgent need to reinform producers about the risk of highly oestrogenic subclovers to farm productivity and animal welfare. This is especially vital in today's market, where consumer discretion is demanding a more ethical livestock sector (Martin 2009).

As part of this project the team worked with all aspects of the sheep meat and wool industry to improve understanding of the distribution, causes, costs and management options of oestrogenic pastures and conducted research to improve the reliability of pasture renovation, which is the best long-term option for improving animal productivity and welfare.

One of our project team members undertook a course in Design Led Thinking (DLT) in Brisbane during the development of Phase 2, which informed our approach at the start of the project. We presented at two high-impact industry forums in 2019. In 2020, due to COVID shutdowns and restrictions, we presented online at two annual conferences, a webinar and a scientific conference. We regularly posted on Twitter, and producers and consultants also used Twitter to engage with us directly.

The extension and adoption relating to the project were highly successful. The program interacted directly with 975 people. The number of enquiries coming into the team increased as the extension multiplied over time. The project was featured in media such as "The Land", the UWA Institute of Agriculture Annual Report, the Perennial Pastures Society newsletter, "Farming Ahead" and on their MLA website.

During 2019, we disseminated 230 free pasture kits with subclover identification sheets to producers; 103 were returned, many containing samples from multiple paddocks or, from consultants, multiple farms. The offer of free testing kits was well-subscribed in 2020. We distributed 80 kits and 56 were returned; again, many had more than one sample enclosed. All producers received their pasture identification and laboratory results. Of the samples received, 60% had higher formononetin levels in the green leaf than the current safety threshold (0.2% of leaf dry weight). Dinninup was the most common oestrogenic subclover in the pasture samples. We found no relationship between formononetin and pasture phosphorus, potassium and sulfur, as indicated by leaf nutrient levels. We found that waterlogging can increase levels of formononetin.

The strong feedback from the livestock and seed industries indicated the need for an oestrogenic subclover Ute Guide: this will be released in late 2021. We also produced two oestrogenic subclover fact sheets for the sheep meat and wool industry. The University of Western Australia (UWA) version was later updated by MLA and is available on their website.

We discussed the issue of oestrogenic subclovers with the Australian Seed Federation (ASF), including the need for testing oestrogenic contaminants in non-certified commercial seed. The ASF agreed, and the issue will be highlighted nationally. We also developed a new seedling morphological identification tool to speed up the subclover certification process significantly. This should reduce the inflow of oestrogenic subclovers back into the system.

At the UWA Ridgefield Future Farm near Pingelly in the medium rainfall zone of WA, we successfully reduced the oestrogenic seed bank over three years on a commercial scale under continuous cropping with in-crop chemical control of broadleaf weeds and subclover seedlings. The control of subclover seed set in the cropping phase is an option often overlooked by producers but will increase the chances of successful pasture renovation. Encouragingly, based on our results from the submitted pasture samples, many producers reported that they were commencing a renovation program. Our cost-benefit analysis estimated the impact of remediating oestrogenic pasture—to achieve 10% more lambs and 1% less ewe mortality—on overall farm profit. The probable effect of clover disease ranged from $10–51 per hectare per year of gross margin per 10% change in lambing percentage. The range of response, $10-51/hectare, provides an estimate of the investment which could be justified to mitigate the effects of clover disease.

A paper that contains data on oestrogenic subclover which was produced in collaboration with project staff has just been accepted by the journal *Crop and Pasture Science*: "Diversity for morphological traits and isoflavone contents among ecotypes of *Trifolium subterraneum* L. ssp. *yanninicum* and their relationships with site of origin" by Enkhbat, Nichols, Foster, Ryan, Inukai and Erskine.

Pasture renovation with new non-oestrogenic subclover cultivars, or other species, is the best long-term option for increasing the productivity and returns from oestrogenic pastures. Future extension priorities should focus on improving the success and reliability of pasture renovation.

Objectives

Objective 1

Use a Design Led approach to develop and implement an extension and adoption plan to address oestrogenic induced reproductive failure in meat sheep

One of our project team members undertook a course in Design Led Thinking (DLT) in Brisbane in 2018 at the start of the development of the Phase 2 project. We used DLT to inform our approach to the project.

This may involve but is not limited to:

a) *Five practical farmer workshops per year in association with producer groups and consultants across five states*

COVID-19 reduced opportunities to undertake workshops and field days, however, we were still worked closely with producers, consultants and researchers via online technology. These included:

The Association for Sheep Husbandry, Excellence, Evaluation and Production (ASHEEP); Department of Primary Industry and Rural Development (DPIRD) in WA; Sheep Connect, Ag KI (Kangaroo Island) and Department of Primary Industries and Regions in South Australia (PIRSA); Perennial Pastures Society (PPS) and Southern Farming Systems(SFS) in Victoria; Local Land Care Services (LLS) and Department of Primary Industries (DPI) in New South Wales (NSW);and the University of Tasmania.

b) *A minimum of four social media releases per year aimed at informing farmers and advisors about the project*

We established a Twitter account and connected with southern Australian producers with interest in low Merino lambing rates as our initial contact point. We regularly posted, and producers and consultants used this forum to engage with us directly. Two of our social media posts reached 30,000 potential followers.

c) *Presentations at a minimum of two high-impact industry forums per year (e.g. Agribusiness Update and Livestock Update)*

In 2019, we presented at the PPS in Victoria and the ASF committee meeting in Perth. In 2020, we presented online at two annual conferences, Grasslands Society of Southern Australia and PPS. The project leader was the panellist for the webinar hosted by the New South Wales DPI on *"Oestrogenic sub clover pastures: identification and potential sheep health issues".*

Objective 2

Implement a monitoring and evaluation plan to record and highlight the results, outcomes and impact of the extension and adoption plan

The extension and adoption relating to the project was highly successful, with 975 people interacting directly. Beyond these numbers, it is difficult to gauge the impact of social and print medias; however, the number of enquiries coming into the team increased with extension over time. The project featured on other media channels such as "The Land", UWA Institute of Agriculture Annual Reports, PPS newsletter, and "Farming Ahead".

Objective 3

Use farm consultants to distribute sampling kits to farmers with at least 100 kits returned, clovers identified, and oestrogen levels measured in the laboratory and farmers informed of their result

There was a very enthusiastic response by producers to have their pasture samples identified and analysed for isoflavones. We disseminated 310 free pasture kits with subclover identification sheets to producers; 159 were returned, and many contained multiple paddock samples or samples from several farms (from consultants). All producers received their subclover identification and laboratory results. Of the pasture samples received, 60% had higher formononetin levels in the green leaf than the 'safe' threshold (0.2% of leaf dry matter). The most common oestrogenic subclover was Dinninup; its formononetin levels mostly varied from ~ 0.35% to 2.5%, with one outlier at 3%. Some cultivars selected for low formononetin had levels above the safe limit in a significant number of samples, notably Seaton Park and, especially, Trikkala. Enhanced formononetin under waterlogging may be one cause, but further investigation is required. The kits were an excellent strategy to extend our message nationally to the sheep meat and wool industry and increase the awareness of producers, veterinarians and agronomists on oestrogenic subclovers and provide the first real measure across southern Australia.

Objective 4

Produce a Subterranean Clover Cultivar Guide, using photos from Phase 1, covering identification of all common subterranean clover cultivars, including their oestrogen levels, and best alternatives for use in renovating high-oestrogen pastures.

After receiving user responses, the feedback from the livestock and seed industries was a preference for an oestrogenic subclover Ute Guide rather than an updated Subterranean Clover Cultivar Guide; hence, we focused on producing a Ute Guide, which will be released in late 2021.

Objective 5

Update yearly a handout, targeted at farmers and advisors, outlining the problem (in terms of clinical and subclinical impact on livestock), how to identify it, and how to mitigate it using pasture renovation

We produced two oestrogenic subclover fact sheets for the sheep meat and wool industry. The UWA version was later updated by MLA and is available on their website. P.PSH.1138 - here

Objective 6

Communicate to seed industry about oestrogenic clovers

There is an unintentional inflow of oestrogenic subclover cultivars onto farms due to the sowing of non-certified commercial seed. In 2020, we discussed the issue with the ASF and the need to undertake testing for oestrogenic subclover contaminants in commercial seed. They agreed to highlight the issue nationally. They will also encourage customers to buy certified subclover seed.

Objective 7

Complete case study of a high-oestrogen pasture renovation

At the UWA Ridgefield Future Farm, on a medium rainfall site (winter dominant, annual average 425 mm), we reduced the oestrogenic soil seed bank on a commercial scale under continuous cropping (with in-crop control of broadleaf weeds and subclover seedlings) over three years from an estimated 220 kg/ha to just 9 kg/ha by the end of March 2021. Further research is needed to identify economic options for renovating oestrogenic pastures, especially where large seedbanks of oestrogenic subclover exist and where cropping options are limited, such as in higher rainfall zones or permanent pastures that cannot be cropped due to frequent rocks, trees or waterlogging.

Objective 8

Quantification of clinical and subclinical cost of oestrogenic clovers for livestock

Our cost-benefit analysis estimated the impact of remediating oestrogenic pasture—to achieve 10% more lambs and 1% less ewe mortality—on overall farm profit. The potential effect of ‘clover disease' expressed as a decline in lambing percentage ranged from $10–51 per hectare per year of gross margins per 10% change in lambing percentage.

Objective 9

A peer-reviewed paper for a scientific journal ready for approval by MLA

One paper, to which members of the project team contributed, has been accepted and contains data on oestrogenic pastures: Title: "Diversity for morphological traits and isoflavone contents among ecotypes of *Trifolium subterraneum* L. ssp. *yanninicum* and their relationships with site of origin." Enkhbat, Nichols, Foster, Ryan, Inukai, and Erskine. *Crop and Pasture Science*.

Methodology

- We developed and distributed free sample kits and identification kits for producers and consultants which enable them to submit pasture samples for visual identification and laboratory isoflavone level analysis
- We re-established the isoflavone analysis laboratory at UWA to measure formononetin levels in subclover samples.
- Field trials were conducted over three years to establish whether seed banks dominated highly oestrogenic subclover can be reduced to manageable levels by cropping and thereby facilitate effective renovation with new cultivars.
- We developed the first visual identification tool to distinguish problematic highly oestrogenic cultivar Dwalganup from Dalkeith at the seedling stage.
- An easily accessible fact sheet was developed for online reference (via MLA website) to inform the sheep meat and wool industry about 'clover disease', oestrogenic cultivars and adaptions and remediation.
- We expanded conventional communication strategies (workshops and field days) to include webinars, scientific conferences, Zoom meetings, newsletters, industry annual reports and social media via Twitter to stimulate awareness and engagement and provide solutions to the sheep meat and wool industry on identifying, managing and remediating highly oestrogenic subclovers in pastures.

Results/key findings

- The project has substantially raised awareness of the continuing issues of highly oestrogenic subclovers in 975 producers, consultants, veterinarians and students. This included training to identify oestrogenic cultivars and how to remediate the issue in the short and long term; many more were alerted to the need to monitor their pastures through social media.
- Around 600 leaf samples submitted by producers were tested in the re-opened isoflavone diagnostic centre at UWA. All producers received their pasture identification and laboratory results, demonstrating the extent of highly oestrogenic subclover pastures across southern

Australia. The kits were an excellent strategy to extend information on oestrogenic subclovers nationally to the sheep meat and wool industry.

- Of the subclover samples submitted, 60% contained higher formononetin than the safe threshold of 0.2% of green leaf tissue. Dinninup was the most common oestrogenic subclover these samples. Several cultivars released as safe, notably Trikkala, had a substantial number of samples over the safe limit. Large variation was present in the level of all isoflavones, including formononetin for individual cultivars, especially Dinninup.
- A contributing factor to safe cultivars being over the limit and high variation in formononetin is likely waterlogging. There was no evidence of suboptimal nutrition being a factor, although previous research suggests it is possible. Thus, it seems that unknown factors are impacting formononetin levels in field samples. Screening of new cultivars for formononetin levels under commercially relevant field conditions is therefore highly recommended.
- The soil seedbank of oestrogenic clovers can be very greatly reduced by three years of cropping with in-crop herbicide control of oestrogenic subclover seedlings. Sowing of a low-formononetin cultivar directly following an oestrogenic pasture is likely to result in considerable oestrogenic subclover contaminating the pasture into the future.
- The cost of losses from the occurrence of highly oestrogenic subclovers was calculated using modelling at $10–51 per hectare (gross margin per 10% change in lambing percentage) per season. We then modelled the effects of clinical and subclinical costs of oestrogenic subclovers for livestock, using a simplified farm system with 30% oestrogenic pastures occur across the whole farm. The outcome suggested a $14,000 decline in enterprise profit, incurred largely through reduced sheep turnoff (–$9,900) and wool income (–$4,400).
- There is an unintentional inflow of oestrogenic subclovers into non-certified commercial seed. New subclover identification tools developed as part of this project will aid the quick identification of oestrogenic subclover contaminants in seed lots.
- The scale of the oestrogenic subclover problem is substantially larger than many producers, consultants or veterinarians realise; as such, we expect the demand for information and services to continue to increase in future years.

Benefits to industry

The project alerted producers, consultants and researchers across southern Australia to the problem of oestrogenic subclovers through workshops, laboratory results, identification tools, presentations, pasture walks and social media. Thus, the sheep meat and wool industry is now much more informed about ‘clover disease’. Future extension will be aided by: the oestrogenic subclover factsheet; the oestrogenic subclover Ute Guide; the map of southern Australia indicating where clusters of oestrogenic subclover are present.

The sample kits enabled many producers to be informed about the presence of oestrogenic subclover in their pasture and identified the cultivars present. The results suggested a significant proportion of pastures across southern Australia likely contain oestrogenic subclover and that Dinninup is the most common cultivar that producers need to know how to identify.

We communicated with producers, and the pasture seed industry, about the need to renovate with certified seed and developed a method to identify and visually separate seedlings of the highly oestrogenic Dwalganup from Dalkeith: this should reduce the inflow of oestrogenic subclover into the system.

We demonstrated that the soil seedbank of oestrogenic clovers can be very greatly reduced by three years of cropping with in-crop herbicide control of oestrogenic subclover seedlings. Our techniques will guide producers to renovate successfully.

Future research and recommendations

Future research

- A more comprehensive field survey of New South Wales is needed to better under the occurrence of oestrogenic subclover.
- Further information is required to determine which environmental and management factors can cause the level of formononetin in subclover to increase in pastures under field conditions.
- Further economic modelling analysis should be undertaken to compare the full costs and benefits that result from pasture improvement by renovating with new cultivars.
- Research is needed to develop an accurate in-field test for pasture formononetin level. If successful, the need to identify subclovers visually or submit pasture samples to a laboratory will be removed and producers will be able to quickly act to address the issue by, for example, removing livestock from the pasture or strategic grazing.
- Research is also needed to develop a simple in-field test to be used by livestock veterinarians to determine equol levels in sheep grazing oestrogenic subclover.
- Development of an herbicide-resistant subclover is urgently needed in the medium and high rainfall zones where producers cannot crop due to waterlogging and thereby reduce the soil seedbank of oestrogenic subclovers.

Recommendations

- The UWA isoflavone laboratory was re-opened as part of the project. However, the future of this service is uncertain. There is an alternative service available in south-western Victoria, but it is more expensive and uses a methodology that needs more interpretation by the producer. Equivalent laboratory services do not currently exist in New South Wales, South Australia or Tasmania. We therefore recommend that the UWA isoflavone laboratory be further funded to ensure producers can submit samples of subclover for determination of formononetin level. This information is needed to provide certainty for producers with diagnosing the problem prior to taking action to avoid or remediate through pasture renovation.
- Producers be encouraged to undertake ewe pregnancy scanning and be better informed that unexpectedly low and variable conception rates and ewes tending to conceive later in the joining period can be signs of impact from highly oestrogenic subclover.

- All non-certified commercial subclover seed should be tested for contamination by highly oestrogenic subclover, that is, the four common cultivars of Dinninup, Geraldton, Dwalganup and Yarloop, and the results included on the bag label.
- Information of the issue of oestrogenic pastures and subclover identification be part of the Lifetime Ewe Management accreditation course.
- An E-learning package on oestrogenic subclover, including identification, animal productivity and welfare impacts, and remediation should be provided to universities and the sheep meat and wool industry.
- We recommend that new breeding material be tested for formononetin levels when grown under a range of relevant field environments and management practices.
- Finally, we strongly recommend a further widespread industry extension-focused project to: disseminate the findings from this project; emphasise the need for isoflavone testing on subclover in older pastures; inform producers of best-practice pasture renovation techniques; and inform producers of how to best manage oestrogenic pasture prior to renovation. It is crucial that this also include further training of producers and advisers on identification of oestrogenic subclover. To avoid reoccurrence of a knowledge gap in a further 10-20 years it is crucial that legacy materials are produced and that a program is instituted to further train advisers in all states.

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1. Background

1.1 Introduction

Subterranean clover (*Trifolium subterraneum* L.) or 'subclover' is the most commonly grown annual pasture legume in the farming systems of southern Australia. There are many cultivars of subclover. However, while new cultivars are regularly released, old cultivars remain common on many farms. Unfortunately, some older cultivars contain high concentrations of oestrogenic compounds that can cause two infertility syndromes in sheep: (1) Short-term infertility, which can be resolved by removing the animal from the oestrogenic pasture; (2) Permanent infertility, commonly known as 'clover disease', which is associated with a variety of serious disorders, including dystocia and uterine prolapse, often leading to ewe mortality and postnatal lamb mortality. In severe cases, there can be permanent loss of ewe fertility, milk secretion in maiden ewes and wethers, false bladders in wethers and barren ewes with fluid in their mammary glands at lamb marking (Adams 1987). However, ewes suffering from permanent infertility rarely present clinical signs at joining because they show normal oestrous cycles. The condition, therefore, often goes unnoticed by producers, especially in Merino and Merino cross ewes.

1.2 Knowledge gaps

'Clover disease' was first identified in the 1940s (Bennetts et al. 1946), with the cause established to be high levels of the phytoestrogen formononetin in the green leaves of subclover (Shutt and Braden 1968). There is extensive literature over more than 60 years reporting on the deleterious effects of oestrogenic pastures on livestock in southern Australia and New Zealand. The oestrogenic potency of high-formononetin cultivars of subclover was confirmed based on clinical symptoms (Davies and Bennett 1962; Adams et al. 1988) and their impact on sheep reproduction (Davies et al. 1970; Marshall 1974; Rossiter and Marshall 1974). In response, all subsequent cultivars released by breeding and selection programs should now be screened to ensure low formononetin levels. The problem of 'clover disease' was assumed to be resolved; consequently, communication about the problem declined. However, it is now clear—as confirmed by the surveys and field days conducted as part of this project—that a substantial and widespread knowledge gap developed. Contributing to this knowledge gap is the generational change in the sheep meat and wool industry since oestrogenic subclover was first recognised and new low-formononetin cultivars were first released. In addition, the classic symptoms of 'clover disease' are now less common, with a lower prevalence of uterine prolapse and masculinised genitalia and behaviour that characterised the disease until the 1970s (Adams 1990). However, unexplained occurrences of high numbers of dry ewes and dystocia are often reported.

