Recurrent selection in perennial ryegrass (*Lolium perenne* L.) for reduced levels of ergovaline with particular emphasis on the effect of other ergot alkaloid concentrations

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James Clifford Sewell

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Recurrent selection in perennial ryegrass (*Lolium perenne* L.) for reduced levels of ergovaline with particular emphasis on the effect of other ergot alkaloid concentrations

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Perennial ryegass (*Lolium perenne* L.) has coevolved with symbiotic *Epichloë* fungal endophytes (*Neotyphodium lolii*) to form a mutualistic association. Infection with endophyte imparts unique bioactive properties which increase the host plants tolerance to a range of biotic and abiotic stressors. Different endophyte strains produce different classes of secondary metabolites and concentrations in conjunction with their host. Ergovaline is the end product of the ergot alkaloid pathway and is considered one of the primary secondary metabolites responsible in animal toxicosis. It is desirable when breeding for new ryegrass-endophyte associations to either eliminate, or reduce the level of ergovaline expression, while maintaining adequate levels to provide protection against invertebrate attack. Ergovaline levels have previously been shown to be a highly heritable trait for selection. However the effect of recurrent selection for reduced ergovaline on the regulation of other intermediate ergot alkaloids has not been established. The effect of recurrent selection on associated peramine concentrations and mycelial density and whether environment plays a significant role in the synthesis of the intermediate ergot alkaloid pathway is also largely unknown. An experiment was designed to determine whether ergovaline and other intermediates in the ergot alkaloid pathway are under independent or associated control. An initial base population was developed by interpollinating drought surviving genotypes infected with the novel endophyte strain 'AR5'. Recurrent selection primarily for reduced ergovaline was then applied for two cycles. Three populations of the recurrent selection cycles (base, first cycle and second cycle generations) were evaluated in a field experiment at Leigh Creek (south-west Victoria, Australia) over 1 year in 2012-13. A split-split plot design allowed the populations to be subjected to different environment (dryland and irrigation) and nitrogen (plus and minus) treatments. Herbage samples were collected for high performance liquid chromatography (HPLC) to determine ergovaline concentration and liquid

chromatography-electrospray ionization mass spectrometry (LC-MS/MS) to detect peramine and the intermediate ergot alkaloid pathway concentrations. Enzyme-linked immunosorbent assay (ELISA) was also used to quantify mycelial density. The expected profile of ergot alkaloid pathway alkaloids were detected including chanoclavine, lysergyl alanine, ergine and ergovaline. Ergovaline concentation was inconsistent across the two selection cycles and showed a significant (P<0.05) increase in the first cycle across all environments and treatments, conflicting with the predicted outcome. Ergine and peramine both significantly (P<0.05) increased in concentation following reselection for reduced ergovaline. When the ergot alkaloids are presented as percentage components of the biosyntheic pathway, it did however show that recurrent selection reduced both ergovaline and lysergyl alanine, and simultaneously increased ergine. It appears that there is a biochemical shunt in the pathway from ergovaline towards ergine, rather than a down-regulation of the whole pathway. An alternative ergot alkaloid biosynthetic pathway is proposed. These results support the hypothesis that in this host ryegrass-AR5 population, the ergot alkaloids are under independent host genetic control, and the pathway is inefficient as the intermediates do not convert directly to ergovaline, but accumulate as ergine. Large environmental effects were observed in ergot alkaloid concentations within environments and between nitrogen treatments, with significantly greater (P<0.05) concentrations in the dryland environment. There also appears to be no effect from recurrent selection on mycelial density in this host-endophyte association. Limitations of this particular study are discussed and possibilties for future work are proposed.

Keywords: Perennial Ryegrass, endophyte, *Lolium*, *Neotyphodium*, ergot alkaloid, ergovaline, ergine, peramine, mycelial density, biosynthetic pathway.

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Chapter 1

Introduction

Perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.) are amongst the most important grass species sown in the temperate, high rainfall (>600 mm) zone of south-eastern Australia. Perennial ryegrass occurs in 6 million hectares of grasslands (Hill and Donald 1998) and is the predominant grass in the cool, temperate, winter-spring rainfall, southern regions, particularly in the state of Victoria. Like several other temperate grasses, perennial ryegrass and tall fescue have co-evolved with the asexual *Neotyphodium* fungal endophyte (Schardl *et al.* 2004), which is classified as part of the *Epichloë* genus (Leuchtmann *et al.* 2014) and in perennial ryegrass the association is with the *Epichloë festucae* var. *lolii* (=*Neotpyhodium lolii*). This grass-host association is asymptomatic and generally regarded as mutualistic, where both the grass and fungus benefit from the association.

For the grass host, infection with endophyte imparts unique bioactive properties which increase its tolerance to a range of biotic (e.g. insect predation) and abiotic (e.g. soil water deficit) stresses (Malinowski and Belesky 2000; Rolston *et al.* 2002; Popay and Bonos 2005; Easton 2007). Although endophyte-infection enhances plant performance, some endophyte strains and their associated secondary metabolites cause ill health and productivity losses in grazing livestock (Fletcher 2012). Perennial ryegrass cultivars infected with the naturalised wild-type (WT) endophyte strain(s) cause livestock to suffer a range of disorders, commonly referred to as perennial ryegrass toxicosis (PRGT). Symptoms of PRGT include occurrence of perennial ryegrass staggers (Fletcher and Harvey 1981), heat stress (Fletcher 1993) and a number of subclinical disorders such as poor animal live-weight gains, reduced milk production and reduced fertility (Carpenter *et al.* 2003; Bluett *et al.* 2005; di Menna *et al.* 2012). Recent estimates placed the economic cost of PRGT to the Australian Sheep Industry resulting from clinical, subclinical and indirect effects on animal performance at $100 million year^{-1} (Leury *et al.* 2014).

Neotyphodium endophytes may produce at least four classes of bioactive alkaloids which include: lolines (saturated aminopyrrolizidines); lolitrems (indole-diterpenes); peramine (pyrrolopyrazine); and the ergot alkaloids (Schardl *et al.* 2011). Most endophytes produce a subset of the four classes. For example, *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon and Hanlin associated with tall fescue typically produces ergot alkaloids, lolines and peramine, whereas *Neotyphodium lolii* (Latch, Christensen and Samuals) Glenn, Bacon and Hanlin associated with perennial ryegrass typically produces lolitrems along with peramine and the ergot alkaloid ergovaline

(Panaccione *et al.* 2003). Ergot alkaloids are produced by several fungi representing two different orders (Panaccione 2005). These include various ergot fungi in the genus *Claviceps* (Flieger *et al.* 1997) and several already highlighted in the genera *Epichloë* and *Neotyphodium*.

Different endophyte strains produce different expressions of the alkaloid profile and concentrations in conjunction with their associated host-plant (Easton *et al.* 2002). The naturally occurring WT endophyte evolved to produce two of the major alkaloids associated with animal toxicosis, which include the neuromuscular toxin – lolitrem B; and the end product of the ergot alkaloid biosynthetic pathway – ergovaline. Ergovaline is an ergopeptide derivative of lysergic acid and additionally simpler ergot alkaloids have been detected in grass-endophyte associations and pathway biosynthesis (Panaccione *et al.* 2003). Ergovaline is considered the main ergot alkaloid responsible for most animal health problems associated with perennial ryegrass (Lane 1999). One of the effects of ergopeptines is they act as vasoconstrictors, which are implicated in thermo-regulatory dysfunction and contribute to poor animal performance (Cosgrove and Hume 2005). However, a number of studies have indicated that ergovaline confers protection to the host-plant by providing strong resistance against invertebrate pests such as African Black Beetle (*Heteronychus arator*) (Wheatley *et al.* 2003; Hume *et al.* 2007) and root aphid (*Aploneura lentisci*) (Popay and Gerard 2007) in both Australia and New Zealand. Conversely, the peramine class of alkaloid has no known detrimental effects on animal health (Fletcher 2012) and has strong pest resistant properties, especially as a feeding deterrent to Argentine Stem Weevil (*Listronotus bonariensis*) (Popay and Wyatt 1995).

Considerable progress has been made in the development and commercial release of new strains of 'novel' endophytes that continue to produce beneficial alkaloids that confer agronomic advantage in grazing systems, but do not produce toxins associated with animal toxicosis (Bluett *et al.* 2005). Given the animal health concerns with ergovaline, it is desirable to develop perennial ryegrass-endophyte cultivars that eliminate any presence of the ergot alkaloids, or produce low levels of the end-product ergovaline to maintain sufficient levels for pest protection. The genetics of the grass-host has a significant influence on the expression of the secondary metabolites associated with endophyte (Tian *et al.* 2013). Selection for reduced levels of ergovaline in host plant WT populations is highly heritable in perennial ryegrass (Easton *et al.* 2002) and tall fescue (Adcock *et al.* 1997; Hill *et al.* 2002). This is typically achieved through recurrent selection of a population with individual genotypes that phenotypically express low ergovaline concentration over a number of generations. Interestingly however, ergot alkaloid producing fungi such as those associated with perennial ryegrass (*N.lolii*) typically produce a characteristic profile of several ergot alkaloids rather than a single pathway end product (Panaccione 2005). Most of the attention in alkaloid toxicity has been attributed mainly to the end-product ergovaline (Fleetwood *el al.* 2007). There is a suggestion

however that these intermediate ergot alkaloids, such as lysergic acid derivatives and simples amides, may also contribute and have a significant role in animal toxicosis (Hill *et al.* 2001).

The ergot alkaloid pathway appears unusually inefficient in that certain intermediates do not flow rapidly through the pathway to an ultimate end-product (Panaccione 2005). Intermediate alkaloid products may accumulate in concentrations along the pathway to the same level as the end-product, contributing to a greater percentage component in the pathway. Furthermore, there is evidence that pathways in certain endophyte fungi either completely down regulate, or contain pathway shunts, where intermediates may be diverted to alternative products (Panaccione *et al.* 2003). If the intermediate alkaloids and alternative products in the ergot alkaloid pathway are not rapidly converted to the ultimate end-product, ergovaline, then the accumulation of other components in the pathway suggests they provide some benefit to the association than those conferred by ergovaline (Panaccione 2005).

There is a paucity of studies on the effect that recurrent selection for reduced expression of ergovaline in perennial ryegrass has on the other ergot alkaloids in the biosynthetic pathway. An objective of this research is to determine what effect recurrent selection has on the other ergot alkaloid intermediates in the pathway in a perennial ryegrass population infected with the novel endophyte strain 'AR5' which fails to produce lolitrem B. An understanding of this would provide evidence as to whether the expression of other ergot alkaloids is under independent genetic control rather than associated genetic control. Initially a base ryegrass-AR5, first and second cycle population must be developed so they can be evaluated simultaneously in a field experiment to collect herbage samples for chemistry analysis.

It is hypothesized that concentration of ergovaline in the ryegrass-AR5 base population will be reduced successfully through two cycles of recurrent selection. If there is inefficiency of the ergot alkaloid pathway in this particular ryegrass-endophyte association due to strong host genetic control, and not all intermediate alkaloids are converted to the end-product ergovaline, then there must be an accumulation of these other ergot alkaloid components somewhere in the pathway. Therefore it is also hypothesized that recurrent selection is having an effect on these intermediates by increasing their concentrations. It is not clear if these are in the form of other ergopeptines or simple amides, or whether it is early or late down-regulated effects or shunts in the pathway. The study will also try and understand if recurrent selection has any indirect effect on peramine concentration and fungal colonisation of the host plant, otherwise referred to as mycelial density.

Two of the most common abiotic stresses in Australian perennial ryegrass grazing systems include water deficit (low or inconsistent rainfall) and nitrogen deficiency (Waller and Sale 2001). Therefore two environment (irrigation and dryland) and nitrogen (high and low nitrogen input) treatments will

be evaluated within the field experiment to attempt to establish what effect the treatments have on the synthesis and expression of the secondary metabolites, ergot alkaloid intermediates, peramine and mycelial density. It is hypothesised that environment will have a significant influence on ergovaline by increasing the concentration under stress. If reduced ergovaline concentrations are observed, it is thought that mycelial density will have also simultaneously decreased, unless other component pathway intermediates have accumulated. If there is no decrease in mycelial density, this should provide further evidence of an inefficient pathway that is closely regulated and that accumulation of particular intermediates is controlled and not random. The final objective is to generate a terminal population of a low ergovaline producing perennial ryegass-AR5 cultivar to be used in future breeding and evaluation programmes with potential for commercialisation in the Australian marketplace.

Chapter 2

Review of Literature

2.1 Perennial ryergass in Australia

Perennial ryegrass (*Lolium perenne* L.*)* is one of the most important forage grasses for high rainfall (> 600 mm) and irrigated pasture zones of south-eastern Australia. It is a prolific tillering, tussock forming grass with a fibrous and relatively shallow root system. Perennial ryegrass is considered a highly desirable species because of its ease of establishment, high herbage yield potential, high nutritive value, familiarity in farming systems, and ease of management in relation to other pasture crop species (Reed 1996; Fulkerson and Donaghy 2001). It is the most popular forage grass in many dairy, beef cattle and sheep production systems (Sandral and Kemp 2013).

Perennial ryegrass requires moist and relatively fertile soil conditions to achieve optimum production and persistence in long-term and permanent pasture systems. It is generally best adapted to regions that experience a 7 month growing season in temperate to Mediterranean-like climates. Hill and Donald (1998) estimated that perennial ryegrass is present in over 6 million hectares (ha) of grasslands in Australia, but is predominantly sown in the cooler south-eastern states with winter and spring dominant rainfall patterns. A recent survey by Donald *et al.* (2012) showed perennial ryegrass dominated the percentage of total grazing area of pasture type across both Victoria (4 million ha) and Tasmania. Perennial ryegrass sown with subterranean clover (*Trifolium subterraneum)* or white clover *(Trifolium repens*) covered 19.2% and 29.3% of grazing land respectively in Victoria, compared to 1% in South Australia (SA) and New South Wales (NSW). Western Australia (WA) did not register on the survey. However historically, there have been significant areas sown in the subtropical transition zones of coastal NSW and south-eastern Queensland (QLD) where irrigation is present for year-round production (Cunningham *et al.* 1994).

