Plant Secondary Compounds (PSCs) affect *in vitro* maturation and fertilisation of oocytes and subsequent embryo development

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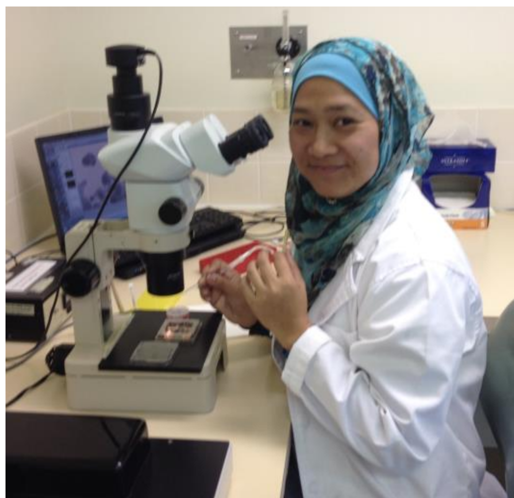

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To my dearest Wan M. Imran, Wan Aisya Sofia and Wan M. Adam

Thesis Declaration

I, Anna Aryani Amir, certify that:

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The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee. Approval #: [RA/3/500/70 and RA/3/500/71].

The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals. The following approvals were obtained prior to commencing the relevant work described in this thesis:

1. Completed the Ethics and Regulations On-Line module and attended the Programme in Animal Welfare, Ethics and Science (PAWES) course
2. Permission to Use Animal (PUA) approval under the university's Scientific Establishment Licence issued by the Department of Agriculture and Food, Western Australia, 6 September 2013. It valid for 5 years which is until 6 September 2018
3. Certified a Completion of Induction as a requirement to perform any work related for sample collection in the abattoir at Thomas Foods International (TFI) site Murray Bridge, Adelaide. Induction was performed twice and valid for 2 years (19 June 2014-18 June 2016, 20 February 2017-19 February 2019)

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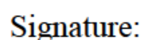
This thesis contains work prepared for publication, some of which has been co-authored.

Signature: 

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Summary

Forage plants are the major source of feed supply in many sheep production systems, including most of those in Australia. Alternative, novel plant species that are fully adapted to Australia's Mediterranean climate have the potential to be used as a major feed source for livestock during 'feed gap' in summer and autumn when traditional species are dead and of little value. However, PSCs (Plant Secondary Compounds) in these forage plants could disrupt reproduction so, as a 'duty of care', early detection of their potential effects on reproduction could avoid another 'clover disease' caused by the phyto-oestrogen isoflavones. One possibility for early and rapid screening of novel plants is to evaluate their effects on oocytes and embryos *in vitro*.

This thesis explores the use of artificial reproductive technology (ART) as a tool for screening for potential PSCs that will inhibit oocyte and embryo development *in vitro*. My research sought to: (i) evaluate the feasability of using ART as a tool for screening plant extracts, extract fractions, and identified PSCs, by studying the effect of isoflavone (a known PSC) on fertilisation and blastocyst development; (ii) use ART to screen common and novel Australian forages, some with known effects on reproduction; (iii) use ART to determine the bioactive fractions and compounds in *B. pelecinus* that affect sheep fertility. The test substances were incorporated into the medium used for *in vitro* maturation of ovine oocytes. They were not present in the media used for *in vitro* fertilisation (IVF) and embryo culture. I recorded the subsequent effects on fertilisation rate (after IVF) and embryo development [blastocyst rate, blastocyst efficiency, total cell number (TCN)]. Having established the protocol, I screened methanolic plant extracts, explored fractions of the extract of one bioactive plant, and the PSC in the extract that was responsible for its bioactivity.

To validate the *in vitro* technology, I studied three isoflavone compounds that are known to inhibit sheep reproduction (genistein, biochanin A, formononetin) by adding them separately to oocyte maturation medium. At 25 µg mL^{-1}, a concentration that is feasible in sheep grazing a clover-dominant pasture, I observed decreases in cleavage rate after *in vitro* fertilisation, and a reduction in blastocyst hatching. Lower concentrations of isoflavone had no detectable effects. Furthermore, biochanin A was found to reduce embryo TCN, specifically at the hatched blastocyst stage. These findings explain clover disease and how isoflavone can effect the fertilisation and

embryo development in sheep, and also provide evidence that ART can be used to screen plant extracts.

I then evaluated the effects of crude methanolic extracts of *Bituminari butuminosa, Medicago sativa, Chicorium intybus, Trifolium subterraneum, Trifolium pratense, Biserrula pelecinus* and *Eremophila glabra*. When the extracts were present during *in vitro* oocyte maturation, they did not disrupt subsequent fertilisation and embryo development to the blastocyst stage. Blastocyst TCN was lowered by *M. sativa* and elevated by *C. intybus* but, overall, blastocyst development was not interrupted. Interestingly, *B. pelecinus* appeared to improve fertilisation and embryo development. It appears that none of the seven forage plants that we tested will disrupt reproductive success *in vivo*, giving us confidence to pursue them further for industrial application.

In the next stage of *in vitro* screening, where I focussed on *B. pelecinus* because of the interesting effects it presented in the previous study. There were two experiments: in Experiment 1, I screened fractions of the extract of *B. pelecinus* and, in Experiment 2, we tested a compound, loliolide, that we isolated and identified in the active fraction.

For Experiment 1, *B.pelecinus* extract was fractionated using rapid silica filtration (RSF) with solvents of varying polarities. Seven fractions were produced and tested, with one reducing cleavage rate by about 10% and three others reducing blastocyst development by 14-22%. Most interesting was fraction BP6 which increased blastocyst hatching rate, confirming the unexpected result seen with the crude methanolic extract.

For Experiment 2, fraction BP6 was further purified by semi-preparative HPLC and the compound loliolide was found to be abundant. Pure loliolide was then added to the oocyte maturation medium, and found to increase hatching rate, consistent with the effect of fraction BP6. All oocytes supplemented with *B. pelecinus* fractions and all loliolide concentrations reached the final stage of embryo development-blastocyst hatching with no effect on TCN. This study shows the value of ART in the purification and identification of bioactive PSCs from forage plants.

Collectively, this research demonstrates that ART is a suitable tool for screening novel forage plants for potential problematic PSCs that might affect oocyte maturation and, subsequently, fertilisation and embryo development. In the screening of common and novel forage plants, I identified *B. pelecinus* as a plant that might benefit sheep reproduction in the field because it contains a PSC, loliolide, that increases fertilisation

rate and embryo development. A systematic screening procedure based on *in vitro* ART can be used to assess novel forage plants before they are committed to production systems, although, having identified plants that have effects *in vitro*, it is important to recognize the need to transfer the hypotheses to *in vivo* studies. The specific compound responsible for the reproductive bioactivity in *B. pelecinus*, loliolide, certainly needs to be investigated further.

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Authorship declaration

This thesis contains three manuscripts that are prepared for publication:

(1) **Amir, A.A**. (80%), Kelly, J.M. (10%), Kleemann, D.O. (4%), Durmic, Z. (1%), Blache, D. (1%) and Martin, G.B. (4%). Phyto-oestrogens affect fertilisation and embryo development *in vitro* in sheep. *Reproduction, Fertility and Development*, in press. DOI: 10.1071/RD16481 (Chapter 3)

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We hereby declare that the individual authors have granted permission to the candidate (Anna Aryani Amir) to use the results presented in these manuscripts.

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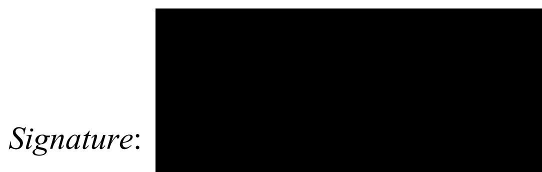
Signature:  *Date*: 11 January 2018

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Publications

Journal papers

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Chapter 1

General Introduction

Forage is an important source of nutrition for grazing sheep because the supply of energy and protein is involved in the management of growth, production and reproductive performance (Martin *et al*., 2004). The productivity of grazing sheep has been successively improved by the introduction, over many years, of variety of new forage plants, amongst which the leguminous species have perhaps been the most successful. Although the contribution of the various forages to improvement in reproductive output has often been studied, relatively little attention is generally paid to the plant secondary compounds (PSCs) contained in the forages and their deleterious effects that they might have on the health and reproduction (Wina *et al*., 2005). Therefore, it is important to detect such problems before a new forage is introduced. The extent of the risk is perhaps best exemplified by 'clover disease', a disease phenomenon in which there was a massive, national deterioration of fertility in the Australian sheep industry, from the 1940s to the 1960s (Adams, 1995). Ewes grazing clover-dominant pastures showed infertility due to the presence of a family of phyto-oestrogens, the isoflavones, that disrupted gonadotrophin secretion, oestrus and sperm transport, and caused uterine prolapse and dystocia, with the nation-wide outcome being very low lambing rates (Adams, 1988). Therefore, there is a need to acknowledge our "duty of care" in forage development programs to ensure an analysis of the risks for health and reproduction (Revell and Revell, 2007).

The concept of "Clean, Green and Ethical management" proposed by Martin *et al.* (2004), a vision designed to direct the development of animal industries by placing pressure on chemical inputs (clean), environmental impact (green) and animal welfare (ethical), has also been a driver in the development and adoption of new forage species. In particular, research effort has been directed towards mitigating methane emissions from ruminant production systems, and to reducing gastrointestinal parasite burdens that are now very difficult to control because the parasites have developed resistance to oral anthelmintic drenches. Almost certainly, the advantages of these novel plants are due to bioactive plant secondary compounds that have anti-methanogenic and anthelmintic properties. Do these compounds also affect reproduction?

The risks of new forages for reproductive performance in sheep and other grazing animals have typically been assessed through *in vivo* observations of oestrus, ovulation rate, fertility and fecundity (Lightfoot *et al*., 1974; McDonald, 1981; Ramírez-Restrepo and Barry, 2005; Blache *et al*., 2008; King *et al*., 2010; Oldham *et al*., 2013). Some forage plants and their active metabolites are known to directly affect fertilisation and embryo development (McEvoy *et al*., 2001, Lightfoot *et al*., 1974; Trenkle and Burroughs, 2012), but it is difficult to measure the effect of grazed plants on oocytes, fertilisation and embryos *in vivo*. Therefore, attention has been turned towards *in vitro* (Leite et al., 2004; Spinaci et al., 2008, Rajabi-Toustani et al., 2013).

Artificial Reproductive Technologies (ART), such as *In Vitro* Maturation, Fertilisation and Culture (IVMFC), have been used to test for toxic effects of plants on the development of oocytes and embryos. Studies using plant extracts have revealed effects on oocyte maturation (Zargari *et al*., 1995; Rajabi-Toustani *et al*., 2013; Tavana *et al*., 2012), meiosis progression (Barakat *et al*., 2015) and embryo development (Leite *et al*., 2004; Tavana *et al*., 2012). Furthermore, *in vitro* studies using purified PSCs were also used to identify effects on oocytes and embryos (Wang *et al*., 2005; Spinaci *et al*., 2008; Santos *et al.*, 2014).

In this thesis, I have tested the general hypothesis that PSCs which have the potential to disrupt reproduction in sheep can be detected using *in vitro* reproductive technologies because they will inhibit the maturation and fertilisation of oocytes, and subsequent embryo development. I established the system using the phyto-oestrogens that are responsible for ‘clover disease’, by adding isoflavones to the medium used for *in vitro* maturation of the oocytes, and then measuring the consequences for fertilisation and blastocyst development. I then used this system to test several common and novel forages, beginning with simple methanolic extracts of the plants. Finally, for *Biserrula pelecinus*, I tested fractions of the extract and a single bioactive compound that was apparently responsible for the effect of the species.

Chapter 2

Literature Review

Background

Australia is a large and efficient producer and exporter of red meat, with the industry generating $15 billion in export revenue, and it is the second largest sheepmeat exporter behind New Zealand (State of the Industry Report, Meat and Livestock Australia, 2017). Australian domestic expenditure was about $2.3 billion on lamb and $88 million on mutton in 2016-2017 (Fast Facts 2017, MLA). Australia has laid out a Sheepmeat Industry Strategic Plan 2015-2020 (SISP 2020), supported by an annual investment of $58 million, that aims to increase net industry income to $728 million by 2020 and $3.49 billion by 2030 (Sheepmeat Industry Strategic Plan 2015-2020, Sheepmeat Council of Australia, 2015), driven by expected high demand in both the domestic and export markets.

To help meet these demands, the sheep industry needs to increase the animal productivity and one important aspect is by improving the reproductive performance through supplementation with high quality forages. High quality forages are essential because the production system is based on grazing year-round. The development and breeding of forages have historically started by importing species already established in the world's temperate and tropical regions, and such programs often produced cultivars that improved animal production under Australian conditions.

Studies of new forages produce findings that have potential advantages for reproductive performance, such as increase in ovulation rate, oocyte quality, embryo production, lambing rate, and milk production. However, the disastrous outbreak of 'clover disease' in the mid 1900s led to a focus on plant secondary compounds (PSCs) in forages that can affect health and productivity. These concerns led Revell and Revell (2007) to introduce the term 'duty of care' into pasture science to ensure appropriate testing before the commercialisation of new species and cultivars.

This review of the literature is divided into three main sections: the first will describe plant secondary compounds (PSC); the second will describe the problems with PSCs interfering with reproduction in the ewe; the final section describes the *in vitro* control of reproduction, from oocyte maturation to fertilisation and subsequent culture of embryos (IVMFC), and explores how this technology can be used as a tool to assess the potential for interference by PSCs. Overall, this review will show how PSCs from

forages can affect sheep reproductive performance at the *in vivo* level and how, IVMFC can be used as a screening tool in testing the effect of PSCs on oocytes and pre-implantation embryos to prevent problems in the sheep industry.

2.1 What are Plant Secondary Compounds (PSCs)?

Natural products research has often improved various aspects of human life and remains a major avenue for discovering bioactive compounds, such as novel plant secondary compounds (PSCs). Plant synthesize a vast variety of organic compounds and they are classified as 'primary' and 'secondary' compounds. Primary compounds play essential roles associated with photosynthesis, respiration, growth and development. Other compounds, referred to as plant secondary compounds (PSCs), can accumulate in surprisingly high concentrations in some species and are now attracting attention because they play key roles in protecting plants from herbivores, microbial infection and UV radiation, in attracting pollinators, and as signal molecules in the formation of nitrogen-fixing root nodules in legumes. Natural products such as spices, flavouring agents, cosmetics, perfumes and dyes are also examples that are used around the world in our everyday lives. On the other hand, some are recognised as toxins, including pesticides, yet continue to interest us and encourage us to explore further.

Chemists have done much work focusing on the isolation and structure of the metabolites using modern separation and spectroscopic techniques. However, these days, chemists are working with other disciplines to explore the origin of the bioactivity. Jerrold Meinwald, Goldwin Smith Professor of Chemistry at Cornell University and a pioneer in the field of chemical ecology, commented in an interview "Chemists need to talk to biologists, who could offer valuable guidance about where to start. Good field biologists are likely to notice interactions that might provide clues to interesting chemistry" (Rouhi, 2003). Through successful collaboration among different research areas, more active metabolites have been discovered and with beneficial applications. The fungal population of the Earth has been estimated to comprise 1.5 million species, of which only 100,000 have been described. Similarly, for flowering plants, only half of the predicted total of 500,000 species has been described. The exploration of new species and their PSCs will continue.

But how are biologically active metabolites detected? Traditional medicines were derived from groups of plants that showed beneficial effects and were later developed into remedies. Other clues might come from observations by scientists in the field who are in a good position to recognize interactions among organisms. These days, however,

the process is more organised with the screening of large numbers of organisms for useful effects and for new metabolites, such as PSCs, that display pharmacological properties. The detection of active PSCs is the starting point for a strategic approach in the search for compounds that might have useful properties. They are structurally diverse, many are distributed among a very limited number of species within the plant kingdom, and they can be diagnostic in chemotaxonomic studies. In-depth examples of the important effects of PSCs in humans and animals, and their roles in human medicine and livestock production, will be presented in Section 2.1.1.

2.1.1 Traditional medicine

Traditional medicine is a broad term used to define any non-Western medical practice (Bannerman RHO, 1983). Literature from the 1930s through to the 1970s contains reports of interesting biological activities in extracts, but the studies often did not continue through to identification of the active principles (Fabricant and Farnsworth, 2001).

Organized traditional medical system

For thousands of years, Ayurveda, Kampo, Unani and traditional Chinese medicine, have flourished. These systems are still in place today because of their organizational strengths, although western science views this system as lacking in credibility. Fabricant and Farnsworth (2001) have reviewed various systems of traditional medicine and their utility in drug discovery. They have successfully discovered and identified around 122 compounds obtained from 94 species of plants that have been used globally as drugs, and 80% of these plants have ethnomedical use identical to the current use of the plant. The authors also proposed an approach that could be used by developing countries that lack sophisticated types of research to experimentally pursue plants as a source of medication (Farnsworth *et al*., 1985) (Figure 2.1).

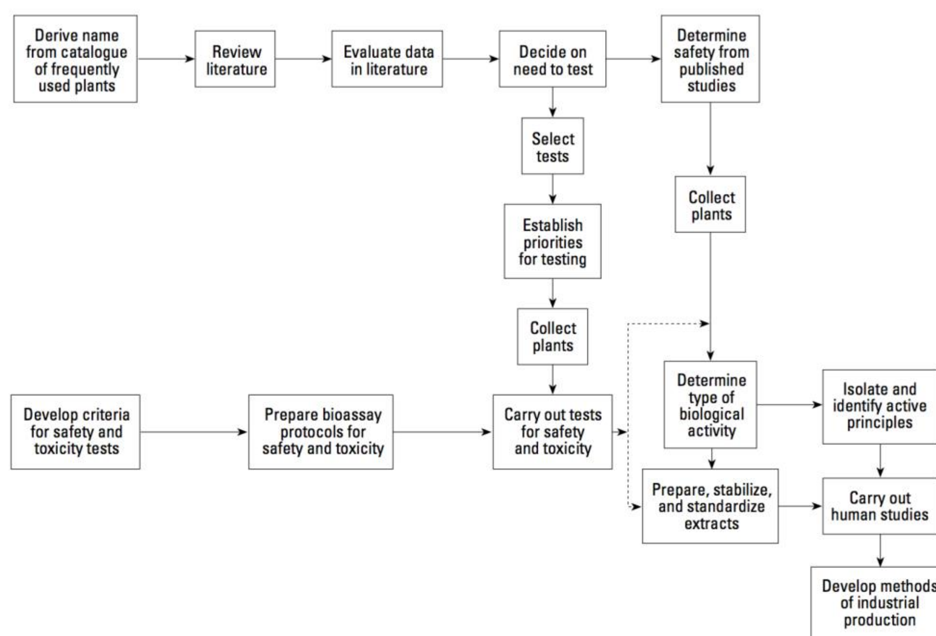

Figure 2.1. Flow chart of sequence for the study of plants used in traditional medicine; from Farnsworth *et al*. (1985).

Indigenous Australian medicine

Indigenous Australians have strong spiritual, physical, social and cultural connections with nature, often evidenced in their artwork. They have explored the healing qualities of the plants in Australia for over 40,000 years. Some of these plants have been shown to produce useful biologically active compounds (Pennacchio *et al*., 1995; Wickens and Pennacchio, 2002) that have antibiotic and cardioactive properties (Pennacchio *et al*., 2005). The leaves of *Eremophila maculata*, for example, were used in the preparation of poultices for treating headaches and blisters for colds (Cunninghams *et al*., 1981) and we now know that an ethanol extract of these leaves inhibits the growth of Gram-positive bacteria (Palombo and Semple, 2001; Pennacchio *et al*., 2005) probably because it contains a variety of molecules, including: prunasin, a toxic cyanogenic glycoside (Finnemore and Cox, 1929), two sesquiterpenes, (-)-10-11-dehydromyoporone and (-)-10,11-dehydromyodesmone (Blackburne *et al*., 1972), and also an alkaloid (Aplin and Cannon, 1971). Indigenous Australians have also been routinely using *Acacia auriculoformis* phyllodeshas as antiseptic cleanser and treatment for skin rash (Barr, 1993), probably because it inhibits the growth of the Gram-negative bacterium, *Escherichia coli* (Pennacchio, 2005). Although saponin was successfully isolated from these species, it is not clear whether this PSC is responsible for the antibiotic activity (Sarkar *et al*., 1998). Studies are still needed to confirm whether these PSCs are scientifically related to the apparent effects, but the potential for learning from indigenous medicine is clear.