Thus, knowledge on identifying and remediating oestrogenic subclover pastures has greatly diminished, with producers often attributing poor livestock productivity and health issues to other causes. Overall, as an industry, we have lost sight of this critical cause of reproductive wastage.

1.3 Scale of the current problem

Low-formononetin subclover cultivars have been sown widely across southern Australia. However, old high-formononetin cultivars are well-adapted to the environment and pasture management regimes of southern Australia and remain widespread (Cocks et al. 1982). In 2002, an MLA report estimated that more than 10 million sheep were affected by oestrogenic subclover across Australia (MS.009 (August 2002)). Adams (1995) estimated a similar number, with 10–15 million sheep of the national flock likely affected to varying degrees. No assessment of the prevalence of oestrogenic subclover cultivars has occurred since Adams (1995).

1.4 Detecting 'clover disease'

1.4.1. On-farm

For ewes, 'clover disease' is most often suspected after an increase in dry ewe percentage at scanning, unexpected low lambing percentages, uterine prolapse or ewe and lamb mortality around birth. However, to confirm the diagnosis, the subclover cultivars present in the pasture must be identified. Subclover cultivars are distinguished by characteristic flowering times, leaf marks and other aspects of shoot morphology. Dwalganup, Dinninup and Yarloop cultivars have been associated with serious 'clover disease' problems and Geraldton has been implicated in less severe cases. Other potentially oestrogenic cultivars are Howard and Tallarook, but both have been grown only to a limited extent (Nichols et al. 1996). However, many divergent strains or mutations of subclover have developed over time, some with moderate levels of formononetin (Cocks and Philips 1979): these can be morphologically identical to cultivars with safe formononetin levels, for example, Eden Valley in South Australia (a high formononetin variant of Mt. Barker). Ultimately, the level of formononetin must be confirmed in the laboratory.

1.4.2. In the laboratory

For ewes, 'clover disease' can currently be diagnosed by microscopic examination of sections of the cervix collected at the abattoir. This is a specific diagnostic test for 'clover disease', but requires a specialised procedure and the death of the animals.

For subclover plants, the levels of formononetin and the other two isoflavones commonly found in the leaves of subclover (biochanin A and genistein) can be determined using thin layer chromatography (TLC) according to a modified version of Beck (1964). A maximum formononetin content (0.2% of leaf dry weight) is used as a guideline in the subclover improvement program. However, no commercial laboratories are offering this as a cost-effective test, apart from the UWA isoflavone laboratory, which was re-established as part of this project.

1.5 Preventing 'clover disease'

1.5.1. Livestock

There is no cure for permanently infertile ewes. Even with short grazing periods, subclinical infertility may occur due to the selective grazing behaviour of sheep. The proportion of subclover in pasture, the cultivar and, likely other environmental conditions, determine the oestrogenic potency of a subclover pasture. However, if a problem is suspected, the effects of 'clover disease' can be reduced by strategic grazing to minimise formononetin intake and the adverse effects on livestock. Other measures include: i) avoiding grazing most valuable livestock, such as ewe weaners or younger ewes, on potent oestrogenic pastures (but can be grazed with terminal lambs; ii) limited grazing by wethers to avoid high exposure but monitor for health; and iii) awareness that high formononetin levels can be maintained in hay dried quickly or in silage. Additionally, the proportion of feed intake that contains formononetin can be diluted by: i) providing alternative feed sources, e.g. shrubs or grasses, in adjacent paddocks; ii) sowing other species into the pasture, e.g. rye grass or oats, to dilute the intake of subclover; and iii) retaining grasses and/or broadleaf weeds in the pastures i.e. don't grass clean.

1.5.2. Pastures

Subclover leaves are only oestrogenic while green, and the oestrogenic potency is fairly constant during the growing season up to flowering when it often declines. However, predicting when the level of formononetin in subclover cultivars is considered 'safe' for grazing animals is challenging, given previous published reports that variation in climate, soil conditions and plant nutrition may affect formononetin levels and pasture composition. For instance, waterlogging and severe phosphorus deficiency have been reported to more than double leaf formononetin in subclover (Rossiter and Beck 1966; Francis and Devitt 1969; Schoo and Rains 1971). Efforts to prevent 'clover disease' are based on reducing the overall oestrogenic subclover content of the pasture. Methods for achieving this are discussed in the next section. However, replacing high-formononetin subclover cultivars with new low-formononetin cultivars is preferred.

1.6 Eliminating oestrogenic subclovers – on farm

The complete removal of old highly oestrogenic subclover cultivars from the soil seed bank has proven difficult because they are well-adapted to the grazing environment. Dinninup, for instance, was much more prevalent than expected, given the number of sowings (Adams et al. 1988).The old highly oestrogenic cultivars are somewhat less palatable compared to lower formononetin subclover cultivars (Rossiter 1974; Cocks et al. 1982), increasing their proportion in pasture biomass over time. The agronomic success of programs for replacing highly oestrogenic cultivars with new low-formononetin cultivars will depend heavily on the relative 'competitive abilities' of the current pasture (which often has large reserves of hard seed in the soil) *versus* the replacement cultivars. There is, however, solid evidence that replacement can be achieved. For example, the newer cultivar Seaton Park is a good competitor and can maintain good purity when sown on old pastures of the highly osteogenic Dwalganup, and other cultivars increased substantially in Dwalganup pastures under conditions of high grazing pressure (Davies et al. 1970). Similarly, the highly oestrogenic cultivar Yarloop can be displaced by a range of cultivars, such as Trikkala (Beale and Crawford 1982). Many of the new low-formononetin cultivars have improved persistence and competitive vigour compared with older cultivars, and there will be a dilution of the older cultivars in pastures with the new replacement cultivar. However, it is essential that pasture renovations give the new cultivars the best possible chance to dominate the pasture and thus reduce the chances of the old high-formononetin cultivars returning to dominance over time.

1.7 Eliminating oestrogenic subclovers – supply chain

Seed certification is a formal program for the production of high-quality seeds of genetically distinct pasture cultivars made available to the industry. The Australian Seeds Authority (ASF) is the peak body for seed certification and authorises certification agencies to implement both OECD Seed

Schemes and the Australia Seed Certification Scheme in Australia. In Western Australia, seed certification is administrated by the Department of Primary Industries and Regional Development. Under these programs, seed producers produce certified seed by following scheme rules that ensure traceability throughout the production cycle. Sowing authenticated pedigree seed, undergoing field inspections during the growing season, grading seed at authorised seed processors, inspection of seed in an ISTA (International Seed testing Authority) accredited laboratory and unique labelling are features of the schemes. Certification is an officially recognised method for maintaining the varietal identity of seed on the open market. Seed needs to meet very high standards to be certified. Certification is also based on the concept of truth in labelling. If purchasing certified seed, the buyer can be assured that the seed in the bag will be the variety named on the label and have a high degree of genetic purity. Certified seed is also grown, processed and tested to meet several other quality standards, including purity of clean seed relative to dirt, high germination percentages, and a minimum of other crop and weed seeds present. Traditionally, seed certification schemes are undertaken in the field, based on morphological traits like leaf markings and pigmentation of flower calyces and stipules as well as flowering time. Correct identification is vital to seed certification officers, with flowering currently the phenological stage recommended for attempting subclover cultivar identification in the field.

The need for correct subclover cultivar identification is significant for this project and the ASF. However, there is concern that while the seed certification procedure has been highly successful, it is subject to limitations; for example, it is very difficult to distinguish highly oestrogenic Dwalganup plants in a stand of Dalkeith. Thus, many paddocks could not be certified with seed crops only labelled as “Dalkeith /Dwalganup”. Dalkeith can be distinguished biochemically from Dwalganup due to its much lower formononetin level, but for the last 20 years, there has been no laboratory testing service available. Across southern Australia, clean land with no previous subclover contamination is difficult to access, so seed producers face difficulties obtaining pure subclover pastures. Typically, there is more than one subclover cultivar in the background at planting of long cultivated land; sometimes, one or more of these are oestrogenic. If undetected, we continue to have an inflow of oestrogenic subclovers like Dwalganup and others into the system when trying to renovate pastures with new non-certified low-formononetin subclover cultivars.

2. Objectives

2.1. Objective 1 - Extension and adoption

Extension and adoption through outreach

Objective 1a. Use a Design Led approach (DLT) to develop and implement an extension and adoption plan to address oestrogenic induced reproductive failure in meat sheep

We discussed the best way to use DLT to frame the research in this project and work with industry and business to ensure the outcomes from our work have maximum impact on the livelihoods of the producers. There was a consistent approach to feedback between scientists and producers so that project findings could be translated into maximum value for producers. One of our project team members undertook a course in DLT in Brisbane in 2018 at the start of the development of the Phase 2 project. Consequently, DLT helped to refine our approach to tackling the issue. We endeavoured to have a DLT workshop session in Perth with producers and researchers, but there was no mutually suitable time prior to the onset of COVID-19. However, DLT enabled us to approach the project differently at the start of the project.

Objective 1b. A minimum of four social media releases per year aimed at informing farmers and advisors about the project

We regularly posted on Twitter, and producers and consultants also used Twitter to engage with us directly. Presence at field days, workshops and forums was also reported on social media. During the project, we increased our tweets and mentions by more than 40% each and increased our followers to more than 480.

Objective 1c. Presentations at a minimum of two high-impact industry forums per year (e.g. Agribusiness Update and Livestock Update)

In 2019, we presented at the PPS in Victoria and the ASF committee meeting in Perth. In 2020, due to COVID-19 restrictions, we presented online at two annual conferences, the Grasslands Society of Southern Australia and PPS again. In addition, the project leader was the panellist for the webinar hosted by the DPI New South Wales on *"Oestrogenic sub clover pastures: identification and potential sheep health issues".*

2.2. Objective 2 - Extension and adoption outcomes

Implement a monitoring and evaluation plan to record and highlight the results, outcomes and impact of the extension and adoption plan

The extension and adoption relating to the project was highly successful, with 975 people interacted with directly. Beyond these numbers, it is difficult to gauge the impact of social and print media; however, the number of enquiries coming into the team increased with extension over time. The project has been featured on other media channels, such as "The Land", UWA Institute of Agriculture Annual Reports, PPS newsletter, and in "Farming Ahead"; it also featured on the MLA website.

2.3. Objective 3 - Pasture sampling kits

Use farm consultants to distribute sampling kits to farmers with at least 100 kits returned, clovers identified, and oestrogen levels measured in the laboratory and farmers informed of their results (Year 2)

There was a very enthusiastic response by producers to the opportunity to have their pasture samples identified and analysed for isoflavones. During 2019, we disseminated 230 free pasture kits with subclover identification sheets to producers; 103 were returned, and many contained samples from multiple paddocks or, submitted by consultants, from multiple farms. The offer of free testing kits was also well-subscribed in 2020 with 80 kits distributed; 56 were returned and, again, many had more than one sample enclosed. This highlights the acute need to keep the UWA isoflavone analysis laboratory open for industry beyond the end of the project.

All producers received their pasture identification and laboratory results. Of the pasture samples received, 60% of the samples had a greater formononetin level in the green leaf than the threshold (0.2% of leaf dry matter). Our survey also showed that many Trikkala samples had up to 2–3 times higher levels of formononetin than desirable: the reasons for this are unknown but likely include waterlogging.

The kits were an excellent strategy to extend our message nationally to the sheep meat and wool industry and increase the awareness of producers, veterinarians and agronomists around oestrogenic subclover. The results confirmed that old high-formononetin subclover cultivars remain prevalent across southern Australia.

2.4. Objective 4 – Ute Guide

Produce a Subterranean Clover Cultivar Guide, using photos from Phase 1, covering identification of all common subterranean clover cultivars, including their oestrogen levels, and best alternatives for use in renovating high-oestrogen pastures

After receiving user responses, the feedback from the livestock and seed industries was a preference for an oestrogenic subclover Ute Guide rather than an updated Subterranean Clover Cultivar Guide; hence, we focused on producing a Ute Guide, which will be released in late 2021.

2.5. Objective 5 - Handout

Update yearly a handout, targeted at farmers and advisors, outlining the problem (in terms of clinical and subclinical impact on livestock), how to identify it, and how to mitigate it using pasture renovation

A fact sheet was produced by UWA for field days and to be included with the free sample kits for producers. Over 600 fact sheets were distributed at field days and industry forums. This factsheet was later modified and uploaded to the MLA website. It describes the problem, its history and impact on livestock and how to ameliorate the problem in affected paddocks. Images of the five major subclover cultivars which contain high-formononetin are included.
https://www.mla.com.au/globalassets/mla-corporate/research-and-development/final-reports/2019/p.psh.1138-oestrogenic-clovers-factsheet.pdf

2.6. Objective 6 - Seed industry communication

Communicate to seed industry about oestrogenic clovers

We have been in discussions since 2019 with the seed industry and the ASF, locally and nationally, about the unintentional inflow of oestrogenic subclovers such as Dwalganup into commercial seed lots. To highlight the issue, in 2019, we grew and inspected 13 random samples of uncertified Dalkeith seed lines from Western Australia (non-certified seed sold is estimated at 90% in Western Australia) to visually identify any oestrogenic contaminants. We found six seed lines contained oestrogenic subclovers (between 5 and 45%, confirmed by isoflavone analysis), mostly Geraldton and Dwalganup. However, even at 5%, it is unlikely that these oestrogenic contaminates will remain at a low level for several years after sowing the pasture, as these oestrogenic subclovers are very competitive.

2.7. Objective 7 - Renovation case study

Complete case study of a high-oestrogen pasture renovation

At the UWA Ridgefield Future Farm, on a medium rainfall site (winter dominant, annual average 425 mm), we reduced the seed bank of highly oestrogenic old subclover cultivars, which dominated the pasture, on a commercial scale under continuous cropping (with in-crop subclover and broadleaf weed control) over three years from an estimated 220 kg/ha to 9 kg/ha. Further research is needed to identify economic options for renovating oestrogenic pastures, especially where large seedbanks of oestrogenic subclover exist and cropping options are limited, such as in higher rainfall zones or permanent pastures that cannot be cultivated due to frequent rocks, trees or waterlogging.

2.8. Objective 8 - Cost of oestrogenic pastures

Quantification of clinical and subclinical cost of oestrogenic clovers for livestock

Our cost-benefit analysis estimated the impact of remediating oestrogenic pasture—to achieve 10% more lambs and 1% less ewe mortality—on overall farm profit. The potential effect of clover disease expressed as a decline in lambing percentage ranged from $10–51 per hectare per year of gross margins per 10% change in lambing percentage.

2.9. Objective 9 - A peer-reviewed paper

A peer-reviewed paper for a scientific journal ready for approval by MLA

One paper has been accepted for publication in *Crop and Pasture Science* journal that contains data on oestrogenic pastures: Title: "Diversity for morphological traits and isoflavone contents among ecotypes of *Trifolium subterraneum* L. ssp. *yanninicum* and their relationships with site of origin." Enkhbat, Nichols, Foster, Ryan, Inukai, and Erskine.

3. Methodology

3.1 Objective 1 - Extension and adoption

Use a Design Led approach to develop and implement an extension and adoption plan

Methods:

Design Led Thinking (DLT) is a problem-solving approach popular in the extension and adoption of new practices or technology, focusing on questioning, interaction and feedback. In this project, DLT was modified through time constraints and the advent of the coronavirus pandemic, but the principles guided the construction and operation of the adoption activities.

Comments:

Design Led Thinking (DLT) courses were attended in Brisbane in 2018 at the start of the development of the Phase 2 project that helped to refine our approach to tackling extension and adoption, using producer surveys to provide regular feedback. We endeavoured to have a DLT workshop session in Perth with producers and researchers, but there was no mutually suitable time before the onset of COVID-19. However, DLT informed our approach to the project.

O1a. "*Five practical farmer workshops per year in association with producer groups and consultants across five states and smaller field days in South Australia and New South Wales where high levels of oestrogenic clovers are known to occur".*

Methods:

Visual identification guides, lectures, observation methods and field sampling techniques were developed for the sampling kits, workshops, field days and walks. These were then expanded and adapted for online presentation during lock-down periods when COVID-19 reduced opportunities to undertake face-to-face workshops and field days during 2020 and 2021.

Additional assistance was provided by consultants and researchers from ASHEEP and DPIRD in WA, Sheep Connect, AgKI, and PIRSA in South Australia, PPS and Southern Farming Systems in Victoria, LLS and DPI in New South Wales, and the University of Tasmania via online technologies.

O1b*. "A minimum of four social media releases per year aimed at informing farmers and advisors about the project"*

Methods:

We established a Twitter account and connected with southern Australian producers with an interest in, or concern about, low Merino lambing rates as our initial contact point. Management of the Twitter account required ongoing maintenance and feedback.

Comments: We regularly posted on Twitter, and producers and consultants used Twitter to engage with us directly. Presence at field days, workshops and forums was also reported on social media.

O1c. *"Presentations at a minimum of two high-impact industry forums per year (e.g. Agribusiness Update and Livestock Update)".*

Methods: PowerPoint presentations were developed for delivery at conferences or by webinar and Zoom. These presentations led the audience through the history of the issue, the past and current situation (from surveys), ways to identify potential problems, clover cultivar identification, pasture management and options for strategic grazing management or renovation.

Comments: In 2019, we presented at the PPS in Victoria and the ASF committee meeting in Perth. In 2020, due to COVID-19 restrictions, we presented online at two annual conferences, the Grasslands Society of Southern Australia and the PPS again. In addition, the project leader was a panellist for the webinar hosted by the DPI New South Wales on "*Oestrogenic sub clover pastures: identification and potential sheep health issues*".

3.2 Objective 2 - Extension and adoption outcomes

"Implement a monitoring and evaluation plan to record and highlight the results, outcomes and impact of the extension and adoption plan"

Methods: A strategic plan was developed to identify the most appropriate regions, producer groups, government and private organisations in each state for contact and interaction, using the survey data as the initial basis. Record keeping established the numbers, location and (where appropriate) contact details of interaction with producers and consultants, with additional data added each year.

Comments: The extension and adoption relating to the project was highly successful. This can be demonstrated by the program's reach, which has interacted directly with 975 people. Beyond these numbers, it is difficult to gauge the impact of social and print media; however, the number of enquiries coming into the team continues to increase with extension over time. The project has been featured on other media channels, such as New South Wales press, "The Land", Institute of Agriculture UWA Annual Reports, PPS newsletter, an article in "Farming Ahead" and on the MLA website.

3.3 Objective 3 – Pasture sampling kits

"Use farm consultants to distribute sampling kits to farmers with at least 100 kits returned, clovers identified, and oestrogen levels measured in the laboratory and farmers informed of their result".

Methods:

We designed a sampling kit including visual identification guide with instructions for the correct way in which samples of suspected high-formononetin subclovers could be taken in the paddock. The kits were published, assembled in padded post bags, already return addressed and pre-paid. Samples returned by mail were stored in a cold room, before determination of the levels of isoflavones, cultivar identification and phosphorus, potassium and sulfur leaf analysis.

Isoflavone contents (formononetin, genistein and biochanin A) were measured using the semi-quantitative technique of Beck (1964) and Francis and Millington (1965). Samples of six leaf discs (6 mm diameter) were collected from healthy leaves for analysis. Isoflavones were then extracted in alcohol and subjected to thin-layer chromatography. Duplicate samples taken for dry weight determination were oven-dried at 60°C for 48 hours. Isoflavone contents were calculated as a percentage of leaf dry matter.