2.2 Limitations of perennial ryegrass

One of the defining limitations of perennial ryegrass in temperate grassland pasture systems in Australia is its poor persistence in relation to three other commonly sown pasture species: tall fescue *(Festuca arundinacea*); phalaris (*Phalaris aquatica*); and cocksfoot (*Dactylis glomerata*). A review by Waller and Sale (2001) provided a thorough description of these constraints and grouped them into four major contributing factors which included: 1) climatic; 2) edaphic (soil physical properties, soil nitrogen and phosphorus status); 3) biotic; and 4) the influence of grazing management. Their review highlighted an important concept often neglected in industry that persistence should not be classified as a single standalone trait, but should be considered as a complex range of characteristics

and multiple stress events that all ultimately contribute to the pastures longevity for both agronomic and economic performance.

The effect of climate on perennial ryegrass persistence is often associated with extended periods of moisture deficit during dry summer-autumn periods. These periods have been commonly blamed for decline in plant densities of perennial ryegrass (McWilliam 1978; Anderson *et al.* 1999). It is also generally accepted that growth of perennial ryegrass is restricted by high temperature (Vough and Marten 1971; McWilliam 1978) which further contributes towards poor persistence. For example, optimal growth for perennial grass has been reported to be between 17-21°C (Baker and Jung 1968) with growth ceasing above 30°C (McWilliam 1978). As a cool season grass, perennial ryegrass is not well adapted to elevated temperatures that occur in many regions of Australia.

The influence of grazing management through defoliation, treading and excreta return can also have detrimental effects on perennial ryegrass persistence. For example, over-grazing or heavy stocking densities over the summer-autumn period can reduce the competiveness of perennial ryegrass (Brougham 1960). This leads to poor persistence especially when pastures are confounded by moisture stress (Beattie 1994). Fulkerson and Donaghy (2001) outlined that the act of defoliation (either through grazing or mechanical cutting hay/silage) is a major imposition on ryegrass with its impact greatly dependent on the severity and timing of defoliation. Furthermore, a study by Cullen *et al.* (2006) showed the physiological response of perennial ryegrass under continuous grazing over dry summers was to continue to support shoot growth at the expense of roots, in contrast to the response seen in phalaris. Nevertheless, best practice grazing management can be adopted to overcome these limitations through the removal of livestock or controlled rotation grazing to maintain ground cover, conserve root reserves and minimise plant deaths to keep high plant densities in the sward (Reed *et al.* 2013).

Breeding and producer education efforts around germplasm/cultivar development and grazing management have made considerable inroads to resolve the climatic, edaphic and management challenges. However biotic stresses have also been attributed as a key driving factor for perennial ryegrass survival and associated productivity in the review by Waller and Sale (2001). Arguably, these cannot always be addressed successfully from a management perspective. Examples of these biotic factors include invertebrate insects, competition from weeds, ryegrass disease such as crown rust (*Puccinia* spp.) and contribution of the associated asymptomatic endophyte (*Neotyphodium lolii*). Extensive research has been conducted on the role of symbiotic fungi in temperate grasses to increase pasture persistence and production in Australia (Hume and Sewell 2014) while mitigating associated animal health issues (Cosgrave and Hume 2005). This review examines the biotic stress tolerance mechanisms of endophyte in grazing systems and how selection breeding methodologies

can exploit the host-endophyte relationship to maintain agronomic performance, but also reduce the negative animal productivity implications often associated with these host-endophyte interactions.

2.3 Perennial ryegrass improvement and breeding in Australia

Perennial ryegrass occurs in nature as a diploid (2*n* = 2x = 14) obligate out-breeder which can suffer serious inbreeding depression (Cunningham *et al.* 1994). As a diploid it contains two sets of seven chromosomes in each cell of every plant. Since the 1950s, treatment with the chemical colchicine allowed for increasing the ploidy level and creation of allotetraploids (2n = 4x = 28). Recently, these have been further developed through selection to achieve genetic gain in many public and private plant breeding programmes.

Numerous reviews have described the genetic improvement and the future breeding objectives for perennial ryegrass improvement in Australia (Cunningham *et al.* 1994; Reed 1996; Reed *et al.* 2001). Since the introduction of perennial ryegrass into the country, the activity and efforts in the improvement and development of this species is relatively recent (c. 1970s). Cunningham *et al.* (1994) reviewed the history of perennial ryegrass improvement in Australia, the performance of introduced cultivars, breeding objectives and the effect of endophyte on agronomic and animal performance. These authors outlined a detailed timeline of the history of perennial ryegrass improvement in each state of Australia, from the first introduction of perennial ryegrass pasture grasses in c.1860, the Victorian Department of Agriculture commencement of selection and evaluation of introduced and naturalised populations of pasture species in 1928, and finally, the recent improvement programmes within Victoria by both public and private sectors. The cultivar (cv.) 'Victorian' perennial ryegrass was based on local ecotypes from the western and central areas of Victoria and was first certified for commercial release in 1936 (Drake 1942). Reed *et al.* (2001) highlighted that cv. 'Victorian' is the most commonly sown cultivar, or ecotype, across the 6 million ha of grasslands in Australia.

Within Australian ryegrass breeding programmes, the breeding objectives and focus was historically based on improvement of agronomic traits such as yield, adaptation to multiple environments, persistence, seasonal growth and disease resistance (Smith *et al.* 1997). Little attention was given to selecting and actively improving the host-endophyte association, therefore the endophyte-infected ryegrass populations were likely to be indirectly affected by any selection pressure. Early work on the host-endophyte association had previously been limited by a multitude of factors including: a lack of awareness of the implications and associated animal health issues attributed with naturalised Wild-Type (WT) endophyte - a link not discovered until the early 1980s (Fletcher and Harvey 1981); a poor understanding of the science or agronomic advantages conferred by endophyte-infection; and an inability or lack of methodologies to easily identify or quantitatively measure endophytes and

associated secondary metabolites produced. Therefore, prior to the discovery of the influence and importance of endophyte in ryegrass and tall fescue pastures, any reports of results from breeding and evaluation experiments need to be interpreted with caution.

2.4 Endophyte in perennial ryegrass

2.4.1 Overview

Many cool season grasses form a mutualistic symbiotic relationship with an ascomycete fungus, in particular with the genus *Epichloë* and derived species. Both the fungus and the host grass benefit from this relationship and this forage grass-endophyte association is the most intensively studied (Easton 2007). The hosts of these fungi include perennial ryegrass (associated with *Neotyphodium lolii)* and tall fescue (associated with *Neotyphodium coenophalium*). Endophyte resides within the intracellular spaces of the leaf sheath and pseudo-stem of the plant and does not invade the cell wall (Schmid and Christensen 1999). The host plant provides the endophyte with protection, nutrition and a unique means of dispersal (Prestidge and Ball 1993). Endophytes are transmitted through seed and complete their entire life cycle within the plant; therefore reproduction is asexual and maternally inherited in the seed embryo and is not observed in pollen (Easton and Fletcher 2007).

Perennial ryegrass, like many other grass species, has coevolved with these symbiotic fungal endophytes. The naturally occurring endophyte present in many current populations of perennial ryegrass in Australia is often referred to as WT endophyte; other common names include Standard Endophyte (SE) or High Endophyte (HE). The WT endophyte is very widely distributed in both sown and naturalised perennial ryegrass pastures. For example, Reed *et al.* (2000) undertook a survey of 56 populations of 'Victorian' and 45 populations of cv. 'Kangaroo Valley' perennial ryegrass sampled from old pastures within the recognised zone of naturalisation for both ecotypes. These workers subsequently found all populations were infected with WT endophyte, with the mean frequency within populations of 'Victorian' and 'Kangaroo Valley' ecotypes being 88% and 93% respectively.

2.4.2 Agronomic advantages of endophyte

Natural selection favours endophyte-infected plants and the association with a host plant is beneficial as it imparts unique bioactive properties which increase the plants' tolerance to a range of biotic (e.g. insect predation) and abiotic (e.g. soil water deficit) stresses (Malinowski and Belesky 2000; Rolston *et al.* 2002; Popay and Bonos 2005; Easton 2007). Agronomic performance of endophyte-infected grass cultivars has been studied in many different countries, with reports of agronomy experiments and cultivar evaluations predominantly cited in New Zealand (NZ), northern United States of America (USA) and Australia. Biotic tolerance is largely driven by a degree of protection, either directly against, or as a feeding deterrent to a range of invertebrate insects that

are known to influence the survival and production of perennial ryegrass in Australia. These major pests observed include Argentine stem weevil (*Listronotus bonariensis*) (Popay and Bonos 2005), African black beetle (*Heteronychus arator*) (Reed 2002), root aphid (*Aploneura lentisci*) (Hume *et al.* 2007) and pasture mealey bug (*Balanococcus poae*) (Pennell *et al.* 2005).

Hume and Sewell (2014) recently reviewed the literature for Australia and presented new data to examine the agronomic effects of endophyte in both the establishment phase, both in mature pasture swards. All regions studied reported significant (P<0.05) positive responses to endophyte-infection, while a further 10 out of the 18 experiments reported either higher yields and greater plant or tiller densities than endophyte-free (nil endophyte) plots within the same cultivar. The magnitude of the advantages ranged from +7% to +212%. For example, yield advantages for cultivars infected with WT endophyte in south-east QLD were +6%, +31% and +44% for the first, second and third year respectively (Lowe *et al.* 2008). In experiments which reported full seasonal data, the endophyte effects were greatest in the summer and autumn period (Launders *et al.* 1996; Wheatley 2005; Lowe *et al.* 2008). No experiments reported statistically significant (P<0.05) negative yield responses to endophyte-infection.

Since the release of the continental tall fescue cv. 'Demeter' in the 1936, tall fescue sold in Australia has largely been free of WT endophyte (Easton *et al.* 1994). Naturalised tall fescue has been identified in some pockets around Australia (i.e. roadsides and riverbanks in Tasmania, and small areas of grazed pastures in SA, Victoria and NSW) that are infected with the WT strain toxic to livestock (Guy and Davies 2002). The agronomic impact of endophyte-infection for the novel endophyte strain (AR542) in tall fescue was first tested in 2000 at three sites in NSW and QLD with favourable results observed in the first year (Hume and Sewell 2014). Further trials were established with independent researchers evaluating similar cultivars with and without this endophyte. A total of 30 experiments were sown and Hume and Sewell (2014) showed endophyte-infection was advantageous to agronomic performance over the nil (no endophyte) treatment of the same cultivar for the majority of the experiments analysed (24 out of 30).

2.4.3 Secondary metabolites and animal toxicosis

Neotyphodium endophytes produce at least four classes of bioactive alkaloids: lolines (saturated aminopyrrolizidines); indole-diterpenes (the lolitrems); ergot alkaloids; and peramine (pyrrolopyrazine) (Panaccione *et al.* 2003; Schardl *et al.* 2011). Different endophyte strains produce different concentrations of the alkaloid profiles in conjunction with their host plant. The naturally occurring WT endophyte of perennial ryegrass evolved to produce two major toxic alkaloids – lolitrem B and ergovaline, both of which are implicated in a range of animal disorders commonly referred to as perennial ryegrass toxicosis (PRGT). This strain also evolved to produce the

invertebrate toxin, peramine, but no adverse effects on animal health have been reported with this particular chemical group. The effects of endophyte in pastoral livestock systems are less well studied in Australia as opposed to the USA (predominantly focussed on tall fescue) and NZ (predominantly focussed on perennial ryegrass). Despite the small number of studies in Australia, significant effects have been identified (Hume and Sewell 2014). Considerable progress has been made in the identification and commercial release of new strains of 'novel' endophytes (i.e. AR1 and AR37) that continue to produce beneficial alkaloids to confer agronomic advantage, but do not express those associated with animal toxicosis (Bluett *et al.* 2005).

Following the link between ryegrass staggers and presence of endophyte by Fletcher and Harvey (1981), a number of other disorders were suspected or became apparent in affected animals (di Menna *et al.* 2012). These associated 'subclinical' animal health problems reported from grazing endophyte-infected ryegrass included weight loss and poor growth rates (Fletcher 1983), scouring with accumulation of dags and danger of fly strike and heat stress in sheep (Fletcher *et al.* 1999). Recent studies have provided preliminary data showing increased water intake in sheep from grazing WT-infected ryegrass compared to a novel endophyte-infected ryegrass (AR37) of the same variety (Sewell *et al.* 2009). Findings from a recent 3 year farmlet grazing study in south-east Australia (Dookie, Victoria), with two breeds of sheep (crossbred and merino), found sheep grazing the WT-infected pasture had lower live-weight gains, increased rectal temperatures and increased respiration rates in comparison to those grazing the same ryegrass cultivar infected with the novel AR1 or AR37 endophyte strain (Leury *et al.* 2014). The effects were significant ($P<0.05$) when they occurred from mid-summer onwards on dry standing feed, but were variable and appeared dependent on a range of factors including environment and feed availability. These workers clearly demonstrated that WT endophyte can have significant impacts on sheep productivity and physiology. Leury *et al.* (2014) concluded that WT endophyte was considered to be an unacceptable option, with AR1 better than AR37 from an animal health perspective.