Traditional fertility medicine

In 1985, the WHO Special Programme of Research and Training in Human Reproduction set up the *Task Force on Plants for Fertility Regulation* that showed an early interest in selecting traditional plants based on ethnomedical claims related to human reproduction, such as contraception, abortion, ecbolic, emmenagogue (Fabricant and Farnsworth, 2001). The program was terminated before any of the identified plants reached the stage of clinical examination, but researchers from around the world continue to explore and study traditional plants related to fertility.

Iranian Traditional Medicine (ITM) was based on historical texts such as the Al-Qanun Fi Al-Tibb (The Canon of Medicine) by Avicenna, Kholaseh Al-Hekmat Zakhireh Kharazmshahi and Exir Azam (Dehkordi Jafari *et al*., 2015). A review of 39 valid references of ITM that was written between 815 and 1901 AD listed 180 medicinal plants that might affect the male reproductive system. After excluding plants that had compounds with toxic side effects, and then filtering out controversial statements that lacked a scientific basis, 36 herbal compounds remained that showed beneficial effects on male fertility and libido (Nejatbakhsh *et al*., 2016). Of the 36 selected herbs, *C. borivilianum* (safed musli), *C. rotundus (nut sedge)*, *Trachyspermum ammi* (ajowan), *Alhagi persarum manna* (camel thorn), *Alyssum alyssoides* (yellow alyssum) and *zingiber zerumbet* (bitter ginger) were associated with improvement of connective tissue repair and regeneration, and also the repair of internal hernias (Hosein Farzaei *et al*., 2014). Other herbs contained active compounds that overcome azoospermia, and improve erection, ejaculation and libido (Nejatbakhsh *et al*., 2016).

In India, the national fertility rate is still high and is causing long-term population growth, so the Indian government puts a lot of effort into educating its people regarding family planning programs and into the promotion of contraception. A large number of plants in India have been studied with a view to derive contraceptive products for both males and females. A contraceptive cream called *Consap*, containing spermicidal saponins extracted from *Sapindus mukorossi*, has been licensed to Hindustan Latex Limited (Mumbai) for marketing (Ansari *et al.*, 2017). Saponins alter the glycoproteins associated with the lipid bilayer of the plasma membrane of the spermatozoa (Dhar *et al*., 1989). Meanwhile, *Carica papaya* was found to have potential as a dual contraceptive, being effective in both sexes. The seed and fruit cause abortion in women (Tang, 1979), and the chloroform extract was a powerful antifertility agent in rats and

rabbits, mainly due to a mild estrogenic property that inhibited the cauda epididymal sperm motility (Ansari *et al.*, 2017).

2.1.2 Livestock production

Digestive system - digestion, bloat and methane emission

Herbivores rely on microbial fermentation of plant material in the gut as a source for energy and protein. Studies have shown that PSCs can play a role in ruminal fermentation and utilization of fibre, by altering the anatomy and physiology of the gut wall (Robins and Brooker, 2005) or by binding to digestive enzymes to reduce their activity (Reed, 1995). One example is *Acacia aneura*, a shrub legume that contributes to livestock nutrition in semi-arid, arid and tropical regions, and is important for the maintenance of body mass during droughts (Pritchard *et al*., 1992).

Prolonged ingestion of *A. aneura* tannins was found to show striking histological difference in the abomasal and intestinal displaying open gastric pits (Robins and Brooker, 2005). Furthermore, tannin also inhibits gut enzyme activity and affect gut permeability, causing decreased passage of nutrients through the gut wall (Walton *et al*., 2001). Condensed tannins, also known as proanthocyanidins (PA), are flavonoid polymers and are known to be toxic to ruminants (Reed, 1995). Proanthocyanidins (PA) are not absorbed by the rumen but affect the mucosa of the digestive system and, when present at high concentrations, decrease the absorption of essential amino acids. Methionine is one of the susceptible amino acids and a decrease in methionine will increase the toxicity of other plant compounds, such as cyanogenic glycosides, because methionine is involved in the detoxification of cyanide via methylation to thiocyanate (Reed, 1995). In ruminants, bloat is a widespread disorder caused by stable foam in the reticulo-rumen that forms when fermentation is rapid and the gases are entrapped (Table 2.1). The frothy mass at the dorsal sac of the rumen compresses the heart and lungs, and will lead thus lead to anoxia and death of cattle and sheep (Mangan, 1988). Legume forages such as white clover, red clover and lucerne are found to release large amounts soluble leaf 'cytoplasmic' protein, which is an active foaming agent that can cause bloat. However, non-bloating forages, such as *L. corniculatus* and sainfoin, contain condensed tannin that can destabilize plant protein foams (Jones *et al*., 1973). A minimum requirement of condensed tannin (about 5g/kg DM) has a high probability of preventing bloat (Li *et al*., 1996).

Table 2.1. Types of tannin and their nutritional effects; from Mangan *et al*., 1988.

Plant source	Tannin identified	Type	Principal nutritional effect	References
Sorghum bicolor L. Moench (grain)	Proanthocyanidin, catechin	Condensed	Weight loss in children Lower DMD, PER, weight gain in rats	Asquith and Butler (1986) Maxson *et al.* (1973)
Onobrychis viciifolia L. (leaves)	Prodelphinidin	Condensed	Protection of leaf protein from rumen degradation, bloat control	Osbourn *et al.* (1971) Jones *et al*. (1976)
Lotus pedunculatus L. (leaves)	Proanthocyanidin	Condensed	Protection of leaf protein from rumen degradation, inhibits rumen carbohydrate digestion, reduced weight gain in sheep	Barry and Manly (1986)
Acacia nilotica (leaves)	(+)-Catechin mono- and digallates	Hydrolysable	Precipitation of leaf proteins, inhibition of rumen fermentation	Self *et al.* (1986)
Camellia sinensis (green tea)	(-)-Epicatechin, (-)-epigallocatechin gallates	Hydrolysable	Precipitation of milk and other dietary proteins	Bradfield and Bate-Smith (1950)
Leucaena leucocephala (leaves)	Gallotannins Catechins	Hydrolysable Condensed	Inhibits digestibility	D'mello and Fraser (1981)
Quercus robur (leaves, callus)	Gallotannins, ellagitannin	Hydrolysable	Inhibits of tryptic digestion of protein	Fenny (1969) Haddock *et al.* (1982)
	Catechins	Condensed		
Lespedeza cuneata (leaves)	Prodelphinidin	Condensed	Inhibits rumen fermentation Depresses growth in rats and chickens	Bell *et al.* (1965)
Ceratonia siliqua (carob pods)	Galloyl-D-glucose, flavan-3-ol-gallates	Hydrolysable		Tamir and Alumot (1970) Joslyn *et al.* (1968) Haddock *et al.* (1982)

DMD: dry matter digestibility; PER: protein efficiency ratio

In addition to risks to health, PSCs can help manage the production by ruminants of methane, one of the primary greenhouse gases that the major source of anthropogenic global warming in agriculture (Wood and Knipmeyer, 1998). As the microbes in the rumen process plant material and provide energy for the animal in the form of volatile fatty acids (VFA), they also produce methane as a by-product. Animals grazing pasture legumes emit less methane than animals grazing pasture grasses because the legumes contain less fibre and are more digestible (McDonald *et al*., 1988). However, PSCs

could also help explain these effects because tannins can reduce methane production by reducing the population of methanogens, the principal cause of methanogenesis in the rumen (Jayanegara *et al*., 2015). Condensed tannins (CT) extracted from the bark of the *Acacia mearnsii* (Black wattle tree) were found to reduce methane losses in dairy cows but unfortunately also reduce milk yield when fed at the necessarily high dietary concentration (Grainger *et al*., 2009). Meanwhile, *in vitro* fermentation experiments using the ruminal fluid of Holstein cows has shown that *Yucca schidigera* extract decreases, whereas *Quillaja saponaria* extract increases, methane emission due to the different chemical nature of saponin in the plants (Pen *et al*., 2006). *Yucca* saponin has a steroidal nucleus while *Quillaja* saponin has a triterpenoid nucleus (Rochfort *et al*., 2008).

Wool and skin

Australia is one of the largest wool producers and exporters in the world, producing 25% of the greasy wool on the market, with high demand leading to an export value estimated to be around $3 billion in 2015-2016 (Department of Agriculture and Water Resources, 2016). Wool growth is sensitive to the absorption of protein and the overall health of the animal, so there is some concern that it might be affected by PSCs. Studies have shown that *Lotus corniculatus* can be used to improve reproduction and wool production (Min *et al.*, 1999; Ramírez-Restrepo and Barry, 2005), probably because it contains condensed tannins (CT) that increase the availability and absorption of essential amino acids (EAAs) from the small intestine (Reis, 1979). In long term grazing experiments with sheep, the increase in EAA absorption increased wool growth by 12% during summer and milk protein secretion by 14% in mid- and late lactation during spring (Wang *et al*., 1996a, 1996b).

Plant-sourced medicines can also be used to treat wounds and treat disorders in skin, such as eczema, irritation, inflammation and warts. In a survey, 128 plant species were identified was useful for treating veterinary diseases in the Ruoergai region of the Sichuan province (China), where local people used medicinal plants to maintain livestock health for thousands of years. This information was passed orally from generation to generation, and much of it was recorded in books (Shang *et al*., 2012). The aerial parts of *Senecio diversipinnus* (common Chinese name, Yi Yu Qian Li Guang; common Tibetan name, Ye Ge Xing) is used to treat eczema and dysentery while the aerial parts of *Elsholtzia densa* (common Chinese name, Mi Hua Xing Ru; common Tibetan name, Qi Rou Se Bu) was used to treat itchy skin, inflammation, and

other problems such as diuresis and parasitic diseases (Shang *et al*., 2012). Due to the rapid progress of urbanization in China, modern chemical drugs have become more widely used by veterinary practitioners and the application of traditional plants is gradually decreasing. However, more studies are need to identify the biologically active molecules in traditional plants that are can help in treating skin diseases.

Reproduction

PSCs may have positive and negative effects on reproductive processes in both male and female animals. In males, some plant extracts can decrease sperm production by damaging germ cells (Randel *et al*., 1992) while others increase sperm counts (Kistanova, 2005). One long-known example is gossypol, a toxic compound produced in the pigment glands of the root, leaves, stems and seeds of the cotton plant genus, *Gossypium* (Berardi and Goldblatt, 1969). Chenoweth *et al.* (1994) discovered that Brahman bulls receiving 8.2 g free gossypol per bull daily in cottonseed meal showed depressed sperm counts largely due to increased frequency of abnormal mid-pieces. These mid-piece abnormalities were similar to those seen in rats producing immotile sperm due to mitochondrial damage in the tail (Randel *et al.*, 1992). In bovine females, gossypol was found to affect *in vitro* ovarian steroidogenesis (Gu *et al*., 1990) as well as oocyte cumulus expansion and nuclear maturation (Lin *et al*., 1994).

A large number of plants contain compounds that have oestrogenic effects because their structure is similar to that of the natural animal oestrogen, 17β-oestradiol (E_2). The presence of oestrogenic substances in plants was first reported by Loewe and Spohr in 1926 (cited by Trenkle and Burrough, 2012) and, subsequently, many more plant species were found to have oestrogenic activity (Bradbury and White, 1954). Plant oestrogenic molecules, usually known as phyto-oestrogens, can disrupt the morphology and physiology of reproductive organs and alter sexual behaviour (Adams, 1995). Phyto-oestrogens are produced by numerous *Leguminosae* and grasses that are commonly consumed by livestock and humans (Table 2.2). Ewes grazing oestrogenic pastures, such as subterranean clover (*Trifolium subterraneum*), show reductions in oestrus, ovulation rate and egg fertilisation (Adams, 1995). The effects of phyto-oestrogen a major aspect of this thesis, so they will be considered in more depth below (Section 2.2.1).

Table 2.2. Common plants that contains oestrogenic compounds; from Kaldas *et al*. (1989).

Alfalfa	Coffee	Parsley	Sage
Apple	Fennel	Peas	Sesame
Barley	Garlic	Rape	Soybean
Carrot	Green beans	Red beans	Soya sprout
Cherry	Marijuana	Rice	Wheat
Clovers	Oats	Rye	Yeast

2.2 Forage PSCs and their effect on reproduction in the ewe

The ability of nutrition to improve reproduction has probably been known since domestication, and was only described in the scientific literature in the 1800s (for a review, see Clark, 1934). Nutrition affects all aspects of the reproductive process, from puberty to gametogenesis, in both rams and ewes, although ewes require more nutritional investment in reproduction so their responses to nutritional signals are more complex (Scaramuzzi *et al*., 2006). Nutrition can be used to manipulate folliculogenesis, the oestrous cycle, ovulation rate, pregnancy, fetal development and lamb survival (Figure 2.2).

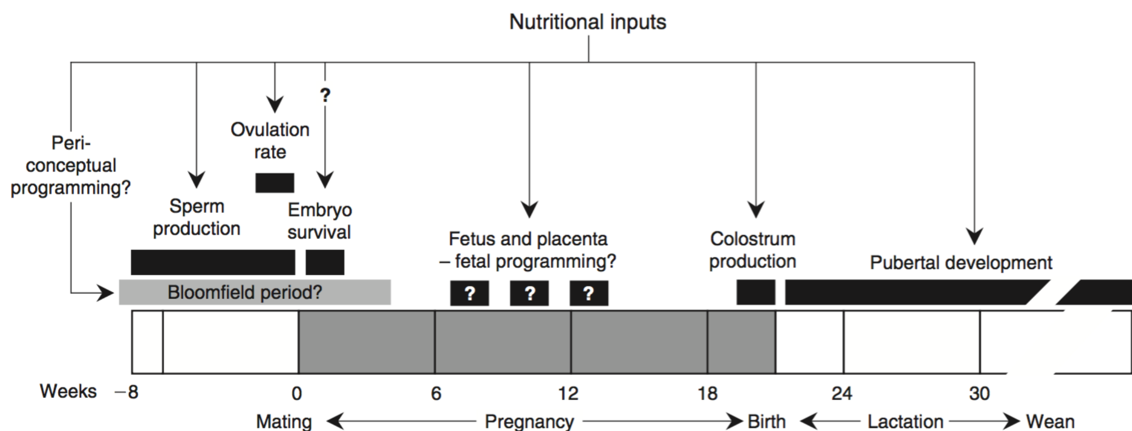

Figure 2.2. Nutritional inputs during the period of the reproductive process may affect the performance of the sheep; from Martin *et al*. (2004).

There are many well-documented studies demonstrating the value of adding nutritional input by introducing new forages to grazing sheep. However, it should be a concern that, in addition to their nutritional value, the forage plants also contain PSCs that can affect reproductive performance in sheep, as exemplified graphically by 'clover disease', a major problem for the sheep industry.

2.2.1 Clover disease - a massive impact in sheep reproduction

Subterranean clover ('subclover', *Trifolium subterraneum;* Figure 2.3) was first introduced into Western Australia for pasture improvement in the 1940s. Not long after, improved subterranean clover pastures were found to be associated with fertility problems in sheep (Bennetts *et al*., 1946), with ewes showing severe prolapse of the uterus (Figure 2.4), abnormal cervical morphology, difficult birth (dystocia) and very low lambing rates. Sheep that grazed subclover for a few weeks and then moved to non-oestrogenic pastures were found to show temporary infertility, whereas sheep grazing subclover-dominant pastures for long periods became permanently infertility (Underwood EJ, 1951). The outcomes were disastrous for the sheep industries in Australia and New Zealand (Adams *et al*., 1988; Smith *el. al*., 1979). The cause was found to be compounds in the clover that have effects similar to those of the natural oestrogens produced in humans and animals (Nilsson A, 1961; Batterham *et al*., 1965; Francis and Millington, 1965). These oestrogenic compounds were subsequently identified as 'phyto-oestrogens'.

Figure 2.3. Subterranean clover (*Trifolium subterraneum*).

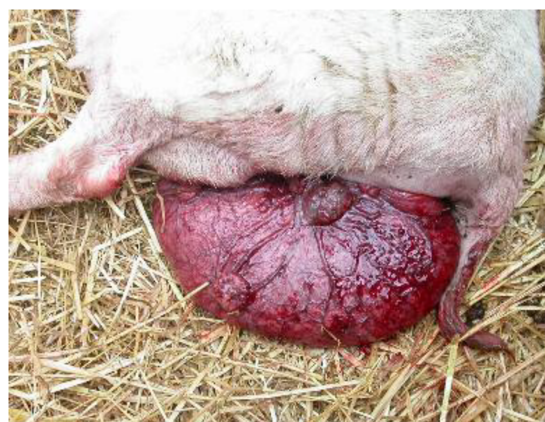

Figure 2.4. Prolapse of the uterus.

Phyto-oestrogens are non-steroidal substances, with a structure similar to that of 17β-oestradiol (Figure 2.5), and that fall into three groups: the isoflavones, coumestans and lignans (Trenkle and Burroughs, 2012). The isoflavones family is responsible for clover disease, with the prominent members in subclover being genistein, biochanin A and formononetin (Pace *et al*., 2011; Figure 2.5).

Oestrogen **Biochanin A**

Genistein **Formononetin**

Figure 2.5. Structural formulas of the natural oestrogen (oestradiol-17ß), formononentin, biochanin A and genistein.

Early studies on rodents showed that genistein and biochanin A were the most active oestrogenic compounds. However, later studies in sheep found that formononetin was more strongly correlated with infertility than genistein and biochanin A because of metabolic processes in the rumen (Shutt *et al*., 1967). Rumen microorganisms metabolise formononentin to equol, which is the predominant oestrogenic metabolite (Shutt *et al*., 1968; Trenkle and Burrough, 2012). Meanwhile, genistein and biochanin A are metabolised to p-ethylphenol which was thought to have no hormonal activity (Batterham *et al*., 1965; Lundh *et al*., 1990). Earlier finding in isoflavone have also found that genistein can be destroyed by a polyphenol oxidase released by crushed clover leaves (Francis and Millington, 1965) while biochanin A are demethylated in the rumen (Nilsson, 1961) before further degradation. More recently, an *in vitro* study showed that, similar to oestrogen, p-ethyphenol plays a role in stimulating the production of prostaglandin–F2α in the corpus luteum (Woclawek-Potocka *et al*., 2006). Furthermore, although genistein and biochanin A have low oestrogenic activity, both metabolites can still be detected within hours after being consumed by sheep and will not decrease until 2-4 days later (Lindsay and Kelly, 1970). Therefore, although isoflavones are metabolised in ruminants, more studies need to be done on the traces of isoflavone that remain in the system and the effects of their metabolites.

Phyto-oestrogens cause adverse effects and thus considered as ʻendocrine disruptorsʼ. However, phyto-oestrogens also have potential benefits for health especially in human medicine, lowering the risk of breast cancer and heart disease, and reducing menopausal symptoms and osteoporosis (Patisaul and Jefferson, 2010). For example, genistein in soy was found to lower the growth of breast cancer cells in Asian women by inhibiting protein tyrosine-kinase (PTKs), and enzyme that plays an important role in growth and proliferation of tumour cells (Paul and Mukhopadhyay, 2004). By inhibiting the PTKs pathways, genistein thus has the potential to slow down breast cancer tumorigenesis and preventing it from spreading (Piotek *et al.*, 1993). However, the impact of soy isoflavone in humans is still unclear and seems to differ with ethnicity, with the protective effect of soy only found in pre-menopausal Caucasian, but not Asian, women (Trock *et al*., 2006).

2.2.2 PSCs and the oestrous cycle – oestrus and ovulation

In the natural breeding season, oestrus occurs on average every 17 days. However, a range of 14-20 days is regarded as normal due to the influence by breed, stage of the breeding season, or environmental stress. The oestrous cycle of the ewe can also be manipulated by feeding a forage that contains PSCs. In Britain, *Brassica napus* (rape) and *Brassica oleracea* (kale) are common forage plants for ruminants but have goitrogenic effects and also reduce reproductive performance. Prolonged feeding of kale to ewes resulted in anaemia and a shorter oestrus, and affected embryo survival and implantation (William *et al*., 1965).