For shoot nutrient analysis, samples were dried at 70 °C for 72 hours to constant weight, and ground to a fine powder using a Geno/Grinder 2010 (Spex SamplePrep, Metuchen, NJ, USA). Weighed subsamples, c. 100 mg, were digested using a concentrated HNO_3–$HClO_4$ (v/v = 3:1) mixture. Elemental levels were determined by an inductively coupled plasma-optical emission spectrometer (ICP–OES, OPTIMA 5300 DV, Perkin–Elmer, Shelton, CT, USA).

All results on cultivar identification and formononetin levels were disseminated back to producers by email.

Comments: During 2019, we distributed 230 free pasture kits with the subclover identification sheets to producers: 103 were returned, and many contained samples from multiple paddocks or farms. Based on the interest of producers, we then decided to offer this service again in 2020 and distributed 80 kits with 56 returned: again, many had more than one sample enclosed. All producers received their pasture identification and laboratory results before the autumn break of the following year.

3.4 Objective 4 - Ute Guide

Produce a Subterranean Clover Cultivar Guide, using photos from Phase 1, covering identification of all common subterranean clover cultivars, including their oestrogen levels, and best alternatives for use in renovating high-oestrogen pastures.

Feedback from the livestock and seed industries revealed a preference for an oestrogenic subclover Ute Guide, similar to others used in farming for weed identification, rather than an updated Subterranean Clover Cultivar Guide. We therefore decided to focus on a Ute Guide, while continuing to work on updating the existing cultivar guide (Nichols et al. 1966) when time permitted. For both guides, new photographs were produced using cultivars grown specifically for this purpose at the UWA Shenton Park Field Station. Key features of these were photographed at key phenological stages.

Comments. The anticipated release date for the Ute Guide is late spring 2021. Work is continuing on the new Subterranean Clover Cultivar Guide.

3.5. Objective 5 - Handout

"Update yearly a handout, targeted at farmers and advisors, outlining the problem (in terms of clinical and subclinical impact on livestock), how to identify it, and how to mitigate it using pasture renovation".

Method: A simple fact sheet was designed.

Comment: We produced two oestrogenic subclover fact sheets for the sheep meat and wool industry. The UWA version was later updated by MLA and has been uploaded to their website.

https://www.mla.com.au/globalassets/mla-corporate/research-and-development/final-reports/2019/p.psh.1138-oestrogenic-clovers-factsheet.pdf

3.6. Objective 6 - Seed industry communication

Communicate to seed industry about oestrogenic clovers.

Method 1: The Australian Seed Federation (ASF) in Western Australia was approached to investigate whether seed from highly oestrogenic subclover cultivars were contaminating seed from non-certified sources. Random samples of non-certified seed were germinated and grown at the UWA Shenton Park Field Station. Joint meetings were held to develop a strategy and determine the need for a national information program. Protocols were developed for a future communication strategy with ASF.

Comments: Productive discussions occurred with ASF (Western Australia) and nationally on the need to seek future funding to test for oestrogenic subclover contaminants in non-certified commercial seed. ASF agreed to encourage customers to buy certified subclover seed and highlight the issue of contamination by highly oestrogenic cultivars nationally.

Method 2: Development of a visual identification chart to distinguish seedlings of low- and high-formononetin subclover cultivars with similar morphology, such as Dalkeith and Dwalganup. This chart precisely details the morphological markings in emerging seedlings in paddocks or controlled conditions, such as when seeds are germinated and grown out prior to certifying seed lines.

Comment: This new seedling morphological identification tool has been well-accepted by ASF (WA) and used in the glasshouse to differentiate cultivars. It will be available for use by the ASF nationally.

3.7. Objective 7 - Renovation case study

3.7.1 Renovation case study overview

A field trial was set up at the UWA Ridgefield Future Farm near Pingelly WA, on a medium rainfall site (winter dominant, annual average 425 mm), to assess whether a new low-formononetin subclover cultivar could be established as the dominant subclover in a paddock dominated by old high-formononetin cultivars.

Eight pasture paddocks were assessed for potential renovation sites, with two selected for the treatments and a third used as the control. The three paddocks were small and adjacent. Each paddock was maintained for three years (March 2018 to March 2021). The treatments were: 1) sown to low-formononetin Urana cultivar in Year 1 and managed for two additional years; 2) sown to oats each year for three years; and 3) managed as usual (i.e. high-formononetin cultivars undisturbed).

Cultivar Urana was chosen for the renovation paddock due to its low formononetin level and, importantly, no leaf marking, which makes it easy to distinguish from the other highly oestrogenic cultivars in the seed bank.

3.7.1 Soil seed bank assessment

Subclover burrs were sampled from each paddock in March 2018 to quantify the size of the subclover seed bank (Fig. 1A). Quadrants, 1 m by 0.20 m (20/paddock), were randomly sampled and bulked. The burr were then threshed to extract seed. Seed from each paddock were grown in a glasshouse to identify the subclover cultivars using leaf marks. In April 2018, we confirmed highly oestrogenic subclover cultivars in many pastures at the farm, with Geraldton, Dwalganup and Dinninup present. These are all highly hard-seeded cultivars and thus persistent, as seeds remain viable and available to germinate for up to four or more years. In March 2019, 2020 and 2021, i.e. prior to the season break and growing season, we again harvested burr to determine the size of the seed bank and its varietal composition.

At each sampling time, all seed was cleaned and weighed. A subset of the seed from each of the three treatments was hand processed and grown in the glasshouse for identification using seedling morphology. Prior to sowing in the glasshouse, seeds were scarified for 10 seconds using a brass cylinder lined with fine sandpaper driven by compressed air. The air compressor pressure was 30 kPa (200 psi). Seeds from each plot were sown in hydrated jiffy peat pots (size 522 Jiffy Products Ltd, Norway) containing potting mix in May each year. Each seedling tray contained 30 peat pots, with four trays per plot (minimum of 120 seedlings each treatment). Group C Rhizobium inoculant was applied by watering can three days after sowing, by adding 40 mL inoculum to 10 L water (one watering can for seven trays). Peat pots were watered when required. After six weeks, all seedlings were visually identified in each tray (Fig. 1B). The occurrence of each cultivar as a seedling was assumed to reflect its occurrence in the seed sample.

Comments: The field sampling burr method and growing of the seed in the shadehouse were successful each year.

3.7.2 Agronomic management

Year 1 (2018)

We sprayed two small paddocks (0.5 ha) containing highly oestrogenic subclover with glyphosate twice in June, one and three weeks after the break of the season (see Year 1 methodology box below); one was sown to Urana (Fig. 1C) and one was sown to oats. A third paddock was left undisturbed as the control. The oats crop had in-crop broadleaf weed control applied once in August.

Urana established well (Fig. 1D); seedling counts undertaken in late July (20 quadrats per plot) showed 189 seedlings per m^2. For the oats paddock, the background oestrogenic subclover that geminated in the crop was five plants per m^2. Overall, the oats and Urana paddocks were well-managed, with the Urana pasture grazed to control weeds and encourage seed set. Both paddocks were fertilised in spring and grazed over summer to remove any crop or pasture residues. The control paddock also established well in early June with 1075 seedlings per m^2 and was managed and grazed as part of normal farm operations. The oats paddock had good crop establishment with few subclover seedlings. Broad leaf herbicides were later used to ensure no subclover set seed.

Methodology Summary: Year 1

The following methods were used to establish the Urana subclover and oats paddocks in 2018:

Urana

- Pre-sowing knockdown (glyphosate): 2.5 L/ha (twice)
- Urana subclover sown: 20 kg/ha with group C rhizobia (dry inoculant)
- Superphosphate: 150 kg/ha at sowing
- Superphosphate and potash (3:1): 100 kg/ha applied in spring

Oats

- Pre-sowing knockdown (glyphosate): 2.5 L/ha (twice)
- Oats sown: 120 kg/ha
- Superphosphate and urea: 150 kg/ha at sowing
- In-crop broadleaf weed control (dicamba): 250 mL/ha in August
- Superphosphate and nitrogen fertiliser: 100 kg/ha in spring

Figure 1. Aspects of the pasture renovation study at the UWA Ridgefield Future Farm in 2018. (A) Sampling paddocks in March 2018 to determine the size of the subclover seed bank. (B) Trays containing subclover seed from each paddock being grown out to determine the cultivar. (C) Sowing the low-formononetin subclover cultivar Urana into subclover pastures dominated by high-formononetin cultivars (note that the paddock was sprayed three days prior with glyphosate for the second time). (D) Excellent establishment of Urana with good weed control.

Year 2 (2019)

The renovation site was resown in 2019 (see Year 2 methodology box below). The first paddock was again sown to oats. The second paddock was again resown to Urana (Fig. 2A–C). The control paddock was again maintained under normal farm management.

In July, the Urana paddock had 140–170 Urana seedlings per m^2 across 20 quadrants. The control paddock established well and had 1088 subclover seedlings per m^2. The oats paddock had only six seedlings per m^2. The site was well-managed, and pasture grazed to encourage seed set in the subclover paddock and grazed for weed control (Fig. 2C). It was useful to have the new cultivar (Urana) characterised by its lack of leaf marks (Fig. 2D). The oats paddock established and grew well and contained few weeds or subclover plants (Fig. 3). The unrenovated control paddock was heavily grazed and continued to be dominated by highly oestrogenic cultivars and weeds (Fig. 4).

After the 2019 growing season, all paddocks were lightly grazed over summer to remove dry residue and oat stubble and encourage subclover germination in 2020.

Methodology Summary: Year 2

The following methods were used to establish the subclover and oats paddocks in 2019:

Urana

- Presowing knockdown (glyphosate): 2 L/ha (twice)
- Urana subclover sown: 20 kg/ha with group C rhizobia (dry inoculant)
- Superphosphate: 100 kg/ha at sowing
- Post-sowing pre-emergent insecticide (bifenthrin): 40 mL/ha
- Superphosphate and potash (3:1): 100 kg/ha in spring

Oats

- Presowing knockdown: glyphosate 2 L/ha (twice)
- Oats sown: 120 kg/ha
- Gusto Gold fertiliser (10:13:11:6 N:P:K:S): 100 kg/ha
- In furrow application of the fungicide Uniform for control of rhizoctonia
- Post-sowing pre-emergent insecticide (bifenthrin): 40 mL/ha
- In-crop broadleaf weed control (dicamba): 250 mL/ha in August
- Superphosphate and nitrogen fertiliser: 100 kg/ha in spring

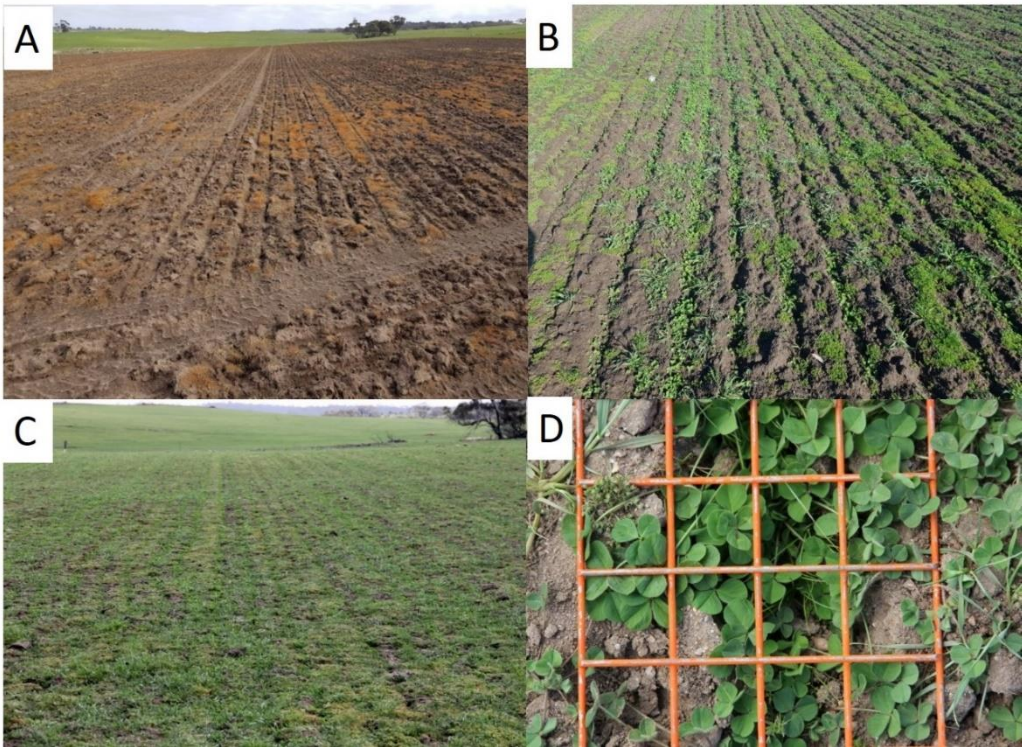

Figure 2. Aspects of the pasture renovation study at the UWA Ridgefield Future Farm in 2019. Paddock resown to Urana subclover. (A) June: excellent control of weeds and background highly oestrogenic subclover. (B) July: good density of Urana and some weeds, including annual ryegrass. (C) September: excellent weed control one week post-grazing. (D) July: dominance of Urana (no leaf marks) but some highly oestrogenic Dinninup present in centre.

Figure 3. Oats renovation paddock in 2019 with excellent seedling emergence and control of highly oestrogenic subclover and weeds. (A) Post-sowing. (B) Late August. (C, D) September.

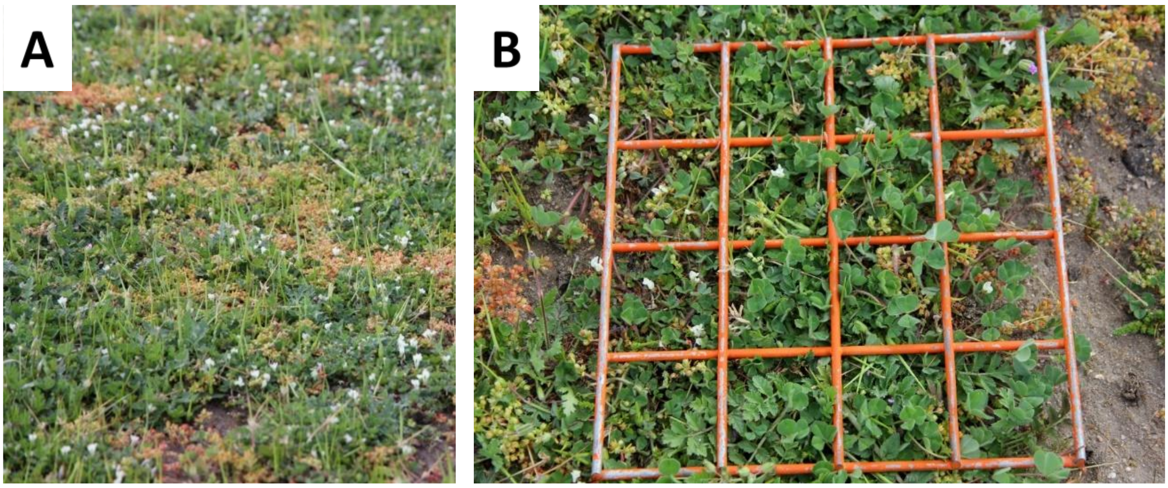

Figure 4. Unrenovated control paddock in October 2019 showing high occurrence of (A) weeds and (B) highly oestrogenic cultivars.

Year 3 (2020)

The renovation site was resown in 2020 (see Year 3 methodology box below). Both renovation paddocks were sprayed twice with glyphosate after the break of the season. In June, the pasture paddock was oversown with Urana, and oats were sown in the cropping paddock for the third time (Fig. 5). Urana establishment was very good with 125–150 seedlings per m^2 (Fig. 5). The Urana was not grazed until mid-winter to maximise leaf production (Fig. 5C). In the oats paddock, there were again very few subclover plants, with only two plants per m^2 (Fig. 5D). The control paddock was again subjected to normal farm management only; there were 1040 subclover seedlings per m^2.

Methodology Summary: Year 3

The following methods were used to establish the subclover and oats paddocks in 2020:

Urana:

- Pre-sowing knockdown (glyphosate): 2.5 L/ha (twice)
- Urana sub clover sown: 20 kg/ha with group C rhizobia (dry inoculant)
- Superphosphate: 150 kg/ha at sowing
- Post-sowing pre-emergent insecticide (bifenthrin): 40 mL/ha
- Superphosphate and potash (3:1): 100 kg/ha in spring

Oats:

- Knockdown (glyphosate): 2.5 L/ha (twice)
- Oats sown: 120 kg/ha
- Superphosphate and urea: 150 kg/ha each
- Post-sowing pre-emergent insecticide (bifenthrin): 40 mL/ha
- In-crop broadleaf weed control (Dicamba): 250 mL/ha in August
- Superphosphate and nitrogen fertiliser: 100 kg/ha in spring

Grazing pressure was reduced after the first flowers became visible on the Urana plot as constant removal of flowers can significantly reduce seed production. Adequate super and potash fertiliser were also applied in spring along with 100 kg/ha of nitrogen to the oats plot. We harvested the renovation plots for seed yield in summer (March 2021) and grew the subclover seed in the glasshouse for identification. Harvested seed was grown in the glasshouse again to assess the proportions of Urana subclover and oestrogenic subclover left in the seed bank. The subclover and oats plots were grazed for short and intense periods over summer before seed harvesting.

Comments: Preparation for renovation should start the year before sowing the new, low-formononetin subclover cultivar or oats. The year before a paddock is to be renovated, spray topping (before flowering) will help lower the seed bank before renovating with a new cultivar. The relatively late maturity of Urana was not ideally suited to this site, with dry springs in 2019 and 2020 likely reducing seed set. However, the ease of distinguishing it from the established highly oestrogenic cultivars at the site, due to its lack of leaf marks, was beneficial.

Figure 5. Aspects of the pasture renovation study at the UWA Ridgefield Future Farm in 2020. (A) Sowing Urana subclover in June. (B) Urana renovation paddock successfully established with excellent density. (C) Grazing of Urana to control grasses and weeds and encourage seed set. (D) Oats crop with little survival of highly oestrogenic subclover following broadleaf weed control.

3.8. Objective 8 - Cost of oestrogenic pastures

Quantification of clinical and subclinical cost of oestrogenic clovers for livestock.

Note: Methods 1: The first modelling was a cost-benefit analysis that estimated the impact of remediating oestrogenic pasture—to achieve 10% more lambs and 1% less ewe mortality—on overall farm profit

Methods 2: The MERINO program developed by Morley (1985) is designed to assess the physical and economic consequences of a change in management decisions in a Merino production system in the higher regions of southern Australia. This model has been used in sheep consulting practices where its predictions almost always follow expected patterns. Its profitability prediction to increased reproductive performance closely follows that estimated by more complex models of sheep production systems (Watts, 1987).

We used the same parameters as an average southern Australian mixed farm with 30% of the pasture biomass as oestrogenic subclover over the whole farm to model the economic consequences of suboptimal fertility. That is, where low conception rates and incidence of lamb and ewe mortality reduce productivity and profit.