There has been considerable research in NZ comparing the effects of WT, novel endophytes (AR1 and AR37) and nil endophyte treatments in perennial ryegrass on the health and productivity of dairy herds. For example, Bluett *et al.* (2005) showed a significant ($P<0.05$) milk production advantage of 8.9% to cows grazing novel AR1 (absence of lolitrem B or ergovaline) ryegrass (cv. 'Bronsyn') over those on WT endophyte-infected ryegrass. Furthermore, incidence of ryegrass staggers was reported on the WT-infected ryegrass treatments. In an earlier study a direct relationship between milk production from cows grazing endophyte-infected and endophyte-free grass could not be determined (Thom *et al.* 1994). However, Thom and co-workers more recently showed in short term trials, milk yields from cows grazing WT were reduced by 6-26% compared with those grazing AR1 (Thom *et al.* 2010) during the summer-autumn period. In Australia, Valentine *et al.* (1993) reported a

4-14% reduction in milk yield for cows grazing irrigated pastures with 88% WT endophyte-infected (frequency) ryegrass within the same nil cultivar (cv. 'Ellett') with only a 1% infection. A recent study by Moate *et al.* (2012) found no significant differences (P>0.05) in milk, fat or protein production in cows grazing WT, AR1 or AR37 infected ryegrass. However, ryegrass staggers was observed in a small number of cows in the first year on the WT ryegrass treatment. All evidence clearly indicates that WT endophyte is unacceptable in any grazing situation and the associated toxins lolitrem B and ergovaline need to be either reduced, or completely eliminated, in forage improvement programmes while still maintaining agronomic reliability.

2.4.4 Lolitrem B

The indole-diterpene chemical alkaloid group act as neuromuscular tremogenic toxins which affect the central nervous system of mammals and are toxic to insects. Specifically lolitrem B has been identified as the main causative agent of ryegrass staggers in perennial ryegrass (Gallagher *et al.* 1984). Natural outbreaks of perennial ryegrass staggers is characterised by tremors, staggering gait, convulsions and collapse. Symptoms are all exacerbated by exercise and have been observed in sheep (*Ovis aries*), cattle (*Bos taurus*), horses (*Equus cabaallus*), donkeys (*Equus africanus asinus*), deer (*Odocoileus virginanus*), alpaca (*Vicugna pacos*) and goats (*Capra hircus*) all grazing ryegrass endophyte-infected pastures (di Menna *et al.* 2012). di Menna *et al* (2012) highlighted that it is important to determine which of the indole-diterpenes produce tremors in animals since it is possible other compounds could make significant contributions to the incidence of ryegrass staggers. For example, Fletcher *et al.* (1993) reported that neurotoxins other than lolitrem B may cause staggers in grazing lambs, following removal of lolitrem B from the ryegrass-endophyte association in a grazing study. It was not identified whether those particular causative toxins acted individually or synergistically with other metabolites. However, concentration of paxilline (tremogenic indole-diterpene) was strongly correlated with the late development of ryegrass staggers in lambs grazing the lolitrem B free ryegrass-endophyte cultivar. That study provided a clear indication that alkaloids secondary to the main causative agent (in this case lolitrem B) may have greater implications in animal health or productivity not typically captured in agronomy experiments. Therefore quantifying intermediate metabolites is an important area requiring more emphasis, especially in relation to biosynthetic pathways interaction within specific alkaloid chemical groups.

2.4.5 Ergovaline and other ergot alkaloids

Ergovaline, the major ergopeptide alkaloid in endophyte-infected perennial ryegrass, is considered the principal cause of increased heat stress in sheep and cattle. According to Lane (1999), it is the main ergot alkaloid responsible for most animal health problems associated with perennial ryegrass. The ergopeptines act as vasoconstrictors, which are implicated in thermo-regulatory dysfunction and

in turn contribute to poor animal performance (Cosgrove and Hume 1995). Heat stress symptoms in sheep and lambs have been recognised on grazing endophyte-infected and endophyte-free ryegrasses, as well as on strains with lolitrem B eliminated. The associated animal toxicosis has also been recognised through reduced serum prolactin levels causing fertility issues (Piper and Fletcher 1990; Carpenter *et al.* 2003), increased rectal temperatures (Fletcher 1993) and respiratory problems (Fletcher *et al.* 1999). For example, Fletcher (1993) showed there was a significant difference (P<0.05) in respiration rate and body temperature between lambs grazing WT-infected and endophyte-free treatments.

A key finding from controlled short term indoor feeding experiments was that ewes fed ergovaline at different stages of pregnancy had reduced feed intake from which they did not recover after ergovaline was removed from the diet (Leury *et al.* 2014). These experiments also showed that sheep on switched diets also lost weight at a quicker rate and lamb growth rates also tended to be slower on ergovaline and ergovaline/nil treatments. Furthermore, ergovaline during winter can increase heat load in sheep, even under thermo-neutral conditions with concentrations similar to what might be found *in situ*. However, this result was not thought to be detrimental to productivity and was supported when ergovaline and lolitrem B were fed for several weeks. However, rectal temperatures and faecal moisture were found to increase when the two alkaloids were fed in combination. This suggested strong synergistic effects between the two alkaloid toxins.

The adverse effects of WT endophyte on productivity of dairy cattle has been previously discussed (Valentine *et al.* 1993; Bluett 2005; Thom 2010; Moate *et al.* 2012). Another Australian study undertaken in NSW found significant detrimental effects of high ergovaline in silage fed to dairy cattle. Lean (2001) reported that a significant (P<0.05) decrease in milk production by 4.6 litres per cow per day was associated with high concentrations of ergovaline in ryegrass silage fed to dairy cattle. In addition, milk somatic cell count increased significantly (P<0.05) over a comparable period, reproductive performance declined and the body conditions score and coat condition of the cows were also adversely effected. The levels of ergovaline in the silage tested up to 1.75 ppm (Lean 2001).

A series of range finding experiments and case studies of the fescue foot and summer syndrome toxicities relative to the ergovaline alkaloid found in endophyte-infected tall fescue, and lolitrem B present in endophyte-infected perennial ryegrass, was conducted in Arkansas (USA) by Tor-Agbidye *et al.* (2001). The authors determined critical thresholds for ergovaline is 0.4 to 0.75 ppm in cattle and 0.5 to 0.8 ppm for sheep. An earlier report by Aldrich-Markham and Pirelli (1995) further explained that subclinical effects on livestock performance can result from measureable changes in physiological function that could occur at 0.2 to 0.3 ppm and better explain a lower threshold for

horses of 0.3 to 0.5 ppm. Lolitrem B levels of 1.8 to 2.0 ppm in feed for both livestock classes are approximated thresholds (Tor-Agbidye *et al.* 2001).

The ergopeptide alkaloid ergovaline has been identified and acknowledged as the primary toxin causing fescue toxicosis in livestock; predominantly cattle grazing WT-infected tall fescue (Hill 2005). The effects of endophyte-infected tall fescue on animals have been widely studied in the USA and shown to cause the associated problems such as fescue foot, fat necrosis and fescue toxicosis. Fescue foot is a gangrenous condition of the feet and possibly tips of the ears or tail and commonly occurs in cattle grazing fescue in the cool season of the year, when ambient temperatures are low. A study by Solomons *et al.* (1989) found ergotamine, ergosine and agroclavine (precursors in the ergot alkaloid pathway of *N.coenophialum* to ergovaline) resulted in vasoconstriction which reduced blood flow to extremities of the animal. However, loline and loline derivative alkaloids had no effect on the veins. Fat necrosis in cattle has been documented in the literature since the early 1900s (Stuedemann and Thompson 1993). It is a condition whereby the presence of hard or necrotic fat form in the adipose tissue of the abdominal cavity.

Fescue toxicosis is the third disorder from associated WT endophyte tall fescue, which is also referred to as 'summer slump', 'summer syndrome' or 'summer-autumn ill-thrift'. The signs of this include reduced animal performance and intake, lethargy, intolerance to excessive environmental temperatures, excessive salivation and rough hair coats (Stuedemann and Thompson 1993). There are confounding factors in diagnosing summer-autumn ill-thrift syndrome due to a number of other factors other than endophyte-infected tall fescue, such as mycotoxins or other secondary metabolites of the pastures (eg. perloline derived alkaloids). Recently a survey review conducted in Tasmania (Australia) focussed on the summer-autumn ill-thrift of Tasmanian beef herds grazed on ryegrass pastures. In that study it was found that mycotoxins (i.e. endophyte) are likely to be major contributors to ill-thrift in cattle over the autumn period and further research in this area was recommended (Sherriff *et al.* 2014).

It appears the attention and effects of ergot alkaloid toxicity have been attributed mainly to the end product ergovaline (Fleetwood *et al.* 2007). However, many researchers have suggested that intermediate ergot alkaloids may contribute and play a significant role in animal toxicity, such as the intermediate lysergic acid derivatives and simple amides (Hill *et al.* 2001), rather than solely the ergopeptines. Hill *et al.* (2001) argued that this distinction of ergovaline as the candidate toxin is without the appropriate toxicological or physiological studies to support the hypothesis. Reed *et al.* (2011a) also postulated that other endophyte metabolites in the biosynthetic pathways, aside from the lolitrem B and ergovaline end products, may be involved in Australian cases of acute PRGT.

A series of experiments by McLeay *et al.* looked at the pathophysiological effects of the ergot alkaloids, specifically ergotamine and ergovaline, on sheep. Ergotamine is thought to be less potent than ergovaline; although it has been shown to have similar effects that ergovaline has on the cardiovascular, pulmonary function and body temperature (McLeay *et al.* 2002). Both ergopeptides were found to increase blood pressure without associated effects on heart rate (and decreased respiration rate) but the depth of respiration increased. Body temperature of the animals rose markedly and remained elevated for at least 10 hours after the dose of ergopeptides, with the effect of ergovaline greater than ergotamine. McLeay and Smith (2006) further demonstrated that disruption of digestion may occur in animals grazing endophyte-infected pasture that has high ergopeptide content. This 'excitatory' effect was shown that it can be mediated through intrinsic nerves and muscle receptors, but peramine, on the other hand, was shown to have no effect (Poole *et al.* 2009).

A study by Piper *et al.* (1997) found little effect of ergovaline on rats (*Rattus norvegicus*) feed intake, weight gain, or serum prolactin when fed endophyte-free seed. However, diets without added ergovaline of endophyte-infected seed decreased all the described measurements, to which Hill *et al.* (2001) suggested ergot alkaloids other than ergovaline have a significant role in fescue toxicosis. Those workers subsequently presented three major reasons why the associated alkaloids contributing to toxicosis are other ergot alkaloids: 1) monoclonal antibodies specific to the ergot alkaloids reverse fescue toxicosis (Hill *et al.* 1994); 2) urinary alkaloid excretion of ergot alkaloids is inversely proportional to average daily gain of cattle (Hill *et al.* 2000); and 3) tall fescue containing novel endophytes without ergot alkaloids result in superior animal performance (Bouton *et al.* 2002).

Other ergot alkaloids in the biosynthetic pathway might work synergistically with ergovaline to subsequently cause full expression of toxicity in grazing animals. For example, Gadberry *et al.* (2003) fed lambs synthetic ergovaline on an endophyte-free tall fescue seed diet. This research supported the fact that ergovaline is a fescue toxin; however, it also suggested a synergistic effect. The synthetic ergovaline treatment was compared to lambs on an endophyte-infected tall fescue seed, endophyte-infected perennial ryegrass seed and a nil endophyte seed diet. As expected there was a dramatic response of animal effects on lambs grazing endophye infected seed, but well above the ergovaline fed lambs. Interestingly, lambs fed the endophyte-infected perennial ryegrass seed had a lower toxic response than those grazing the endophyte-infected tall fescue seed, even though the perennial ryegrass seed had a higher concentration of ergovaline. The authors explained this discrepancy between ergovaline content and toxicity effect was due to the greater ergine (a simple lysergic acid amide) concentration in the endophyte-infected tall fescue seed compared with the perennial ryegrass endophyte-infected seed. High-Performance Liquid Chromatography (HPLC) determined concentrations of ergine against ergovaline in the endophyte-infected seed were 1.26

and 2.50 ppm for perennial ryegrass and tall fescue respectively, while very high for ergovaline being 16.10 and 5.2 ppm respectively.

Precursor ergot alkaloids have also been observed at high levels in other pasture species. For example, drunken horse grass (*Achnatherum inebrians*) also forms a mutualistic symbiosis with a *Neotyphodium* endophyte (*N.gansuense*) which has been reported to be toxic against grazing livestock and expresses high levels of the ergot alkaloids ergine and ergonovine (Zhang *et al*. 2011). An experiment in China investigated different defoliation heights and cutting intervals on ergine concentrations and reported ergine levels up to 180 ppm (Zhang *et al.* 2011). Furthermore, ergine has been found in high concentrations (10 - 20 ppm reported) in sleepygrass (*Stipa robusta*), several varieties of morning glories (*Calystegia* spp.) and Hawaiian baby woodrose (*Argyreia nervosa*) all infected with *Epichloë* endophyte (Chao and Der Marderosian 1973; Petroski et al. 1992). Ergine is also a component of the alkaloids contained in the *Claviceps purpurea* (ergot) fungus which grows in the heads of infected ryegrass and tall fescue plants. Although there are differences in the primary ergopeptide produced by *Neotyphodium* spp. and *C.purpurea*, the mode of action of these ergopeptides is similar and the clinical presentation of toxicity in animals can be indistinguishable (Canty et al. 2014).

Hill and colleagues have highlighted that little attention is given to various forms of ergot alkaloids in endophyte-infected tall fescue or their ability to cross digestive barriers resulting in toxicosis. An experiment by Hill *et al.* (2001) showed that the simple amide alkaloids are more likely to cross digestive barriers than the ergopeptine alkaloids, and that the transport mechanism is an active process. The authors concluded that the ergot alkaloids with greatest absorption potential were lysergic acid, lysergol and ergonovine, but those ergopeptide alkaloids implicated as the candidate toxin (i.e. ergovaline) had a lower absorption potential. Therefore, ruminant absorption (the primary site of absorption) of ergot alkaloids is active rather than passive, and favours the ergoline alkaloids (i.e. ergine) over the ergopeptine alkaloid transport, especially lysergic acid (Hill 2005). The review by Hill (2005) offered a new hypothesis which was other non-ergovaline bioactive ergot alkaloids are responsible for fescue toxicosis based on the chemical structures of the major classes of ergot alkaloids. This greatly influences the ability of them to transport across gastric tissues, and thus, increase their toxicity potential to livestock.