Targeted feeding with pastures, such as birdsfoot trefoil (*Lotus corniculatus*) has generally been applied to increase ovulation rate in sheep (Ramírez-Restrepo and Barry, 2005; Viñoles *et al*, 2009). This response was thought to be caused by the condensed tannins in *Lotus corniculatus* that increase the absorption of essential amino acids (EAAs) in the small intestine (Waghorn *et al*., 1987). On the other hand, Barry and McNabb (1999) reported that increasing condensed tannin to more than 50g/kg DM would decrease food intake, potentially reducing ovulation rate, suggesting that the intake of *Lotus corniculatus* needs to be moderated.

Ovulation rate in sheep can also be increased by grazing lucerne (*Medicago sativa*; Robertson *et al*., 2015), but lucerne has also been shown to depress ovulation rate due to the presence of coumestan, a phyto-oestrogen produced by a foliar disease during the late summer/autumn in New Zealand (Smith *et al*., 1979). Low reproductive rate has

also been observed in ewes consuming red clover (*Trifolium pretense*) that contains isoflavones (Kelly *et al.,* 1980).

Sexual behaviour can also be affected by phyto-oestrogen exposure. The duration of oestrus averages about 30 hours in the ewe, with a range of 20-42 hours, some of the variation being associated with breed (Thimonier, 1979) and age, with shorter durations in young maiden ewes and old multiparous ewes, and under environmental stress (Doney *et al*., 1976). Ewes with clover disease exhibit oestrus later than normal ewes, and also show less soliciting behaviour (Adams, 1983). These behavioural changes are due to decrease in nuclear receptor occupancy by oestrogen-receptor complexes (Kaldas *et al*., 1989).

2.2.3 Effects of PSC on reproductive organs, fertilisation and embryo loss

The impact of PSCs on the development and quality of oocytes and early embryos *in vivo* has mostly been studied in mice (Fredrick *et al*., 1981; Jefferson *et al*., 2002, 2006, 2007). Mice fed with coumestrol and later mated produced oocytes with extensive vacuolization and a reduced cleavage rate, and degenerate embryos with an uneven distribution of cytoplasm, asymmetrically sized blastomeres, leading to a reduction in litter size (Fredrick *et al*., 1981). At birth, mice have large oocyte clusters that dissociate into individual oocytes with granulosa cells during the first week of life (Pepling *et al*., 2001). The process of oocyte differentiation is disrupted by genistein, leaving the oocytes in clusters known as multioocyte follicles (MOFs) with two or more oocytes surrounded by a common follicular envelope of granulosa cells (Jefferson *et al.*, 2002, 2006, 2007). Therefore, the presence of MOFs later in life is an indication of exposure to PSCs such as genistein.

Oestrogen plays a major role in the control of the development of the sexual organs and the secondary sexual characteristics, so it is easy to see how phyto-oestrogenic PSCs can be major disruptors of reproductive function at many anatomical and behavioural sites.

At the level of the ovary, for example, β-sitosterol, a dietary phytosterol, inhibits follicular development and alters the size distribution of follicles in the ewe (El Samannoudy *et al*., 1980). In the rat, intraperitoneal administration of *Dieffenbachia amoena*, an indigenous plant in South Africa containing high concentration of phyto-oestrogen, inhibits follicular maturation (De Pasquale *et al*., 1984). Although *Dieffenbachia amoena* is not consumed by sheep, it is an example of a PSC that inhibits

the functional activity of the follicular structure that produces oestrogen and causes temporary and reversible interruption to the oestrous cycle.

Phyto-oestrogen can also affect the uterus in ewes. The weight of the increases with clover disease, again reflecting a typical oestrogenic mechanism and implying oestrogen-receptor mediation. El Samannoudy *et al*. (1980) injected lambs daily with β-sitosterol, and found that uterine weight increased during the two weeks of treatment, then decreased over the six-week period after the end of treatment. β-sitosterol disrupts the distribution of alkaline phosphatase activity in the uterus, as well as in the zona pellucida and ovary (El Samannoudy *et al*., 1980). Uterine alkaline phosphatase has been implicated in metabolic transformations involved in the developing preimplantation embryo in sheep and rabbits (Murdoch *et al*., 1970).

Phyto-oestrogen can also affect the morphology of the vulva in the ewe, leading to a masculinized appearance with enlarged clitoris and the fusion of the lips at the central commissure (Figure 2.6). Therefore, vulva conformation is one of the indicators used to diagnose the degree of oestrogen-induced transdifferentiation (OIT) in ewes (Adams, 1990).

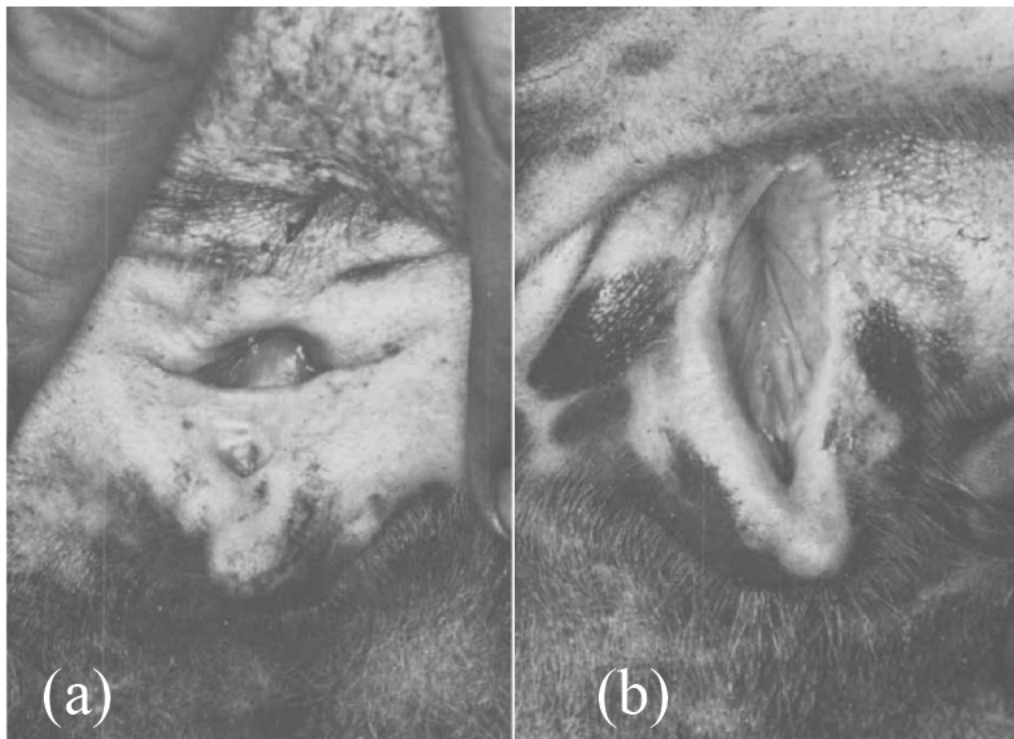

Figure 2.6. Fusion of the labia in the ventral part of the external genitalia of the ewe with clover disease (a) compared with control (b); from Adams, 1979b.

In the cervix of ewes with clover disease, the folds are fused together, resulting in a loss of cervical crypts, and the coiled tubular glands grow down into the *lamina propria*, so they look like uterine glands (Adams, 1988). Furthermore, the cervical mucus also changes, developing a molecular structure that reduces the formation of 'strands' or 'threads' (measured as 'spinnbarkeit') reducing its ability to guide the sperm through the cervix (Adams, 1976). The combination of loss of mucus structure and the irreversible re-differentiation of the cervix, so that it becomes more like uterus, results in failure of sperm transport (Adams, 1995), explaining why fewer sperm are found on the zona pellucida of eggs recovered from the oviducts of ewes with clover disease (Lightfoot and Wroth, 1974; Kaldas *et al*., 1989).

Embryonic losses, abortion and birth defects in healthy sheep are usually low but are possible to happen due to infectious diseases, nutritional deficiency and toxins contain in forages. After ovulation, progesterone secretion from the developing corpora lutea increases for the next 8-9 days and, if fertilisation has been successful, continues into pregnancy. In the absence of an embryo, prostaglandin F2α is released by the uterus around 13 days after oestrus, inducing luteal regression and a decline in progesterone secretion that permits the processes that lead to another ovulation. Prostaglandin production can be stimulated by consumption of locoweed, leading to foetal death and abortion (Welch *et al*., 2009).

In fact, several plants have been found to cause embryonic death, abortion and still births in livestock (Table 2.3). *Veratrum californicum* (false hellebore) is commonly found in the wetlands of the western rangelands of America and prodices the highly toxic steroidal alkaloids, cyclopamine (11-deoxojervine), cycloposine (3-glucosyl-11-deoxojervine) and jervine, that induce embryo mortality during the first month of gestation and also neural tube defects that result in lethal cyclopic facial abnormality, limb deformities and collapsed trachea (Binns *et al*., 1965; Keeler *et al*., 1985).

Table 2.3. Plants that cause abortion in livestock; from Welch *et al*. (2009).

Plant	**Toxicant**	**Effect**	**Species**
False hellebore (*Veratrum californicum*)	Cyclopamine	Embryonic death	Sheep
Snakeweeds (*Gutierrezia sarothrae* or *microcephala*)	Unknown	Abortion when grown on sandy soils	Cattle, sheep, goats
Locoweeds (*Oxytropis* and *Astragalus spp*.)	Swainsonine	Abortion, embryonic death	All livestock
Hairy vetch (*Vicia villosa*)	Unknown	Abortion	Cattle
White popinac (*Leucaena leucocephala*)	Mimosine	Infertility, abortion	Cattle, pigs

Tall fescue (*Festuca arundianea*)	Ergot alkaloids	Abortion, still births	Horses
Nitrate-containing plants	Nitrate	Abortion, fetal death	Ruminants

2.3 *In Vitro* Maturation, Fertilisation and Culture (IVMFC)

Late in the 19th Century, Walter Heape recognized that an understanding of fertilisation in mammals would be greatly aided by observing the process *in vitro*, and that to do so would require the development of conditions similar to those in the oviduct. Work in this area led to the successful transfer of embryos between rabbits (Heape, 1890). He mated an Angora doe and an Angora buck, retrieved two embryos by flushing the oviduct, and then transferred them to the oviduct of a Belgian hare which had mated a few hours previously. The hare produced six young, two of which were Angoras. This study showed that the recovery and transfer of preimplantation embryos did not affect their development.

The next step in our understanding of mammalian fertilisation arose through the demonstration of sperm capacitation (Chang, 1951; Austin, 1951) followed by the first demonstration of IVF resulting in the birth of young rabbits in 1959 (Chang 1968,1977). Capacitation is a physical change that the sperm must undergo before fertilizing the egg. In the next section of this review, I will explain briefly each stage need to for successful *in vitro* maturation, *in vitro* fertilisation *and in vitro* culture (IVMFC), and also how we might study the effects of active PSCs using IVMFC as a screening tool.

2.3.1 *In Vitro* Maturation (IVM)

IVM is a process where immature oocytes harvested from the ovary are cultured in a medium that provides an environment designed to mimic the environment that would have been provided by the follicle in which the oocyte would normally have matured *in vivo*. The IVM culture medium can only effect maturation of those harvested oocytes that had reached metaphase II and were thus capable of undergoing fertilisation, but it can also influence subsequent embryo development (Bavister *et al*., 1992). A simple culture medium usually comprises bicarbonate buffered saline with pyruvate, lactate and glucose, plus serum or albumin, and antibiotic (penicillin, streptomycin and gentamicin). Some media are also more complex with added amino acids, vitamins and purines. Among the complex media used for IVM, tissue culture medium 199 (M199) is one of the most widely used and has been the standard for IVM of cattle and sheep oocytes in Ireland and Australia (Lu and Gordon, 1987; Lu and Polge, 1992; Walker *et*

al, 1996 and Kelly *et al*, 2007). In addition to the maturation medium, a successful IVM requires control over osmolarity, water quality, maturation time, temperature and gas phase and somatic cell support.

The ideal morphological features of matured oocyte are an expanded and clear cumulus cell mass, changes in the dimensions of the perivitelline space and expulsion of the first polar body into that space. Crosby (1969) showed that most sheep oocytes reach metaphase II after about 24 hours of *in vitro* maturation, and Staigmiller and Moor (1984) were the first to show that, under appropriate IVM culture conditions, sheep oocytes can acquire full development competence. The success rate of ovine oocytes in reaching metaphase II is improved by adding fetal calf serum (FCS) to the medium (Agrawal and Polge, 1988), by controlling ovary transportation temperature and oocyte recovery method (Mehmood *et al*, 1992; Baldassare *et al*., 1994) and by supplementating the medium with gonadotrophins (O′Brien *et al*., 1994; Pugh *et al*., 1991).

Oocyte recovery

Oocytes are aspirated using either an appropriate pipette or a syringe and needle (Leibfriend and First, 1979, Xu *et al*., 1988; Walker *et al*., 1996; Kelly *et al*., 2007). One of the difficulties in recovering the oocytes lies in the fact that the oocytes are retrieved from only 30-60% of the punctured follicles, with wide variation among recovery techniques, as demonstrated for bovine oocytes (Table 2.4).

Table 2.4. Total oocytes and proportion of acceptable quality oocytes recovered from bovine ovaries; from Gordan, I.,1994.

Authors	Total no. per ovary	Acceptable quality (%)
Aspiration		
Katska and Smorag (1984)	10.2	45.0
Iwasaki *et al.* (1987)	9.4	56.4
Suss *et al*. (1988a)	11.5	46.5
Sato *et al.* (1990)	10.0	80.0
Lonergan (1992)	9.7	31.1
Lu and Polge (1992)	12.2	-
Follicle dissection		
Katska (1984)	-	63.2
Lonergan (1992)	17.2	60.7
Ovary slicing after aspiration		
Iwasaki *et al.* (1987)	13.0	29.7
Sato *et al.* (1990)	16.0	76.9
Ovary slicing		

Suss and Madison (1983)	20-30	-
Xu *et al*. (1992)	55.5	70.3
Hamano and Kuwayama (1993)	31.6	-
Carolan *et al*. (1994)	44.2	-

Oocyte recovery is three times faster with follicle aspiration than with the dissection method, but speed has no merit unless it associated with a high yield of good embryos. Thus, Lu *et al* (1987) suggested that dissecting intact follicles prevents disruption of the cumulus cells. This view was supported by comparison of aspiration and dissection in cattle (Jiang *et al*., 1992) and sheep (Wahid *et al*., 1992). Finally, oocytes can be recovered after slicing, cutting or dissection of the ovary, before or after aspiration. Several studies showed that oocyte yield increased threefold with cutting and slicing compared to aspiration (Hamano and Kuwayama, 1993; Carolan *et al*., 1994). However, combining the slicing of ovaries after aspiration of follicles showed no merit in terms of oocytes recovered or their quality.

2.3.2 *In Vitro* Fertilisation (IVF)

After around 24 hours of maturation, oocytes that matured successfully can be fertilized *in vitro* (IVF), a complex procedure that, in addition to appropriate IVM, requires sperm selection, sperm capacitation, and an appropriate IVF medium. Ejaculated semen, whether fresh or frozen-thawed, is a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells, various microorganisms and debris, so it needs to be processed to provide only motile and viable spermatozoa. The most common methods for separating motile and non-motile sperm are "swim-up" (Shabankareh and Akhoondi 2012; Wani *et al*., 2012; Shirazi and Motaghi, 2013) and the Percoll density gradient centrifugation (Li *et al*., 2006; Garcia-Garcia *et al*., 2007; Wan *et al*., 2009; Heidari *et al*, 2013 Wang *et al*, 2013). A comparison of these techniques with glass wool filtration showed higher rates of cleavage and blastocyst formation using Percoll density gradient (62% and 18%) than with swim-up (50% and 11%) or glass-wool filtration (44% and 8%; Rho *et al*., 2001).

After sperm selection, and before IVF, sperm are incubated for 15 to 40 mins with capacitation agents in the IVF medium. Capacitation agents include i) oestrous sheep serum (OSS; Berlinguer 2012, Catalá *et al*., 2012, Shirazi and Motaghi 2013); ii) heparin (Heidari *et al*., 2013; iii) heparin and PHE (penicillamine, hypotaurin and epinephrine; Shabankareh and Akhondi, 2012); vi) heparin and serum (Cox and Alfaro, 2007); vii) heparin, PHE and serum (Wan *et al*., 2009).

2.3.3 *In Vitro* Culture (IVC) of Embryos

The last stage of embryo production *in vitro* is the culture of the presumptive zygotes to reach the final blastocyst stage, about 6-8 days after IVF in ruminants (Gardner *et al*., 1994). Embryo culture *in vitro* requires an appropriate environment so that the early embryo can undergo several cleavage divisions and therefore reach the blastocyst stage of development. Generally, the presumptive zygotes are washed and then cultured in IVC medium (usually SOF medium plus serum) under mineral oil in a humidified atmosphere 5% O_2 at 37-38°C. The percentage of oocytes that yield blastocysts varies greatly between laboratories and experiments, perhaps due to variations in incubation conditions.

2.3.4 Using IVMFC to study effects of PSCs on embryo development

Various natural metabolites in shrubs and plants can impair or improve reproduction in ruminants, through actions on the endocrine system, or on oocytes and embryos. The outcomes can profoundly affect fertility, as seen with 'clover disease', a historical catastrophe caused by the ingestion of PSCs by female sheep (Section 2.2.1). The issues cross into human health also because, in women, exposure to toxic environmental and dietary compounds can impair reproduction and cause prolonged infertility.

To assess the risks and understand the mechanism of action of such chemicals, *in vitro* models have been used to evaluate the effects of compounds found in food, using bovine and porcine models (Table 2.5). The compounds of interest are added to the medium used for oocyte maturation, and then removed before IVF. After IVF, oocyte 'competency' (ability to be fertilised and develop an embryo) is assessed. Clearly, *in vivo* studies cannot be eliminated, but *in vitro* testing has become the approach of choice for assessing PSCs and plant extracts in the 21st century.

Table 2.5. Compounds tested *in vitro* using bovine and porcine models; from Santos *et al*. (2014).

Compound	**Species**		**Exposure**			**Oocytes**		**Blastocyst**	**Reference**
	Bovine	Porcine	IVM	IVF	IVC	MII	Fertilisation		
Biphenol		X	X			X			Mylnarcikova *et al*., 2009
α-Chaconine	X		X	X	X			X	Wang *et al*., 2005
Daidzein		X	X			X	X	X	Galeati *et al*., 2014
Deoxynivalenol		X	X			X	X	X	Schoevers *et al*., 2010
Flavanones		X	X			X	X	X	Solak *et al.,* 2014
Genistein	X		X	X	X	X	X		Lazzari *et al*., 2008
α-Solanine	X		X	X	X			X	Wang *et al*., 2005
Solanine-N-oxide	X		X	X	X			X	Wang *et al*., 2005
Zearelanone	X		X			X		X	Tagaki *et al*., 2008
		X	X			X		X	Malekinejad *et al*., 2007

α-Zearalenol	X	X			X	Malekinejad *et al.*, 2007

During maturation of the oocyte and surrounding cumulus cells, the oocyte goes through meiosis and is susceptible to epigenetic alteration. Therefore, many of the compounds of interest are anti-oxidants. For example, *Papaver rhoes* L., an annual herb indigenous to Iran, with sedative, narcotic and emollient effects, is widely used for treating inflammation, diarrhoea, sleep disorders (Zargari *et al*., 1995; Rajabi-Toustani *et al*., 2013). The phenolic compounds in *Papaver rhoes* L. are known to neutralize free radicals (Du *et al*., 2009) and, when it is included in the IVM medium, it improves maturation of sheep oocytes (Rajabi-Toustani *et al*., 2013). Similarly, *Crocus sativus* L. (commonly known as saffron), a perennial stemless herb widely cultivated in Iran, India and Greece, also has antioxidant properties. Tavana *et al*. (2012) showed that the addition of an aqueous extract of to IVM medium improves mouse oocyte maturation and embryo development. These benefits have been ascribed to the radical-scavenging activity of crocin and crocetin derivatives found in saffron. Another antioxidant plant, *Moringa oleifera*, a native to Asia and Africa, improves the progression of meiosis, increases calcium ion (Ca^{2+}) and improves protein synthesis during the maturation of sheep oocytes (Barakat *et al*., 2015).