Comments: There has been little new research on the subclinical cost of oestrogenic subclover with ewes, but the previous models are still relevant. We note that these models do not consider increased pasture production or other quality effects (increased disease tolerance, improved winter vigour and seed yield) likely to result from pasture improvement by renovating with new low-formononetin cultivars.

3.9. Objective 9 - A peer-reviewed paper

A peer-reviewed paper for a scientific journal ready for approval by MLA.

Method: Team collaborators have identified various parts of the project that will be written up as scientific journal papers for submission to peer-reviewed journals of international standing.

One paper with team member contribution has been published in **Crop and Pasture Science**: it contains data on formononetin levels in subclover subspecies *yanninicum*: **Title**: "Diversity for morphological traits and isoflavone contents among ecotypes of *Trifolium subterraneum* L. ssp. *yanninicum* and their relationships with site of origin."

4. Results

4.1 Objective 1 – Extension and adoption

1a. Use a Design Led (DLT) approach *to develop and implement an extension and adoption plan to address oestrogenic induced reproductive failure in meat sheep*

We discussed the best way to use DLT to frame the research in this project and work with industry and business to ensure that the outcomes from our work have maximum impact on the livelihoods of the producers. For instance, discussions with people in the sheep meat and wool industry (producers, extension personnel, veterinarians, agronomists) indicated that close to 100% were not familiar with the issue of oestrogenic subclover pastures and 'clover disease'. Many were unaware that subclovers can contain oestrogenic compounds or the implication of this for ewe health and reproduction. Few producers or advisers could identify subclover cultivars in the field, but field days demonstrated that producers and researchers could be upskilled quickly through informed extension. Many producers at field days were concerned about their low lambing percentages and unsure as to the cause. Thus, discussions with industry and close in-field engagement with producers (e.g. observing and identifying pasture species in the field) identified the problem being addressed in the project. The project was deliberately producer-centred so that the solutions to the problem could be readily translated into practical actions on-farm.

Extension and communication through the pasture community included partnering with producers, seed producers, university researchers, consultants, veterinarians, industry representatives and government employees (NSW DPI, Local Land Services New South Wales, PIRSA, Sheep Connect in South Australia, Agriculture Victoria, Perennial Pasture Systems, Southern Farming Systems in Victoria and the University of Tasmania) was determined the most effective outreach method. We also engaged Merino producers and prominent meat producers. An extension program was developed that centred on outreach activities with these partners. Extension examples are shown in Appendix 1.

1b. Social media. *A minimum of four social media releases per year aimed at informing farmers and advisors about the project*.

Using social media, we reached a much wider audience of producers than would have been possible with just traditional field days and workshops alone. We increased our tweets and mentions by more than 40% each and increased our followers (Appendix 1). Presence at field days, workshops and forums were highlighted on social media. Producers and consultants used Twitter to engage with us directly, posting photos of subclover plants and asking questions on oestrogenic subclovers and renovation (Appendix 1). Many of our responses were retweeted. Two of our social media posts reached 30,000 potential followers. Overall, the tweets stimulated conversation on subclover identification and pasture renovation within the pasture community. Due to the widespread social media attention, we received requests on Twitter from producers and agronomists across southern Australia for assistance with the identification and management of oestrogenic pastures. Overall, the more attentive approach afforded by the increased reliance on social media led to deeper learning opportunities for producers, greater engagement and a flow-on effect that stimulated conversations on media forums such as Twitter.

In addition to Twitter, the project was featured on other media channels, including New South Wales press “The Land”, UWA media release and Institute of Agriculture Annual Reports, MLA website, PPS newsletter, and “Farming Ahead” by the Kondinin Group.

c. ***Presentations at a minimum of two high-impact industry forums per year*** *(e.g. Agribusiness Update and Livestock Update).*

In 2019, we presented the findings at the PPS in Victoria and the ASF committee meeting in Perth. In 2020, due to COVID-19 restrictions, we presented online at two annual conferences, the Grasslands Society of Southern Australia and PPS. We also presented a one-hour webinar on 5 May 2021 on “Oestrogenic clovers – identification and remediation” with a Q&A feedback session for producers and industry. The webinar has been uploaded to the MLA website for producers and has led to subsequent producer enquires about subclover identification. In February 2021, we presented at the 33rd Australian Association of Animal Science online conference in Perth. We have also included a project update for the UWA Institute of Agriculture (IOA) annual research report.

4.2 Objective 2 – Extension and adoption outcomes

Record and highlight the results, outcomes and impact of the extension and adoption plan.

The extension and adoption relating to this project was highly successful and primarily demonstrated by the immediate reach of the program (975 people – Appendix 1). Beyond these numbers, enquiries have continued to increase as the direct extension effect has multiplied. This is partly due to the many researchers, community and grower group leaders, and veterinarians who were impacted by the program and provided the information developed through the project to the broader farming community and producers. For example, we helped train a commercial agronomist in South Australia to identify oestrogenic subclovers and provided seed of five oestrogenic subclovers as a living reference and teaching guide. Similarly, we had regular and valuable discussions with David Woodard from PIRSA, and we attended field days and undertook paddocks inspections together in South Australia and Victoria in 2018.

4.3 Objective 3 – Pasture sampling kits

Use farm consultants to distribute sampling kits to farmers with at least 100 kits returned, clovers identified, and oestrogen levels measured in the laboratory and farmers informed of their results (Year 2).

During 2019, we disseminated 230 free pasture kits with subclover identification sheets to producers. Of these, 103 were returned, and many contained samples from multiple paddocks or more than one farm. The inclusion of samples from multiple paddocks (up to 10) highlighted the enthusiasm of producers. All results and interpretation were disseminated to producers. The offer of free testing kits was well-subscribed in 2020, with 80 free pasture kits disseminated and 56 kits returned. Again, many returned kits contained many samples from multiple paddocks or more than one farm. In both years, many returned samples contained more than one cultivar or variant of subclover, typical of older pastures; these were analysed separately. For instance, many samples from New South Wales contained multiple older subclover types such as Woogenellup, Mt. Barker, Seaton Park, and Bacchus Marsh, and highly oestrogenic subclovers like Dinninup and local variants that we did not recognise as known cultivars.

It must be noted that the survey was not a random survey that captured the overall prevalence of highly oestrogenic subclovers: producers that already suspected a problem were most likely the ones who returned the kits. The number of kits returned, and number of samples analysed, was quite low for most states relative to the area sown to subclover. For instance, in New South Wales, most kits were returned from only a few districts, such as those around Yass, Bowning and Galong (Fig. 6). Thus, the results presented in this section should not be used to make definitive conclusions about differences between states or the overall prevalence of highly oestrogenic subclover.

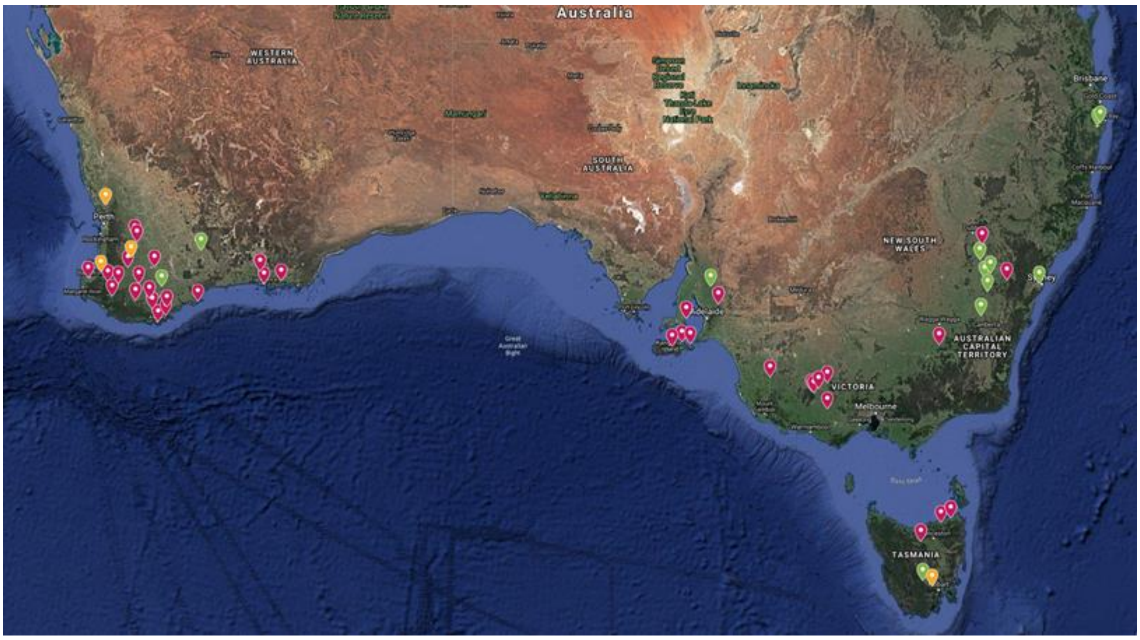

Figure 6. Sample collection sites across southern Australia, mapped to the closest town with traffic light label pins (green, <0.2; orange, 0.2-0.3; red, >0.3% percentage of leaf dry weight as formononetin). Note that each pin can represent more than one sample.

In total, 577 samples were analysed; of these, 348 (60%) contained levels of formononetin above the safe level (0.2% of leaf dry weight) (Table 1). South Australia had the highest average formononetin level across all samples and the highest average among samples over the safe limit. While further sampling is required (especially New South Wales) to confirm these differences among states, it suggests South Australia as a priority for further extension activity, followed by Western Australia, Victoria, New South Wales and Tasmania. Figure 6 highlights the regions of greatest concern with clusters identifiable in the far south-west corner of Western Australia, near Kangaroo Island and proximal mainland areas in South Australia, across Victoria, and in northern Tasmania. However, it also highlights regions that still need to be investigated; these should also be targeted for future extension.

Table 1. Formononetin levels (% leaf dry weight) in the samples returned from the pasture sampling kits disseminated in 2019 and 2020, showing state of origin and the number (and %) of samples where formononetin was above the safe level (0.2% of leaf dry weight).

	All samples received			Samples with formononetin >0.2% leaf dry weight		
State	Number	Formononetin (%)	Standard error	Number (and %)	Formononetin (%)	Standard error
NSW	100	0.25	0.041	27 (27)	0.77	0.123
SA	133	0.90	0.058	107 (80)	1.09	0.058
TAS	80	0.18	0.030	20 (25)	0.50	0.089
VIC	72	0.37	0.046	43 (60)	0.54	0.065
WA	192	0.70	0.039	151 (79)	0.87	0.039
All samples	**577**	**0.55**	**0.024**	**348**	**0.87**	**0.030**

There was a large number of subclover cultivars in the samples returned from the kits (Table 2). The cultivars were identified based on their leaf morphology and their formononetin level. The most common cultivar identified was, by far, Dinninup. It was found in samples from all states and was particularly dominant in South Australia and Western Australia. The four other old cultivars known to be associated with 'clover disease' due to high formononetin levels were also present: Dwalganup, Geraldton, Yarloop and, in high rainfall regions, Tallarook. The relatively low occurrence of relatively modern low-formononetin cultivars indicates that producers were motivated to submit samples based on their suspicions that highly oestrogenic subclovers were present or because they simply did not know what cultivars were present. However, it should be noted that the data in Table 2 are influenced by the low sample size and the concentration of samples from only a few regions. For instance, surveys by project team members prior to this project found that Dwalganup was quite common around Wagga Wagga in New South Wales where few samples were received.

Table 2. Subclover cultivars in the samples returned from the pasture sampling kits disseminated in 2019 and 2020, showing state of origin – known oestrogenic cultivars bolded.

South Australia		Western Australia		Victoria		Tasmania		New South Wales	
Bindoon	2	Dalkeith	2	**Dinninup**	13	Bacchus Marsh	1	**Dinninup**	11
Dinninup	65	**Dinninup**	90	Mt Barker	3	**Dinninup**	2	**Dwalganup**	2
Dwalganup	5	**Dwalganup**	7	Seaton Park	4	**Geraldton**	2	Mt Barker	3
Esperance	1	Esperance	1	Trikkala	10	Howard	1	Seaton Park	4
Geraldton	7	**Geraldton**	12	Unknown	2	Mt Barker	2	**Tallarook**	1
Mt Barker	3	Gosse	2	Woogenellup	11	**Tallarook**	3	Trikkala	3
Seaton Park	2	Mt Barker	4			Unknown	5	Woogenellup	2
Trikkala	8	Seaton Park	17			Woogenellup	4	**Yarloop**	1
Unknown	1	Trikkala	8						
Woogenellup	7	Unknown	1						
Yarloop	6	Woogenellup	5						
		Yarloop	2						

All data obtained from each cultivar identified are graphed in the scatterplots in Figure 7; the mean for each cultivar is also indicated. The results of a one-way analysis of variance on these data are contained in Table 3. As expected, the highest average formononetin level occurred in Yarloop, followed by Dinninup, Dwalganup and Geraldton; there was insufficient samples of Tallarook to include. One interesting aspect of these data is the high variability; this is particularly evident for Dinninup which mostly varied from around 0.35% to 2.5% with a single outlier approaching 3%. Yarloop was also highly variable; although sample size was quite small. These results, coupled with those in Table 2, suggest that Dinninup is the oestrogenic clover of greatest concern across southern Australia, especially in South Australia and Western Australia. Dwalganup and Dinninup are early to mid-maturing and have a high hard seed level. These traits will aid them to survive false breaks at the start of the season and set more seed in a dry springs than later-maturing cultivars with lower levels of hard seed. Thus, a number of factors could act individually or in combination to result in dominance by these two cultivars including; lower palatability than other cultivars with lower formononetin in the same pasture, false breaks, dry springs, a long cropping phases or droughts.

All remaining cultivars had a level of formononetin was that significantly lower than that of the four known oestrogenic cultivars. However, it is notable that some of these “safe” cultivars also had samples where formononetin content was above 0.2%. Woogenellup and Mt Barker had a smaller proportion of samples where F was above 0.2%. However, concerningly, Seaton Park and Trikkala, had around 50% of samples above 0.2%. Although, it must once again be noted that, these samples likely came from producers who suspected a problem and hence are likely not representative of the pastures of these cultivars across southern Australia. Even so, it is worth noting that Seaton Park was re-selected for low formononetin content in 1988 (Seaton Park LF: suffix later dropped), following the finding that more than one version of the cultivar existed, with some being moderately oestrogenic (Rossiter et al. 1985). It is therefore likely that some of the submitted samples were from pastures sown to the original higher formononetin cultivar. Trikkala has been widely sown in high rainfall zones (>400 mm) since the late 1970s as a replacement for the highly oestrogenic cultivar Yarloop (Francis 1976); both Trikkala and Yarloop belong to subspecies *yanninicum* and have good tolerance of winter waterlogging.

All three isoflavones have previously been reported to increase in response to environmental stresses, including severe deficiencies of soil phosphorus (Butler et al. 1967; Rossiter 1970), sulfur (Schoo and Rains 1971) and nitrogen (Rossiter 1969), as well as waterlogging (Francis and Devitt 1969). Indeed, severe P deficiency increased formononetin levels up to five-fold in an environmentally controlled glasshouse (Rossiter and Beck 1966). Reduced growth due to phosphate deficiency and waterlogging are thought to increase the levels of carbon substrate in leaves (Rossiter and Beck 1966; Barta 1988), which could make more substrate available for isoflavone synthesis (Jones and Hartley 1999). Francis and Devitt (1969) reported that waterlogging decreased shoot and root dry matter markedly and increased leaf formononetin more so in waterlogging-intolerant than waterlogging-tolerant subclover cultivars.

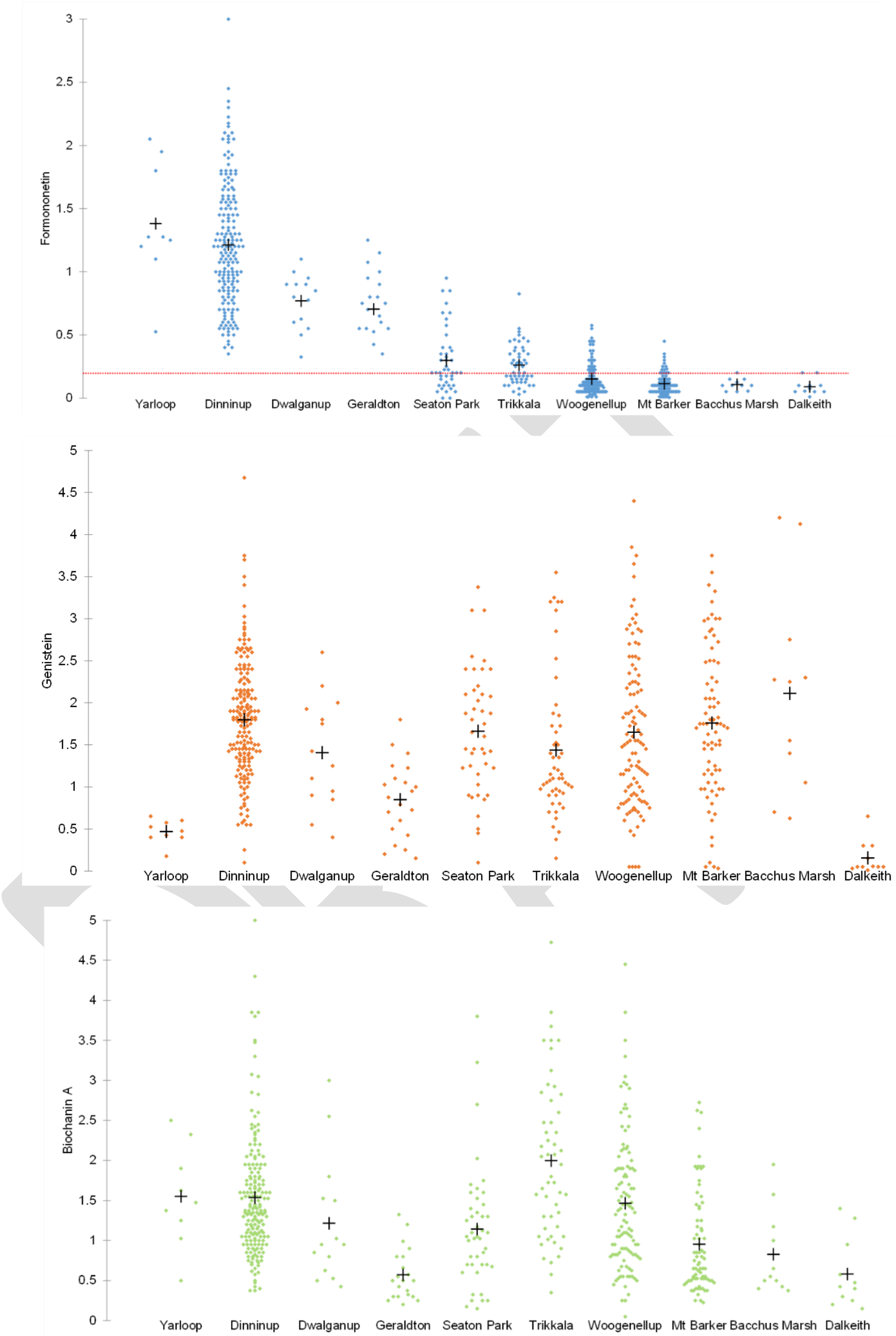

Figure 7. Scatterplot of the percentage leaf dry weight consisting of the three major phytoestrogens (formononetin, biochanin A, genistein) in the ten most common subclover cultivars returned by producers (mean indicated by black cross). The red line indicates the level of formononetin considered safe (0.2%).