2.5 Variation of ergot alkaloid concentration

Concentrations of ergovaline (and other ergot alkaloids) in herbage is influenced by a number of different contributing factors. Although the focus of this review is the host genetic influence on alkaloid concentrations, specifically the ergot alkaloids, concentration of many other alkaloids is also greatly affected by environmental factors. This includes temperature, moisture stress and nutrient

status, as well as the developmental stage of plant growth and distribution within the host plant. Because of the combination of these factors, Easton (2007) explained that differences between field work results across studies can be difficult to interpret.

It is well documented that secondary metabolite concentrations within endophyte-infected plants is known to considerably vary seasonally, from high concentrations in summer to low concentrations in winter. A number of studies conducted in New Zealand and Australia have clearly demonstrated this (Ball *et al.* 1995; Fletcher *et al.* 2001; Reed *et al.* 2015) in different environments and different endophyte types such as WT and novel endophyte strains. For instance, Fletcher *et al.* (2001) compared two populations of perennial ryegrass, one infected with WT strain and the other with a novel ergovaline producing strain, AR6, in Canterbury, New Zealand. The concentrations of ergovaline, lolitrem B and peramine were analysed over 3 years in the basal and total herbage fractions. The results showed an annual cycle of variation in alkaloid concentration from a winter-spring minimum, to a summer or early autumn maximum. There was a greater range of variation for lolitrem B and ergovaline than for peramine (Fletcher *et al.* 2001). For example, Figure 2.1 showed ergovaline for total herbage fraction ranged from less than 0.5 ppm in winter and spring to a highly variable annual peak in early-late summer which ranged from 1.4 ppm (Dec-94) to 1.8 ppm (Feb-93). Although a more detailed annual cycle of alkaloid concentration, these data supported earlier work by Ball *et al.* (1995) for ergovaline. Furthermore, the cv. 'Nui' AR6 population accumulated higher ergovaline concentrations in the summer and Figure 2.1 also showed that for the 3 years of the experiment, the maximum summer concentration ranged from 3.1 ppm (Feb-93) to 5.5 ppm (Mar-95).

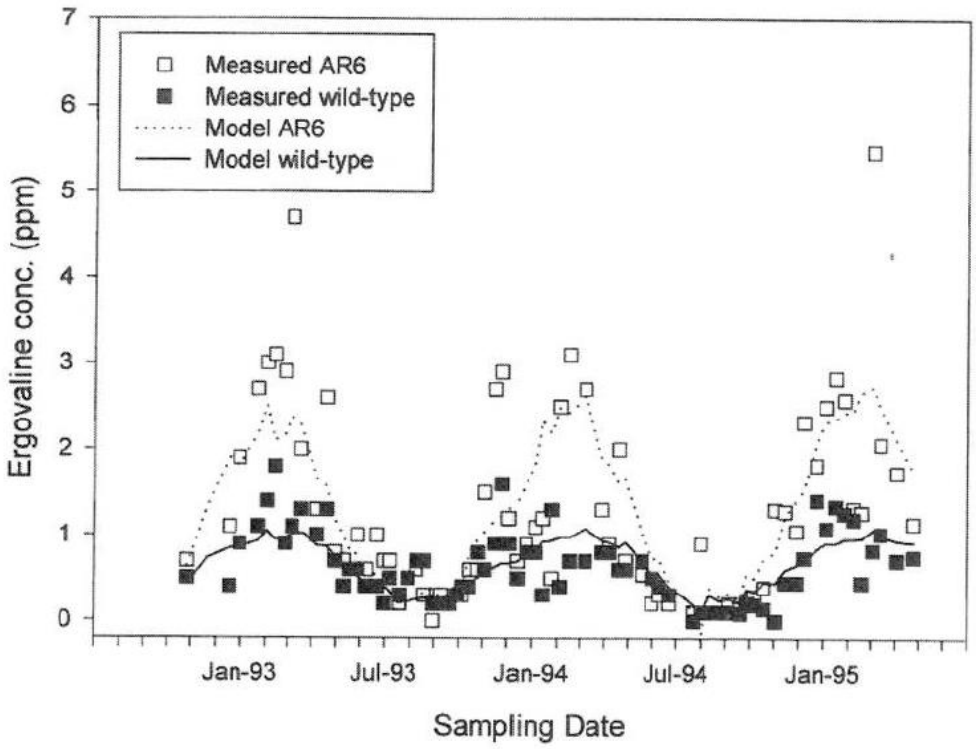

Figure 2.1 Seasonal variation of ergovaline concentrations in total herbage fractions from pasture of Nui perennial ryegrass infected with either WT or AR6 strain. The filled boxes – WT; unfilled boxes – AR6. The models are also depicted, with WT – solid line; AR6 – dotted line. (Fletcher *et al.* 2001).

A statistical modelling approach by Fletcher and colleagues attempted to explain the relationship for variation of alkaloid concentration to climatic data collected throughout the experiment. This showed that climatic variation accounted for 42% and 66% of the variation in ergovaline and peramine respectively (Fletcher *et al.* 2001). Specifically for ergovaline, the soil temperature (1 m) was the only significant ($P<0.05$) parameter in the model for both the WT and AR6 strains for total herbage (Figure 2.1) for the annual cycle. In addition, solar radiation and deep soil temperature (10 m) were significant ($P<0.05$) parameters in the modelling, both with negative coefficients for peramine and lolitrem B respectively. The authors concluded that to reproduce seasonal variations in the alkaloid concentration, models that are more complex in structure than simple regression models might be required.

Alkaloid concentration is known to be much higher in the crown, basal tissue and pseudo-stem of the host plant than the leaf blade (Keogh and Tapper 1993). Alkaloid concentrations within the different plant segments are factors which can contribute to the observed variations (Keogh and Tapper 1993). Watson *et al.* (1999) also reported that ergovaline levels were significantly higher during late spring, summer and autumn than levels in winter for both years of the experiment, similar to the pattern reported by previous work (Ball *et al.* 1995; Fletcher *et al.* 2001). In addition, the authors broke

down the plant into individual fractions of sheath and leaf blade and further reported a significant (P<0.001) seasonal effect on ergovaline levels in the ryegrass leaf sheath and leaf blade components. During the periods of elevated ergovaline production, the levels were two times higher in the sheath than the leaf blade over both years. This supported earlier work by Lane *et al.* (1997a) where wide variation in ergovaline concentration between individual plants was observed (n = 7, separated into root, crown, lower tiller and upper tiller fractions, and at different growth stages of vegetative, reproductive and intermediate tillers). The ergovaline concentrations were found to be highest in the crown and the inflorescence, which are associated with the plant fractions most important for survival and reproduction. In vegetative tissues, the gradient for ergovaline was leaf < vegetative pseudo-stem < crown, with minimal occurrence in the root tissue (Lane *et al.* 1997a).

The concentration of ergovaline has potential to express two periods of peak alkaloid production throughout the season. In Australia, a study by Reed *et al.* (2011b) reflected the earlier findings in New Zealand of high concentrations in late-spring, summer-autumn and low in winter. However Reed *et al.* (2011b) subsequently reported that ergovaline peaked twice during the sampling period, in November-December and also during mid-autumn. This study investigated the association between alkaloid concentration and weather observations on swards sown from two cultivars of WT-infected perennial ryegrass. Another study in south-west Victoria (Ballarat), Australia, reported a similar trend with two peaks of ergovaline at similar periods of the year in both a WT strain and an ergovaline producing novel AR5 endophyte strain (Mason *et al.* 2013). Another study in south Gippsland (Victoria, Australia), had also shown similar trends. While the peaks were not as pronounced as the first two studies, the seasonal variation within the different endophyte alkaloids was consistent with all previous experiments (Moate *et al.* 2012). Reed *et al.* (2011b) attributed the first peak in the late-spring, early summer period to seed-head emergence and development, which had been previously reported that ergovaline concentration increased in the inflorescence (Lane *et al.* 1997b). This first peak by Reed *et al.* (2011b) was observed earlier in the year than Mason *et al.* (2013), however this could be explained by the use of later maturing cultivars in the latter experiment and the Ballarat location is considered to have a longer growing season. Reed *et al.* (2011b) attributed the second peak (mid-autumn) to the new regrowth which was stimulated by successive rainfall events, coinciding with the traditional 'autumn break'. Ergovaline declined as the mean daily maximum temperature increased over the preceding 5 days.

Different endophyte strains that express different secondary metabolite profiles also show distinct differences in seasonal patterns for alkaloid concentrations under Australian conditions. This was shown in a recent study by Moate *et al.* (2012) where the expression of lolitrem B in WT showed distinct seasonal patterns with peak concentrations in excess of 1.0 ppm occurring between January and March of each year (Figure 2.2). Ergovaline was similar, with the concentration in the WT

elevated during the summer and autumn (> 0.25 ppm), but the peaks and troughs in concentration of ergovaline were less distinctive than for lolitrem B. This study also reported on two other novel endophytes AR37 and AR1 in the same background cv. 'Samson'. In contrast to the WT endophyte expression of lolitrem B and ergovaline, the peramine concentration showed little seasonal variation in WT and AR1 treatments. Interestingly, novel endophyte AR37 pastures also showed the peak concentration of total alkaloid (epoxy-janthritrems) were significantly (P<0.05) greater during February and March than any other time of the year. For example, Figure 2.2 showed it had similar timing in seasonal fluctuations of other alkaloids, predominantly lolitrem B in the same experiment, and previously reported studies. Furthermore it showed the greatest seasonal variation of all the four alkaloids measured in this study (Figure 2.2).

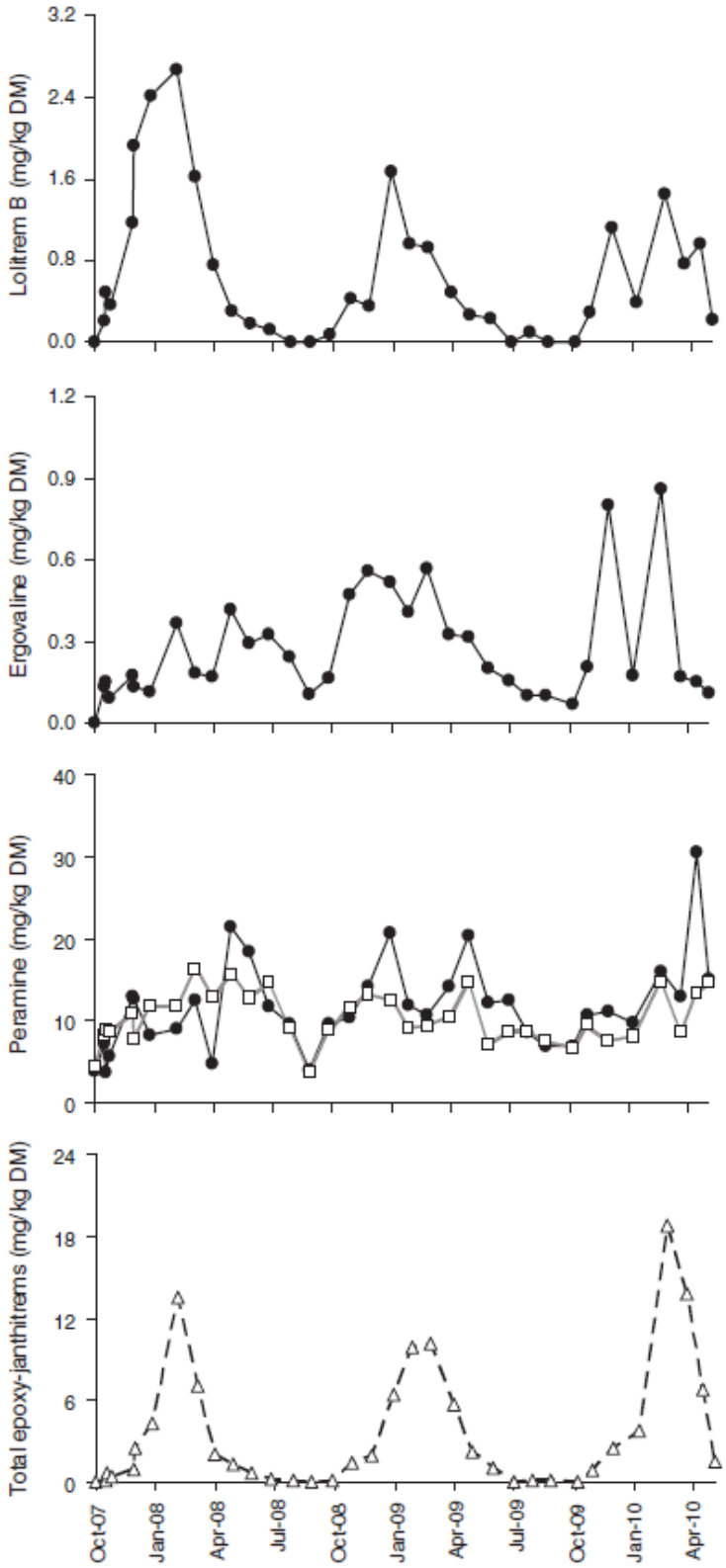

Figure 2.2 Mean alkaloid concentrations in perennial ryegrass infected with either wild-type (●), AR1 (□) or AR37 (Δ). (Moate *et al.* 2012).