In addition to improvements in oocyte competency, plants can be toxic for the embryo developing *in vitro*. In two-cell mouse embryos, culture with an aqueous extract of the leaves of *Indigofera suffruticosa* can prevent normal development to morula and blastocyst (Leite *et al*., 2004).

Effects of PSCs on oocyte maturation and embryos using IVMFC

In addition to testing plant extracts, *in vitro* studies have been used to test specific compounds isolated from plants of interest. For example, green tea contains the polyphenol, (-)-epigallocatechin-3-gallate (EGCG) that has been shown to help prevent cancer, HIV, neurological damage, and DNA damage (Jankun *et al*., 1999; Liang *et al*., 2010; Yu *et al*., 2010; Li *et al*., 2011; Morley *et al*., 2005). Dietary supplementation with catechins from green tea reduces lipid oxidation in pig meat and the cholesterol content of egg yolk (Mason *et al*., 2005; Uruganbayar *et al*., 2006). In the context of reproduction, high levels of consumption of green tea during around fertilisation and early embryo development has been linked to neural tube defects (Correa *et al*., 2000), whereas EGCG has negative effects on pig granulosa cells (Basini *et al*., 2005). These interesting findings led to tests of the effects of green tea polyphenol on *in vitro*

maturation of pig and bovine oocytes. Wang et al. (2007) demonstrated that the presence of green tea polyphenol (99% catechin derivatives) during IVM culture enhanced pronucleus formation and subsequent embryo development to blastocyst stage, as well as increasing intercellular GSH concentration. Interestingly, the outcomes did not further improve as the polyphenol concentration increased, an observation confirmed by Spinaci *et al*. (2008) who found, in fact, that a high concentration of polyphenol reduced oocyte competence and embryo development.

Phyto-oestrogens are among the most studied of plant secondary compounds because of their potentially dramatic effects on human and animal reproduction (reviews: Adams, 1995; Jefferson *et al*, 2002, 2006, 2007). Daily exposure or consumption of oestrogenic compounds found in the diet, particularly soy products, is thought to impair oocyte and embryo developmental competence. In this case, it is clear that IVMFC is essential for assessing the risk and understanding the mechanism of action. In soy, the major class of phyto-oestrogen is the isoflavone family and most attention has been paid to genistein and daidzein. The presence of a low level (5 μM) of genistein and daidzein during maturation of denuded mouse oocytes did not block progress through germinal vesicle (GV) break down and first polar body extrusion to arrest at metaphase II (MII); moreover, the distributions of actin microfilaments, cortical granules and metaphase spindle were all normal (Yoshida and Mizuno, 2012). This finding agrees with the result obtained porcine oocytes, in which low concentrations did not affect nuclear maturation or fertilisation rate, although progesterone production by cumulus cells was reduced (Galeati *et al*., 2014). However, a high concentration of genistein inhibited the maturation of pig oocytes *in vitro* (Jung *et al*., 1993) and, in mouse oocytes, inhibited germinal vesicle breakdown, first polar body extrusion, and, at the highest concentration (250 μM), induced cell mortality (Van Cauwenberge and Alexandre, 2000).

In addition to these oocyte studies, the effect of isoflavone of the blastocyst was also tested using a whole-embryo culture (WEC) model. When mouse blastocysts were incubated in the presence of 25 and 50 μmol/L genistein, dose-dependent effects were observed: an increase in apoptosis, a decrease cell number, and a developmental delay after implantation (Chan *et al*., 2007). In addition, with *in vitro* WEC, genistein combined with bisphenol-A led to neural tube malformation, especially forebrain abnormalities, and other structural damage in the central nervous system (Xing *et al*., 2010). Obviously, consumption of a high-isoflavone diet could be disastrous for development of oocyte, embryo and fetus.

Gap in knowledge

There is great interest in the search for new forage plants that are safe for sheep to consume, and that offer a wide variety of benefits such as mitigation of methane emissions, inhibition of gastro-intestinal worms, and high-quality feed in the dry season. However, as part of the process of assessment of forages, there is an emphasis on avoiding disturbance of reproductive performance because it is well known that plants can produce problematic secondary compounds, such as the isoflavones, that have a history of causing disastrous infertility in ewes, such as with 'clover disease'.

Studies of the effects of plant secondary compounds in new forage plants and their potential effects on reproduction in sheep are slow and expensive, so there is an obvious need to determine whether *in vitro* reproductive technology could be used to screen common forage plants with a known history in disrupting reproduction, or novel plants that have not yet been tested for effects on reproduction. However, it is difficult to predict whether the *in vitro* approach will be effective. Even for clover disease, a phenomenon that was studied extensively at the time it was prevalent, and where have great detail about the effects on the cervix, uterus, and endocrine system, there were very few studies of effects on oocyte maturation and fertilisation, or subsequent embryo development. This is a clear knowledge gap.

There is, therefore, a need to determine whether oocytes and embryos are affected by PSCs *in vitro* before we can contemplate establishing an *in vitro* screening system. We can do thus with IVMFC and, having validated the approach, the technique can be used to look deeper into how extracts of forage plants, and putative bioactive compounds from those forages, affect the maturation of oocytes, or fertilisation and subsequent embryo development.

Therefore, the general hypothesis tested in this thesis was that PSCs that have the potential to disrupt reproduction in sheep can be detected using *in vitro* reproductive technologies because they will inhibit the maturation and fertilisation of oocytes, and subsequent embryo development *in vitro*. Within this context, specific hypotheses were developed and tested in four experiments: Experiment 1) Establishing the IVMFC technique and using it to test PSCs that are known to disrupt fertility for their effects on the maturation and fertilisation of ovine oocytes, and subsequent blastocyst development; Experiment 2) Using the *in vitro* system, test extracts of common and novel forage plants to identify plants that could disrupt reproduction in sheep;

Experiment 3) Explore in depth *in vitro* any plant identified in Experiment 2 as having an effect on oocyte maturation and fertilisation, and subsequent embryo development, and attempt to identify the bioactive PSC.

Chapter 3

Phyto-oestrogens affect fertilisation and embryo development *in vitro* in sheep

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Abstract

Phyto-oestrogens such as isoflavones are natural compounds that can profoundly affect reproductive function. In the present study, we tested whether including isoflavone compounds (genistein, biochanin A, formononetin) in the maturation medium would affect the outcomes for ovine oocytes *in vitro*. A factorial design (3 isoflavones x 5 concentrations: 0, 2.5, 5, 10, 25 µg mL^{-1}) was used and the entire protocol was repeated four times. Cumulus-oocyte-complexes were randomly allocated to the treatments, then fertilized and cultured in vitro. Compared with control (0 µg mL^{-1}), the lower concentrations of isoflavone (2.5, 5 and 10 µg mL^{-1}) had no detectable effect on the rates of cleavage or embryo development, or on embryo total cell counts (TCC). However, the highest concentration (25 µg mL^{-1}) of all three isoflavones exerted a variety of effects ($p < 0.05$): genistein decreased cleavage rate, blastocyst rate and blastocyst efficiency (blastocysts produced per 100 oocytes); biochanin A decreased cleavage rate and blastocyst efficiency; and formononetin decreased blastocyst rate and blastocyst efficiency. Biochanin A (25 µg mL^{-1}) reduced embryo TCC specifically at the hatched blastocyst stage ($P < 0.05$). We conclude that the presence of isoflavones at 25 µg mL^{-1} during IVM decreases the cleavage rate and inhibits blastocyst hatching.

Introduction

The importance of nutrition in the management of reproductive performance has long been recognized in ruminant industries, but in recent years the value of forages is being considered to encompass more than just the supply of energy and protein (Bickell *et al.* 2010). In particular, there is great interest in the ability of alternative forage species to provide plant secondary compounds (PSCs) that reduce greenhouse gas emissions and improve health (Kotze *et al*. 2009; Durmic and Blache 2012; Durmic *et al.* 2012; Payne *et al.* 2013; Li *et al*. 2014). However, before such forages are incorporated into a

grazing system, it is critical that we test whether they cause problems in reproduction, a lesson learned from the 'clover disease' that caused uterine prolapse and severe infertility due to disruptions in gonadotrophin secretion (Adams and Martin 1983) and the function of the cervix (for a review, see Adams 1995). Revell and Revell (2007) therefore proposed the need for a 'duty of care' involving analysis of a broad variety of risks for animal productivity and health during the development of novel forages.

'Clover disease' emphasises the importance of phyto-oestrogens, non-steroidal oestrogen-like substances, such as the isoflavones, which have been studied intensively, as documented in over 10,000 peer-reviewed papers (Baber 2010). Three isoflavones, namely biochanin A, formononetin and genistein, are found in forages such as clovers and alfalfa (Trenkle and Burroughs 2012). In the rumen of sheep, genistein and biochanin A are broken down to non-oestrogenic products so their oestrogenic activity is much reduced, but their oestrogenic effects can still be detected within hours after they are first consumed (Lindsay and Kelly 1970; Adams 1995). Formononetin can be detected in the plasma of the ewe (Shutt *et al*. 1967) but, importantly, it is metabolised to equol, a more oestrogenic compound that is absorbed rapidly through the rumen wall (Lundh *et al*. 1990; Adams 1995). Thus, formononetin is considered to be the main cause of clover infertility (Adams 1995).

PSCs could also affect the processes of fertilisation, embryo survival and fetal development (McEvoy *et al*. 2001). Studies of oocytes flushed from ewes with clover disease demonstrated a lower fertilisation rate and fewer spermatozoa on the zona pellucida (ZP) due to disruption of the sperm transport function of cervical mucus (Lightfoot *et al.* 1974; Kaldas and Hughes 1989; Adams 1995; Trenkle and Burroughs 2012). Measurement of these processes in grazing animals is difficult so *in vitro* techniques are preferred for screening new forages (Leite *et al*. 2004; Wang *et al*. 2005, 2007; Spinaci *et al.* 2008; Rajabi-Toustani *et al*. 2013). Therefore, in the present study, we used *in vitro* technology to test the hypothesis that oocyte maturation and fertilisation, as well as subsequent early embryo development, will be inhibited by genistein, biochanin A and formononetin, PSCs that are known to interfere with reproductive function in grazing sheep.

Materials and methods

The experimental protocol was approved by the Animal Ethics Committee of the University of Western Australia (RA/3/500/70 and RA/3/500/71) according to the recommendations of the Australian National Health & Medical Research Council.

Experimental design

We used a 3 x 5 factorial design with three isoflavones (genistein, biochanin A, formononetin) added to the IVM medium at five concentrations (0, 2.5, 5, 10, 25 μg mL^{-1}). Dimethylsulfoxide (DMSO; 1 : 200) was used to dilute the isoflavones and was therefore used as the control treatment (0 μg mL^{-1} isoflavone). A comparison between control treatments with and without DMSO was also included in the study. The experiment was replicated four times. Unless specified otherwise, all chemicals and isoflavones were purchased from Sigma (St. Louis, MO, USA).

Collection of ovaries and oocytes

Ovaries from adult ewes were collected from an abattoir and transported to the laboratory in warm (33°C) phosphate-buffered saline (PBS). Cumulus-oocyte complexes (COCs) were aspirated from follicles ≥ 2 mm in diameter using an 18-g needle and a vacuum pump (Cook Australia, Queensland, Australia) with a pressure equivalent to 25 mm Hg. The COCs were collected into 2 mL aspiration medium, composed of HEPES-buffered TCM199 supplemented with 2% (v/v) ovine oestrus serum (SS), 100 IU mL^{-1}heparin (Pharmacia and Upjohn), 100 μg mL^{-1} streptomycin sulphate (CSL), and 100 IU mL^{-1} penicillin G (CSL Limited). With the aid of a stereo-microscope (×40 magnification, Olympus, Tokyo, Japan), unexpanded COCs were recovered, and atretic oocytes and those with signs of expanded cumulus were discarded.

IVM

The IVM procedures in the present study were similar to those outlined by Walker *et al.* (1996) and Kelly *et al.* (2007). Briefly, COCs were rinsed in IVM medium (sodium bicarbonate-buffered TCM199 supplemented with 20% (v/v) SS, 5 μg mL^{-1} FSH (Folltropin; Bioniche), 0.1 IU mL^{-1} human chorionic gonadotrophin (hCG; Chorulon; Intervet) and 1 μg mL^{-1} 17β-oestradiol and were then matured in groups of 20-25 per well in four-well culture dishes (Nunc Inc; IL) containing 500 μL maturation medium

covered with 300 μL mineral oil for approximately 24 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. The COCs were allocated equally among treatments.

Sperm preparation and IVF

After maturation, COCs were gently stripped of excess cumulus cells in 400 IU mL^{-1} hyaluronidase using a fine-bore pipette, and washed three times in IVF medium: (synthetic oviduct fluid (SOF) supplemented with 2% (v/v) SS). Up to 25 COCs per well were placed into four-well culture dishes containing 450 μL IVF medium overlaid with 300 μL mineral oil. Motile spermatozoa were obtained using the swim-up procedure, in which 200 μL frozen-thawed semen, pooled from two rams of proven fertility, was layered under 1 mL IVF medium in a 14-mL tube (Falcon; Becton Dickinson, Melbourne, Victoria). Approximately 0.5 x 10^6 spermatozoa were placed in each well and co-incubated with the COCs for 24 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air.

In vitro culture

After approximately 24 h, remnant cumulus cells were removed by gentle pipetting with a fine-bore pipette and the presumptive zygotes were washed three times in IVC medium (SOF containing 8 mg mL^{-1} bovine serum albumin (Fraction V; Invitrogen) and amino acids at concentrations found in the oviduct fluid of sheep; Walker *et al.* 1996; Kelly *et al.* 2007). Presumptive zygotes (n = 20-25 per well) were cultured in 600 μL IVC medium under 300 μL mineral oil in a humidified atmosphere of 5% CO_2 : 5% O_2 : 90% N_2 at 38.8°C. Oocytes that failed to divide ('single cell') were removed after 24 h, allowing the cleavage (fertilisation) rate to be determined. The remaining embryos comprised two to eight cells. We did not record the exact number for each stage, only whether they had cleaved so we could calculate cleavage rate.

Embryo assessment

Embryo development was assessed on Day 7, where Day 0 was the day of IVF. Blastocysts (the blastocoel accounts for more than half the volume of the embryo), expanded blastocysts (the blastocoel volume is larger than in the early embryo and the ZP is thinning), hatching blastocysts (the trophectoderm has started to herniate through the ZP) and totally hatched blastocysts (the blastocyst has completely escaped from the ZP) were collected and stained (Figure 3.1). The blastocyst rate was defined as the number of blastocysts plus expanded blastocysts expressed as a percentage of the number of cleaved oocytes; hatching rate was defined as the number of hatching plus

totally hatched blastocysts as a percentage of the total number of blastocysts; and blastocyst efficiency was defined as the number blastocysts produced expressed as a percentage of the original number of COCs. Embryos were mounted on a microscope slide in a drop of glycerol containing Hoechst 33342 (1 mg mL^{-1}) and covered with a coverslip. The total cell count (Figure 3.1) was recorded using a fluorescence microscope (Olympus Optical Co Ltd, Tokyo, Japan).

Statistical Analysis

The effects of isoflavone compounds on the IVM and developmental competence of oocytes were analysed as a factorial design (3 isoflavones x 5 concentrations x 4 replicates). Categorical data for embryonic development (cleavage rate, blastocyst rate, hatching rate, blastocyst efficiency) were analysed using the CATMOD procedure in SAS (SAS Institute). Continuous data (cell number) were analysed using the GLM procedure in SAS. Isoflavone concentration and replicate were initially included in the model (Model 1). A further analysis was used for cell number to determine whether isoflavone concentration was independent of stage of development (blastocyst, expanded blastocyst, hatched blastocyst; Model 2). Probability values < 0.05 were considered significant.

Results

There were no significant differences between the -DMSO and +DMSO control groups for any of the measures (cleavage rate, blastocyst rate, hatching rate or blastocyst efficiency). This allowed direct comparison of treatments with the -DMSO control group (Table 3.1).

Addition of 25 µg mL^{-1} genistein to the IVM medium significantly decreased cleavage rate, blastocyst rate and blastocyst efficiency (Table 3.1). Biochanin A at 25 µg mL^{-1} significantly decreased cleavage rate and blastocyst efficiency compared with the control group and the other concentrations of biochanin A (2.5, 5 and 10 µg mL^{-1}). Formononetin had no effect on the cleavage rate or hatching rate at any concentration, although blastocyst rate and blastocyst efficiency were both decreased with formononetin only at the highest concentration (25 µg mL^{-1}; Table 3.1).

Total cell number was decreased by the highest concentrations of biochanin A and formononetin but not genistein (Table 3.2 (Model 1, unadjusted for stage of development) and Table 3.3). Even after adjustment of cell number for stage of

development (Table 3.2, Model 2), there was a significant ($P < 0.001$) interaction between stage of development and biochanin A concentration (Table 3.2), with cell number remaining constant at the blastocyst stage but being reduced by high concentrations of biochanin A at the hatched blastocyst stage (Table 3.4).

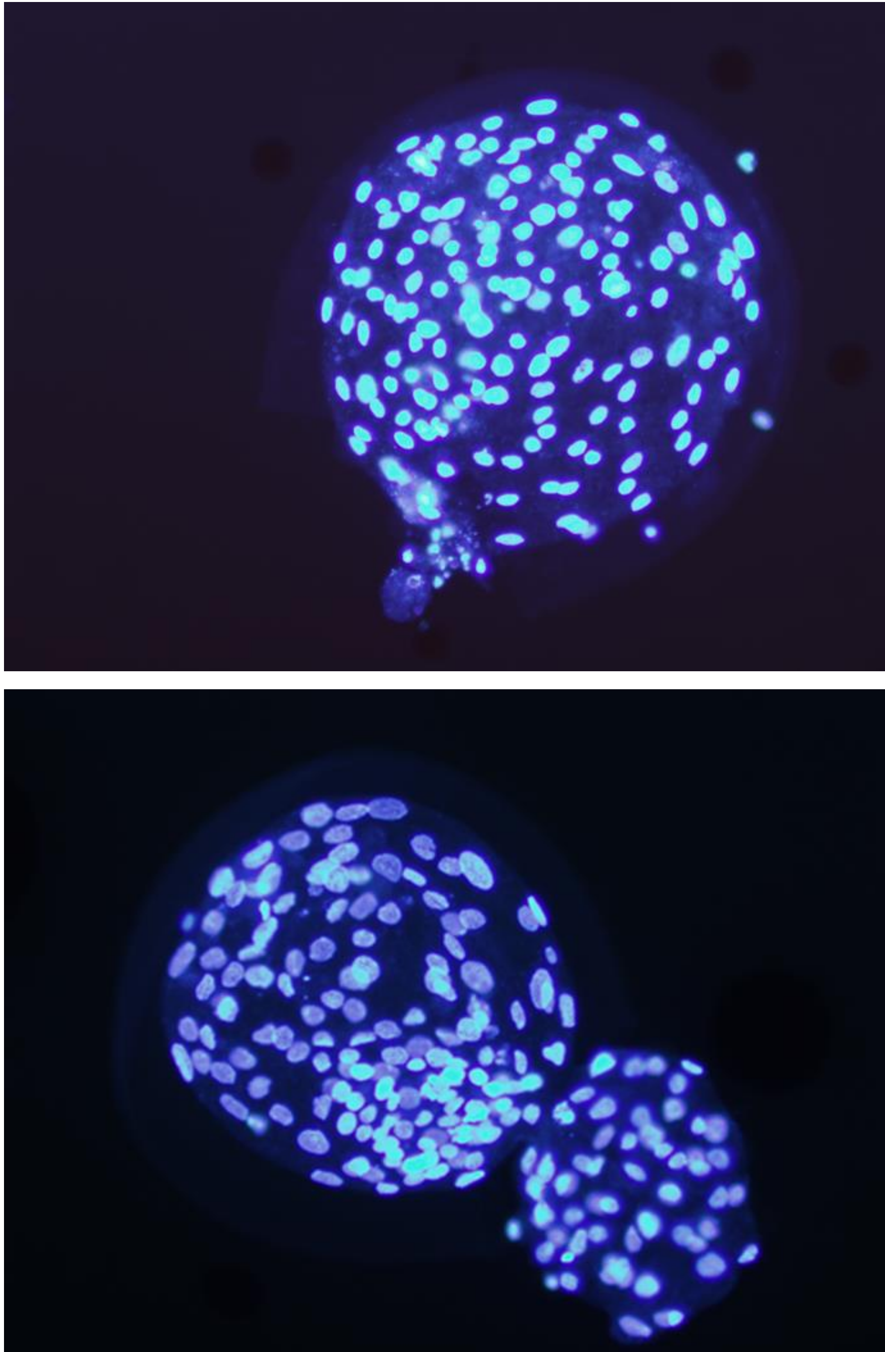

Figure 3.1. An expanded ovine blastocyst (above) and a hatching ovine blastocyst (below) with the inner cell mass (ICM) revealed by fluorescence microscopy (20X magnification).