Table 3. Outcome of the one-way analysis of variance on the impact of cultivar on the percentage of leaf dry weight consisting of the three major phytoestrogens (formononetin, biochanin A, genistein). Post-hoc pairwise comparisons were made using the Games Howell test: means in the same column with different letters differ at P<0.05. s.e. = standard error.

Cultivar	Formononetin			Cultivar	Biochanin A			Cultivar	Genistein		
	Mean	s.e.			Mean	s.e.			Mean	s.e.	
Yarloop	1.38	0.11	A	Trikkala	2.00	0.11	A	Bacchus Marsh	2.11	0.24	A
Dinninup	1.21	0.02	A	Yarloop	1.55	0.25	AB	Dinninup	1.80	0.06	A
Dwalganup	0.77	0.08	AB	Dinninup	1.54	0.06	AB	Mt Barker	1.76	0.09	A
Geraldton	0.70	0.07	B	Woogenellup	1.47	0.07	B	Seaton Park	1.66	0.12	A
Seaton Park	0.30	0.05	C	Dwalganup	1.22	0.20	BC	Woogenellup	1.65	0.07	A
Trikkala	0.26	0.04	C	Seaton Park	1.14	0.11	BC	Trikkala	1.44	0.11	A
Woogenellup	0.15	0.03	D	Mt Barker	0.95	0.09	BC	Dwalganup	1.41	0.21	A
Mt Barker	0.11	0.04	D	Bacchus Marsh	0.83	0.23	BC	Geraldton	0.85	0.17	A
Bacchus Marsh	0.11	0.10	D	Dalkeith	0.58	0.23	C	Yarloop	0.47	0.26	B
Dalkeith	0.09	0.10	D	Geraldton	0.57	0.17	C	Dalkeith	0.15	0.24	C
	F	Pr > F	R^2		F	Pr > F	R^2		F	Pr > F	R^2
	143	**<0.0001**	0.71		13	0.000	0.18		11	0.000	0.16

In response to these previous reports, we examined the relationship between formononetin and the levels of phosphorus, potassium and sulfur in the subclover shoots for the samples returned with the kits (Fig. 8). In each case, however, there was no relationship with formononetin. This was despite a reasonable number of samples being less than 0.2% of dry weight as phosphorus, suggesting phosphorus was limiting growth (Weir and Cresswell, 1994). Indeed, Reed (2020) in a glasshouse subclover phosphorus response experiment found that low phosphorus only resulted in a significant increase in formononetin once below 12 mg P/kg, that is, very much below the external critical P level of 20 mg P/kg (soil P required to achieve 90–95% of maximum yield; Simpson et al. 2015). Even so, it may still be worth noting a potential link between low phosphorus and formononetin to producers as there is an increased awareness of the economic loss and environmental degradation that can be caused by excessive phosphate use. This awareness could lead to efforts to improve the phosphate use efficiency of pastures (Weaver and Wong 2011) through reducing fertiliser use to in order to maintain pastures at the external critical P. There may also still be some very low P soils across southern Australia. For instance, rocky hilltops where fertiliser is not easily spread.

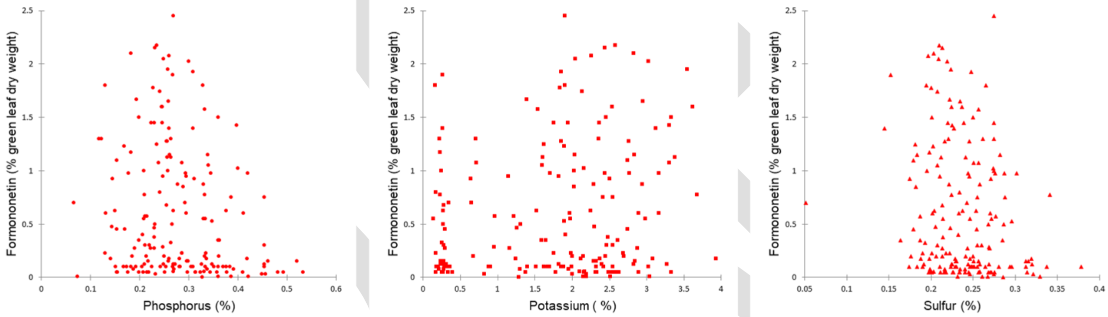

Figure 8. Relationship between the percentage of green leaf dry weight containing formononetin, and shoot phosphorus (P), potassium (K) and sulfur (S) levels).

The level of potassium and sulfur in green leaves that indicates subclover plants as likely being deficient is these elements is <0.8% and <0.22% respectively (Weir and Creswell 1994). Very few of our samples are therefore deficient in potassium (Fig. 9). However, around one-third are perhaps deficient in sulfur. In spite of this, there was no relationship with formononetin for either potassium or sulfhur. Thus, it seems that factors other than nutritional deficiencies are responsible for the high degree of variation in formononetin for individual cultivars.

We did examine experimentally the impact of waterlogging on the level of formononetin in subclover. Reed (2020), in a glasshouse experiment, found that waterlogging increased formononetin from around 0.05% to 0.2% in two low-formononetin cultivars from around 0.8% to 1.2% for Dinninup and 1.6% to 1.9% in Yarloop. In a paddock which experienced extended waterlogging in low lying areas in 2021, formononetin levels in Dinninup were 0.86% in plants not experiencing waterlogging and 1.5% in plants experiencing waterlogging (Gereltsetseg Enkhbat, unpublished data). Further work on waterlogging and formononetin in subspecies *yanninicum* is currently underway and preliminary results suggest potential for increased formononetin under waterlogging (Gereltsetseg Enkhbat, unpublished data).

In summary, it seems likely that some of the variation in formononetin levels for each cultivar is likely due to environmental factors. Waterlogging seems certain to increase formononetin levels if it occurs, but the role of low phosphorus in current pastures is less clear. The discrepancy between our results and those in the older literature likely reflect older studies between carried out with extremely low levels of soil available P: levels that are rarely encountered in pastures today. However, these are many other factors that merit investigation including stresses such as insect damage and herbicide application.

4.4 Objective 4 – Ute Guide

Produce a Subterranean Clover Cultivar Guide, using photos from Phase 1, covering identification of all common subterranean clover cultivars, including their oestrogen levels, and best alternatives for use in renovating high-oestrogen pastures

The strong feedback from the livestock and seed industries was the preference for an oestrogenic subclover Ute Guide rather than an updated Subterranean Clover Cultivar Guide; hence, we focused on producing a Ute Guide, which we expect to be released in late 2021.

The pictorial guide is designed to assist with the identification and management of oestrogenic subclover cultivars. Five cultivars of subclover were selected for inclusion in this guide due to their high levels formononetin: Dwalganup, Geraldton, Yarloop, Dinninup and Tallarook. In the Ute Guide, they are arranged in order of time to first flowering. The primary identification tools in the Ute Guide are photographs and illustrations. The text is organised around a general description, followed by highlights of key features to aid in correct identification. Photographs of each cultivar are shown in order according to season. This is important as leaf marks and other characters change from winter to spring. Seedling descriptions and photographs are provided to enable identification at early growth stages to ensure effective and timely control or avoidance of oestrogenic subclovers. After the five main oestrogenic cultivars are individually described, they are compared to non-oestrogenic cultivars that are morphologically similar (Fig. 9).

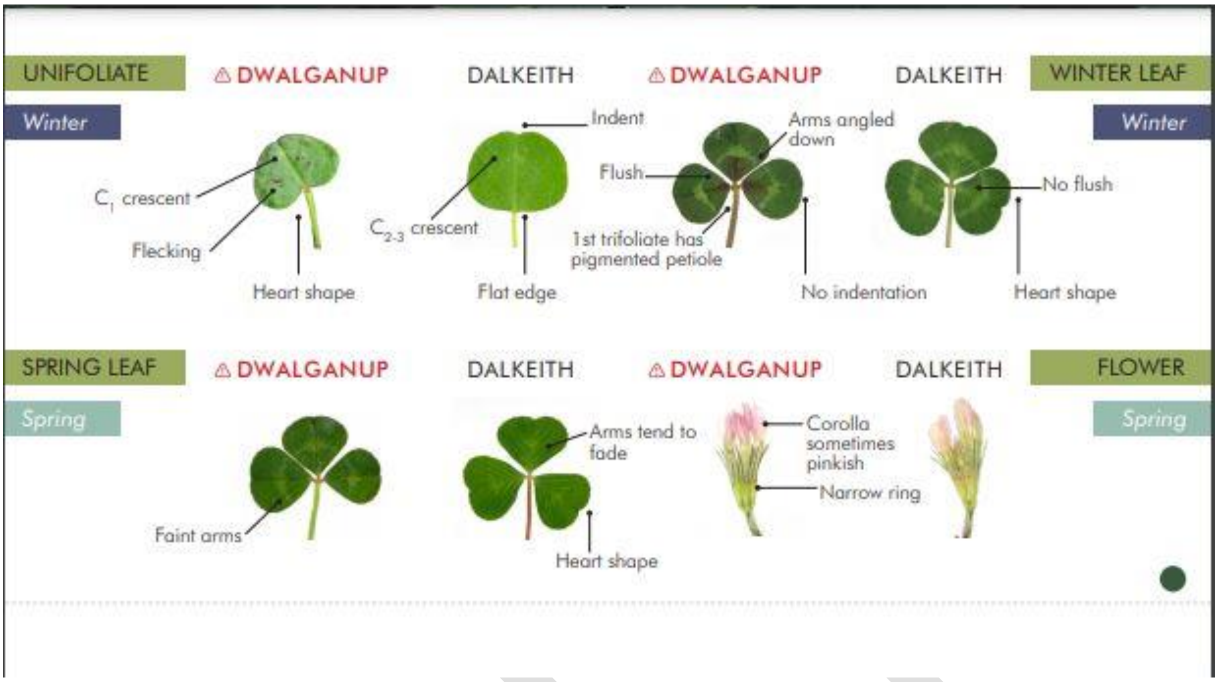

Figure 9. An excerpt from the oestrogenic subclover Ute Guide highlighting how to distinguish the highly oestrogenic cultivar Dwalganup from the non-oestrogenic cultivar Dalkeith, which is morphologically similar.

Significant progress has been made on the new Subterranean Clover Cultivar Guide, which will provide a detailed description of all registered cultivars of subclover (Fig. 10). This Guide will replace the outdated 1996 version containing 26 cultivars. Since 1996, 27 new cultivars have been released; the new guide lists 53 cultivars, both public and privately bred, their time of maturity, growth habit, pest and disease tolerance, hard seed level, isoflavone level, and a morphological description of each cultivar, including leaf mark, calyx tube colour, leaf flecking and flush. Seedling descriptions have been included for the first time to help with cultivar identification. The Guide will be ~140–150 A4 pages with two pages per cultivar, plus supporting text and tables. The completed Guide should be available in 2022 and become the definitive guide for subclover in Australia for the next 15–20 years.

Figure 10. Subterranean Clover Cultivar Guide thumbnail imagery of spring leaf and flower colour.

Image supplied by Department of Primary Industry and Rural Development**.**

4.5 Objective 5 - Handout

Update yearly a handout, targeted at farmers and advisors, outlining the problem (in terms of clinical and subclinical impact on livestock), how to identify it, and how to mitigate it using pasture renovation.

4.6 Objective 6 – Seed industry communication

"*Communicated to seed industry about oestrogenic clovers – engagement to be document and summarised in project reporting*."

Since 2019, we have been in discussions with the seed industry and the ASF, locally and nationally, on the unintentional inflow of oestrogenic subclovers, such as Dwalganup, into commercial seed lots. To highlight the issue, in 2019, we grew and inspected 13 random samples of uncertified Dalkeith seed lines from Western Australia (non-certified seed sold is estimated as being 90% of the total in WA) to see if we could visually find any oestrogenic contamination (Fig. 11). We found six seed lines containing oestrogenic subclovers (between 5 and 45%, confirmed by laboratory analysis), mostly Geraldton and Dwalganup. We note that contamination levels as low as 5% are of concern as over many seasons, the contaminants may come to dominate due to the competitive and persistent nature of highly oestrogenic subclovers.

Figure 11. Non-certified Dalkeith samples grown to check for genetic purity. Each white tag is an oestrogenic subclover contaminant.

We presented these data and our new seedling identification tools at the ASF Western Region conference in June 2019 in Perth (Fig. 13). The Chief Executive Officer of ASF agreed to highlight the issue nationally, encourage customers to buy certified subclover seed and increase awareness of the risks of purchasing seed of unknown provenance.

Few subclover cultivars were thought to have unique characteristics that can be used to identify cultivars as young seedlings. As a result, plants have needed to be grown through to maturity in the field to determine if highly oestrogenic subclovers are present as contaminants, delaying seed certification and, potentially, seed sales. This is particularly problematic when a common highly oestrogenic cultivar is morphologically similar to the desired cultivar; for example, Dwalganup and Dalkeith. However, as a result of our seedling morphology study, we now have a rapid and accurate method for the seed industry to identify and separate these two cultivars at the seedling stage (Fig. 12), providing the seed certification authorities with a new tool, which will significantly speed up the certification process and reduce the inflow of oestrogenic subclovers into the system. All seedling images will be available for the ASF and consultants to use and will be included in the new oestrogenic clover Ute Guide.

Figure 12. Key morphological differences between Dwalganup (highly oestrogenic - top) and Dalkeith (low formononetin - bottom) at the seedling stage. Previously, these cultivars were visually indistinguishable as these differences had not been documented.

We trained ASF seed certification officers in Perth in the new seedling identification skills (Fig. 13) and worked with private seed certification consultants to help identify difficult samples in the field.

Figure 13. Seed certification officer using the seedling identification tools to confirm genetic purity of commercial seed lots.

4.7 Objective 7 – Renovation case study

“*Complete case study of a high-oestrogen pasture renovation*.”

4.7.1 Urana renovation paddock

The initial seed bank in the pasture plot before sowing to Urana in March 2018 was 176 kg/ha, with subclovers dominated by oestrogenic Geraldton (55%), Dwalganup (33%) and Dinninup (12%) (data not shown). The subsequent seed yields in March 2019, 2020 and 2021 were 245, 217 and 260 kg/ha, respectively (Fig. 14). However, the seed yields from 2019 onwards in the renovated block contained both Urana and background hard-seeded oestrogenic subclovers. These were present in the pasture each season and contributed to the seed bank. Geraldton was the most common oestrogenic subclover in the Urana pasture treatments found visually in the seedlings grown in the glasshouse. By the end of March 2021, the estimated oestrogenic subclover component in the Urana pasture block had declined from 100% in 2018 to 35% (91 kg/ha) of the total seed bank (Table 4). However, overall, the Urana seed bank was still quite low after three years of renovation at only 169 kg/ha.

Table 4. Subclover seed bank (kg/ha) in March each year and the percentage of each cultivar present in the Urana pasture plot. Urana was sown in March 2018 and the background seedbank comprised three old cultivars high in formononetin: Geraldton, Dwalganup and Dinninup.

	2018		2019		2020		2021	
	Seed	%	Seed	%	Seed	%	Seed	%
Urana	0	0	102	42	78.1	36	169	65
Geraldton	98	55	61.2	25	67.2	31	39	15
Dwalganup	58	33	49.0	20	49.9	23	33.8	13
Dinninup	21	12	31.8	13	21.7	10	20.8	8
Total seed	176		245		217		260	
Oestrogenic		100		58.4		64		35

4.7.2 Oats paddock

By the end of March 2021, the oestrogenic subclover seed bank under continuous cropping (with in-crop weed control) had fallen from 220 kg/ha to just 9 kg/ha (Fig. 14). A selection of seed from each treatment was grown in the glasshouse to visually identify the proportion of the seed bank containing each cultivar, which showed that the proportion of each oestrogenic subclover cultivar had changed little over the three years. Geraldton remained the dominant subclover at 61% of the seed bank followed by Dwalganup (22%) and Dinninup (16%) (data not shown). Management during cropping, particularly double knockdown weed control at the start of the season and in-crop weed control, very greatly reduced the size of the oestrogenic subclover seed bank by the end of Year 3.

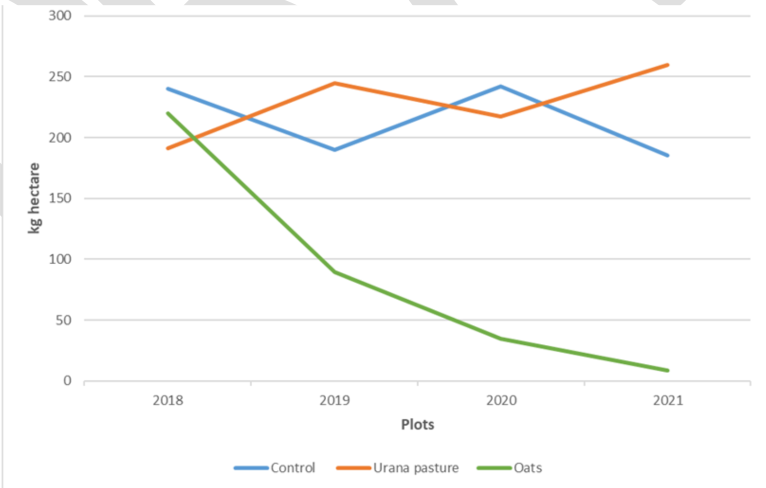

Figure 14. Soil seed bank of oestrogenic subclover in the control, oats and Urana blocks from March 2018 to March 2021.

4.7.3 Control paddock

The subclover seed bank in the unrenovated control paddock declined from 240 kg/ha in 2018 to 165 kg/ha in 2021, which was likely due to seasonal variation and some overgrazing at flowering. The subclover seedbank continued to be dominated by highly oestrogenic subclovers, mostly Geraldton. There was a low incidence (<5%) of a cultivar that was either Daliak or Esperance in Year 3.

In summary, pasture-on-pasture renovation, even with a modest oestrogenic seed bank in a medium rainfall zone, is unlikely to be a viable method for controlling oestrogenic subclovers. Even when combining high sowing rates (20 kg/ha) and double knockdowns at the break of the season for three years, our results show the high level of persistence of highly oestrogenic cultivars. The proportion of the subclover soil seed bank containing oestrogenic subclover in March 2021 in the Urana plot was still high at 31% and considered unsafe for ewes. However, this result could have been improved by using a different cultivar. Urana requires an average annual rainfall >400 mm in the Western Australian wheatbelt, more than was received in the three years of the study. The relatively short seasons at Pingelly resulted in suboptimal seed set for Urana. It is likely better suited to areas further south with a growing season of 5–7 months, extending well into mid-October. Izmir is an earlier season cultivar (similar to Geraldton), which has greater potential to complete seeding before soil moisture shortages in late spring/early summer and be competitive with Geraldton. Izmir was sown in the pasture and oats blocks in 2021 and has established well, flowering by mid-August.