Water deficit and addition of nitrogen (N) based fertiliser have been shown to be associated with increased ergovaline concentrations. Lane *et al.* (1997b) demonstrated this in a series of studies

looking at the environmental impact (N, water, temperature) on the concentration of alkaloid in WT-infected ryegrass 'Nui'. For example, in the seed production plot experiment the herbage ergovaline concentrations in late summer and autumn were significantly higher (P<0.05) in the applied N treatment across 2 years. The un-irrigated pasture also had higher levels of ergovaline than irrigated treatments. Lolitrem B concentration mirrored the ergovaline results for both applied N and un-irrigated treatments, however; only the irrigation treatment significantly reduced (P<0.05) the peramine concentration and was not affected by N treatment. A second small plot experiment showed the seasonal herbage increase in ergovaline was much greater with applied N and this carried through to the end of summer regrowth material (Figure 2.3). N was applied in this experiment at 175 kg N/ha from late winter to early summer, or was left untreated. However, no consistent pattern was reported for peramine and lolitrem B in the pseudo-stem and was consistently lower with the addition of N. A further experiment in the greenhouse was not consistent with the results from their field experiments, showing no significant differences (P>0.05) with added N on ergovaline. However the water deficit plants reflected what was reported in the field. These studies concluded that peramine and lolitrem B concentrations may also be affected by environmental growth conditions, but no consistent pattern was reported.

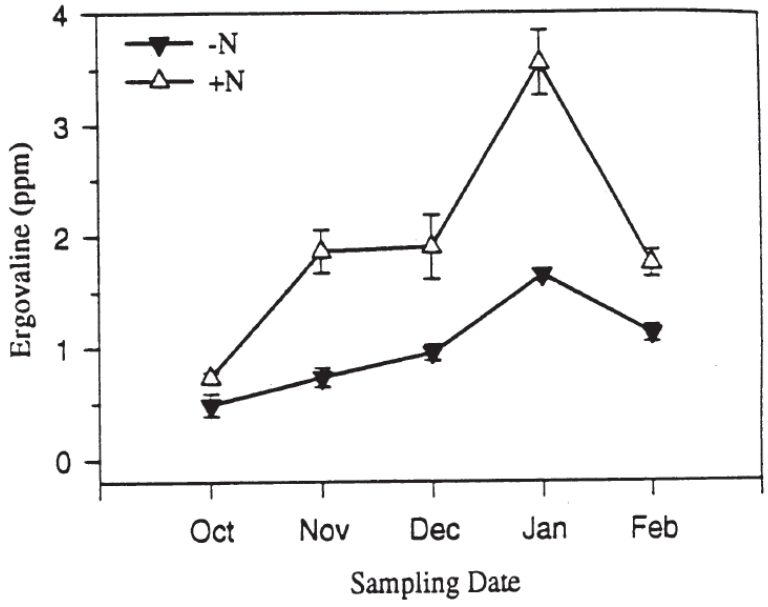

Figure 2.3 Effect of added nitrogen on ergovaline (vegetative pseudostem) in a small plot experiment, located in Manawatu, New Zealand. (Lane *et al.* 1997b).

Management of the grazing swards over the critical peak periods of alkaloid concentration is reported to influence ergovaline concentration. Reed *et al.* (2011a) showed that where growth was allowed to continue through the summer period (i.e. low grazing pressure systems), the ergovaline concentration remained relatively low (<0.7 ppm). However, in hard grazed systems (coinciding with

sward height to 30 mm), ergovaline concentration was higher (up to 1.1 ppm). This could be considered to reflect the higher percentage of pseudo-stem in the samples, or, a plant-endophyte association response to the stress of overgrazing. That study however was limited by no reported measurements of sward height, or feed availability at each sample date. This information may have provided more robust data around the grazing management contribution to total variation of ergovaline.

Higher temperatures, longer and more severe periods of moisture deficit and high solar radiation experienced in southern Australia (i.e. Victoria) in comparison to other countries may further exacerbate the alkaloid concentration and interaction of ergovaline and other ergot alkaloids. In two separate studies, Reed *et al.* (2011b) and Reed and Mace (2013) have shown that ergovaline concentrations reported from New Zealand are usually lower than those recorded in Australia, whereas lolitrem B is noticeably higher in New Zealand. For example, typical concentrations for WT-infected perennial ryegrass have been reported to be around 0.2 - 1.5 ppm (Easton *et al.* 1996; Fletcher *et al.* 2001), whereas Reed *et al.* (2011a; 2011b) and Woodburn *et al.* (1993) reported ergovaline concentrations of up to 3.0 ppm in Victoria, with up to 4.3 ppm in green tissue. A subsequent review of recent data and a new experiment by Reed and Mace (2013) investigated the unique, severe expression of perennial ryegrass toxicosis in south-east Australia by analysing alkaloid concentrations of collected samples at four Victorian sites. The authors concluded that the ergovaline:lolitrem B ratio (expected to be greater in Victoria than reported overseas studies i.e. NZ and Oregon, USA) seems likely to be more important than other metabolites in explaining the different expression of PRGT between the regions. Furthermore, they suggest there is a synergistic effect where the animal's tolerance of lolitrem B may be lowered with exposure to elevated ergovaline concentrations.

2.6 Ergot alkaloids

Ergot alkaloids are among the most important natural pharmaceuticals and toxins in human history (Schardl *et al.* 2006). Ergovaline is the end-product of the ergot alkaloid pathway, abundantly produced by endophyte and is frequently studied in *Neotyphodium*-grass interactions. Ergot alkaloids are complex compounds classified into four groups: clavines; lysergic acid; simple lysergic acid amides; and ergopeptines (Schardl *et al.* 2006). Ergovaline is an ergot-peptide derivate of lysergic acid and additional simpler ergot alkaloids have been detected in endophyte-grass interactions (Panaccione *et al.* 2003) which are collectively referred to as clavines (Bush *et al.* 1997). This is composed of D-lysergic acid linked via an amide bond to a three membered peptide derived from L-alanine, L-valine and L-proline (Schardl *et al.* 2006) as described in the biosynthetic pathway (Figure 2.4). The ergot alkaloid pathway, structures and synthesis of the pathway involves multiple

steps, most of which have been identified (Wang *et al.* 2004). This pathway is summarised in Figure 2.4 which also includes additional branches and compounds.

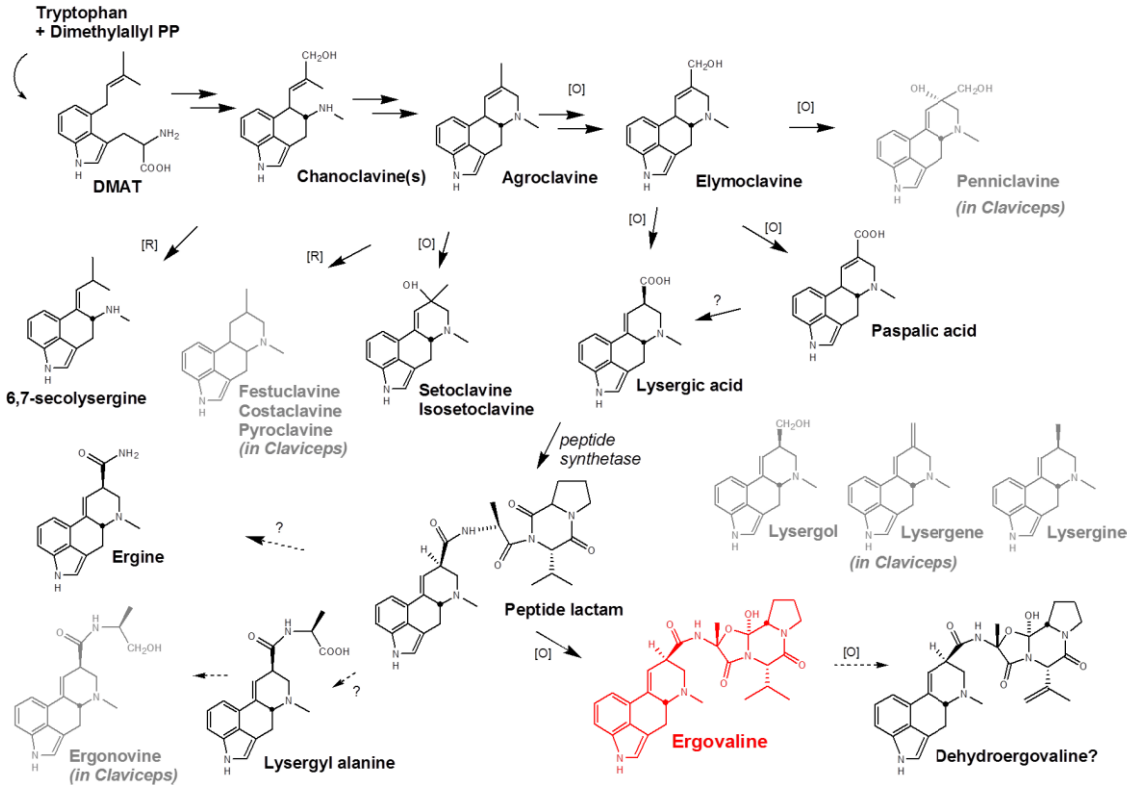

Figure 2.4 Common names and structures of key intermediates and products of the ergot alkaloid pathway. The clavines and some derivatives may also be present and add to bioactivity affects. Some of the related specifically *Claviceps* compounds (grey) are also shown for structure comparison. Adapted from Tapper *et al.* (2004).

The first step in the biosynthesis of ergot alkaloids is the addition of the prenyl group from dimethylallyl diphosphate (DMAPP) to the indole ring of L-tryptophan, forming dimethylallyl tryptophan (DMAT) catalysed by DMAT synthase (DMATrp) (Panaccione *et al.* 2003; Wang *et al.* 2004; Fleetwood *et al.* 2007; Schardl *et al.* 2011). DMATrp is encoded by the gene *dma*W (Gebler *et al.* 1992; Tsai *et al.* 1995) and has been characterised from a range of ergot alkaloid producing fungi *C. fusiformis, C.purpurea, Aspergillus fumigatus* and *N.lolii* (Tsai *et al.* 1995; Wang *et al.* 2004).

An experiment by Wang *et al.* (2004) confirmed the determinant step of biosynthesis of the ergot alkaloids and included an insightful review linking the discovery of each known step of the early pathway and nearly half a century of intensive investigation into the precursors, enzymes and pathways involved. This included the first proposed notion that the ergoline ring system was derived from prenylated tryptophan by Mothes *et al.* (1958) (cited by Wang *et al.* 2004), through to Tsai *et al.* (1995) who cloned *dma*W gene encoding the DMAT enzyme and confirmed the biochemical activity of the gene product. Wang *et al.* (2004) set out to study *N.lolii* in perennial ryegrass and test the hypothesis that DMATrp is required for ergot alkaloid production. This experiment was a genetic

test, cloned by degenerative polymerase chain reaction (PCR) and disrupting *dma*W gene, and introducing the wild type *dmaW* discriptant into the host ryegrass plants to subsequently test for expression of clavinet and ergot alkaloids. The mutant failed to produce any detectable ergovaline or simpler ergot and clavinet alkaloids, which confirmed the biosynthetic role of DMAT.

There are other steps involved in the biosynthesis of compounds which are encoded in the 11 genes of the ergot alkaloid synthesis (*eas*) gene cluster (Schardl *et al.* 2011). This gene clustering and intermediary pathway is described in step by step detail by Schardl *et al.* (2006) and more recently by Young *et al.* (2015). An overview by Fleetwood *et al.* (2007) provided a genetic foundation for elucidating biochemical steps in the ergovaline pathway, the ecological role of individual ergot alkaloid compounds and the regulation of their synthesis *in planta*. The second step in the ergot alkaloid biosynthesis is the *eas*F gene encoding DMATrp N-methyl transferase (Rigbers and Li 2008). The formation of chanoclavine isomers requires two oxidation steps to result in chanoclavine I. This is composed of D-lysergic acid linked via an amide bond to a three membered peptide derived from L-alanine, L-valine and L-proline (Schardl *et al.* 2006). In later steps in the pathway, the formation of the simple amides (i.e. ergine) and ergopeptines contain amide linkages from lysergic acid and require the activity of peptide synthases for their formation (Panaccione *et al.* 2003; Panaccione 2005). Ergot alkaloids range in complexity from simpler tricyclic alkaloids such as chanoclavine or 6,7-secolysergine to more complex tetrapcyclic alkaloids with tripeptide-derived side chains such as the ergopeptines (Figure 2. 4). Panaccione (2005) described that they are often classified into three groups – clavines, simple amides or lysergic acid and ergopeptines.

2.6.1 Breeding for low ergovaline producing endophyte-host assocations

Considering the impact of ergovaline and the associated animal toxicosis caused by the ergot alkaloid class of secondary metabolites, it is desirable to have non ergot alkaloid producing grass species or cultivars. Due to the significant agronomic advantages of cultivars described with host-endophyte associations that may produce this toxic alkaloid, it has been considered that an appropriate strategy is to produce low levels of this alkaloid within forage grass-endophyte cultivars. This is to maintain high levels of adequate biotic and abiotic stress tolerance. There have been a number of attempts in both ryegrass and tall fescue to achieve this utilising various strains of endophyte.

The original strategy for a novel ryegrass-endophyte association was to discover, or select, a strain of endophyte which did not produce lolitrem B, yet still produce peramine which had no known detrimental effects on animal health (Fletcher 2012). Tapper and Latch (1993) reported the first novel endophyte which would fulfil the criteria required. This was subsequently inoculated into several endophyte-free perennial ryegrass cultivars. Other novel endophyte-ryegrass cultivars were sown into multiple evaluation trials for grazing trials (sheep primarily) and invertebrate pest

monitoring by AgResearch, New Zealand. Fletcher (2012) described that no ryegrass staggers were observed in these initial grazing trials which was expected because of the absence of lolitrem B in this specific ryegrass-endophyte association. Furthermore, Popay and Latch (1993) outlined that the same cultivars had good resistance against a number of ryegrass pests, and attributed this result to the continued expression of peramine alkaloid.