Table 3.1. Effect of isoflavones in the maturation medium, over a range of concentrations (µg mL^{-1}), on rates of ovine oocyte cleavage and embryo development *in vitro*.

Treatment (µg mL^{-1})	No of oocytes	Cleavage rate (% of oocytes cultured)	Blastocyst rate (% blastocysts per cleaved oocyte)	Hatching rate (% blastocysts formed)	Blastocyst efficiency (% blastocysts per total oocytes cultured)
Control + DMSO	77	90.9	51.4	13.9	46.8
Control - DMSO	111	91.9	61.8	25.4	56.8
Genistein					
Control - DMSO	111	91.9^{a}	61.8^{a}	25.4	56.8^{a}
2.5	103	90.3^{a}	60.2^{a}	17.9	54.4^{a}
5.0	100	87.0^{ac}	59.8^{a}	26.	52.0^{a}
10.0	100	91.0^{a}	68.1^{a}	25.8	62.0^{a}
25.0	102	80.4^{bc}	45.1^{b}	13.5	36.3^{b}
Biochanin A					
Control - DMSO	111	91.9^{a}	61.8^{a}	25.4	56.8^{a}
2.5	90	95.6^{a}	52.3	15.6	50.0^{a}
5.0	99	89.9^{a}	64.0	17.5	57.6^{a}
10.0	98	92.9^{a}	53.8	20.	50.0^{a}
25.0	101	57.4^{b}	55.2	12.5	31.9^{b}
Formononetin					
Control - DMSO	111	91.9	61.8^{a}	25.4	56.8^{a}
2.5	100	90.0	50.0^{a}	26.7	45.0^{ab}
5.0	107	89.7	50.0^{ab}	27.1	44.9^{ab}
10.0	98	90.8	48.3^{ab}	27.9	43.9^{ab}
25.0	101	93.1	44.7^{b}	11.9	41.6^{b}

Comparisons between controls with + or – DMSO, within columns, with different superscripts differ significantly ($P<0.05$).

Within isoflavone within a column: values with different superscripts differ significantly ($p < 0.05$).

Table 3.2. Least-squares analysis of variance giving probability values for cell number in ovine embryos subjected to various concentrations of isoflavones *in vitro.*

Source of variation	df	Biochanin A	Formononetin	Genistein
(1) Isoflavone concentration	4	0.0456	0.0414	0.5239
Replicate	3	0.0003	0.1441	0.0019
(2) Isoflavone concentration	4	0.0043	0.7120	0.9111
Stage of development (SD) [A]	2	<0.0001	<0.0001	<0.0001
Isoflavone concentration x SD	8	0.0001	0.2960	0.9118
Replicate	3	0.0049	0.3464	0.1148

(1), (2) Models analysed in procedure GLM in SAS. Model 1 includes isoflavone concentration only, while Model 2 includes isoflavone concentration, stage of development, and their interaction.

[A] Stages of development were blastocyst, expanded blastocyst and hatched blastocyst

Table 3.3. Least-squares means (± SEM) for total cell number, combined for all stages of ovine embryo development (combined blastocyst, expanded blastocysts, hatched blastocysts), after treatment with a range of concentrations of isoflavones *in vitro.*

Isoflavone concentration ($\mu g\ mL^{-1}$)	Cell number for total embryos		
	Biochanin A	**Formononetin**	**Genistein**
Control	104.5 ± 5.5 ^{a} (51)	105.1 ±5.8 ^{a} (51)	103.9 ± 5.9 (51)
2.5	104.2 ± 6.2 ^{ac} (40)	103.1 ± 8.4 ^{a} (45)	101.2 ± 7.4 (53)
5.0	108.6 ± 5.3 ^{a} (55)	101.5 ± 7.1 ^{a} (48)	106.4 ± 5.4 (52)
10.0	83.0 ± 7.3^{b} (46)	91.2 ± 5.8 ^{ab} (42)	91.4 ± 7.4 (61)
25.0	84.9 ± 7.5 ^{bc} (32)	76.9 ± 8.0 ^{b} (45)	96.3 ± 6.7 (39)

Within isoflavone, values with different lower case letters differ significantly ($P < 0.05$).

Numbers in parentheses are the number of embryos.

Table 3.4. Least-squares means (± SEM) for cell number for individual stages of development of ovine embryos following the inclusion of various concentrations of Biochanin A in the culture medium. The effect of the interaction (concentration x stage of development) was significant (P < 0.001; see Table 3.2).

Biochanin A (μg mL^{-1})	Stage of development		
	Blastocyst	Expanded blastocyst	Hatched blastocysts
Control	56.8 ± 8.2 ^{a} (12)	99.3 ± 5.7 ^{ab} (25)	153.5 ± 7.5 ^{a} (14)
2.5	65.1 ± 8.2^{a} (12)	112.1 ± 6.2 ^{a} (21)	140.9 ± 10.7 ^{a} (7)
5.0	68.9 ± 6.9^{a} (17)	109.7 ± 5.2 ^{a} (29)	147.7 ± 9.5^{a} (9)
10.0	61.6 ± 7.7^{a} (21)	80.2 ± 9.0^{b} (14)	136.8 ± 10.9^{a} (11)
25.0	75.6 ± 10.9^{a} (8)	97.4 ± 7.2 ^{ab} (17)	73.9 ± 11.8^{b} (7)

Values, within stage of development, with different lower case letters differ significantly (P < 0.05).

Discussion

Genistein, formononetin and biochanin A can all reduce the rates of IVF of sheep oocytes and inhibit subsequent embryo development, helping to explain embryonic loss in sheep consuming phyto-oestrogens (Adams 1995). The observations for formononetin and biochanin A are novel for any species, and those for genistein support previous studies on the process of maturation in oocytes from the sow and cow (Van Cauwenberge and Alexandre 2000, Santos *et al*. 2014). In rodent models, complementary studies have been restricted to the developing ovary following neonatal exposure to genistein, with observations of adverse effects on ovarian differentiation and an increase in the number of multi-oocyte follicles before puberty (Jefferson *et al*., 2007; Zhuang *et al*., 2010). Conversely, in 3- to 11-month-old rats, genistein appears to increase 'ovarian longevity' by conserving primordial follicles and total surviving follicles (Zhuang *et al*., 2010). Another *in vitro* study of the maturation of oocytes in the maturing germinal vesicle suggested that genistein had no negative effects during development to second metaphase (Yoshida and Mizuno, 2012).

The range of concentrations used in the present study (2.5-25 μg mL^{-1}) was chosen on the basis of a previous in vitro study using polyphenols from green tea in pig oocytes (Spinaci *et al.* 2008). The highest concentrations may exceed values seen *in vivo*, for example, Shutt *et al.* (1967) found a plasma concentration of isoflavone up to 7 μg mL^{-1} in sheep consuming oestrogenic clovers, within the range tested in the present study. However, concentrations of isoflavones within oestrogenic clovers can vary with

genotype, season and fertilizer practices (Adams 1995), so testing for effects at higher levels is also warranted. Although there seem to be no data on the concentration of phyto-oestrogens in follicular fluid of sheep consuming oestrogenic clovers, there is a strong correlation ($r = 0.9$) between the blood and follicular fluid concentrations of endocrine disruptors, in humans at least (Petro *et al*. 2012).

Cleavage rate declined after IVF when a high isoflavone concentration (25 µg mL^{-1}) was present in the maturation medium, suggesting effects during the maturation of ovine oocytes. The mechanism behind this decline is not clear although oocyte maturation consists of events that occur in the both nucleus and cytoplasm, and the environment supplied for *in vitro* oocyte maturation can affect subsequent embryo development and events that occur after fertilisation (Rose and Bavister 1992). In granulosa cells harvested from porcine follicles, the addition of genistein (>1 µM and 500 nM) to the medium has been shown to inhibit cytochrome P450 cholesterol side chain-cleavage (P450scc; and thus the conversion of cholesterol to pregnenolone), as well as 3β-hydroxysteroid dehydrogenase (3β-HSD; and thus the conversion of pregnenolone to progesterone; Piasecka-Strader *et al.* 2014; Tiemann *et al*. 2007). Numerous studies in a variety of mammalian species suggest certain hormones are needed during oocyte IVM for fertilisation and developmental competence (Armstrong *et al*. 1991). Granulosa cells are metabolically coupled to the oocyte via gap junctions throughout the growth of the oocyte and during the initial stage of maturation (Brower and Schultz 1982; De Loos *et al.* 1991). Granulosa cells are responsible for conveying nutritional and regulatory elements responsible for growth, maintenance of meiotic arrest and the maturation and development of the oocyte (Brower and Schultz 1982). In mature granulosa cells, FSH stimulates the production of inhibin α, follistatin and activin β_A (Tuuri *et al.* 1996; Welt and Schneyer 2001). Inhibin and activins are paracrine factors that modulate follicle growth and steroidogenesis, whereas follistatin regulates the paracrine and autocrine actions of activins (Guillette and Moore 2006). Phyto-oestrogens can disrupt the gonadotrophin-oestrogen-inhibin/activin system by inducing gene expression for inhibin α and follistatin during folliculogenesis, leading to the formation of multi-oocytic follicles (Guillette and Moore 2006). In rodents, multi-oocytic follicles are associated with a decreased IVF success rate and increased embryonic loss (Iguchi *et al.* 1990; Guillette and Moore 2006).

It is also possible that isoflavones, because they have a molecular structure that is similar to oestradiol, as well as oestrogenic activity, are affecting the function of the mitochondria in the gamete. Mitochondria are needed to provide adequate ATP to fuel the oocyte during the first few days of embryonic development (Lin *et al.* 2004; Thouas *et al*. 2005) and it has been shown that the cleavage rate can be compromised when oocytes are exposed to chemicals that impair mitochondrial function (Chiaratti *et al*., 2011). In an *in vitro* study, Kotwicka *et al.* (2016) found that a high concentration of 17ß-oestradiol decreased mitochondrial membrane function in spermatozoa, leading to an excessive influx of free calcium ions into the mitochondria. Mitochondria could be reservoirs for oestrogen (Kotwicka *et al*. 2016). The mechanisms through which isoflavones affect the function of cell organelles and cumulus cell hormone synthesis during oocyte maturation warrant further investigation because of the consequences for subsequent embryo development.

High concentrations of all three isoflavones in the maturation medium reduced blastocyst rate and blastocyst efficiency, but total cell number was only reduced by formononetin and biochanin A. Moreover, for biochanin A, in the present study the effect on total cell number seems to depend on the phase of blastocyst development, being only observed in hatched blastocysts. Effects on the later stages of embryo development are critical because a high rate of cell division is associated with high embryo viability, so blastocyst cell number, particularly inner cell mass (ICM) cell number, is related to pregnancy outcome (Lane and Gardner 1997). Theoretically, the ICM will develop into the main part of the fetus so cell count is now widely used for blastocyst selection and prediction of clinical pregnancy (Hardarson *et al*., 2003; Kovacic *et al*., 2004). Blastocyst development rates and total cell number are recognized as good indicators of oocyte developmental capability; they indicate that the oocyte has the ability to successfully undergo the change from maternal to genomic activation (Bavister *et al*., 1992; Newmark *et al*., 2007).

In conclusion, exposure of ovine oocytes to isoflavones during IVM affects subsequent IVF and embryo development. In addition, when maturing oocytes were exposed to biochanin A, downstream effects were seen in the developing embryo, as evidenced by changes in total cell number. Thus, the ingestion of large quantities of isoflavones could thus reduce reproductive efficiency in sheep through these processes, as well as the processes already documented by Adams (1995), assuming the isoflavone concentrations bathing the oocyte and embryo reach the levels tested here. The

developmental capacity of embryos derived from IVM and IVF oocytes is a more definitive way to assess the normality of maturation (Rose and Bavister, 1992). More studies are needed to determine the concentrations of isoflavones in follicular fluid, oocytes, embryos and semen, as well as molecular studies on oocytes and embryos, in sheep consuming forages that contain phyto-oestrogens. This examination of the effects of isoflavones on the development of ovine oocytes clearly demonstrates the value of in vitro techniques for assessing the risks of novel forages for reproduction.

Chapter 4

Extracts of forage plants affect developmental competence of ovine oocytes *in vitro*

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Abstract

Forage plants may contain secondary compounds that disrupt reproduction in ruminants so, as 'duty of care', proposed new forage species need to be tested for harmful effects on reproduction before industrial release. We evaluated the effects of *Bituminari butuminosa, Medicago sativa, Chicorium intybus, Trifolium subterraneum, Trifolium pratense, Biserrula pelecinus* and *Eremophila glabra*, on the *in vitro* developmental competence of ovine oocytes. Crude methanolic extracts of each plant were added to the medium (final concentrations: 0, 50 or 100 μg dry extract per mL) used for *in vitro* maturation of cumulus-oocyte complexes derived from abattoir-sourced adult ewe ovaries. After *in vitro* fertilisation, we quantified cleavage rate, blastocyst rate, hatching rate, blastocyst efficiency, and total blastocyst cell number (TCN). Extract from *B. pelecinus* increased cleavage rate at 50 μg mL^{-1} ($P<0.05$) and blastocyst rate and blastocyst efficiency at 100 μg mL^{-1} ($P<0.05$). The other plant extracts did not affect these measures. TCN was affected by stage of development and treatment, but not by the interaction between stage and treatment. Within treatments, TCN was increased by *C. intybus* (at both 50 and 100 μg mL^{-1}) but decreased by *M. sativa* (at both 50 and 100 μg mL^{-1}; $P<0.05$). We conclude that methanolic extracts of forage plants, present during *in vitro* oocyte maturation, did not disrupt subsequent fertilisation and embryo development until the blastocyst stage. On the contrary, *B. pelecinus* appears to improve fertilisation and embryo development. Overall, these observations suggest that these plants will not disrupt *in vivo* oocyte maturation but further testing is still required, especially for the other stages of the reproductive process.

Introduction

Forage plants are primary sources of nutrients for growth and development in ruminants, but they may also contain secondary compounds such as isoflavones (biochanin A, formononetin, genistein, daidzein), saponin, and coumestans, that may

negatively affect animal health and reproduction (McEvoy *et al*., 2001, Wina *et al*., 2005). For example, as discovered in the 1940s, in 'clover disease' in sheep, the consumption of red clover (*Trifolium pretense*) causes infertility due to the presence of phyto-oestrogens that affect embryo survival and fetal development (Adams 1995; McEvoy *et al*., 2001).

Other forage plants have also been shown to affect reproduction in sheep and other animals (Table 4.1). In addition to these mainstream forages, some recently introduced, novel forage species are thought to contain bioactive substances. For example, *Biserruala pelecinus*, a self-generating annual legume with anti-methanogenic properties, introduced into Western Australia during the 1990s where it has become popular with sheep producers (Banik *et al*., 2013) was later linked to outbreaks of photosensitivity caused by secondary metabolites (Kessell *et al*., 2015). Similarly, *Eremophila glabra* is also reported to have anthelmintic properties and is seen as a critical factor in the drive for greenhouse gas mitigation (Kotze *et al*., 2009). For both species, the potential bioactive substances have not yet been identified and there has been little study of their effects on reproductive performance.

Table 4.1. Effect of feeding mainstream forages on sheep reproductive performance.

Plant species	Effect on reproduction	References
Trifolium subterraneum (subclover)	Infertility, dystocia, perinatal lamb loses	Lightfoot *et al*., 1974; Reid, 1981; McDonald, 1981; Adams, 1995; Pace *et al*., 2011
Trifolium pratense (red clover)	Reduce reproductive rate Infertility	Kelly *et al*., 1979, 1980; McDonald, 1981; Mu *et al*., 2009; Mustonen *et al*., 2014
Bituminari butuminosa (tedera)	Increase weight and body condition score	Diawara *et al*., 2001; Diawara and Kulkosky, 2003; Oldham *et al*., 2013
Chicorium intybus (chicory)	Short term grazing increases ovulation rate	King *et al*., 2010
Medicago sativa (lucerne)	Short term grazing increases ovulation rate	King *et al*., 2010
	Depressed ovulation rate but did not affect embryonic loss or number of embryos	Ramon *et al*., 1993

Assessment on the impact of ingestion of forage plants on reproductive performance has typically relied on *in vivo* observations of oestrus, ovulation rate, fertility and fecundity (Blache *et al*., 2008; Pace *et al*., 2011). However, there is growing evidence that adverse effects of forages on early embryonic development are programmed even before fertilisation in ruminants (Ramírez-Restrepo and Barry, 2005; King *et al*., 2010), with recent *in vitro* studies focusing on effects on oocyte quality and embryo development (Leite *et al*., 2004, Wang *et al*., 2007, Spinaci *et al*., 2008; Rajabi-Toustani *et al*., 2013).

Using the *in vitro* approach, we have recently shown that isoflavones, the family of phyto-oestrogens responsible for 'clover disease', disrupted fertilisation and embryo development in sheep (Chapter 3; Amir *et al*., 2018a). These findings encouraged us to investigate other mainstream pastures and novel forages in a similar manner.

In the present study, we have added plant extracts of common and novel forages that contain unknown secondary compounds to ovine *in vitro* maturation medium to test for potential effects on reproduction. The methanolic extracts of five common forage plants, with a known history of effects on reproduction, and two novel forage plants that have not yet been tested for effects on reproduction, were expected to reduce cleavage rate, blastocyst rate, blastocyst efficiency and total cell number (TCN).

Materials and methods

Procedures were approved by the Animal Ethics Committee of the University of Western Australia (RA/3/500/70 and RA/3/500/71). Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO, USA).

Plant material and crude extract preparation

The information on plant material is listed in Table 4.2. The samples consisted of mixtures of leaves, stem (up to 5 cm long) and flowers. Fresh weight was measured before the plant materials were stored at –20 °C. The samples were freeze-dried, weighed and milled to powder using a grinder fitted with a 1 mm sieve (CYCLOTECH 1093 Sample Mill; Tecator, Hoganas, Sweden). Powdered material was stored in sealed containers at room temperature until use. The powder was extracted using a modified methanol extraction procedure (Sweeney *et al*., 2001; Billo *et al*., 2005; Mothana and Lindequist, 2005): 0.5 g was macerated in 5 mL 70% ethanol for 3 h on an orbital shaking (Certomat® MO II, Sartorius Stedim Biotech GmbH, Goettingen, Germany) at 100 rpm and then centrifuged (Alleygra® X-12R Centrifuge, Beckman Coulter Inc., California, USA) at 3000 rpm for 10 min at room temperature (22 ºC). The collected supernatant was transferred to a fresh tube, labelled and set aside. The pellet underwent a second extraction by maceration in 2 mL 70% ethanol for an hour, and centrifuged at 3000 rpm for 10 mins at room temperature (22 ºC), after which the supernatant was again collected. Both supernatants were pooled and the mixture was evaporated under a slow stream of compressed air for 3-4 days. Prior to inclusion in the IVM medium,

extracts were reconstituted in 1 mL DMSO to make a stock for preparation of the final dilutions.

Table 4.2. Mainstream and novel forage plants used in the present study.

Plant species *Latin name* (common name)	**Accession**	**Rep stage**	**Location**	**Date/Year**
Novel forages				
Biserrula pelecinus (biserrula)	Casbah	Vegetative	Ridgefield, WA	26 Aug 2014
Eremophila glabra (tar bush)	45599	Reproductive	Ridgefield, WA	May 2014
Mainstream forages				
Trifolium subterraneum (subclover)	Urana	Reproductive	Waite, SA	4 Sept 2013
Trifolium pratense (red clover)	Rubitas	Reproductive	Waite, SA	17 Dec 2012
Bituminari butuminosa (tedera)	DAFWA	Reproductive	Medina Research Station, WA	9 Jul 2008
Chicorium intybus (chicory)	Choice	Reproductive	Waite, SA	10 Jan 2013
Medicago sativa (lucerne)	Aurora	Reproductive	Waite, SA	2012

Experimental design

The extracts were added to the *in vitro* maturation (IVM) medium and cumulus-oocyte complexes (COCs) collected from abattoir-sourced ovine ovaries were exposed to plant extracts only during the IVM phase. After IVM, mature oocytes underwent *in vitro* fertilisation (IVF) and embryo culture (IVC) procedures, and cleavage rate, blastocyst developmental rate and blastocyst total cell number (TCN) were quantified. COCs were randomly allocated among 16 IVM wells with plant extracts added at 50 or 100 µg mL^{-1}. There were two control wells, one with and one without DMSO (1 in 1000). The experiment was replicated 4 times. As described previously (Amir *et al.*, 2018a; published), we measured cleavage rate (fertilisation), blastocyst rate, hatching rate, blastocyst efficiency, and blastocyst total cell number (TCN).