Many producers fail in their first attempts to renovate their oestrogenic pastures. In the years prior to renovation, producers should prioritise reducing seed set of oestrogenic cultivars. The successful renovation of highly oestrogenic subclover pastures at the UWA Ridgefield Future Farm by cropping can be used as a successful model for similar regions and could be applied across southern Australia. In our method, the oestrogenic subclover seed bank must be methodically reduced over several seasons via cropping, with oestrogenic subclovers controlled by herbicides at the break of every season prior to sowing.

Every effort must be made to prevent subclovers from re-seeding. However, controlling subclover seed set in the cropping phase is often overlooked; failure to do so can significantly reduce the chances of successful renovation. To further reduce subclover seed set under crops, crops should be grown that allow the use of heavy rates of broadleaved herbicides and produce a dense leafy canopy to shade out the subclover. Post-harvest management is also often overlooked and should avoid moving sheep from other oestrogenic subclover pastures directly onto the crop stubble as undigested subclover seed in sheep droppings will likely contaminate the renovation cropped area. Based on the lessons learned from this case study, we have written a short step-by-step guide for pasture renovation by cropping to remediate pastures dominated by highly oestrogenic subclover (Appendix 2). Further short examples of successful renovations are in Appendix 3.

4.8 Objective 8 – Cost of oestrogenic pastures

Quantify the incidence of clinical and subclinical cost of oestrogenic clovers for livestock.

4.8.1 Estimate the cost of oestrogenic subclovers in 2021 pastures

Many producers tolerate the non-clover content (weeds and grasses) to avoid the impact of highly oestrogenic subclovers on ewe fertility and survival, despite the reduction in productivity. When subclover content is low in such pastures (<20%), there is little suggestion of any clinical (overt) signs of 'clover disease'. However, the subclinical effect on ewe fertility remains. The first model estimated the impact of remediating oestrogenic pasture—to achieve 10% more lambs and 1% less ewe mortality—on overall farm profit. The potential effect of 'clover disease', expressed as a decline in lambing percentage, ranged from $10–51 per hectare of gross margins per 10% change in lambing percentage. The largest effect occurred in composite flocks with a high proportion of output as lamb sales. The smallest effect was observed in fine wool merino flocks where a high proportion of income was achieved through wool sales. The second model here uses an average farm with 30% oestrogenic types in pastures across the whole farm. This short analysis models the economic consequences of this suboptimal fertility. Experiential knowledge of sheep managers and veterinary clinicians broadly agree that there is little clinical disease in a modern mixed composition pasture with about 20% subclover content.

The work of Lloyd-Davies et al. (1979) in the 1960s at Glen Lossie Research Station at Kojonup positively correlated ewe pregnancy rates and subsequent lambing percentages of ewes grazing oestrogenic pastures. Clinical 'clover disease' symptoms were absent in the mixed sward plots (Lloyd-Davies et al., 1970). Based on this, we extracted the fertility data from Lloyd-Davies et al. (1979) for the nil subclover and 30% Yarloop pastures (Fig. 15). Note that while formononetin can have adverse effects on wethers, the results and discussion used here are limited to their effect on ewe reproductive performance and related economic losses.

In this run of the MERINO model farm— 6600 DSE Merino enterprise—the outcome suggests a $14,000 decline in enterprise profit, incurred largely through reduced sheep turnoff (–$9,900) and reduced wool income (–$4,400). Input costs do not much vary. On a gross margin basis, the negative effect of 30% oestrogenic subclover in pastures appears to be around $22/ha or $2.30/DSE (4% decline). However, successive runs of the model showed that increasing subclover contents in the pasture worsened the outcome.

This simple analysis does not consider the opportunity cost of those oestrogen-affected ewes being slightly lower DSE equivalence, whereby oestrogenic subclover pasture is run at a slightly reduced stocking rate due to the impact on ewe fertility (more dry ewes). The analysis also does not consider increased ewe mortalities in years when climate favours subclover growth above average. It does not consider the opportunity cost of maintaining pastures of suboptimal productivity. This cost is perhaps the largest cost of 'clover disease' in today's high production systems. Further economic modelling analysis should be undertaken to compare the full costs and benefits of increased pasture production and other quality effects (increased disease tolerance, improved winter vigour and increased seed yield) that result from pasture improvement by renovating with new cultivars.

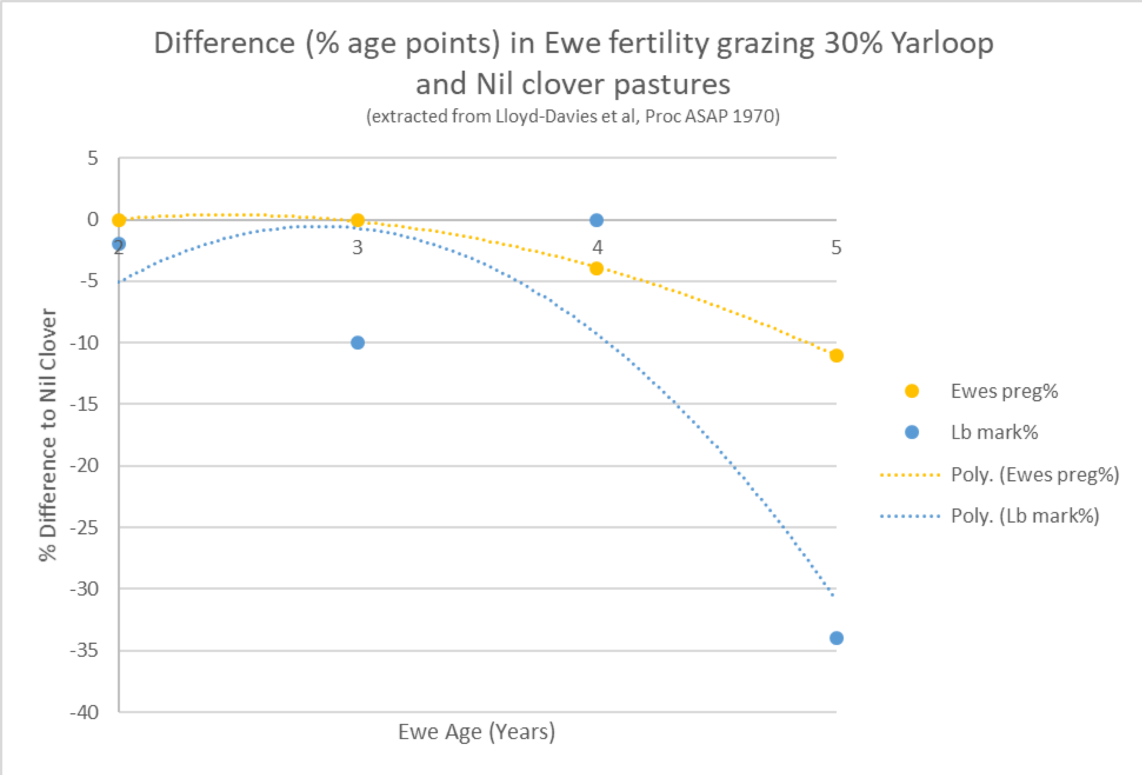

Figure 15. Scatterplot of lamb marking percentage and pregnant ewe percentage versus ewe age taken from Lloyd-Davies et al. (1970). Dashed lines are polynomial curves of best fit.

In summary:

- The effect of ‘clover disease’ ranged from $10–51 per hectare of gross margins (per 10% change in lamb percentage).
- The economic impact was greatest in flocks with a higher proportion of income from lamb/sheep sales.
- The small economic impact was in flocks produced primarily for wool.
- The calculated change in gross margin helps quantify the reproductive value of improving pastures.

4.9 Objective 9 - A peer-reviewed paper

One paper is published, others are being prepared.

Gereltsetseg Enkhbat, Phillip Nichols, Kevin Foster, Megan Ryan, Yoshiaki Inukai, William Erskine. Diversity for morphological traits and isoflavone contents among ecotypes of *Trifolium subterraneum* L. *ssp. yanninicum* and their relationships with site of origin. *Crop and Pasture Science*; in press.

5. Conclusions

Our national surveys and field days indicated that the knowledge gap in the sheep meat and wool industry about oestrogenic subclover pastures at the start of this project was substantial; this can be mainly ascribed to a new generation of producers and consultants unfamiliar with previous research and development and the failure to continue to extend the knowledge on this uses as occurred in the 1980s and 1990s. The samples submitted through our pasture survey kits confirmed that oestrogenic pastures remain widespread. While 60% of submitted samples had higher formononetin levels than the level considered safe (0.2%), this will be part reflect producers who considered they had a problem being more likely to submit samples. Dinninup was the most common highly oestrogenic subclover present in the samples. We showed that it is possible to renovate pastures to new low-formononetin cultivars by use of a 3-year cropping phase, with in crop herbicide control of subclover seedlings, to greatly reduce the soil seed bank of oestrogenic clovers. New renovation strategies may be needed for producers unable to implement a cropping phase. There needs to be further research and communication to the sheep meat and wool industry on the oestrogenic clover issue to ensure it is addressed now and does not reoccur in the future. One important aspect of this is advising producers on how to proceed once oestrogenic pastures are diagnosed on their property; see below. Overall, it is clear that oestrogenic pastures are common and are an economic cost to the industry. Further extension and research is required to address this issue.

Summary of strategies for producers if oestrogenic subclover pastures are present

Short term

- **No action**: suffer the loss in reproduction and stock
- **Avoidance**: remove sheep from oestrogenic pastures or delay grazing livestock during joining for 4-6 weeks after the end of season (that is, after the pasture has senesced and dried off naturally)
- **Strategic grazing**: use for finishing terminal lambs or wethers but monitor: don't graze young ewes or lambs
- **Dilution**: sow ryegrass or forage oats to reduce intake of oestrogenic subclover
- **Nutrition**: ensure soil nutrition is adequate
- **Waterlogging**: avoid grazing waterlogging pastures
- **Enterprise**: change land use or switch to a greater focus on wool

Long term

- **Diagnosis**: confirm diagnosis using autopsy with assistance from a veterinarian
- **Assessment**: rank each pasture for occurrence of highly oestrogenic subclover
- **Pasture renovation**: follow protocols in Appendix 2.

There are also a number of broad implications for the sheep meat and wool industry from this project. First, there is an increasing demand from the public for more ethical and sustainable animal farming practices, including in relation to animal husbandry and welfare. The industry must be responsive to these demands in order to retain market share and social license to operate. In this context, there is a need to address lamb mortality related to ewes grazing oestrogenic subclover pastures. Second, there remain reports of poor lambing percentages of ewes, particularly in Merino flocks across southern Australia, despite these sheep being reportedly in excellent condition score with good nutrition. Some of these reports come from producers who have participated in Life Time Ewe Management courses. While cases of lambing percentages falling to as low as 10–30% seem less frequent today than in the past, quite a few instances were reported to the project team and were found to be grazing on oestrogenic clovers. In addition, less severe examples still appear still common, and probably are a largely undiagnosed infertility issue. This widespread subclinical infertility is mostly unrecognised by producers because they accept the reduced level of fertility as normal. Further work is required to address this issue. Addressing it successfully will make the livestock production systems more efficient as lost lambs represent a loss of output relative to input.

6. Key findings

Key findings of this study can be summarised as follows:

- The project has substantially raised awareness of the continuing issues of highly oestrogenic subclovers in 975 producers, consultants, veterinarians and students. This included training to identify oestrogenic cultivars and how to remediate the issue in the short and long term; many more were alerted to the need to monitor their pastures through social media.
- Around 600 leaf samples submitted by producers were tested in the re-opened isoflavone diagnostic centre at UWA. All producers received their pasture identification and laboratory results. The survey demonstrated that highly oestrogenic subclover pastures are present across southern Australia. The kits were an excellent strategy to extend information on oestrogenic subclovers nationally to the sheep meat and wool industry.
- Of the subclover samples submitted, 60% contained higher formononetin than the level considered to be safe for livestock (i.e. > of 0.2% of leaf dry weight). Dinninup was the most common oestrogenic subclover in these samples. Several cultivars released as safe, notably Trikkala, had a substantial number of samples over the safe limit. Large variation was present in the level of all three isoflavones including formononetin for the oestrogenic cultivars, especially Dinninup.
- A contributing factor to safe cultivars being over the limit and high variation in formononetin is likely waterlogging. There was no evidence of suboptimal nutrition being a factor, although previous research suggests it is possible. Thus, it seems that unknown factors are impacting formononetin levels in field samples. Screening of new cultivars for formononetin levels under commercially relevant field conditions is therefore highly recommended.

- The soil seedbank of oestrogenic clovers can be very greatly reduced by three years of cropping with in-crop herbicide control of oestrogenic subclover seedlings. Sowing of a low-formononetin cultivar directly following an oestrogenic pasture is likely to result in considerable oestrogenic subclover contaminating the pasture into the future.
- The cost of losses from the occurrence of highly oestrogenic subclovers was calculated using modelling at $10–51 per hectare (gross margin per 10% change in lambing percentage) per season. We then modelled the effects of clinical and subclinical costs of oestrogenic subclovers for livestock, using a simplified farm system with 30% oestrogenic pastures occurring across the whole farm. The outcome suggested a $14,000 decline in enterprise profit, incurred largely through reduced sheep turnoff (–$9,900) and wool income (–$4,400).
- There is an unintentional inflow of oestrogenic subclovers into non-certified commercial seed. New subclover identification tools developed as part of this project will aid the quick identification of oestrogenic subclover contaminants in seed lots.
- The scale of the oestrogenic subclover problem is substantially larger than many producers, consultants or veterinarians realise; as such, we expect the demand for information and services to continue to increase in future years.

7. Benefits to industry

The project has delivered numerous benefits to an industry previously largely unaware of the persistence of 'clover disease'. We have alerted producers, consultants and researchers across southern Australia to the problem of oestrogenic subclovers through the pasture sampling kits, laboratory results, workshops, presentations, pasture walks and social media. Notably, social media enabled us to reach a much wider audience of producers than would have been possible through traditional means. In particular, we communicated with the younger generations in the industry. Thus, the sheep meat and wool industry is now much better informed about 'clover disease'. Future extension will be aided by: the oestrogenic subclover factsheet; the oestrogenic subclover Ute Guide; and, the novel map of southern Australia indicating where clusters of oestrogenic subclover are present.

The sample kits enabled many producers to be informed about the presence of oestrogenic subclover in their pasture and identified the cultivars present. The results, when combined, suggest that there is likely a significant proportion of pastures across southern Australia that contain a level of oestrogenic subclover that may be unsafe for sheep and potentially other livestock. They also suggested that Dinninup is the cultivar that is now most common and thus producers need to know how to identify it.

We addressed the issue of flow of oestrogenic clover back to paddocks through contamination of non-certified seed lots through communication with producers and the pasture seed industry, emphasising the need to renovate with certified seed. We also developed the first method to identify and visually separate seedlings of the highly oestrogenic Dwalganup from Dalkeith to ensure effective and timely control or avoidance of oestrogenic subclover contamination in seed lots. This should reduce the inflow of oestrogenic subclover into the system.

We demonstrated that the soil seedbank of oestrogenic clovers can be very greatly reduced by three years of cropping with in-crop herbicide control of oestrogenic subclover seedlings. Our techniques will guide producers on how to renovate successfully.

Finally, in a supplementary exercise, we undertook studies of ewes on a commercial farm which were frequently grazed on pastures containing highly oestrogenic subclovers to investigate clinical abnormalities (Appendix 5).

8. Future research and recommendations

Future research

- A more comprehensive field survey of New South Wales is needed to better under the occurrence of oestrogenic subclover.
- Further information is required to determine which environmental and management factors can cause the level of formononetin in subclover to increase in pastures under field conditions.
- Further economic modelling analysis should be undertaken to compare the full costs and benefits that result from pasture improvement by renovating with new cultivars.
- Research is needed to develop an accurate in-field test for pasture formononetin level. If successful, the need to identify subclovers visually or submit pasture samples to a laboratory will be removed and producers will be able to quickly act to address the issue by, for example, removing livestock from the pasture.
- Research is also needed to develop a simple in-field test to be used by livestock veterinarians to determine equol levels in livestock grazing oestrogenic subclover.
- There are reports from overseas of temporary infertility of cattle from feeding of red clover silage. Research is merited in Australia on the effect of oestrogenic subclovers on other ruminants.
- Development of an herbicide-resistant subclover is urgently needed in the medium and high rainfall zones where producers cannot crop and there reduce soil seed bank of oestrogenic subclovers.

Recommendations

- The UWA isoflavone laboratory was re-opened as part of the project. However, the future of this service is uncertain. There is an alternative service available in south-western Victoria, but it is more expensive and uses a methodology that needs more interpretation by the producer. Equivalent laboratory services do not currently exist in New South Wales, South Australia or Tasmania. We therefore recommend that the UWA isoflavone laboratory be further funded to ensure producers can submit samples of subclover for determination of formononetin level. This information is needed to provide certainty for producers with diagnosing the problem prior to taking action to remediate through pasture renovation.

- Producers be encouraged to undertake ewe pregnancy scanning and be better informed that unexpectedly low and variable conception rates and ewes tending to conceive later in the joining period, can be signs of impact from highly oestrogenic subclover.
- All non-certified commercial subclover seed should be tested for contamination by highly oestrogenic subclover, that is, the four most common cultivars of Dinninup, Geraldton, Dwalganup and Yarloop, and the results included on the bag label.
- Information of the issue of oestrogenic pastures and subclover identification be part of the Lifetime Ewe Management accreditation course.
- An E-learning package on oestrogenic subclover, including identification, animal productivity and welfare impacts, and remediation should be provided to universities and the sheep meat and wool industry.
- We recommend that new breeding material be tested for formononetin levels when grown under a range of relevant field environments and management practices.
- Finally, we strongly recommend a further widespread industry extension-focused project to: disseminate the findings from this project; emphasise the need for isoflavone testing of subclover in older pasture; inform producers of best-practice pasture renovation techniques; and inform producers of how to best manage oestrogenic pasture prior to renovation. It is vital that this also include further training of producers and advisers on identification of oestrogenic subclover. To avoid reoccurrence of a knowledge gap in a further 10-20 years, it is crucial that legacy materials are produced and that a program is instituted to further train advisers in all states.

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Unless otherwise noted, all photographs are credit of Kevin Foster.

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11. Appendices

11.1 Appendix 1: Extension, social media and extension

How well do you know your paddocks?

30 July 2020

Dr Kevin Foster from UWA (centre) helped identify oestrogenic sub-clovers at Mark Cooper's farm. Dr Foster is pictured inspecting renovated pastures with Aspley BestWool/BestLamb Group members Lawrie and Xavier Close – Mark Cooper (not pictured) is also a member of this group. Image: David Woodard,

Figure A1.1. How well do you know your paddocks - July 2020 https://www.mla.com.au/news-and-events/industry-news/how-well-do-you-know-your-paddocks/

Getting to the root of the problem

30 July 2020

Victorian sheep producer Mark Cooper has a good reason to smile – he's identified the root of his flock infertility problem and can start working towards a solution.

While he's still working out the complexities it brings to his business, putting the spotlight on the decades-old problem of oestrogenic sub-clover has made him optimistic about how to overcome it.