The success of the early 1990s grazing and insect resistant trials resulted in the first novel endophyte ryegrass cultivars being commercialised and the novel endophyte (AR6 strain) was given a trademark name of Endosafe™. However, not long after its commercial release sheep grazing one of the cultivars of Endosafe™ portrayed poor animal health performance in the form of heat stress and lameness. This was directly associated with ergot alkaloid associated toxicosis and subsequent herbage analysis revealed highly toxic concentrations of ergovaline (Fletcher 2012). It could be argued that ergovaline expression was essentially overlooked in any selection of this endophyte because of the clear absence of ryegrass staggers in grazing animals and the toxicity effects of ergovaline were not observed or recorded in the trials in Canterbury (New Zealand). The effects observed in commerce were confirmed under trial conditions and one of the cultivars was withdrawn from the market. However, the case was not the same for a second tetraploid cultivar inoculated with Endosafe™, which produced much lower levels of ergovaline and was considered safe for grazing and was continued to be marketed successfully (Fletcher 2012). Fletcher (2012) highlighted that from the experience with the AR6 strain Endosafe™ in different background host ryegrass cultivars, there was a risk of developing new novel endophyte-grass associations. These risks were not only confined to the variation in the profile of alkaloids present, but also the inconsistencies of the subsequent concentrations expressed due to host-genetic control.

2.6.2 Selection for reduced ergovaline in tall fescue

Genetics of the grass host has a significant influence on the expression of the secondary metabolites associated with the endophyte strain. This association was discussed in a number of studies (Latch 1994; Easton *et al.* 2002; Easton 2007) and demonstrated in experiments at the plant genotype level (Roylance *et al.* 1994; Adcock *et al.* 1997; Bouton *et al.* 2002; Hill *et al.* 2002). Considerable effort has been made in selecting for low ergot alkaloids in WT endophyte in tall fescue in the USA by Hill and co-workers. The focus was predominantly due to the savage nature of the particular WT strain and the severe animal toxicosis associated with grazing this naturalised WT endophyte.

Roylance *et al.* (1994) commented that a breeding strategy designed to mitigate endophyte toxicosis in tall fescue would be conceivable if the other beneficial endophyte products, including peramine, are not indirectly reduced or impacted. The authors suggested that before any manipulation of alkaloid expression is attempted, an understanding is required of how ergovaline and peramine are

regulated within a host plant of endophyte-infected tall fescue. This relationship was investigated by Roylance *et al.* (1994) among progeny of reciprocal crosses between high- and low- ergovaline and peramine plant-endophyte combinations. These researchers found that the endophyte derived alkaloids are independently regulated and are controlled by both plant and endophyte genotype. Therefore, ergovaline and peramine are produced independently from one another; which the authors suggested that selecting and breeding in tall fescue for low ergot alkaloid concentration is unlikely to indirectly effect peramine concentration.

Selection for low levels of ergovaline in endophyte-infected tall fescue is highly heritable. An experiment by Adcock *et al.* (1997) successfully reduced the ergovaline alkaloid concentration by 86% in the low alkaloid population after two generations of selection. The objectives of Adcock's *et al.* (1997) study was to: a) determine maternal and paternal effects as estimates of plant and endophyte genotype interactions for ergot alkaloid phenotype (Experiment 1); and b) calculate the heritability of the ergot alkaloid trait in divergently selection endophyte-infected populations in tall fescue (Experiment 2). In Experiment 1, diallel crosses were made among four endophyte-infected tall fescue plant genotypes at the University of Georgia, USA. In experiment 2, seed from cv. 'Jesup Improved-EI' (endophyte-infected) tall fescue was propagated and 630 randomly selected seedlings were transferred to the field in Georgia (USA) and 45 plants were selected each for low and high alkaloid concentration based on enzyme-linked immunosorbent assay (ELISA) absorbance values. Subsequently each of those two groups was polycrossed (random genetic recombination) separately. This procedure was then repeated for a second cycle the following year based on ergovaline concentration.

The result from Adcock's *et al.* (1997) first experiment suggested large male, female, and male by female interactions. The authors suggest that because endophyte is maternally transmitted, the pollen parent's influence on ergot alkaloid concentrations can only be genetic. If there was no effect, each progeny would have the same concentrations as the female parent. Continued discussion outlined that as a consequence of maternal inheritance of endophyte in seed (i.e. no endophyte in pollen), a potential source of variation is endophyte associated with the female parent. Further to this, the variance in concentration of alkaloid from progeny to that of the female parent may also be attributed in part to genetic recombination of genes from the maternal and paternal, suggesting a second source of variation in alkaloid concentration. The diallel cross results showed that ergot alkaloid concentration in the progeny ranged from 0.07 ppm to 2.4 times the progeny mean, leading to the conclusion that high alkaloid expression is likely only when the endophyte and plant genotype have the genetic capacity to complement each other for alkaloid production (Adcock *et al.* 1997).

The second experiments results clearly demonstrated that progress can be made when selecting for high or low ergot alkaloid containing plant populations. For example, Table 2.1 showed that the means of selected population in selection cycle 1 differed by as much as 30% of the base population. Divergent selection was clearly successful from the base to the first generation and to the second generation. Heritability estimates were 0.56 and 0.49 for the low- and high-alkaloid populations for the first generation respectively, whereas realised heritability's for the second generation were 0.91 and 0.45 for the low- and high-alkaloid containing populations respectively. Given the data presented and calculated heritability, selection for lower levels resulted in greater genetic gain than selection for high levels. Adcock *et al.* (1997) suggested the apparent low-alkaloid trait is controlled by multiple genes that could be additive, recessive, or both, and therefore because of the auto-allohexaploid nature (2n = 6x = 42) of tall fescue perhaps gene action may be difficult to assess.

Table 2.1 Mean ergot alkaloid concentration of population and families within high and low ergot alkaloid populations of tall fescue. (Adcock *et al.* 1997).

		Ergot Alkaloid				
		Generation 1		Generation 2		
Population	N[a]	Raw data (µg/kg)	Transformed [$\log_{10}$(µg/kg)]	N[a]	Raw data (µg/kg)	Transformed [$\log_{10}$(µg/kg)]
Low	37	1231	2.92	48	237	2.13
Base	25	1722	3.08	48	1223	2.92
High	44	2304	3.19	48	1662	3.14
lsd (0.05)[b]	low vs. base	1092	0.12	*lsd* (0.05)	896	0.10
	high versus base	1061	0.11			
	low vs. high	754	0.08			

[a]Number of families used to characterise mean ergot alkaloid concentrations within the low and high population; number of individuals used to characterise alkaloid concentration for the base population of Jesup-Improved.

[b]Least significant difference at the 0.05 level of probability

The conclusions drawn by Adcock *et al.* (1997) are significant for a number of reasons. Firstly, the fact it was easier to make progress towards lower concentration than higher ergot alkaloid concentration (Table 2.1) led the authors to suggest the higher levels are important for persistence in agricultural ecosystems and as a consequence have naturally selected for this. They further acknowledged that little is understood as to what effect breeding might have on this relationship between the plant host-endophyte, and selection for low levels could possibly result in an affiliated selection for plants antagonistic to endophyte, which was also earlier alluded to by Hill (1993). Therefore populations with lower levels of ergot alkaloid may not be as agronomically productive or persistent as the original endophyte-infected base populations and will need field validation to determine this prior to commercial release of low-selected populations. Nevertheless, this study

clearly indicated that host plant genetics were involved with the low alkaloid trait, and was not strictly endophyte regulated.

A subsequent series of field studies was conducted in Georgia (USA) by Hill *et al.* (2002) to examine yield, alkaloid stability, stand survival and animal toxicity in tall fescue bred for high- and low- ergot alkaloid concentration, as well as a comparison with the WT naturalised strain. The tall fescue germplasm used in these experiments had been previously selected in work described by Adcock *et al.* (1997). The low-ergovaline selected population (GA-961) resulted in less plant survival than the WT strain (cv. 'Jesup E+'), but had significantly greater (P<0.05) survival than the endophyte-free strain (cv. 'Jesup E-'). This is clearly demonstrated in Table 2.2 where stand depletion in year one had all selections (both for low- and high-) having better stand survival than Jesup E-. Only the GA-962 (selected for high-ergot alkaloid) was equivalent to Jesup E+ in the second year. Therefore, selection for reduced ergot alkaloid was detrimental in comparison to Jesup E+, but nevertheless was still superior to Jesup E-. The authors highlight that persistence in this case was driven by abnormal dry conditions and aggressive encroachment by bermudagrass (*Cynodon dactylon*). This confirms Hill's (1993) and Adcock's *et al.* (1997) earlier suggestion that selecting for reduced ergot alkaloid concentration may compromise agronomic performance such as persistence in this case.

Table 2.2 Stand survival of tall fescue genotypes in grazed swards. (Hill *et al.* 2002).

	Date Sampled		
Genotypes	7 May 1998	18 May 1999	7 Dec 1999
	%stand[†]		
Jesup E+	97.8	74.3	47.5
Jesup E-	95.5	29.1	10.7
GA-961	98.2	64.0	29.2
GA-962	98.0	82.0	40.3
LSD (P < 0.05)	5.7	15.9	10.3
CV (%)	5.3	28.5	47.5

[†]Percentage of the 10-cm increments of the original 4.5 m long drilled rows in the sward occupied by live tall fescue tillers

The animal grazing experiments produced mixed results for the low-selected ergot alkaloid cultivar (Hill *et al.* 2002). Improved performance in GA-961 (low-alkaloid) was reported in comparison to Jesup E+ (high WT endophyte). However, it was still inferior to the nil endophyte treatment. For example, both animal gain and serum prolactin had significant differences (P<0.05) for the treatments in the lamb grazing experiments. During a 10 week spring period, the study showed symptoms of ergot alkaloid toxicity by the significantly (P<0.05) depressed serum prolactin levels in the cattle grazing both Jesup E+ and GA-961 treatments. Nevertheless, the GA-961 still had

significantly (P<0.05) higher weight gain by 29.3 g/head/day. In addition the level of ergot alkaloids in GA-961 forage were significantly less (P<0.05) than Jesup E+, but still levels were apparently high enough to 'place the animals in a state of toxicity' (Hill *et al.* 2002) given the prolactin results. The authors concluded by questioning whether breeding for reduced alkaloid concentration is an appropriate method given the animal toxicity and stand reduction still observed in the low-alkaloid producing germplasm.

2.6.3 Selection for reduced ergovaline in perennial ryegrass

Large variations within a population exist at each individual genotype for ergot alkaloid concentration in perennial ryegrass. For example, Latch (1994) reported on a study where 19 seedlings of cv. 'Grasslands Nui' perennial ryegrass were infected with one strain of *N.lolii* and ergovaline concentration (in leaf sheath) ranged from 2.5 to 27.2 ppm in each plant, with a mean of 10.7 ppm. Easton and colleagues provided further evidence that host influences on alkaloid concentration are also highly heritable traits in perennial ryegrass and showed significant variation among parents and full-sib families for concentration of ergovaline and peramine.

Easton *et al.* (2002) investigated the host genetic influence on endophyte activity in perennial ryegrass on progeny seedling families of partial diallel cross and their 12 parent clones in a comparative glasshouse experiment. This host influence on both the intensity of infection and alkaloid concentration was evident from the regression of progeny on the mid-parent values. For example, Table 2.3 showed the mean concentration (of two harvests) of ergovaline, peramine and ELISA, correlated with their mid-parent values (r = 0.79, 0.92 and 0.84 respectively, P<0.0001 in all cases). These workers showed that the respective regression coefficients of family means on mid-parent values were not different from 1.0 for ergovaline and peramine (0.91 and 1.04 respectively), which indicated high heritability for infection intensity and alkaloid concentration traits. The result for ergovaline in particular was better explained using parent values from another trial (not presented) with a value of 0.64, an independent assessment of narrow-sense heritability. The authors stated this result is in agreement with the estimate from the analysis of variance.

Table 2.3 Correlation coefficients between alkaloid concentrations and ELISA readings, and between harvests[†]. (Easton *et al.* 2002).

	EV - H1[††]	Per - H1	ELISA	Ev - H2	Per - H2
EV - H1		0.67	0.52	0.73	
Per - H1	0.78		0.56		0.72
ELISA	0.64	0.81			
EV - H2	0.92				0.79
Per - H2		0.84		0.92	

[†]Above diagonal: correlation between plot values, with 70 degrees of freedom. Below diagonal: genetic correlation (pair factor in ANOVA), with 16 degrees of freedom.
[††] EV = ergovaline; Per = peramine; H1 = Harvest 1; H2 = Harvest 2.

Significant general combining ability (GCA) and smaller specific combining ability (SCA) effects showed further evidence of the host genetic control in perennial ryegrass populations. Analysis of variance GCA means square test indicated a major component of the host genotype effect on mycelium density (ELISA) and accumulation of endophyte-derived alkaloid is a simply inherited direct effect of a parent on its progeny (Easton *et al.* 2002). Interaction between the two parents was indicated through a residual SCA for both ergovaline and peramine. Easton and co-workers discussed it was either through dominance or epitasis affects. SCA effects were nonetheless significant (P<0.05), but much smaller and there were no differences between the two seed parent sub-sets within the full-sibling families. Therefore there was no evidence of specific maternal effects.

An extended diallel analysis indicated no directional dominance and that the alleles for high value at different loci may be either dominant or recessive (Easton *et al.* 2002). Nevertheless, the study showed that an additive-dominance model satisfactorily accounted for ELISA in the peramine data, but not ergovaline, as the regression of array covariance on variance was not close to unity. It was discussed that because data for ergovaline was similar to other data for ELISA and peramine, there is limited evidence for epitasis or other complex gene actions.

Easton *et al.* (2002) showed that in perennial ryegrass, additive heritable elements are the major factors in the genetic control of the ergot alkaloid ergovaline. In addition, this control is also exercised over peramine concentration. This was determined for a substantial set of interrelated families and for two harvests in different seasons. This supported the earlier work by Hill and co-workers (Adcock *et al.* 1997; Roylance *et al.* 1994) who concluded that the host plant genome exercised significant control over the concentration of the ergopeptides produce by endophyte in tall fescue.