Collection of ovaries and oocytes

The procedures have been described in detail by Walker *et al.* (1996). In brief: ovaries from adult ewes were collected from a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS) warmed to 33°C; cumulus-oocyte complexes (COCs) were aspirated from follicles ≥ 2 mm diameter using an 18 G disposable needle and a vacuum pump (Cook Australia, Queensland, Australia) with a pressure equivalent to 25 mm Hg; the COCs were collected into 2 mL aspiration medium comprising Hepes-buffered TCM199 containing 2% (v/v) oestrous sheep serum (SS), 100 IU mL^{-1} heparin (Pharmacia and Upjohn, Bentley, Western Australia), 100 µg mL^{-1} streptomycin sulphate (CSL Limited Parkville, Victoria, Australia), and 100 IU mL^{-1} penicillin G

(CSL Limited). Unexpanded COCs were recovered using a stereo-microscope (40 x magnification; Olympus, Tokyo, Japan).

In vitro maturation, fertilisation and culture

The COCs were rinsed three times in aspiration medium, and then twice in maturation medium that consisted of sodium bicarbonate-buffered TCM199 supplemented with 20 % (v/v) SS, 5 μg mL^{-1} FSH (Folltropin, Bioniche, Inc), 1 IU mL^{-1} hCG (Chorulon, Intervet) and 1 μg mL^{-1} oestradiol-17β. Up to 30 COCs were randomly allocated among treatments (0, 50 or 100 μg mL^{-1} plant extract) in four-well culture dishes (Nunc Inc., Naperville, IL) containing 500 μL maturation medium overlaid with 300 μL mineral oil. The COCs were matured for approximately 24 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air.

After maturation, excess cumulus cells were removed by gently pipetting in 400 IU mL^{-1} hyaluronidase, and the COCs were washed three times in synthetic oviduct fluid (SOF) supplemented with 2% (v/v) SS (IVF medium). The COCs were placed in four-well culture dishes containing 450 μL IVF medium covered with 300 μL mineral oil. Approximately 0.5 x 10^6 sperm, obtained by the 'swim up' procedure, were placed in each well and co-incubated with the COCs for 24 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air.

After approximately 24 h, remnant cumulus cells were removed by gentle pipetting and the presumptive zygotes were washed three times in culture medium (IVC) consisting of SOF supplemented with 8 mg mL^{-1} BSA (Fraction V; Invitrogen Corp., Auckland, New Zealand) and amino acids at concentrations of that of sheep oviduct fluid, as previously described by Walker *et al.* (1996). Presumptive zygotes were cultured in 600 μL of IVC under 300 μL mineral oil in a humidified atmosphere of 5% CO_2:5% O_2:90% N_2 at 38.8 °C. Zygotes that failed to divide were removed 24 h later and counted so the cleavage rate could be recorded.

Embryo assessment and staining

Day 7 (Day 0 = day of IVF) embryos within morphological categories (blastocysts, expanded blastocysts, hatching blastocysts and totally hatched blastocysts) were placed in a drop of glycerol containing Hoechst 33342 (1 mg mL^{-1}) on a microscope slide and covered with a coverslip. Nuclei were counted using a fluorescent microscope (Olympus Optical Co Ltd, Tokyo, Japan).

Statistical Analysis

The effects of the plant extract on *in vitro* maturation and developmental competence of oocytes were analysed within plant species at three concentrations (0, 50, 100 µg mL^{-1}) with four replicates. Categorical data for embryo development parameters of cleavage, blastocyst and hatching rate and blastocyst efficiency were analysed using the CATMOD procedure in SAS (Statistical Analysis System, Cary, NC, USA) with extract treatment (within plant species) and replicate (n=4) included in the model. Continuous data such as TCN was analysed using the GLM procedure in SAS. The model included concentration of extract within plant species, embryo stage (blastocyst, expanded blastocyst, hatching blastocyst), the interaction of concentration with embryo stage, and replicate (n=4). Probability values < 0.05 were considered significant. The control that included DMSO was used as the zero concentration (0 µg mL^{-1}) for all methanolic plant extract comparisons because it did not differ (P>0.05) from the control without DMSO for any of the parameters measured (Table 4.3).

Results

The addition of *B. pelecinus* extract to the *in vitro* maturation medium increased (P<0.05) the cleavage rate compared with the control although this effect was only evident at 50 µg mL^{-1} (Table 4.3). Blastocyst rate and the blastocyst efficiency were also increased by *B. pelecinus* extract, but the differences were only significant at 100 µg mL^{-1} (P<0.05; Table 4.3). No effects were observed on any of the measures of embryonic development for the other plant extracts at either concentration.

Blastocyst total cell number (TCN) was increased by *C. intybus* extract, at both 50 and 100 µg mL^{-1}, and decreased by *M. sativa* extract at both 50 and 100 µg mL^{-1}. No other extracts affected TCN (Table 4.3). As expected, TCN significantly (P<0.001) increased as the embryos grew from one stage to the next – for example, LSMeans (± SEM) were 49.7 ± 4.4 cells per blastocyst, 98.5 ± 3.2 cells per expanded blastocyst, and 152.2 ± 4.1 cells per hatching blastocyst, pooled across the *B. pelecinus* treatments. These changes were independent of plant extract treatment as there were no significant interactions between embryo stage and concentration within plant extract.

Table 4.3. Effect of the addition of methanolic extract of various forage plants (0, 50 or 100 µg mL^{-1} medium) on IVM and oocyte developmental competence (cleavage rate, blastocyst development rates and blastocysts total cell number).

Treatment (µg mL^{-1})	Oocytes	Cleavage rate % (n)	Blastocyst rate (blastocysts/cleaved) (%)	Hatching rate (hatched blastocysts/ blastocysts) (%)	Blastocyst efficiency (blastocysts/total oocytes) (%)	Blastocyst total cell number (LSMeans ±SEM)
Control – DMSO	99	86.9 (86)^{a}	61.6 (53)^{a}	39.6 (21)^{a}	53.5 (53)^{a}	100.1 ± 3.4^{a}
Control +DMSO	104	79.8 (83)^{a}	56.6 (47)^{a}	25.5 (12)^{a}	45.2 (47)^{a}	99.2 ± 4.0^{a}
Biserrula pelecinus	105					99.4 ± 4.1^{a}
50		86.7 (91)^{b}	49.5 (45)^{a}	33.3 (15)^{a}	42.9 (45)^{a}	
100	107	86.0 (92)^{a}	75.0 (69)^{b}	29.0 (20)^{a}	64.5 (69)^{b}	98.5 ± 3.3^{a}
Eremophila glabra	112					98.7 ± 3.8^{a}
50		75.9 (85)^{a}	62.4 (53)^{a}	32.1 (17)^{a}	47.3 (53)^{a}	
100	107	79.4 (85)^{a}	47.1 (40)^{a}	42.5 (17)^{a}	37.4 (40)^{a}	102.4 ± 4.4^{a}
Trifolium subterraneum	102					99.9 ± 3.9^{a}
50		84.3 (86)^{a}	53.5 (46)^{a}	30.4 (14)^{a}	45.1 (46)^{a}	
100	102	82.4 (84)^{a}	51.2 (43)^{a}	27.9 (12)^{a}	42.2 (43)^{a}	107.1 ± 4.0^{a}
Trifolium pratense	103					103.3 ± 3.3^{a}
50		88.3 (91)^{a}	58.2 (53)^{a}	22.6 (12)^{a}	51.5 (53)^{a}	
100	107	79.4 (85)^{a}	64.7 (55)^{a}	25.5 (14)^{a}	51.4 (55)^{a}	96.9 ± 3.1^{a}
Bituminaria butuminosa	111					102.5 ± 3.3^{a}
50		81.1 (90)^{a}	61.1 (55)^{a}	32.7 (18)^{a}	49.5 (55)^{a}	
100	108	84.3 (91)^{a}	57.1 (52)^{a}	30.8 (16)^{a}	48.1 (52)^{a}	110.1 ± 3.6^{a}
Chicorium intybus	105					121.4 ± 3.5^{b}
50		86.6 (93)^{a}	58.1 (54)^{a}	18.5 (10)^{a}	51.4 (54)^{a}	
100	117	77.8 (91)^{a}	62.6 (57)^{a}	12.3 (7)^{a}	48.7 (57)^{a}	110.9 ± 3.8^{b}
Medicago sativa	109					91.2 ± 3.4^{b}
50		85.3 (93)^{a}	54.8 (51)^{a}	21.6 (11)^{a}	46.8 (51)^{a}	
100	101	80.2 (81)^{a}	54.3 (44)^{a}	34.1 (15)^{a}	43.6 (44)^{a}	92.5 ± 3.8^{b}

Numbers given in parenthesis are those used to calculate percentage values.
Values, comparing controls plus or minus DMSO, with different lower case letters vary significantly ($P<0.05$).
Values, within plant extract and within columns, with different lower case letters vary significantly ($P<0.05$)

Discussion

In this study of extracts of common forage plants with a history of disruption of reproduction, and novel forage plants that have not previously been tested, the only inhibitory effect detected was with *M. sativa* (at both 50 and 100 µg mL^{-1}), with a 10% reduction in blastocyst cell count, supporting the hypothesis that this species might disrupt reproductive competence in sheep. The other extracts at the concentrations investigated, showed no deleterious effects on any stage from fertilisation through to blastocyst hatching, with some of the species even appearing to improve embryo development. Some of these plants have a history of disruption of reproduction in sheep, so any PSCs that cause such problems might be at a concentration within the plant extract that is too low to negatively affect the oocytes and early embryos. Alternatively, there might be major increases in bioactivity as a result of rumen fermentation. That said, the present study suggests that infertility problems in sheep grazing these forage plants under field conditions are not likely to be caused by effects on oocyte maturation, fertilization and embryo development.

B. pelecinus extract increased cleavage rate, blastocyst rate and blastocyst efficiency. These unexpected stimulatory effects are novel for any species and the first observations of showing that extractable compounds from Australian forages could affect ovine oocyte and embryo development *in vitro*. It is important to remember that the extracts were present only during *in vitro* maturation, so all subsequent outcomes are a consequence of effects exerted during this period. Although unexpected, the consistency of observations, from cleavage to blastocyst suggest, that they are not chance observations. We should therefore look at the process of oocyte maturation for possible sites of action of PSCs. As outlined by Crosby and Moor (1984), maturation involves changes in a wide variety of cellular activities, including transcription, synthesis and post-translational modification of proteins, energy metabolism, membrane transport and cellular co-operation. The oocyte undergoes meiosis, leading to germinal vesicle breakdown and, finally, the extrusion of the first polar body. The cumulus-oocyte complex permits transport of nutrients and messenger molecules to the oocytes (Lawrence *et al*., 1978; Moor *et al*., 1980). Meanwhile, at the molecular level of meiosis, there are interactions among cell cycle molecules and their target substrates in the nucleus and the cytoplasm (Moor *et al*., 1992b). *B. pelecinus* extract appears to affect at least one of these steps during *in vitro* maturation, improving the quality of the

oocytes, with flow-on benefits for fertilisation rate and blastocyst production. However, our observations were *a posteriori* so further studies are required.

In addition to a site of action of PSCs, we also need to consider the chemistry of the extracts if we are to determine the molecules responsible. The choice of solvent determines, to some extent, whether exo- or endo-cellular metabolites are extracted. In the present study, we needed to study a variety of plant species so we selected a general extraction method using a polar solvent, 70% methanol (Houghton and Raman, 1998). Additional studies are therefore needed using solvent in different polarity and collecting plants at different phenology stages. Furthermore, metabolites available in these forages during *in vivo* digestion and/or by liver activity should also be considered.

TCN has been shown to be an indicator of embryo quality (Papaioannou and Ebert 1988; Jiang *et al* 1992) and, in the present study, *C. intybus* increased TCN and produce more morphologically healthy embryos, whereas *M. sativa* appeared to have the opposite effect. However, in both cases, the embryos reached blastocyst stage at a normal rate and there were no negative effects on the cleavage rate and embryo development. Again, it is important to remember that the plant extracts were present only during *in vitro* maturation, and the lack of consist sequential effects suggests that changes in TCN could be chance observations that need further verification.

These studies demonstrate to the value of *in vitro* methodology for studying the effects of plant extracts on reproduction (Barnes, 2000), as an alternative, or at least an initial step, before going to *in vivo* studies (Amir *et al*., 2018a). Similar conclusions have been drawn for the testing of toxins and antioxidants (Wang *et al*., 2007; Spinaci *et al*., 2008; Rajabi-Toustani *et al*., 2013) – indeed, reproductive toxicology studies, using bovine and porcine IVM/IVF models, have proven valuable for assessing risks in humans (Santos *et al*., 2014). Moreover, the present study has suggested positive effects of extracts of *B. pelecinus* and *C. intybus* on fertilisation and embryo development, suggesting that these forages and their PSCs have some potential for improving reproduction. It would clearly be worthwhile identifying the specific compounds in those extracts that are responsible for the effects revealed at the *in vitro* level.

In conclusion, treatment of ovine oocytes, during *in vitro* maturation, with methanolic extracts of selected novel and common forage plants did not disrupt subsequent fertilisation and embryo development to hatched blastocyst stage. In fact, there was some evidence that *B. pelecinus* and *C. intybus* could improve the outcomes, although

these effects need to be confirmed. These studies are helping us fulfil our obligation for a ‘duty of care’ in the development of novel forages (Revell and Revell (2007).

Chapter 5

Components of *Biserrula pelecinus* that affect *in vitro* maturation of ovine oocytes, and subsequent fertilisation and embryo development

In preparation for submission to Animal (2018)

Abstract

As a 'duty of care' for grazing livestock, new pasture legumes need to be tested for their effects on reproduction, so we have been studying *Biserrula pelecinus*. Contrary to expectations, we found that the presence of a crude extract of *B. pelecinus* during *in vitro* oocyte maturation improved the development of sheep embryos (Chapter 4). Here, we take this work to the next stage by attempting to identify plant secondary metabolites produced by *B. pelecinus* that could be responsible for the effect. For Experiment 1, *B. pelecinus* was extracted with 1:1 (v/v) MeOH/$CHCl_3$ and the extract was fractionated using rapid silica filtration (RSF) with solvents of varying polarities. Dried fractions were added, at final concentrations of 0, 100 or 200 µg mL^{-1}, to the medium used for *in vitro* maturation of cumulus-oocyte complexes (COCs) derived from abattoir-sourced adult ewe ovaries. The matured oocytes were then taken through *in vitro* fertilisation and embryo culture so we could quantify cleavage rate, blastocyst rate, hatching rate, blastocyst efficiency, and total blastocyst cell number (TCN). Of seven *B. pelecinus* fractions tested, one fraction reducing cleavage rate by 9.8% and three fractions reduced blastocyst development by 14-22%. However, one of the active fractions (designated BP6) at 200 µg mL^{-1} increased cleavage rate ($P<0.05$). In Experiment 2, fraction BP6 was further fractionated by semi-preparative HPLC and loliolide was clearly the most abundant compound. Pure loliolide at 25 µg mL^{-1} increased hatching rate ($P<0.05$), consistent with the effects observed with fraction BP6. All oocytes supplemented with *B. pelecinus* fractions and loliolide reached the final stage of embryo development-blastocyst hatching with no effect on TCN. We conclude that the ability of *B. pelecinus*, present during *in vitro* oocyte maturation, to improve fertilisation and embryo development, is at least partly due to the presence of loliolide. We now need to test the concept *in vivo*.

Introduction

Dietary factors that affect reproductive performance is an important area of investigation for industries based on grazing sheep, with a focus on the use of timely supplements or leguminous forages that increase the supply energy and protein to ewes and improve reproductive outputs (Martin *et al*., 2004; Viñoles *et al*., 2009). To expand on the options for industry, there is a constant search for new leguminous species. An example is *B. pelecinus*, a native to the Mediterranean areas of Europe and Africa, and the highland areas in Kenya, Ethiopia and Eritrea, where it grows in infertile and sandy acidic soils (Hackney *et al*, 2013). The excellent agronomic qualities of *B. pelecinus* have led to successful adaptation to Australian conditions where it is now an increasingly important feed resource in the sheep industry (Loi *et al*., 2014; Loi *et al*., 2010).

However, new forage plants bring risks due to inadequate knowledge of the plant secondary compounds (PSCs) such as tannins, saponins, terpenoids, alkaloids and flavonoids. Plants produce PSCs as a defence against stressors such as poor soil fertility, lack of water, high temperatures, grazing pressure and microbial and insect attack (Bickell *et al.,* 2010). The risks of PSCs are exemplified by 'clover disease' in which isoflavones, abundant in some cultivars of subterranean clover, cause catastrophic reproductive problems in sheep (Adams, 1983,1995). This experience has led to considerable interest in the potential effects, both positive and negative, of PSCs on reproductive performance in ruminants (Durmic and Blache, 2012), and the concept of 'duty of care' in programs of forage development (Revell and Revell, 2007).

For the PSCs in *B. pelecinus*, recent studies with sheep have focused on aversion behaviour (Thomas *et al*., 2014), photosensitization (Kessel *et al*., 2015), and the potential for reducing greenhouse gas emissions (Banik *et al*., 2016). Aversion seems to be a problem when sheep graze pastures dominate by *B. pelecinus*, not when it is consumed along with another forage and the suspected aversive PSC seems to be diluted or neutralized (Thomas *et al*., 2014). There have also been suggestions that the aversion behaviour is linked to photosensitization observed in sheep grazing *B. pelecinus* (Revell and Revell, 2007; Thomas *et al*., 2014). Photosensitization leads to severe dermatitis and conjunctivitis caused by heightened reactivity to exposure to sunlight following ingestion of compounds that are UV- or light-reactive (Rowe et al., 1989). For *B. pelecinus*, Kessel *et al*. (2015) reported that the photosensitization effect was caused by a primary photosensitizing agent (Type I PS) that was either ingested or

absorbed cutaneously. Attempts to identify the responsible agent(s) in *B. pelecinus* failed to detect any known primary photosensitizing compounds, such as furanocoumarins or dianthrones (Kessell *et al*., 2015). However, the major PSCs in *B. pelecinus* were found to be flavonoid glycosides (Swinny *et al*., 2015).

To date, there have been no studies on the effect of *B. pelecinus* on the reproductive health of sheep, including effects on the processes of fertilisation, embryo survival and fetal development that are known to be affected by PSCs in ruminants (McEvoy *et al.* 2001; Amir *et al*., 2018a). Measurement of these processes in grazing animals is difficult, so in vitro techniques are preferred for screening new forages in oocytes and embryos (Leite *et al.* 2004; Wang *et al.* 2005; Wang *et al.* 2007; Spinaci *et al.* 2008; Rajabi-Toustani *et al.* 2013). We have previously used this approach to study the effects of the isoflavones which are responsible for clover disease (Chapter 3; Amir *et al.,* 2018a). In a follow-up study, we also tested extracts of novel forages that are being introduced into the Australian sheep industry, including *B. pelecinus*, and found that the presence of methanolic extracts during oocyte maturation increased fertilisation rate and blastocyst development (Amir *et al*., 2018b).

Here we further investigate this phenomenon using a fraction of *B. pelecinus* extract in the medium used for *in vitro* oocyte maturation. The main compound in this fraction was identified as the monoterpene lactone, loliolide, which we then tested in pure form. To test whether the fraction and loliolide would affect reproductive outcomes, we measured cleavage rate, blastocyst rate, blastocyst efficiency and blastocyst total cell number (TCN).

Material and methods

The experimental protocol was approved by the Animal Ethics Committee of the University of Western Australia (RA/3/500/70 and RA/3/500/71) according to the recommendations of the Australian National Health & Medical Research Council. Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO, USA).