Challenge

Yarloop was planted on the Coopers' farm before this variety was found to produce oestrogenic compounds, which impact sheep fertility.

"I always knew I had Yarloop sub-clover which had to be managed," Mark said.

"It's the price I paid for having progressive forebears who, in the 1950s and 60s, saw sub-clover was a great productive feedbase."

Although he strategically only grazed wethers or old ewes on the Yarloop, Mark saw a gradual decline in fertility. The problem was most evident in the earlier of his two preg scans on joined ewes when the pregnancy rate could be as low as 60%.

Figure A1.2 Getting to the root of the problem - July 2020 https://www.mla.com.au/news-and-events/industry-news/getting-to-the-root-of-the-problem/

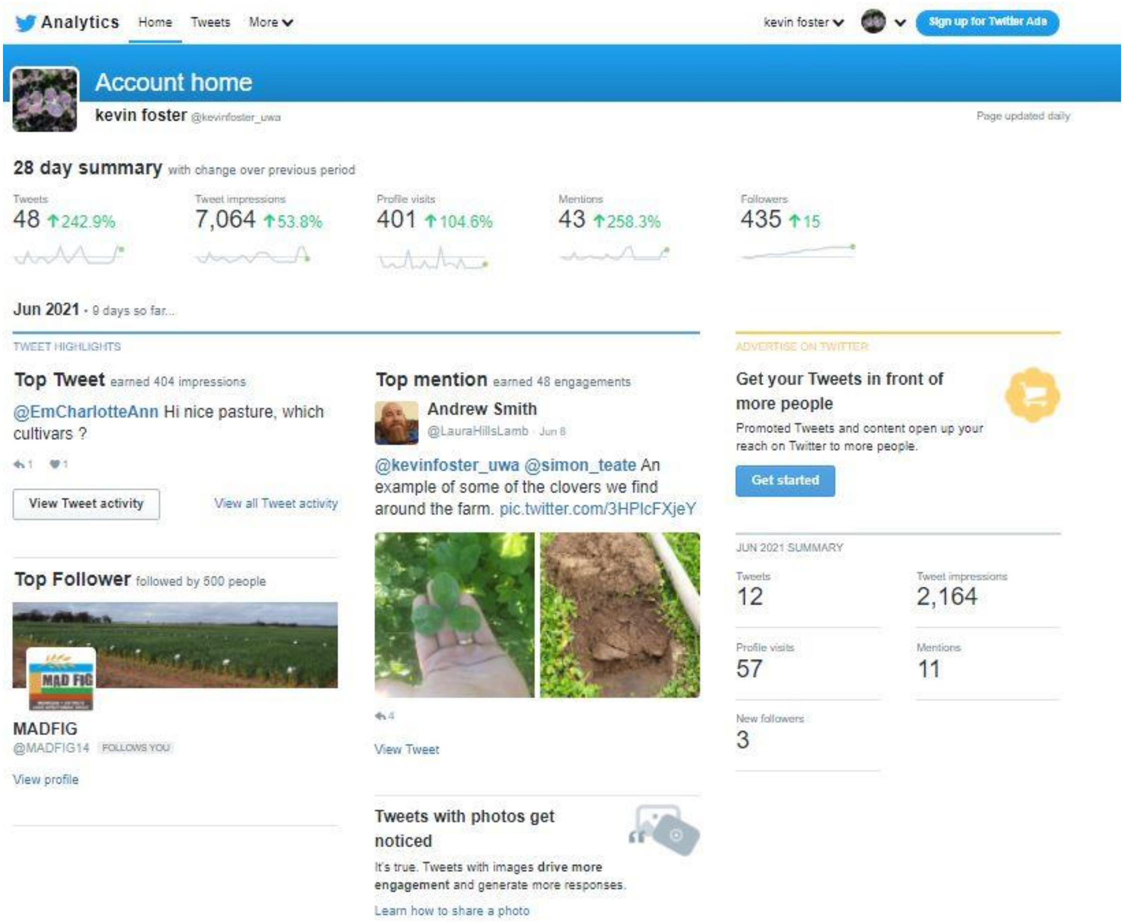


Figure A1.3. Reach on Twitter as of June 2021.

@colmer_tim Follows you

Deputy Vice-Chancellor (Research), The University of Western Australia

Joined July 2020

147 Following 114 Followers

Followed by Otwani Daniel Ajaku, UWA Research, Science at UW...

Tweets Tweets & replies Media Likes

Tim Colmer @colmer_tim · 4h

Important #UWAResearch by Kevin Foster and Megan Ryan with farmers will assist identification of paddocks with oestrogenic sub-clovers. These clovers can cause infertility in ewes. Identification and management tips are available via the MLA web-site:

How well do you know your paddocks? | Me... mla.com.au

Tim Colmer @colmer_tim · 1d

Congratulations to Leigh Simn...

Chris Walsh @chriswa... · 21/9/20

Collecting sub clover samples after a potential ID on oestrogenic types found in paddock - samples in mail and off to @Kevinfoster_uwa for offical ID. Will be keen to see the results 👍 @altmann_craig

Steve Dickson @cropplus1 Following

An independent broad acre cropping and pasture agronomist with a focus on increasing farm productivity, efficiency and long term sustainability.

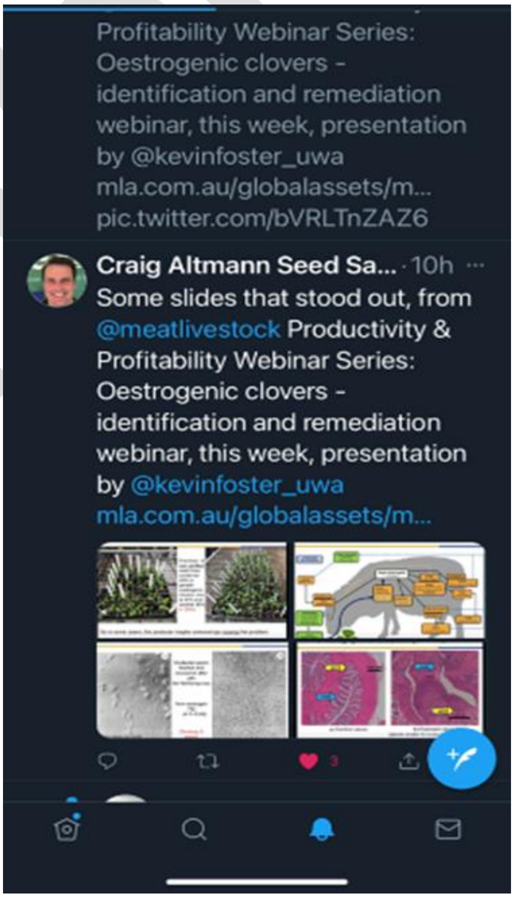
@kevinfoster_uwa and 2 others

Some leaves look a lot like Dinninup sub clover - @meatlivestock publication has images of a handful of the oestrogenic varieties.
I'm still annoyed with the early breeders of these sub clovers & welfare issues.
2008 big sub clover year & many of my ewes prolapsed - Yarloop.

Cut_Out @the_cut_out · 15m

Also had lowest ever lambing percentages that year - only year we didn't preg scan (not my decision!).

Tweet your reply



Profitability Webinar Series: Oestrogenic clovers - identification and remediation webinar, this week, presentation by @kevinfoster_uwa mla.com.au/globalassets/m... pic.twitter.com/bVRLTnZAZ6

Craig Altmann Seed Sa... · 10h

Some slides that stood out, from @meatlivestock Productivity & Profitability Webinar Series: Oestrogenic clovers - identification and remediation webinar, this week, presentation by @kevinfoster_uwa mla.com.au/globalassets/m...

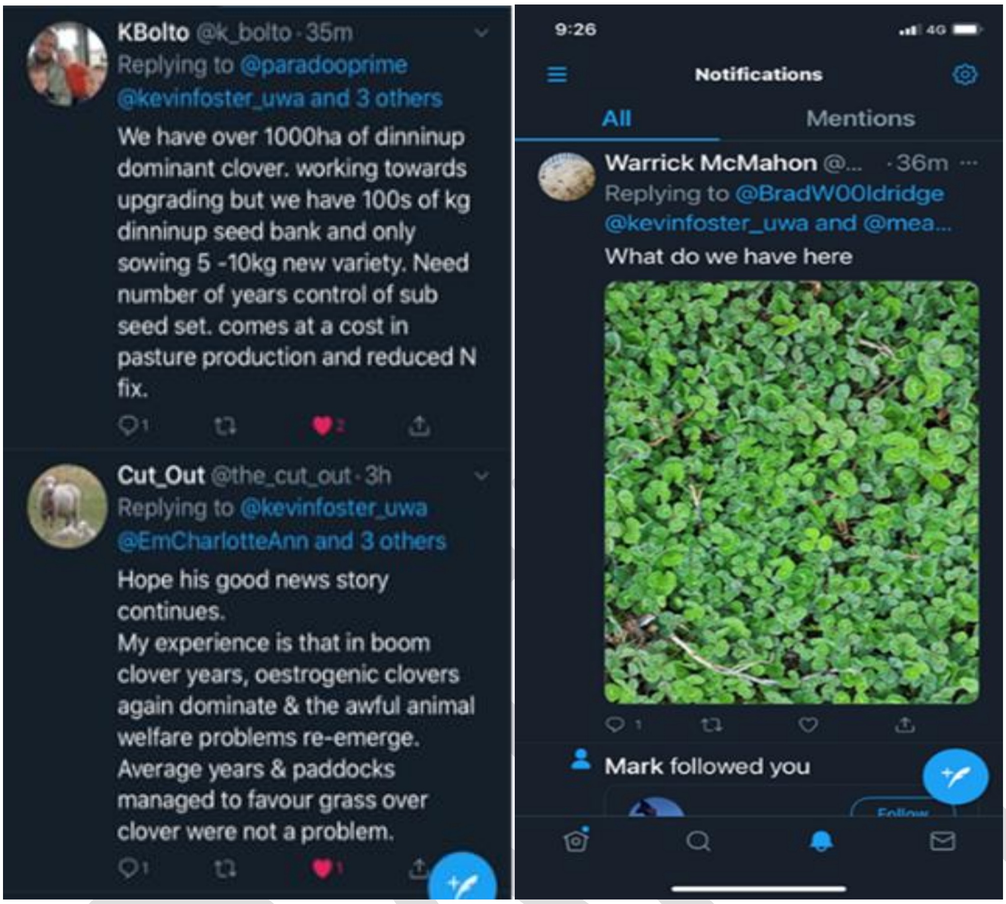


Figure A1.4. Twitter post reach and responses.

Table A1.1. Extension and outreach activities

Financial year	State	Group	Number of attendees	Type of information dissemination
Events with >15 attendees				Code in footer
2019–2020	VIC	Perennial Pastures Society of Victoria Conference	100	T, ID
	WA	WALRC Members forum	15	T, ID
	WA	Dowerin field days	80	ID
	SA	Producers industry forum (PIRSA)	45	T, FW, ID
	NAT	Producer reports meeting	54	ID
	WA	Annual Legume Breeding Australia Open Day	15	T, FW, ID
2020–2021	VIC	Perennial Pastures Society of Victoria Conference	31	T, ID, W
	NSW	Oestrogenic sub clover pastures: identification and potential sheep health issues NSWDPI Webinar	20	T, ID, W
	NAT	Grasslands Society of Southern Australia Conference	180	T, ID, W
	WA	Western Beef Association Field Day	63	T, ID
	NAT	Australian Association of Animal Sciences Webinar	50	W
	WA	UWA field day at Ridgefield Future Farm	84	T, ID, FW
		Reach	737	
Small events and consultation with <15 attendees				
2019–2020	WA	Australian Seeds Authority Meeting	10	T
	VIC	Post-conference producer visits	5	FW, ID
	NSW	Australian Agronomy Conference	10	T, ID
	VIC	Consultants meeting	3	C
2020–2021	SA & VIC	Producers meeting	2	Z, ID
	SA & VIC	PIRSA, producers and agronomists	8	T, ID, Z
	NSW	Producers meeting	2	C
	ACT	Australian Seed Authority committee	5	T, ID
	NSW	Producers meeting	2	C
	NZ	Seed producers meeting	4	T, ID
	WA	Producer meeting	1	C
	WA	Producer meeting	1	C
		Reach	53	
Students				
2019–2020	WA	Students	20	T, FW, ID
	WA	Students	25	T, FW, ID
2020–2021	WA	Students (Muresk Agricultural college)	15	T ID
	WA	Teachers and students	30	T ID
	WA	Researchers	2	T, ID
	WA	Students	25	T, FW, ID
	WA	Researchers, lecturers and students (Murdoch)	30	T ID
	WA	Students (UWA)	20	T, ID
	WA	Students (Curtin)	18	T, ID
		Reach	185	

T = talk, ID = subclover identification, FW = field walk, Z = Zoom meeting, C = conference, W = webinar.

11.2 Appendix 2: Successful pasture renovation by cropping: step-by-step

1) Start preparation the year before.

2) Undertake a comprehensive soil test. Consider this an opportunity to address low pH or nutritional deficiencies with lime and fertilisers. Remember that molybdenum is important for effective nodulation, and subclover has a relatively high requirement for phosphorus.

3) Spray top the existing oestrogenic subclover pasture before flowering to reduce or avoid subclover seed set in the paddock. This will help drive down the seed bank before renovation commences and increase the chances of the newly sown cultivar dominating the pasture.

4) Where possible, crop the paddock or sow to ryegrass for 1–3 years. In each crop, endeavour to:

   - Encourage germination of oestrogenic subclover from the seed bank using shallow tillage (tynes) to maximise soil disturbance early in the season for later herbicide applications or to kill by cultivation.
   - Sow the crop (or ryegrass) at a high rate to ensure a dense leafy canopy to help smother any oestrogenic subclover that germinates.
   - Apply appropriate broadleaf herbicides to prevent any oestrogenic subclover that germinates from setting seed.

5) Sow a new cultivar of subclover (or other pasture legume) and apply management to ensure growth and seed set are maximised in year 1 to ensure it regenerates into a thick sward the following year and outcompete any remaining oestrogenic subclover.

   - Choose a new cultivar to suit your soil type and rainfall zone. Note that newly released subclover cultivars often have improved vigour and disease tolerance to older cultivars.
   - Use good quality certified subclover seed to ensure that you are getting the correct cultivar and avoid reintroducing oestrogenic subclover as a contaminant in uncertified seed.
   - Consider sowing the new cultivar at a high rate (15–20 kg seed/ha) to ensure good ground cover to maximise the chances of it dominating the pasture if the oestrogenic subclovers still germinate from the seed bank.
   - Apply the latest rhizobia strain at sowing to significantly improve nitrogen fixation, and hence productivity. This is particularly important in older paddocks where rhizobia have being lost or become ineffective. Peat-based rhizobia is a cheap and effective option but needs to be incorporated into moist soil soon after it has been applied to seed.
   - A second dressing of superphosphate and potassium in late August or early September may aid seed set.

- Avoid grazing subclover seedlings too early: wait until there are at last 2–3 true leaves.
- Graze in mid-winter and early spring to maximise subclover leaf production as this maximises potential flowering.
- Aim to reduce grazing pressure in spring when subclover begins to flower to maximise seed set.
- Spray for pests as required, e.g. red legged earth mite (*Halotydeus destructor)* especially at the seedling stage

6) Consider the addition of oats or ryegrass (or other forage) to the subclover to compensate for the loss of winter feed in Year 1 of the pasture. Graze the oats hard by early August, when the subclover begins to grow rapidly to lessen the competition.

7) Do not move sheep from dry oestrogenic pastures straight onto crop stubble as subclover seed can pass through the sheep digestion system and contaminate the paddock.

8) Graze subclover stubbles hard over summer to reduce excess litter and to encourage hardseed breakdown to soften the seed for germination the following autumn or winter.

9) For continued peak subclover pasture performance, apply maintenance fertilisers as per soil tests (if needed).

10) Continue to monitor clover composition of the new renovated pastures.

11.3 Appendix 3: Examples of successful renovations

There is evidence for successful pasture renovation of oestrogenic subclover, including in areas where cropping potential is limited. For instance, in 2018–2019, David Woodard (Agricultural consultant Rural Solutions in SA) and Kevin Foster (UWA) visited several properties in the medium to high rainfall zone (>500 mm average annual rainfall) in both South Australia and Victoria. They inspected paddocks with a high subclover content, where most cultivars were the highly oestrogenic Dinninup, Yarloop or Dwalganup. They reported on several case studies.

- In SA, a producer reported that they had previously experienced lambing percentages of 30% of ewes mated. In response, they had destocked the breeding flock and switched to cattle. The producer decided it was worth trying a renovation technique and used sulphonyl urea herbicide to clean up unwanted subclover and weeds during a crop phase. They then resowed the pasture with improved low-formononetin subclover cultivars and grasses. These renovations were successful, and three years after cropping and renovation, the producer returned a flock of ewes to the pastures.
- At Apsley in Victoria, a producer showed them a paddock that had historical lambing percentages of 60–70%. However, it had been relegated to cattle production when highly oestrogenic subclovers had become dominant. The pasture under cattle became grass dominant, but the oestrogenic cultivars of Yarloop, Dinninup and Dwalganup were present, along with some older non-oestrogenic cultivars. The paddocks were successfully renovated with subclover cultivar Trikkala and ryegrass; after three years, the highly oestrogenic cultivars were difficult to find.
- In SW Victoria, they inspected renovated pastures and one unrenovated pasture on a farm. The renovated pastures were dominated by newer cultivars, and oestrogenic cultivars were hard to find. However, the adjacent old unrenovated pasture had high densities of the oestrogenic cultivars Dinninup, Dwalganup and Yarloop.
- In central Victoria, they visited an oestrogenic pasture that had been successfully renovated with a mix of balansa clover and Trikkala subclover. The renovation included a three-year cropping phase with in-crop control of subclover to reduce the size of the seed bank of the old oestrogenic cultivars. The new pastures were well-managed to maximise seed set of the new cultivars in their first year. The oestrogenic subclovers were later found in low numbers in the new renovated Trikkala pasture but common oestrogenic subclovers remained in the adjacent unrenovated paddocks.

11.4 Appendix 4. Producer feedback on renovation intent

Below is a selection of comments, extension and adoption activities provided as project feedback; many indicate an intention to change management and/or renovate.