Easton *et al.* (2002) found no evidence of specific maternal effects. This conflicted with the data from Adcock *et al.* (1997) and Roylance *et al.* (1994) in tall fescue, which showed stronger maternal effect than paternal specifically on ergot alkaloid concentrations and clearly outlined that general genetic effects from both parents are more important than specific maternal affects. It should be highlighted that Easton's *et al.* (2002) data only involved one endophyte strain. In contrast the tall fescue data presented by Adcock *et al.* (1997) and Roylance *et al.* (1994) was in different endophyte genotypes. Importantly their analysis showed the greater maternal effect reflected an influence of endophyte genotype. Easton *et al.* (2002) hypothesised that self-fertilisation could lead to maternal effects. However, the absence of evidence for maternal effects in their work in perennial ryegrass confirmed there was little, or no, self-fertilisation of the parent plants. Another possibility of variation discussed was the potential differences between seed parents of their compatibility with endophyte and subsequent transmission of endophyte to the seed and the seedlings. Because all parents were infected with a common strain of endophyte, there should have been no differences in variation of endophyte type amongst parents in their particular study. It has been shown that parent plants of the same cultivar may vary in their ability to transmit the endophyte strain to their seedling progeny (Hill *et al.* 2005).

Mace and Baker (2012) studied the effect of inter-plant and inter-population variation of peramine, indole diterpenes and the ergot alkaloids (chanoclavine and ergovaline) with the same endophyte WT strain in the same background perennial ryegrass cultivar. Although only two alkaloids in the ergot alkaloid pathway were examined, this relationship was shown to be very different and interesting because they represent the start and the end product of the pathway. The authors suggest further work is warranted on the intermediate alkaloids to better understand the inter-plant variations in different endophyte types.

It could be argued that these recent studies in perennial ryegrass have shown that host plant selection may enable development of pastures with lower levels of the toxic secondary metabolites, but still retain the beneficial attributes required for productivity and persistence. Easton *et al.* (2002) concluded that effective development of low alkaloid levels in new cultivars will depend on genetic expression that will need to be stable in varying environmental conditions.

2.7 Interaction with mycelium density

Expression of secondary metabolite alkaloids and subsequent production throughout the year is strongly associated with the growth of the endophyte within the host plant. Mycelium density or hyphal mass and is analysed in perennial ryegrass either through ELISA technology (Ball *et al.* 1995; Easton *et al.* 2002; Faville *et al.* 2007) or counting of fungal hyphae within grass herbage material (di Menna *et al.* 1992; Norris *et al.* 2007), and tall fescue (Adcock *et al.* 1997). Evidence suggests that

the density of endophyte influences the alkaloid production seasonally, which in turn has undesirable animal toxicity effects. Furthermore it can also contribute to the explanation of reported alkaloid variation in experiments (Reed *et al.* 2011b). Mycelium density has also been determined as a highly heritable trait for selection in both perennial ryegrass (Easton *et al.* 2002) and tall fescue (Norriss *et al.* 2007).

The interrelationships between the endophyte density, lolitrem B and peramine was examined over a 12 month period in 17 individual ryegrass plants infected with WT endophyte by Ball *et al.* (1995). Regression analysis in this experiment showed that the yearly mean concentration of lolitrem B and peramine in individual plants was closely related to the year mean concentrations of the associated endophyte. Among the individual plant samples, the variation in alkaloid concentration of lolitrem B (five-fold) and peramine (six-fold) was associated with variation of hyphal density. For example, Figure 2.5 showed that the yearly mean lolitrem B and peramine concentration in the basal and regrowth was significantly (P<0.001) positively correlated with the yearly mean mycelium density (denoted by '*Acremonium lolii* concentration μg/g'). Seasonal changes in the mycelium density were reported to be similar to other previous studies (Fletcher 1983; Fletcher 1986) which used the ELISA method, and di Menna and Waller (1986) who used the hyphal-count method. Ball *et el.* (1995) discussed that despite the different methodologies used in previous experiments, and that location and timing of sampling varied considerably, it is apparent that mycelium density in perennial ryegrass changes with time and the seasonality of this is generally consistent. This work provides further indication that mycelium density should be an important consideration when selecting or breeding for desirable grass-endophyte associations. The absence of any ergovaline analysis or other ergot alkaloid work is a limitation to this study, which may have provided some beneficial information in breeding selection work on this alkaloid. Nevertheless, Ball *et al. (*1995) still showed that ergovaline concentrations of eight plants in spring were correlated with mycelium density (r = 0.810, P<0.01), but not with the annual means.

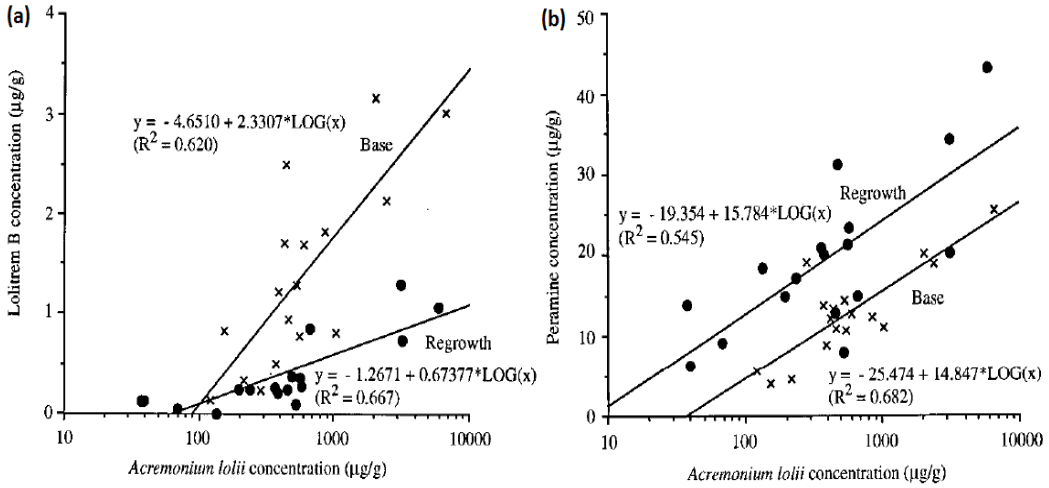

Figure 2.5 Correlations between yearly mean *A.lolii* and (a) lolitrem B and (b) peramine concentrations in basal and regrowth components of individual perennial ryegrass plants. (Adapted from Ball *et al.* 1995).

Changes in temperature have been shown to have a direct effect on mycelium density. di Menna and Waller (1986) showed this in New Zealand where seasonal variation in mean counts of perennial ryegrass endophyte hyphae at three sites was generally related to the variation in mean monthly temperature. According to Ball *et al.* (1995) the concentration of mycelium density seems to be at its peak during plant reproductive development (September to January) which can coincide with periods of high PRGT. However, at a fourth site in di Menna and Waller's (1986) study, the mycelium density counts were reduced during the mid-summer to early-autumn period. Summer moisture stress was thought to be responsible for this decline. Interestingly, Fletcher (1986) showed evidence that increased rainfall enhances the growth of the endophyte and this is possibly what occurred at the other three higher rainfall sites in the previous studies.

It is clear there is an association between certain alkaloids phenotypic responses and their association to mycelium density, therefore alkaloid production is in part dependent on the quantity of mycelium present in the host tissue. For example, Reed *et al. (*2011a) suggested mycelial mass accounted for 19-20% of the variation in the concentration of ergovaline and lolitrem B in perennial ryegrass. There are however conflicting reports on the actual control that mycelium density has on the expression of these alkaloids. Easton *et al.* (2002) showed that using semi-quantitative ELISA, mycelial mass per unit herbage dry weight was under host control, and this accounted for 41% and 65% of variation in ergovaline and peramine concentration respectively. The authors concluded that this was a function of mycelial mass. However, there was no consistent relationship between mycelial mass and ergot alkaloid concentration infected with a common endophyte strain in tall fescue studied by Hiatt and Hill (1997). These authors found no correlation between herbage mass and alkaloid concentration or fungal protein quantity and alkaloid concentration in plants of the

same genotype, suggesting that selection for plants for low alkaloid concentration (i.e. ergot alkaloids) resulted in little or no antagonistic effects on the endophyte-host association expression of mycelial mass.

Lane *et al.* (1997b) reported on the herbage analysis of alkaloid concentration on one sub-experiment looking at seed yield plots. However, there was no discussion of the subsequent seed crop or transmission of endophyte alkaloid concentration or mycelium density to the subsequent seed progeny, which can also be influenced by nitrogen. For example, Stewart (1986) showed that the highest rate (100 kg N/ha) of applied N significantly (P<0.05) reduced the concentration of endophyte mycelium in harvested seed. There was no subsequent effect on the infection rates of the endophyte.

2.8 Aims and significance of this study

Perennial ryegrass is the main cool season perennial pasture species sown in Australia and New Zealand. In *N.lolii*, ergovaline is the primary ergopeptine produced by endophyte strains that have the ability to express this alkaloid. Most attention and effects of ergot alkaloid toxicity has been attributed mainly to the end product ergovaline (Fleetwood *et al.* 2007). However, there is a strong suggestion from the research literature that intermediate ergot alkaloids may contribute to a significant role in animal toxicity, such as the intermediate lysergic acid derivatives and simple amides (Hill *et al.* 2001), rather than solely the ergopeptines. The actual concentrations or effect of selection in breeding programmes on the intermediate ergot alkaloids is not well understood. It is clear that the emphasis in the USA has been on investigating selection in WT-infected tall fescue for reduced ergovaline, due to the severity of associations with high ergovaline and animal toxicosis. In contrast, early studies in New Zealand focussed on reducing or eliminating the Lolitrem B alkaloid in perennial ryegrass due to ryegrass staggers with the exception of the EndoSafe™ (AR6) and Endo5™ (AR5) endophyte strains. However, there are no reported studies on the effects of selection for reduced ergovaline in a perennial ryegrass-AR5 cultivar on the other ergot alkaloid concentrations in the biosynthetic pathway.

It is unclear whether selecting for reduced levels of ergovaline is inadvertently reducing or increasing levels of the other ergopeptines or other intermediate ergot alkaloids. The effect of recurrent selection for ergovaline on the regulation of other ergot alkaloid production modified in response to this selection pressure has not been reported in perennial ryegrass. Furthermore, the effect on the other classes of secondary metabolites, such as peramine alkaloid concentration and mycelial density over multiple selection cycles, has not been determined. The effect of environment on this synthesis of the AR5 ergot alkaloid pathway and peramine through recurrent selection is also unclear.

This study expects to a) contribute to knowledge of whether ergovaline and the other ergot alkaloid secondary metabolites are under independent or associated control in the host perennial ryegrass-AR5 strain endophyte; b) determine the effect that selection for low ergovaline has on the concentration and expression of the other ergot alkaloid metabolites in this biosynthetic pathway; c) determine the concentration of peramine and relationship to the other ergot alkaloids following two cycles of recurrent selection for low ergovaline; d) investigate how the selection for low ergovaline effects mycelial density and determine the influence this may have on other ergot alkaloids; e) determine whether environmental stresses (water deficit) and nutrient (nitrogen) deficit impacts on the synthesis and expression of particular secondary metabolites or densities of mycelial mass; and f) generate a terminal population of a low ergovaline producing AR5 endophyte perennial ryegrass cultivar for further evaluation.

Chapter 3

Formation of the base population and recurrent selection

3.1 Introduction

Perennial ryegrass regional evaluation trials were sown by PGG Wrightson Seeds research and development (then Wrightson Seeds (Australia) Pty Ltd) in the autumn of 2004 and 2005 at two sites in western Victoria (Australia). The 2004 trial was located at Hamilton (-37.7333°S, 142.0167°E, elevation 245m) in the south western beef-sheep agricultural zone averaging 680mm annual rainfall, drill sown on 31st May 2004. The second trial was located at Maryborough (-37.0500°S, 143.7350°E; elevation 249m) in the north western wheat-sheep agricultural zone averaging 525 mm annual rainfall, drill sown on the 16th May 2005. Both sites had contrasting soil types. Hamilton consisted of a heavy cracking grey clay-loam soil derived from basalt rock, with a pH of 5.4 ($CaCl_2$). Maryborough site consisted of a more hostile light sandy-loam soil derived from Ordovician bedrock, with a pH of 4.8 ($CaCl_2$). Both trials were fully randomised complete block designs with four replicates and treatments included new experimental cultivars as well as the most recent commercially available cultivars as controls. Not every cultivar was sown at each site. Both experiments had full establishment and early vigour scores and were managed as dryland yield and persistence trials, using sheep for rotational grazing and spelling (exclusion of sheep) over the dry summer months.

Due to severe drought and above average temperatures during 2005-2006, yield data was limited as both sites suffered from moisture deficit and heat stress over the spring-summer period. Persistence was measured using two different methodologies during the late summer-autumn period of 2006. At Hamilton, final persistence was determined 20 days after a rainfall event using ground cover percentage per meter row, with four measurements per plot. At Maryborough stand decline was observed to be more severe after only one summer. Persistence was measured by percentage of plants alive per plot. Both data sets were analysed by analysis of variance (ANOVA) using Statistix 9.1 statistical package.

This chapter specifically describes the formation of the base population from the selection and inter-pollination of surviving genotypes from the Hamilton and Maryborough trials, the recurrent selection process and results of the two selection cycles for reduced ergovaline to develop the three populations used to determine the ergot alkaloid pathway concentrations described in Chapter 4.