Collection of ovaries and oocytes

Ovaries from adult ewes were collected from an abattoir and transported to the laboratory in warm (33°C) phosphate-buffered saline (PBS). Cumulus-oocyte complexes (COCs) were aspirated from follicles ≥ 2 mm diameter using an 18 g needle

and a vacuum pump (Cook Australia, Queensland, Australia) with a pressure equivalent to 25 mm Hg. The COCs were collected into 2 mL aspiration medium that comprised Hepes-buffered TCM199 supplemented with 2% (v/v) ovine oestrus serum (SS), 100 IU mL^{-1} heparin (Pharmacia and Upjohn; Bentley, Western Australia, Australia), 100 µg mL^{-1} streptomycin sulphate (CSL Limited; Parkville, Victoria, Australia), and 100 IU mL^{-1} penicillin G (CSL Limited). With the aid of a stereo-microscope (40 x magnification) (Olympus, Tokyo, Japan), unexpanded COCs were recovered, and oocytes that were atretic and showed signs of an expanded cumulus were discarded.

In vitro *maturation (IVM)*

We used procedures similar to those outlined by Walker *et al*. (1996) and Kelly *et al.* (2007). In brief: COCs were rinsed in IVM maturation medium: sodium bicarbonate-buffered TCM199 supplemented with 20% (v/v) SS, 5 µg mL^{-1} FSH (Folltropin, Bioniche, Inc), 0.1 IU mL^{-1} hCG (Chorulon, Intervet, USA) and 1 µg mL^{-1} oestradiol-17β. COCs were matured in four-well culture dishes (Nunc Inc., Naperville, IL) containing 500 µL maturation medium covered with 300 µL mineral oil for approximately 24 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. The COCs were allocated equally among treatments.

Sperm preparation and in vitro *fertilisation (IVF)*

After maturation, COCs were gently stripped of excess cumulus cells in 400 IU mL^{-1} hyaluronidase using a fine-bore pipette. They were then washed three times in IVF medium: synthetic oviduct fluid (SOF) supplemented with 2% (v/v) SS. Up to 25 COCs were placed into 4-well culture dishes containing 450 µL IVF medium overlaid with 300 µL mineral oil. Motile sperm were obtained using the 'swim-up' procedure in which 200 µL of frozen-thawed semen, pooled from two rams of proven fertility, were layered under 1 mL of IVF medium in a 14 mL tube (Falcon; Becton Dickinson, Melbourne, Victoria). Approximately 0.5 x 10^6 sperm were placed in each well and co-incubated with the COCs for 24 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air.

In vitro *culture (IVC)*

After approximately 24 h, remnant cumulus cells were removed by gentle pipetting with a fine bore pipette and the presumptive zygotes were washed three times in IVC medium: SOF containing 8 mg mL^{-1} BSA (Fraction V; Invitrogen Corp., Auckland, New Zealand) and amino acids at concentrations found in oviduct fluid of sheep

(Walker *et al*. 1996; Kelly *et al.* 2007). Presumptive zygotes were cultured in 600 µL IVC medium under 300 µL mineral oil in a humidified atmosphere of 5% CO_2:5% O_2:90% N_2 at 38.8°C. Oocytes that failed to divide ('single cell') were removed 24 h allowing the cleavage (fertilisation) rate to be determined. The remaining embryos ranged from 2 to 8 cells. We did not record the exact number for each stage, only weather they had cleaved to give a fertilisation rate.

Embryo assessment

Embryo development was assessed on Day 7 (Day 0 = day of IVF). Blastocysts, expanded blastocysts, hatching blastocysts and totally hatched blastocysts were collected, and stained. Blastocyst rate was defined as the number of blastocysts plus expanded blastocysts expressed as a percentage of the number of cleaved oocytes; hatching rate as defined as the number of hatching plus totally hatched blastocysts as a percentage of the total number of blastocysts; blastocyst efficiency was defined as the number blastocysts produced expressed as a percentage of the original number of COCs. Embryos were mounted on a microscope slide in a drop of glycerol containing Hoechst 33342 (1 mg mL^{-1}) and covered with a coverslip. The total cell count was recorded using a fluorescent microscope (Olympus Optical Co Ltd, Tokyo, Japan).

Experiment 1

Extraction and fractionation of *B. pelecinus*

For Experiment 1, samples of *B. pelecinus* in the vegetative stage collected from *UWA Farm Ridgefield*, Western Australia (32°29'S 116°58'E). The plant material (15.1 g dry weight) was extracted by stirring overnight with 1:1 (v/v) MeOH/$CHCl_3$ (2 × 150 mL). The solvent phase was filtered (Whatman No.1, 32.0 cm) and evaporated to dryness to provide 1.82 g crude extract. High performance liquid chromatography (HPLC) of the crude extract of revealed a complex mixture of compounds (Fig. 5.1), indicating the need for further purification steps.

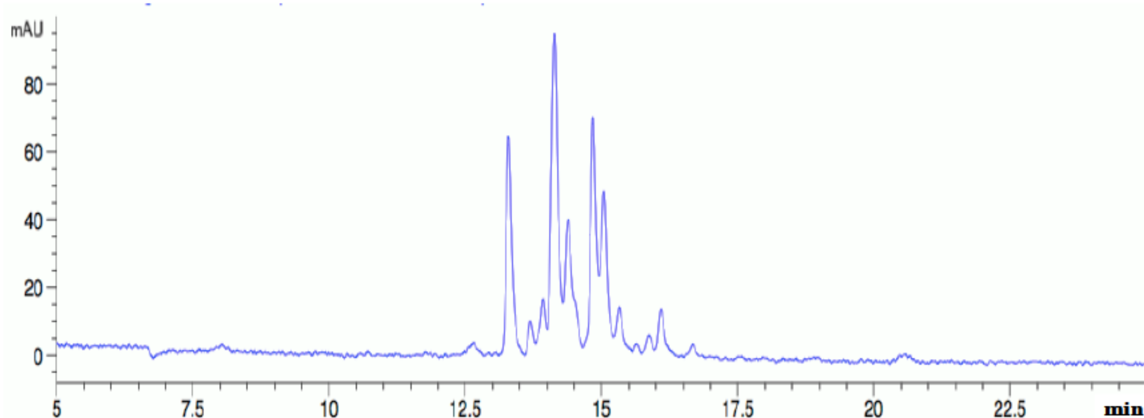

Figure 5.1: HPLC chromatogram of a crude methanolic extract of *B. pelecinus*

To separate the crude extract into fractions, it was dissolved in a minimal amount of MeOH/hexanes and added to a rapid silica filtration (RSF) column (4.5 × 12 cm) containing silica gel (10 g) equilibrated with hexanes. The column was eluted with hexanes (200 mL) followed by solvent mixtures containing increasing concentrations of ethyl acetate (EtOAc) to 100% EtOAc, followed by increasing amounts of methanol (MeOH) in EtOAc to 100% MeOH. Ten fractions were produced (Table 5.1). Each fraction was analysed by HPLC and similar fractions (BP.1 and BP.2; BP.6 and BP.7; BP.9 and BP.10) to reduce the total to 7 fractions for testing for effects on oocyte competence (Table 5.1).

Table 5.1. Yields of fractions from by rapid silica filtration (RSF) of a crude methanolic extract of 15.1 g (dry weight) of *B. pelecinus*

Original Fraction	Elution Solvent	Yield (mg)	Final Fraction
BP.1	100% hexanes	16.5	BP1
BP.2	20% EtOAc/hexanes	35.5	
BP.3	40% EtOAc/hexanes	9.6	BP3
BP.4	60% EtOAc/hexanes	216.1	BP4
BP.5	80% EtOAc/hexanes	78.5	BP5
BP.6	100% EtOAc	8.0	BP6
BP.7	20% MeOH/EtOAc	9.0	
BP.8	50% MeOH/EtOAc	55.9	BP8
BP.9	80% MeOH/EtOAc	562.5	BP9
BP.10	100% MeOH	416.4	

A sub-sample of each final RSF fraction (ca. 10%) was tested in the *in vitro* oocyte maturation system, with a 7 x 3 factorial design (7 fractions at 3 concentrations: 0, 100 and 200 µg mL^{-1}). DMSO was used to dilute the fractions and was therefore used as the control treatment (0 µg mL^{-1}). The most active fractions were to be selected for Experiment 2.

Experiment 2

Fraction BP6 from the RSF separation (16.1 mg) was subjected to semi-preparative HPLC using an Apollo C_{18} column (250 mm × 10.0 mm i.d., 5 µm, Grace-Davison). The sample was dissolved in a minimal amount of methanol and 500 µL was injected. The column was eluted with a gradient mobile phase consisting, beginning with 1% acetonitrile (MeCN) in water (+ 0.1% trifluoroacetic acid) and increasing to 100 % MeCN over 35 min at a flow rate of 4 mL/min. Fractions were collected every 1 min. Allowing for solvent delay and column cleaning, 20 fractions were collected and, based on profile similarity, they were combined to give a total of 6 fractions for further analysis.

One fraction (BP6) was found to be relatively pure with HPLC and contained two compounds, one of which accounted for >80% of the total mass and provided a colourless powder that high-resolution mass spectrometry (HRMS) indicated was a protonated molecular ion $[M+H]^+$ at *m/z* 1967.1178, corresponding with a molecular formula of $C_{11}H_{16}O_3$. Comparison of HRMS data (Table 5.2, Fig. 5.2) and NMR spectroscopic properties (Lee *et al.*, 2001) suggested that the isolated compound was the monoterpene, (-)-loliolide (Fig. 5.3). This was confirmed by comparison with a commercial sample.

Having identified loliolide in the active fraction of extract BP6 of *B. pelecinus*, its effects on oocyte maturation were assessed by adding the pure compound to the IVM medium at five concentrations (0, 2.5, 5, 10 and 25 µg mL^{-1}). We used DMSO (1 in 200) to dilute the loliolide so it was used as the control treatment (0 µg mL^{-1}). A comparison between control treatments, with and without DMSO, was also included in the study. The experiment was replicated four times.

Table 5.2. ^{1}H, ^{13}C chemical shifts compared for (-)-loliolide isolated from *B. pelecinus* with literature values (Lee *et al*., 2001).

	Present work (CD_3OD)		Literature values (CD_3OD)	
C–	δC/ppm	δH/ppm	δC/ppm	δH/ppm
1	37.1	–	37.2	–
2	47.9	1.52, dd, *J*= 3.6, 14.4 Hz. 1.98, dt, *J*= 2.6, 14.6 Hz.	48.0	1.52, dd, *J*= 3.6, 14.4 Hz. 1.98, dt, *J*= 2.5, 13.7 Hz.
3	67.2	4.21, q	67.3	4.21, m
4	46.4	1.73, d, , *J*= 2.41, dt, *J*=2.5, 13.4 Hz	46.5	1.72, overlapped, 2.41, dt, *J*=2.5, 13.7 Hz
5	88.9	–	88.9	–
6	185.2	–	185.7	–
7	113.3	5.74, s ,1H	113.4	5.74, s ,1H
8	172.6	–	174.5	–
9	27.4	1.76, s, 3H	27.5	1.75, s, 3H
10	31.0	1.27, s, 3H	31.0	1.26, s, 3H
11	26.9	1.46, s, 3H	27.0	1.46, s, 3H

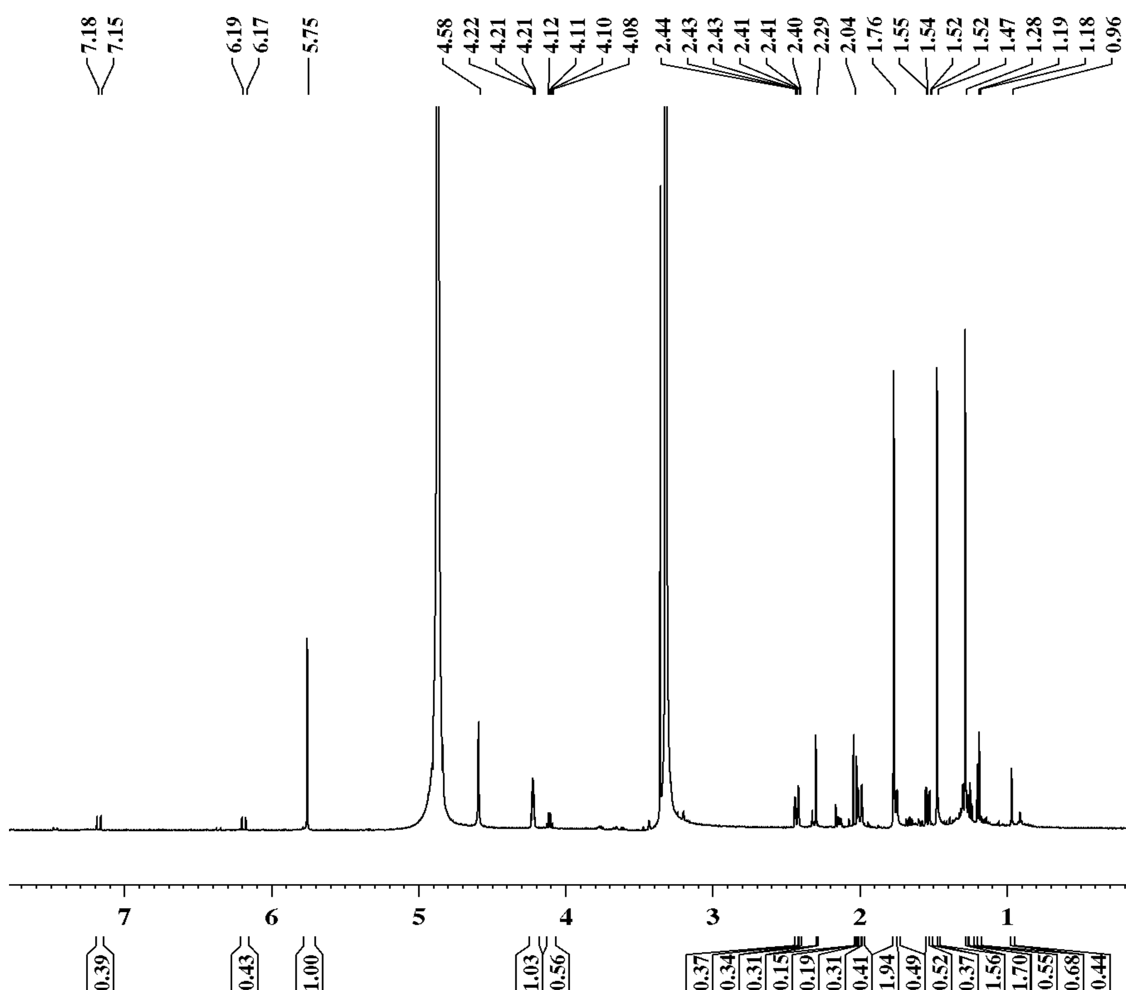

Figure 5.2: ^{1}HNMR spectrum (MeOD) of -(-)Loliolide.

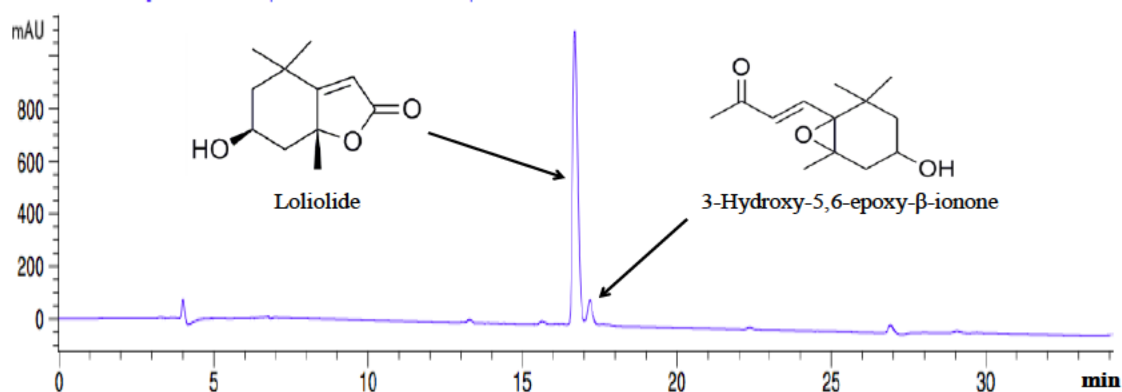

Figure 5.3. HPLC chromatogram of fraction BP6, showing the presence of only 2 major compounds. Loliolide accounted for 80% of the mass.

For the minor compound in the fraction BP6, also obtained as a colourless powder, HRMS returned a protonated molecular ion $[M+H]^+$ of *m/z* 225.1491 corresponding with a molecular formula of $C_{13}H_{20}O_3$. Comparison of the NMR spectroscopic data and EI mass spectrum with literature values (Kim *et al*., 2008) confirmed the compound to be 3-hydroxy-5,6-epoxy-β-ionone (Table 5.3).

Table 5.3. ^{1}H chemical shift for 3-Hydroxy-5,6-epoxy-β-ionone isolated in this study compared with literature values (Kim *et al*., 2008).

	Present work (CD_3OD)	**Literature values (CD_3OD)**
C–	δH/ppm	δH/ppm
1	–	–
2	No	1.60, dd, *J*= 4.3, 12.2 Hz.
		1.28, dd, *J*= 10.3, 12.2 Hz.
3	No	3.77, m
4	No	2.32,dd, *J*=5.2, 9.3 Hz
	1.65, dd, *J*=9.18, 14.29 Hz	1.66, dd, *J*=9.3, 14.7 Hz
5	–	–
6	–	–
7	7.18, d, *J*=15.7 Hz	7.19, d, *J*=15.1 Hz
8	6.19, d, *J*=15.7 Hz	6.21, d, *J*=15.1 Hz
9	–	–
10	2.29, s, 3H	2.30, s, 3H
11	1.18, s, 3H	1.19, s, 3H
12	0.95, s, 3H	0.97, s, 3H
13	1.19, s, 3H	1.20, s, 3H

Statistical Analysis

The effects on *in vitro* maturation and developmental competence of oocytes were analysed as factorial designs: 7 *B. pelecinus* fractions x 3 concentrations x 3 repeats (Experiment 1); 5 loliolide concentrations x 4 repeats (Experiment 2). Data for embryonic development (cleavage rate, blastocyst rate, hatching rate, blastocyst efficiency) were analysed using the procedure CATMOD in SAS (Statistical Analysis System, Cary, NC, USA). Total cell numbers were analysed using the GLM procedure in SAS. Concentration (fraction or loliolide) and replicate were included in the model. Probability values < 0.05 were considered significant.

Results

Experiment 1

There were no significant differences between the two controls (plus or minus DMSO) for any of the measures of oocyte and embryo development (Table 5.4). For fractions BP1, BP4 BP5, and BP8, there were no consistent effects on any measure of embryo development.

Compared with the DMSO control, cleavage rate was almost 10% lower ($P<0.05$) for BP8 at 100 ug mL^{-1} but not at 200 µg mL^{-1} (Table 5.4). No other fraction affected cleavage rate.

At 100 µg mL^{-1}, blastocyst rate was about 22% lower for BP6, and bout 18% lower for BP9, compared with the DMSO control. At 200 µg mL^{-1}, blastocyst rate was about 14% lower for BP3 and 16% lower for BP9 (Table 5.4). Interestingly, for BP6, the effect was significant at 100 but not 200 µg mL^{-1}, and the lower blastocyst rate at 100 µg $mL^{-1}$was reflected in a lower blastocyst efficiency at 100 µg mL^{-1}. With BP6 at 200 µg mL^{-1}, the blastocyst rate was not reduced so more blastocysts were produced leading to a higher hatching rate, compared to the control values, but and the blastocyst efficiency was reduced (Table 5.4).

Experiment 2

There were no significant differences between the two controls (plus or minus DMSO) for any of the measures of oocyte and embryo performance, including total cell number (TCN) (Table 5.5).

There were no effects of loliolide on oocyte and embryo development over the range of loliolide concentrations tested (Table 5.5), with one exception: hatching rate was higher at 25 μg mL^{-1} than at 0, 5 or 10 μg mL^{-1} but the difference was only significant for the comparison of 10 and 25 μg mL^{-1} (Table 5.5).