- "Just as I suspected. We had a few prolapsed uterus at the end of last spring but our lambing percentage is going up. We are in the process of renovating the whole property. Thanks again."
- "I have already started cropping program in one of the problem paddocks and will have 4-5 years of crop now to help remove the problem clover. The two other problem paddocks are not really that suited to cropping and would be interested in following up maybe some other options moving forward that you discussed. I used my oldest ewes to lamb on one of the paddocks and will need to assess what we do with other paddocks. I will also have other unrenovated paddocks that I will assess this year. I will do some survey the coming months on composition to get a better handle on extent of problem >20% or not in which paddocks that I haven't renovated. Thanks and all the best."
- "Thanks for the mammoth effort that you had taken to get my pasture results to me, it has been outstanding and much appreciated. About eight years ago. The paddock which the samples came from was on of the last paddocks that I re pastured, so it had many years of cropping to reduce the seed bed. I noticed that it had a high amount of what I suspected was Dinninup and sent off the samples for confirmation. Once again, I sincerely thank you for the effort that you have taken to communicate your findings. If you have any further information in regards to pasture renovation suggestions, please email them me."
- "Just wanted to thank you both for your contributions Wednesday night, was extremely informative and we had over 100 delegates sign in again to watch. Plenty of questions – we have been struggling to keep to our time limit! I hope this will encourage some more producers to go out and look more closely at their clover. Kind regards.
- "Thanks very much for the email. This information is thoroughly interesting and useful to go forward and do some more investigation. We do suffer from poor lambing rates so this is certainly something to follow up on. Thanks again."
- "That is interesting feedback. I can confirm much of the clover is subjected to extensive water logging in winter. Might try to introduce some more modern and less harmful varieties. I also took your previous advice and consulted an agronomist about the apparently poor N fixing given the extensive clover in the sward. A look at the root nodules showed them to be mostly brown and not the pink/white they should be. So I'll be trailing inoculant on some paddocks to see what happens. Much appreciate you sending me the feedback. Hope the project overall has been a success for you. Regards."
- "I note the Fact Sheet says 'cattle may be affected, but likely this is rare'. Have there been studies that have looked for a link between cattle fertility problems and oestrogenic clovers – but no link was found? Nevertheless, we will try to graze steers on that country, and put in place a plan to crop it and sow it down to low oestrogen cultivars. It seemed from reading the Fact Sheet though, it is difficult to get the oestrogenic clovers out of the system – 'high oestrogenic cultivars can be very persistent'. More thanks."

- “I’m happy to be included in any research projects if required as have collaborated with CSIRO and numerous research institutions in the past (keeps my finger on the pulse with new developments – and it’s interesting!). I think you are spot on with the identifications you previously provided. Kind regards.”
- “Thank you very much for this Kevin. I’ve shared the information with our neighbours and Fiona Leech at LLS in Yass. We greatly appreciate your work!”
- “I tried to go on holiday but had to scramble home after NSW/Vic border suddenly decided to shut. I won’t attempt to travel interstate any time soon. Anyway, MLA have been formatting the factsheet and have made quite a lot of progress. I thought you might be keen to have a look seeing you significantly helped me.”
- “I listened to your excellent report last night on the webinar. I write for the NSW ag publication The Land. I believe this issue is relevant for graziers in the southern part of the state. Also - after last year when so many all over the state imported hay and silage, I found your comment about potential impact on fed livestock very interesting.”
-

11.5 Appendix 5: Ewe clinical investigation

As part of the project, livestock autopsy studies were undertaken using sheep from a commercial property in WA, where pastures were dominated by oestrogenic subclover, to improve the understanding of the physiological and morphological changes in Merino ewes after long-term feeding on oestrogenic subclover and their lambs. Two studies were undertaken; all using animals from the same commercial property. All animal procedures were approved by the Animal Ethics Committee of The University Western Australia (RA/3/100/1657).

Pasture oestrogenicity

Pastures were assessed using the rod point technique where a rod of around 30 cm is placed on the pasture at systematic random intervals and the pasture component at both ends recorded. This sampling was repeated 100 times, yielding 200 random samples per paddock (three paddocks). Components were recorded as weeds, grass or subclover. Subclover was recorded by cultivar based on leaf shape and individual markings. Sixty random samples of subclover, 20 from each paddock, were selected for isoflavone analysis as detailed previously.

Three subclover cultivars were identified, with all represented in each of the three paddocks. Geraldton was the most highly represented cultivar, followed by Dinninup and Dwalganup. The mean formononetin level of subclover was 1.09% ± 0.66 s.e.m, supporting the visual assessment based on leaf shape and leaf marks (K. Foster pers. obs.) that the pastures were highly oestrogenic.

Study 1

Study one was undertaken by UWA Honours Student Mia Kontoolas. In this study, we compared five-year-old ewes from the commercial farm with ewes from Murdoch University in Perth, which were the same age but had never grazed clover (Table A5.1). Ewes were selected from the commercial farm that had suffered infertility for 18 months or two subsequent breeding seasons. This was a pilot study exploring previously established methodology to determine the most reliable and efficient methods of studying the impact of oestrogenic subclover exposure on ewes. Results were used to guide methodology for subsequent analysis.

Table A5.1. Ewe treatment groups in Study 1.

Group	Origin	Treatment	Number	Age (years)	Condition
Control	Murdoch University	Grass-fed only	13	5	Healthy, successfully lambed on last joining
Exposed	Commercial farm	Exposed to oestrogenic subclover for entire life	9	5–6	Infertility for 18 months or two subsequent joinings

Prior to being slaughtered, the general health of all animals was assessed. Ewes were weighed and checked for body condition score to rule out nutritional causes of infertility. External genitalia were photographed and assessed for two measures suggested in previous research:

hypertrophy of the clitoris and labia examined for ventral fusion, both marked as present or absent. Cervical mucus was aspirated with disposable pipette (Fig. A5.1), with approximately 1 mL deposited onto two glass slides. Spinbarkheit was measured as per previous research and the length of cervical mucus stretch was noted (Fig. A5.1).

At slaughter, the reproductive tract was isolated and photographed, and samples taken as detailed in Table A5.2. Paraformaldehyde preserved samples were refrigerated at 0°C for microscopy staining. Stains used were hematoxylin and eosin treatment for cellular aspects.

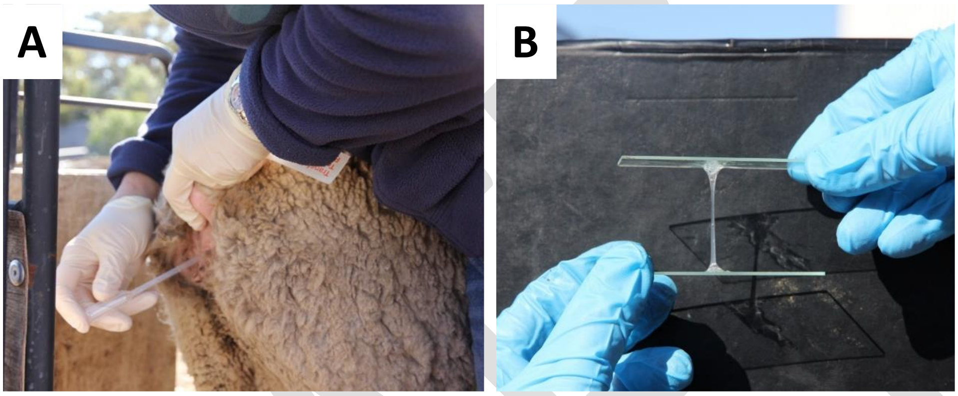


Figure A5.1. (A) Cervical mucus being aspirated with disposable pipette; (B) Spinbarkheit being measured and length of cervical mucus stretch recorded.

Table A5.2. Samples collected post-mortem in Study 1

Organ		Samples taken	Preservation
Cervix	Cranial	5 mm transverse section	Paraformaldehyde
	Mid	5 mm transverse section	Paraformaldehyde
	Caudal	5 mm transverse section	Paraformaldehyde
	OS	Whole	Paraformaldehyde
Ovary	Left	Sliced in half (transverse)	Paraformaldehyde
	Right	Sliced in half (transverse)	Paraformaldehyde
Uterus	Wall	5 mm transverse section	Paraformaldehyde
Fallopian tube	Mid	5 mm transverse section	Paraformaldehyde

Histological examination of infertile ewes exposed to oestrogenic subclover from the commercial farm had the potential for marked cervical damage, even in the absence of outward symptoms. This cohort also showed evidence of cervical gland abnormalities consistent with a reduced conception in fertility (failure to conceive), but not the changes in cervical folds and tissue thickness reported decades previously. This irregularity of symptom progression is similar to pathology reports from veterinarians in the southwest of Western Australia (Besier, pers. comm.). The exposed group had a shorter mean cervix length than the exposed group (p = 0.0004). Exposed ewes had significantly more total glands than the controls (unpaired two-tailed t-test, p = 0.0290) (Figure 8.5). The exposed group had a greater range of gland counts (49–1090) than the control group (16–66.). Hypertrophy of the clitoris was present in 33% of exposed ewes, compared to 16% of controls. Other samples are still being processed.

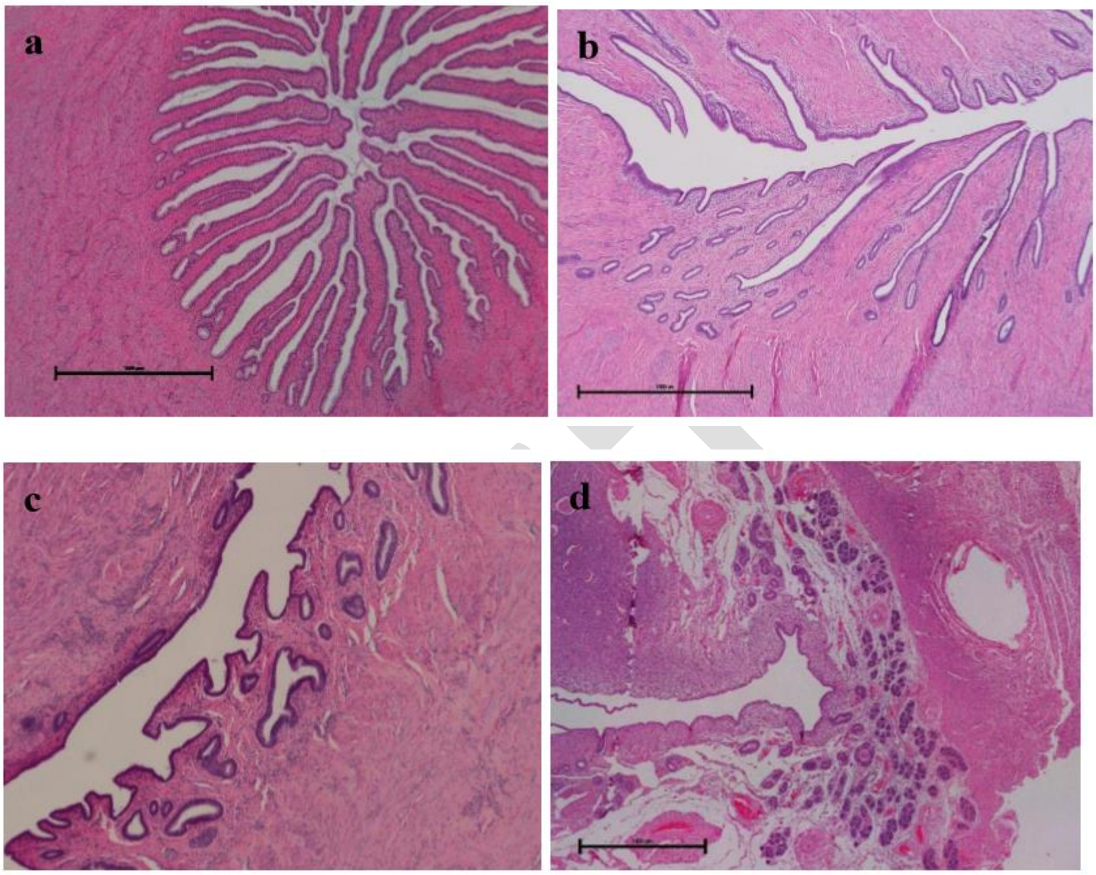


Figure A5.2. Cervix of a control ewe (not exposed to oestrogenic subclover) (a) compared to cervices of ewes exposed to oestrogenic subclover (b, c) which appear more like uterus (d). Control ewe (a) demonstrates normal convoluted crypt and gland growth. Moderately affected exposed ewe cervix (b) and severely affected (c) show increasingly fewer and smaller crypts and increasingly more glands that are tubular rather than round, more like tissue typical of uterus (d). All scale bars represent 1000 µm (figure courtesy of Mia Kontoolas).

Study 2

In Study 2, we again focused on the morphology of the reproductive tract. However, we expanded our sampling to four groups of 5/6 year old Merinos ewes to investigate the change in morphological changes associated with pregnancy and/or exposure to oestrogenic subclover. We used modified methodology as informed by Study 1.

The control group were reproductive ewes with no exposure to oestrogens, again sourced from Murdoch University (Control). The three other groups were sourced from the same commercial farm as Study 1 and comprised ewes exposed to oestrogenic subclover for at least five years and grouped according to reproductive outcomes during the current season: (a) ewes that did not fall pregnant in that season, (b) ewes that fell pregnant but lost their lambs within 48 h after birth, or (c) ewes that raised their lamb (Table A5.3). Samples from all ewes, controls and exposed, were collected on-farm under identical conditions.

Table A5.3. Ewe treatment groups in Study 2.

Group	Origin	Treatment	Number	Age (years)	Condition
Control	Murdoch University	Grass-fed only	12	4	Healthy, successfully lambed on last joining
Dry	Commercial farm		12	5–6	Infertility for 18 months or two subsequent joinings
Lambed and lost	Commercial farm	Exposed to oestrogenic subclover for entire life	12	5–6	Successful conception but lost lamb close to time of birth
Lambed and weaned	Commercial farm		12	5–6	Successful conception, birth and neonatal period

Prior to sampling, the general health of all animals was assessed as per Study 1 (Fig. A5.3A, B). the types of samples and methods of storage are described in Table A5.3 (see Fig. A5.4).

Figure A5.3. (A) Ewes being weighed body condition scored; (B) External genitalia photographed for analysis; (C) Reproductive tract.

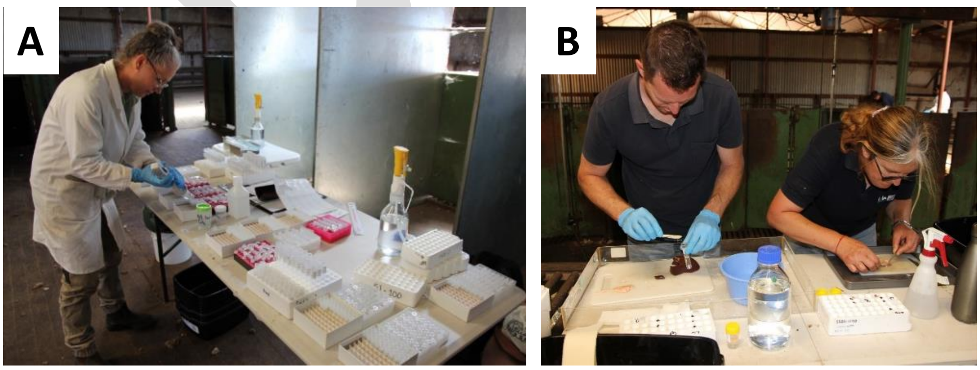


Figure A5.4. Sample collection on-site

Table A5.4. samples collected post-mortem in Study 2. LN-Liquid nitrogen

Organ		Samples taken	Preservation
Cervix	Cranial	5 mm transverse section	Paraformaldehyde, LN, RNA Later
	Mid	5 mm transverse section	Paraformaldehyde, LN, RNA Later
	Caudal	5 mm transverse section	Paraformaldehyde, LN, RNA Later
	Os	Whole	Images only
Ovary	Left	Sliced in half (transverse)	Paraformaldehyde
	Right	Sliced in half (transverse)	LN, RNA Later
Uterus	Wall	5 mm transverse section	
Fallopian tube	Mid	5 mm transverse section	Paraformaldehyde, LN, RNA Later
Bladder	Urine	150 mL	Freezer at –4°C
Liver		4 total from mid-liver	Paraformaldehyde x2, LN, RNA Later
Adrenal gland	Cortex	5 mm transverse section	LN, RNA Later
Adrenal gland	Medulla	5 mm transverse section	LN, RNA Later
Adrenal gland	Peri-renal fat	5 mm^3 section	Paraformaldehyde, LN, RNA Later

The length of the cervix and the diameter at three points (near vagina, middle, near uterus) and the length of the uterus and diameter of each horn were measured from photographs using *IMAGEJ*. The data were expressed as a ratio to live weight and analysed using ANOVA followed by Dunnett's and Tukey's HSD post-hoc tests using SAS by Faustine Chazel (French UWA exchange student) who analysed the morphological characteristics of the cervix and the uterus.

There was an overall effect of exposure to oestrogenic subclovers for all cervix and uterus morphology measures (Table A5.5). The dry ewes had shorter cervix lengths than non-exposed controls and lambed and weaned groups, but similar lengths as the lambed and lost and control groups. The three groups of exposed ewes had smaller diameters of the cervix, in each of the three locations measured, than the controls. Dry ewes had smaller diameters mid-cervix and near the vagina than the lambed and lost and lambed and weaned groups. All of the uterus measurements in the ewes exposed to oestrogenic subclovers were smaller than the control group.

Exposure to oestrogenic subclover over 5–6 years decreased the size of the cervix and uterus of the ewes. Overall, the reduction in size was most pronounced in ewes that did not conceive in the season before measurements (dry). Thus, we cannot conclude if a) pregnancy partially restored the morphological characteristics of the uterus and cervix to that of the non-exposed ewes, or b) if the failure to fall pregnant was due to the different levels of shrinkage that resulted from exposure to oestrogenic subclover for several years prior. Changes in elasticity of the uterus and the cervix might differ between individual ewes, and lead to different reproductive outcomes.

Overall, we conclude from Study 1 and 2 that histological examination of infertile ewes exposed to oestrogenic subclover has the potential for marked cervical damage, even in the absence of outward symptoms and that exposure to oestrogenic subclover reduced the length of the cervix and the uterus compared to the controls. The stored samples from these two studies are resources for future studies and students.

Table A5.5. Morphological characteristics of the cervix and uterus (expressed in cm x 10^2/kg of live weight) of ewes from Murdoch University that had never been exposed to oestrogenic subclover (control) or from a commercial farm that had been exposed to oestrogenic subclover over the last 5 years that were non-pregnant (Dry), pregnant but either lost their lambs within 48 h after birth (LL), or weaned their lambs (RL) during the last breeding season. *: overall effect at $p<0.05$, **: overall effect at $p<0.01$, \$: different to CON at $p<0.05$, # different from NP at $p<0.05$. Data courtesy of Faustine Chazel.

Group	Cervix			
	Length*	Diameter		
		Near vagina**	Mid-cervix**	Near uterus**
Control	13.2 ± 2.6	5.5 ± 1.0	4.9 ± 1.1	4.3 ± 0.7
Dry	10.2 ± 4.0[$]	3.7 ± 1.1[$]	2.5 ± 0.3[$]	2.2 ± 0.3[$]
LL	12.4 ± 1.8	4.2 ± 1.3[$#]	3.2 ± 0.8[$#]	3.0 ± 0.7[$]
RL	13.9 ± 2.7[#]	4.0 ± 1.0[$#]	3.3 ± 0.5[$#]	2.8 ± 0.4[$]

Group	Uterus		
	Length**	Horn diameter	
		Left**	Right**
Control	14.4 ± 0.1	7.6 ± 1.3	3.6 ± 1.1
Dry	7.8 ± 1.3[$]	1.8 ± 0.4[$]	1.7 ± 0.2[$]
LL	9.5 ± 1.6[$#]	4.6 ± 1.1[$]	2.1 ± 0.6[$]
RL	9.4 ± 1.8[$]	4.9 ± 0.9[$]	2.2 ± 0.4[$]