For TCN, there were no significant interactions between loliolide concentration and stage of embryo development. As expected, TCN increased at the embryo developed. Least squares means (±SEM) for TCN were 66.1 ± 3.2 at the blastocyst stage, 109.1 ± 2.4 at the expanded blastocyst stage, and 168.9 ± 3.3 at the hatching stage

.

Table 5.4. Rates of oocyte cleavage and embryo development *in vitro* with oocytes incubated in culture media containing 0, 100 or 200 µg mL^{-1} of various fractions of *Biserrula pelecinus* that had been prepared by rapid silica filtration (RSF).

Treatment	**Dose (µg mL^{-1})**	**Oocytes examined**	**Cleavage rate (%) (cleaved/oocyte)**	**Blastocyst rate (%) (blastocyst/cleaved)**	**Hatching rate (%) (hatching/blastocyst)**	**Blastocyst efficiency (blastocyst/oocytes)**
Control – DMSO	0	120	90.0 (108) [a]	64.8 (70) [a]	24.3 (17) [a]	58.3 (70) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP1	100	132	81.1 (107) [a]	60.7 (65) [a]	18.5 (12) [a]	49.2 (65) [a]
	200	133	83.5 (111) [a]	64.0 (71) [a]	28.2 (20) [a]	53.4 (71) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP3	100	137	79.6 (109) [a]	65.1 (71) [ab]	19.7 (14) [a]	51.8 (71) [a]
	200	133	83.5 (111) [a]	56.8 (63) [b]	25.4 (16) [a]	47.4 (63) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP4	100	134	82.1 (110) [a]	65.5 (72) [a]	23.6 (17) [a]	53.7 (72) [a]
	200	131	73.3 (96) [a]	71.9 (69) [a]	26.1 (18) [a]	52.7 (69) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP5	100	134	78.4 (105) [a]	70.5 (74) [a]	28.4 (21) [a]	55.2 (74) [a]
	200	131	83.2 (109) [a]	67.0 (73) [a]	19.2 (14) [a]	55.7 (73) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP6	100	132	79.5 (105) [a]	48.6 (51) [b]	17.6 (9) [ab]	38.6 (51) [b]
	200	128	79.7 (102) [a]	61.8 (63) [a]	36.5 (23) [b]	49.2 (63) [b]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP8	100	130	76.2 (99) [b]	64.6 (64) [a]	15.6 (10) [a]	49.2 (64) [a]
	200	137	86.9 (119) [a]	60.5 (72) [a]	19.4 (14) [a]	52.6 (72) [a]
Control + DMSO	0	127	85.8 (109) [a]	70.6 (77) [a]	15.6 (12) [a]	60.6 (77) [a]
BP9	100	129	76.7 (99) [a]	52.5 (52) [b]	26.9 (14) [a]	40.3 (52) [a]
	200	130	76.9 (100) [a]	54.0 (54) [b]	24.1 (13) [a]	41.5 (54) [a]

Numbers given in parenthesis are those used to calculate percentage values.

Values, comparing controls with plus or minus DMSO, with different letters differ significantly ($P<0.05$).

Values, within fraction, with different lower case letters vary significantly ($P<0.05$).

Table 5.5. Rates of oocyte cleavage and embryo development *in vitro* with oocytes incubated in culture media containing 0 (Control ± DMSO), 2.5, 5, 10 or 25 µg mL^{-1} of loliolide.

Treatment (µg mL^{-1})	Oocytes tested	Cleavage rate (% of oocytes cultured)	Blastocyst rate (% blastocysts per cleaved oocyte)	Hatching rate (% blastocysts formed)	Blastocyst efficiency (% blastocysts per total oocytes cultured)	Total cell number of blastocysts (LSmeans ± SEM)
Control						
–DMSO	142	82.4 (117) ^{a}	54.7 (64) ^{a}	32.8 (21) ^{a}	45.1 (64) ^{a}	117.7 ± 4.2 ^{a}
+DMSO	161	76.4 (123) ^{a}	56.1 (69) ^{a}	30.4 (21) ^{a}	42.9 (69) ^{a}	109.8 ± 3.7 ^{a}
Loliolide						
2.5	146	81.5 (119) ^{a}	62.2 (74) ^{a}	21.6 (16) ^{ab}	50.7 (74) ^{a}	115.8 ± 4.1 ^{a}
5.0	151	83.4 (126) ^{a}	60.3 (76) ^{a}	19.7 (15) ^{ab}	50.3 (76) ^{a}	114.2 ± 4.4 ^{a}
10.0	132	82.6 (109) ^{a}	63.3 (69) ^{a}	18.8 (13) ^{a}	52.3 (69) ^{a}	118.3 ± 4.4 ^{a}
25.0	142	78.9 (112) ^{a}	56.3 (63) ^{a}	33.3 (21) ^{b}	44.4 (63) ^{a}	112.4 ± 4.0 ^{a}

Numbers given in parentheses are those used to calculate percentage values.

Values, comparing controls with plus or minus DMSO, with different letters vary significantly ($P<0.05$).

Values, comparing loliolide concentrations, with different letters vary significantly ($P<0.05$).

Discussion

The presence during oocyte maturation of the active fraction of *B. pelecinus* extract (BP6), or the loliolide identified in this fraction, produced a similar outcome – an increase in hatching rate. Although extract BP6 caused reductions in other aspects of embryo development, the improvement in hatching rate agrees with the previous observation with a crude methanolic extract, where we observed increases in cleavage rate, blastocyst rate and blastocyst efficiency (Chapter 4; Amir *et al*., 2018b). Stimulatory effects to the crude extract were not predicted *a priori*, but we can now be confident that the effects are repeatable. Moreover, these sorts of effects are novel for any forage species, as well as being the first demonstration that extractable compounds from forage can affect ovine oocyte and embryo development *in vitro*. Importantly, having identified loliolide as the major compound in bioactive fraction BP6, and shown that it has the same effect on hatching rate as BP6 and the crude extract when present during oocyte maturation, we can be quite certain that this PSC explains the improved outcomes in embryo development. Further research with loliolide, and with *B. pelecinus*, is required to identify the molecular mechanisms of action within the oocyte, and to test whether the forage can be used to improve sheep fertility under field conditions.

In addition to the positive effects of BP6 and loliolide, inhibitory effects of other *B. pelecinus* fractions were also observed in Experiment 1, including a 10% reduction in cleavage rate for fraction BP8 at 100 $\mu g\ ml^{-1}$, and 14-22% reductions in blastocyst rate for fraction BP3 (200 $\mu g\ ml^{-1}$), BP6 (100 $\mu g\ ml^{-1}$), BP9 (100 and 200 $\mu g\ ml^{-1}$). Despite these reductions, blastocyst development continued through to hatching. Nevertheless, with feeding *B. pelecinus* under field conditions, the final reproductive outcomes would likely reflect a combination of both stimulatory and inhibitory effects.

In interpreting these observations, it is important to remember the treatments were only present during the maturation of the oocytes, so effects on fertilisation, blastocyst development and TCN are consequences of the actions of the fractions or loliolide on the oocyte before fertilisation. Oocyte maturation, *in vitro* and *in vivo*, involves cellular processes that control meiosis, germinal vesicle breakdown, and the extrusion of the first polar body, any of which could be influenced by any of the PSCs in *B. pelecinus*, with consequences for oocyte quality that flow-on to the pre-implantation embryo or hatching blastocyst.

The polarity of the solvent used for the extraction of plant material determines the types of molecules that are extracted. In Chapter 4 (Amir *et al.,* 2018b), a relatively polar solvent, methanol, was used to extract samples of *B. pelecinus* and provide treatments for the *in vitro* maturation medium. In the present study, this crude methanolic extract of *B. pelecinus* was fractionated by increasing the solvent polarity, beginning with 100% hexanes and then increasing the concentration of ethyl acetate (EtOAc) to 100% EtOAc, followed by increasing amounts of MeOH in EtOAc to 100% MeOH. The final outcome was seven fractions for *in vitro* screening. Loliolide was found in the 100% EtOAc (original BP6) and 20:80 MeOH: EtOAc fractions (original BP7), as found by Malgorzata *et al*. (2015). Obviously, different solvents will isolate different compounds so the process we have used is not exhaustive and there may be other, as yet undiscovered, bioactive compounds in *B. pelecinus*.

The work in this chapter adds to that in Chapters 3 and 4, to re-inforce the general conclusion that *in vitro* reproductive technology is a valuable tool for detecting plant secondary compounds that might affect reproduction in animals grazing novel forages. These technologies were originally developed to overcome infertility problems in humans (Howard WJ Jr.; 1986; Wood and Trounson, 2000) and were later extended to studies of reproductive toxicology in animals (Wang *et al*., 2005, 2007; Malekinejad *et al*., 2007; Spinaci *et al*., 2008; Rajabi-Toustani *et al*., 2013; Galeati *et al*., 2014; Amir *et al*., 2018a,b). Clearly, these technologies offer real opportunities for gaining an understanding of the risks for reproduction, and perhaps for exploring mechanisms of action of specific compounds (Santos *et al*., 2014). In addition, the present study has detected a positive effect of a PSC on oocyte maturation, with beneficial consequences for embryo development, opening up the possibility that forages can be selected for their ability to improve reproduction.

In conclusion, treatment of ovine oocytes, during *in vitro* maturation, with fractions of *B. pelecinus* led to either an increase or a decrease in subsequent fertilisation and embryo development. Most exciting was the identification of loliolide in fraction BP6 that appears to modify the oocyte to allow an increase in blastocyst development. We now need to test the concept *in vivo*.

Chapter 6
General Discussion

In this thesis, I expected to be able to detect PSCs that have the potential to disrupt reproduction in sheep by using *in vitro* reproductive technologies, based on the assumption that they will disrupt the maturation of oocytes, and therefore disrupt fertilisation and subsequent embryo development up to blastocyst stage. The results in Chapter 3 clearly support this general hypothesis, whereas the results in Chapters 4 and 5 suggest some PSCs have no effect while others might even improve the outcome:

1) Isoflavones, a known group of PSCs that cause major reproductive problems in grazing sheep, have the potential to interfere with oocyte maturation, leading to reductions in fertilisation and subsequent embryo development. For these effects to be observed, the isoflavones generally needed to be present at 25 $\mu g\ mL^{-1}$ during *in vitro* oocyte maturation. This concentration appears to be high but is theoretically within the range that might be expected when sheep graze pastures that are dominated by subterranean clover. For the grazing sheep industry, these observations lead to two conclusions: a) we have a greater understanding of how isoflavones can interfere with reproduction; b) *in vitro* reproductive technologies have great potential as a tool for ‘problem detection’ by screening new forage plants before they are introduced into the sheep industry where they might be a risk to animal health and productivity;
2) Our observations suggest that these plants will not disrupt sheep oocyte and embryo development under field conditions; on the contrary, extracts of *B. pelecinus* appeared to improve fertilisation and embryo development. That said, we need to keep in mind that the actions of PSCs will be concentration-dependent and that PSCs structures (and perhaps activities) can be altered by the rumen;
3) In this thesis, the effects of PSCs, fractions and extracts (Chapters 3, 4 and 5) were often only evident at the highest concentration tested. For example, the fractionation of *B. pelecinus* allowed us to be quite certain that loliolide is the PSC that, when present during oocyte maturation, seems to improve fertilisation and embryo development. However, these beneficial effects were observed only in one fraction of *B. pelecinus* (BP6), and only at the highest concentration of loliolide tested (25 $\mu g\ mL^{-1}$). Further research is thus required, perhaps with a

wider range of concentrations, and aiming to describe the mechanisms of action, before we can be certain that sheep fertility can be improved."

These outcomes provide clear evidence that *in vitro* reproductive technologies can be used to detect PSCs that affect oocyte maturation and therefore might disrupt reproduction in grazing sheep. This conclusion supports other studies in which Assisted Reproductive Techniques (ART), such as IVMFC, have been used to test for the effects of exposure to toxins, mainly of dietary and environmental origin, on fertility in women (Santos *et al*., 2014). In this study, through IVMFC, the effects were observed on only a few of the experimental variables (cleavage rate, blastocyst rate, hatching rate, blastocyst efficiency and blastocyst total cell number), with no observation done from 2-cell embryo to morula. Blastocyst development rates and total cell number are recognized as good indicators for determining oocyte developmental capability, showing that the oocyte can successfully undergo the change from maternal to genomic activation. Theoretically, the inner cell mass (ICM) will develop into the main part of the fetus and ICM cell count is now widely used for blastocyst selection and for predicating clinical pregnancy. Compounds that affect oocytes might, through epigenetic processes, also lead to deleterious effects on offspring health that are expressed later in life, a concern for REACH (Registration, Evaluation and Authorization of Chemicals) legislation in Europe that, since 2007/2008, has controlled marketing of chemicals (Lorenzetti *et al*., 2011). Indeed, the entire field of 'developmental origins of health and disease' (DOHAD) is based on this premise (review: Sinclair et al. 2016).

The studies in this thesis also extend the concept of *in vitro* testing to sheep oocytes sourced from abattoir material. This is an important issue because most toxicology studies are done on laboratory rodents, a source of debate for many years (Bremer *et al*., 2007) that led to a directive to decrease the number of animal-based toxicity tests as part of protection of animals for scientific purposes (European Union, 2010). Abattoir-sourced bovine and porcine oocytes have been seen as a solution (Santos *et al*., 2014) and now we can add sheep oocytes to the list.

In addition to studies focussing on toxicology, IVMFC techniques have also been used to test for beneficial effects of plant extracts and PSCs on pronucleus formation, fertilisation and embryo development in animal models, mostly in relation to human health (Leite *et al*., 2004, Wang et al., 2007; Spinaci *et al*., 2008; Rajabi-Toustani *et al*., 2013). As many as 25% of subfertility cases in humans have an unknown etiology and

remain undiagnosed, leading to affected couples attempting to achieve parenthood through IVMFC. Reproductive toxicology tests *in vitro* have been found to offer explanations that are beneficial in human reproductive health, as well as animal health, because of the discovery of toxic effects of drugs, food and environmental pollutants (Santos *et al*., 2014). It is important to measure the contribution of effects on oocyte maturation and early embryonic loss, but other *in vitro* tests are also needed, such those based on embryonic stem cells, cell lines, and sperm. Hormone studies will also help to complete the picture. For the time being, there is no specific consensus on the order for the full range of *in vitro* tests. However, independently of the order of tests, establishing the right IVMFC protocol is an early priority for *in vitro* studies of reproductive toxicology. Thereafter, toxicology effects on oocyte maturation stage, fertilization success and subsequent *in vitro* embryo development can follow. *In vitro* testing of sperm is clearly important and relatively rapid, with toxic effects being immediately evident with standard semen analysis (sperm number; motility; morphology; volume and consistency of ejaculate). Clearly, any *in vitro* study, whether it based on sperm, oocytes or embryos, will provide useful information about the risks of toxic effects of, for example, a novel forage.

We now know that isoflavones (genistein, biochanin A, formononetin) can interfere with oocyte maturation in a way that reduces fertilisation and embryo development (Chapter 3). The presence of biochanin A during oocyte maturation had consequences for total cell number (TCN of hatched blastocysts, suggesting a mechanism specifically associated with blastocyst development. Our observations for formononentin and biochanin A are novel in sheep, while those for genistein support *in vitro* maturation studies using bovine and porcine oocytes (Van Cauwenberge and Alexandre 2000, Santos *et al*. 2014).

Our observations of the effects of isoflavones on oocyte maturation help explain embryonic loss in sheep with clover disease (Adams, 1995), but offer little insight into the mechanisms involved. Isoflavones are oestrogenic compounds that can exert cellular effects similar to those exerted by endogenously-produced oestrogens, such as oestradiol (Adams *et al*., 1988). In addressing this topic, it is important to recognize that, in all the studies described in this thesis, the test substances were only present during oocyte maturation, so any effects on fertilisation or embryo development must be the consequence on effects on the unfertilized oocyte. As sites of action within the oocyte, possibilities include cellular activities related to the process of meiosis, germinal

vesicle breakdown and the production of MII oocytes. We can turn to similar *in vitro* studies in other species, particularly those suggesting that isoflavones disrupt hormone synthesis in the cumulus, the granulosa cells that surround the oocyte (Piasecka-Strader *et al.* 2014; Tiemann *et al*. 2007, Guillette and Moore 2006). Furthermore, mitochondria are thought to be reservoirs for oestrogens, and thus isoflavones, and high concentrations of oestradiol impair mitochondrial function in spermatozoa (Kotwicka *et al*. 2016). Perhaps this concept could be extended to oocyte mitochondria, with consequences for cleavage rate and embryo development. Clearly, in-depth studies are needed if we are to understand how PSCs such as loliolide affect oocyte maturation.

In contrast to the isoflavones, the presence of methanolic extracts of forage plants during *in vitro* maturation did not disrupt fertilisation or embryo development (Chapter 4). Some of the plants tested have a history of disrupting sheep reproduction, so the concentrations of PSCs that cause such problems might be too low within the methanolic plant extract to have detectable effects on the oocyte and developing embryos. As this was the first attempt to study these plant species, we used a general extraction method based on a polar solvent (70% methanol) and studies with other types of solvents might lead to different outcomes. The addition of *B. pelecinus* at 50 µg mL^{-1} increased cleavage rate while 100 µg mL^{-1} increased blastocyst rate and blastocyst efficiency. This first attempt at screening forages to detect disruptive effects on sheep reproduction presents potential good news for industry because, contrary to expectations, fertilisation and embryo development were improved by the extract of *B. pelecinus*, as well as loliolide, the major PSC identified in the extract. There are limitations to this study that constrain interpretation: only a few plant species were tested, and only a single extraction method with a single solvent was used, probably limiting the variety and concentrations of PSCs tested.

Nevertheless, it is clear that *in vitro* studies using crude plant extracts is valuable as an initial step before going into testing fractions or putative active PSCs. The second step, tested for one forage plant only, *B. pelecinus*, allowed us to narrow down the field of metabolites, initially using fractionation with solvents with different polarities, and subsequently further fractionation by semi-preparative HPLC. This process was successful in identifying 2 active compounds in *B. pelecinus*, of which loliolide accounted for more than 80% of the mass. The presence of pure loliolide during *in vitro* oocyte maturation replicated the stimulatory effects of the crude extract of *B. pelecinus* as well as the active fraction of that extract (Chapter 5), although the responses were

only significant at the highest concentration tested (25 μg mL^{-1}). All oocytes supplemented with *B. pelecinus* fractions and loliolide reached the final stage of embryo development-blastocyst hatching with no effect on TCN. *B. pelecinus*, a forage plant that is valued as a source of nutrition for sheep (Loi *et al*., 2010), thus has no known effect on the reproduction but, in fact, might even improve ewe fertility, at least partly due to the presence of loliolide.

Having used *in vitro* studies to detect problems and risks associated with novel forage plants, the next step is to extend the work to *in vivo* studies. It is not clear how these new forages are metabolised by the rumen, so further *in vivo* studies are needed. Furthermore, for interpreting the outcomes of Chapter 3, we need to judge whether the concentrations of isoflavones we used are representative of the concentrations in the bloodstream and reproductive tissues of grazing sheep. This is difficult because so little information is available. Furthermore, the effects of isoflavone, fractions, methanolic extacts and loliolide were often only significant at the highest concentration tested. The only data we could find was by presented by Shutt (1967) who found a plasma concentration up to 7 μg mL^{-1} of isoflavone in sheep consuming oestrogenic clovers. This single value is a third of the concentration that affected oocyte maturation *in vitro*, but provides no indication of the range of concentrations that grazing sheep might experience. Clearly, we need to use modern techniques to determine the concentrations of PSCs in serum, follicular fluid, oocytes, embryos, and seminal fluid, and perhaps test for effects on sperm that parallel those seen in oocytes.

Finally, we need to return to the original premise that led to the studies in this thesis. Revell and Revell (2007) defined four categories for which ‘duty of care’ is needed when commercializing new pasture cultivars, of which the most relevant is “consequences for animal productivity or health”. The value of new forages for animal industry is often assessed by measuring biomass production, nutritive value and palatability. However, there are well-documented cases, such as ‘clover disease’, in which the livestock reproduction and health are severely compromised (Reid, 1981; Adams, 1988, 1995). This historical catastrophe has led to a broadening of the screening processes for new forage species before they are commercialized. Obviously, this is not feasible without specialist skills in animal science and plant chemistry. The work presented in this thesis has demonstrated the potential of reproductive technology in addressing the ‘duty of care’.

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