3.2 Materials and methods

3.2.1 Formation of the base population

The range of different germplasm origin and endophyte types sown in both the 2005 and 2006 field trials showed varying results for persistence. The consequence and explanation of the importance of genetic origin is not discussed in detail here, although Stewart (2006) provides further detailed explanation. Briefly, three key messages were observed from these two experiments: a) the tetraploid material derived from late-maturing north-west Spanish ecotypes (i.e. cv. 'Banquet WT', 'KLp204a AR5', 'Bealey NEA2') performed well at both sites in comparison to the traditional Australian selected types based on northern European ecotypes (i.e. cv. 'Camel nil' and 'Victorian WT') as shown in Table 3.1. The exception to this was cv. 'Avalon' which was significantly (P<0.05) more persistence at the Hamilton site. Interestingly 'Avalon' was originally selected and bred at the Pasture and Veterinary Institute located in a very similar environment and close proximity to the Hamilton site where the 2004 trial was located; b) Maryborough is not conducive to growing perennial ryegrass commercially, however, it provided an important site for accelerated screening for drought and heat stress for a forage breeding programme; and c) there was some observed effects of endophyte at Hamilton with cv. 'Impact' and cv. 'Bronsyn' cultivars infected with WT strains significantly (P<0.05) more persistence than the same cultivars infected with the AR1 strain. This was also observed at Maryborough site, but only for 'Bronsyn' (WT > AR1).

Due to the early stand decline in both trials of over 80% across all the treatments, elite survivors in May 2006 were selected and transplanted from both sites as parent material for new crosses. A total of 18 'KLp204a AR5' (later commercialised as cv. 'Banquet®II Endo5') surviving elite plants were dug (12 from Hamilton, 6 from Maryborough) and transplanted to the PGG Wrightson Seeds Leigh Creek research station (Leigh Creek, south west Victoria) into the field in late autumn 2006. Sixty-five 'Bealey NEA2' plants were selected from the survivors of those plots and eight plants were transplanted around each 'KLp204a AR5' plant using an un-replicated top cross grid formation. All plants were monitored throughout the year for seasonal growth and disease (i.e. crown rust) and then allowed to flower and inter-pollinate within the crossing block using ryecorn (*Secale cereale*) for pollination containment. The female 'KLp204a AR5' plants were synchronised to ensure the surrounding 'Bealey NEA2' plants flowered first to limit any inter-mating. Seed was harvested only from the 'KLp204a AR5' females as individual half-sibling families and stored at -20°C. These female half-sib parents which maternally inherited the AR5 strain endophyte were subsequently combined and formed the 'Base' population for the current experiment. The AR5 host-endophyte strain with the specific genotype association was chosen for this study as it has previously been shown to express low ergovaline, have high levels of peramine and produces no lolitrem B and therefore does not cause perennial ryegrass staggers (Mason *et al.* 2013).

Table 3.1 Final persistence measurements for the cultivars from the 2004 Hamilton trial and 2005 Maryborough sown trial.

Cultivar	Endophyte strain	Persistence† Hamilton	Persistence‡ Maryborough
		22-Feb-06	31-May-06
Avalon	WT	9.00[a]	-
Banquet	WT	7.37[b]	12.25[ab]
KLp204a	AR5	6.67[bc]	10.25[ad]
Barbaria	nil	-	2.19[h]
Bealey	NEA2	7.75[ab]	9.06[ae]
Bolton	WT	-	7.44[cg]
Boomer	nil	3.62[fg]	4.13[gh]
Bronsyn	WT	6.50[bd]	10.81[ab]
Bronsyn	AR1	4.75[eg]	6.44[eg]
Camel	nil	7.00[b]	4.50[fh]
Cannon	nil	4.50[eg]	-
Extreme	AR1	5.00[df]	7.19[cg]
Fitzroy	WT	5.00[df]	12.50[a]
FLp302	AR1	-	7.00[dg]
Impact	WT	5.32[ce]	-
Impact	AR1	3.32[g]	7.88[bf]
Kingston	WT	5.32[ce]	-
Lincoln	WT	3.37[g]	-
Matrix	WT	3.75[eg]	-
Victorian	WT	7.00[b]	10.75[ac]
lsd (0.05)		1.62	3.67

†Measured as Ground Cover Percentage (GC%) per meter row

‡Measured as percentage of plants alive per plot

3.2.2 Recurrent selection for reduced ergovaline

Eight hundred and twenty three individual seeds of the Base population were sown into dry peat-based propagation pots on the 22nd July 2008. They were saturated with water in 100 mm deep stainless steel trays and germinated in a greenhouse at the PGG Wrightson Seeds Leigh Creek research station. At the early two-leaf stage, the trays were placed outside for a period of 4 weeks to induce vernalisation. On the 24th August 2008, the empty jiffy pots (non-germinated seedlings) and weaker seedlings were discarded and all plants trimmed back to a natural height of 60 mm. Seven hundred and ninety plants were transplanted into a field nursery at the PGG Wrightson Seeds Leigh Creek research station on the 16th September 2008 and liberally watered in.

Each genotype was assigned an identification code and scored (1-9 scale) for agronomics such as growth, seed head emergence, aftermath heading and disease (predominantly crown rust) on a 3-4 week rotation. After each assessment, plants were crash grazed by sheep for a period of approximately 3 days, or until all vegetative and seed head material had been removed. Five random tillers from each genotype were sampled on the 6th November 2008 and immuno-blotted to determine presence of endophyte. A plant was determined to have no infection (negatively infected) when none of the five tillers blotted returned a positive result. Twenty-two out of the 790 genotypes were found to be negatively infected (endophyte-free) and those plants were physically removed from the nursery.

One hundred and eighty agronomic superior genotypes were identified and selected for secondary metabolite analysis on the 25th February 2009. Forty vegetative tillers, selected randomly across the plant, were harvested using a scalpel cut 20 mm above ground level. Each sample was labelled and immediately placed into an ice chest in-field and subsequently frozen at -20°C. The frozen samples were then transported to Southern Scientific Services (Hamilton, south west Victoria) for chemical analysis. Ergovaline and ergovalinine determination was performed by High Performance Liquid Chromatography (HPLC) using a modification of the procedure published by Shelby *et al.* (1997) and Shelby and Flieger (1997). The details of the modification were discussed by Reed *et al.* (2004). Briefly, an acetic acid extract of the plant tissue was extracted and cleaned on a mixed-model solid phase extraction cartridge and then eluted in methanol prior to HPLC with fluorescence detection.

Forty of the lowest ergovaline producing genotypes from the 180 plants selected for HPLC analyses were subsequently dug-up and transplanted into a new crossing block for inter-pollination following the same selection criteria and protocols from the Base population. The seed harvested from this new polycross was then bulked and named as the 'Cycle 1' population (C1).

Each individual genotype was indexed on its agronomic score and ergovaline concentration. 40 elite genotypes out of the 180 that all expressed ergovaline concentrations less than 0.4 ppm were those identified for inter-pollination. In late spring, 2010, the elite genotypes were subsequently transplanted to a crossing nursery block 6 m x 6 m, in a randomly assorted grid pattern and fully surrounded by cereal ryecorn for pollen isolation. The plants were observed and scored for stem rust and flowering time, with about 10% early flowerers removed before allowing to inter-pollinate within the polycross block. Each plant was harvested as half-sib families once the seed ripened. Post-harvest, plants were evaluated for further superior agronomic traits including re-growth potential and disease presence and any poor half-sib families were identified and discarded. 20 g of seed from the elite 32 individual half-sib families was bulked to form the C1 population and stored in the freezer at -20°C for future experiments.

Following the harvest of the C1 population (first cycle bulk) , 1018 random individual seeds were subsequently sown into seed raising potting mix, organised by five-leaflet root training cells and propagated in the greenhouse on the 23rd May 2010. In June 2010, 962 individual genotypes were transplanted to the field at the PGG Wrightson Seeds research station and liberally watered in. Similar to the first selection cycle, each individual genotype was subjected to agronomic scoring and assessment for growth, seed head emergence, after-math seed head, disease (predominantly crown rust) on a 3-4 week grazing rotation and crashed grazed by sheep. Each plant was immuno-blotted for presence of endophyte and the endophyte-free plants were discarded. On the 24th February 2011, 130 individual genotypes identified for displaying superior characteristics for agronomics were selected for ergovaline metabolite testing, following the same protocol and HPLC analysis described for the C1 population. Thirty-nine of the lowest producing genotypes from the 130 plants selected for the second cycle of HPLC analyses were subsequently dug-up and transplanted into a new crossing block for interpollination following the same selection criteria and protocols from the base population. The seed harvested from this new polycross in February 2012 was then bulked and named Cycle 2 (C2).

Following identical methodologies to the formation of C1, 39 elite genotypes out of the 130 were selected with a combination of superior agronomics and ergovaline that expressed less than 0.4 ppm ergovaline and were subsequently transplanted to a new polycross block for inter-pollination in late spring 2011. Selection for disease and heading uniformity was continued. Post-harvest regrowth and disease was assessed and poor individual half-sib families were discarded. The final 30 half-sib families were equally balanced by seed weight and combined to form the Cycle 2 (C2) population for further experimental work.

3.3 Results

Figure 3.1 shows the ergovaline (ppm) data for the 180 individual genotypes selected in the Base population. The dataset ranges from 0.03 to 4.06, with the higher frequency of genotypes (30 genotypes) being distributed in the 0.4 – 0.49 ppm range, with 80% of the genotypes less than 0.8 ppm (Figure 3.2). An exponential linear regression model was fitted to the data set (R^2 = 0.91).

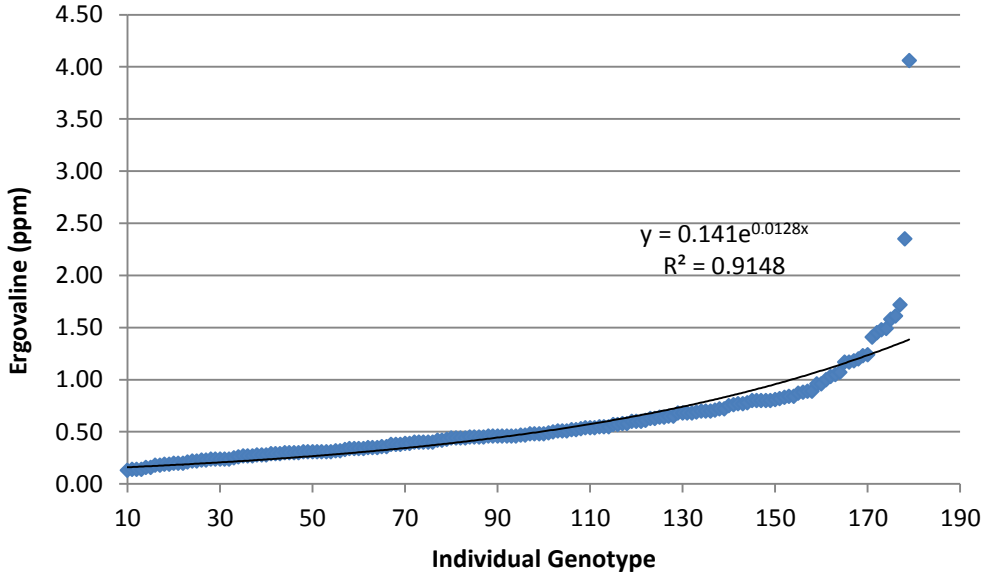

Figure 3.1 Ergovaline concentration (ppm) of the 179 individual genotypes in the first selection cycle of the Base AR5 population.

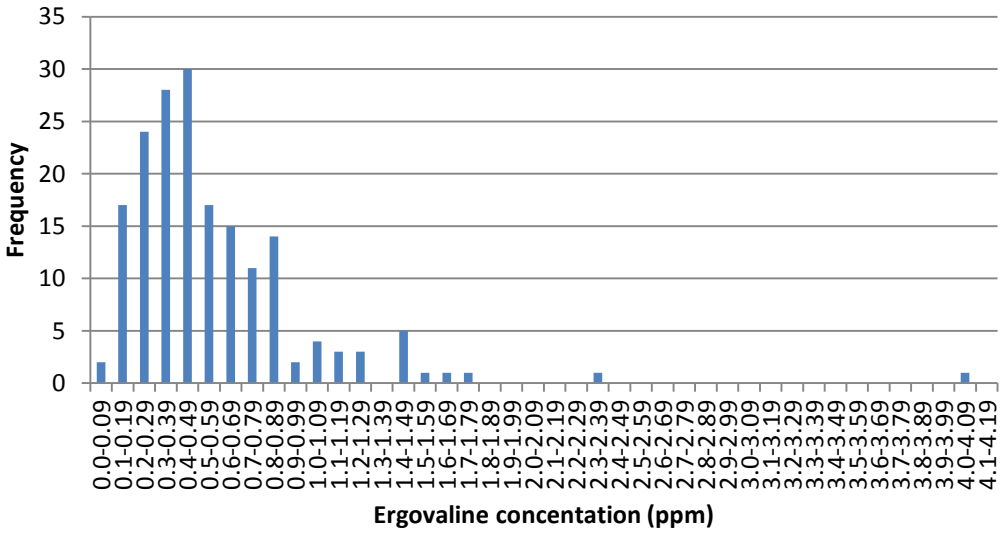

Figure 3.2 Distribution frequencies of the individual genotypes in the first selection cycle for ergovaline concentration. Ranges of 0.1 ppm are depicted.

Figure 3.3 shows the ergovaline (ppm) data for the 130 elite genotypes which expressed a very similar distribution exponential pattern (R^2 = 0.94) to that of C1, with a slightly greater range from <0.1 ppm to 4.58 ppm. The highest frequency (21 genotypes) of ergovaline expression was observed in the 0.30 – 0.39 ppm range (Figure 3.4). This represented 16% of the total population and was one range lower than the first cycle population (Figure 3.2) which represented 17% of the total population. Similarly to the first cycle, 80% of the individual genotypes measured less than 0.8 ppm ergovaline. Interestingly, the second cycle had a greater number of outliers than the first selection cycle, with 8 genotypes above 1.8 ppm in comparison to only 2 above 1.8 ppm respectively.

Although a greater percentage was observed with less than 0.1 ppm ergovaline concentration in the second selection cycle (15%) compared to the first selection cycle (1%).

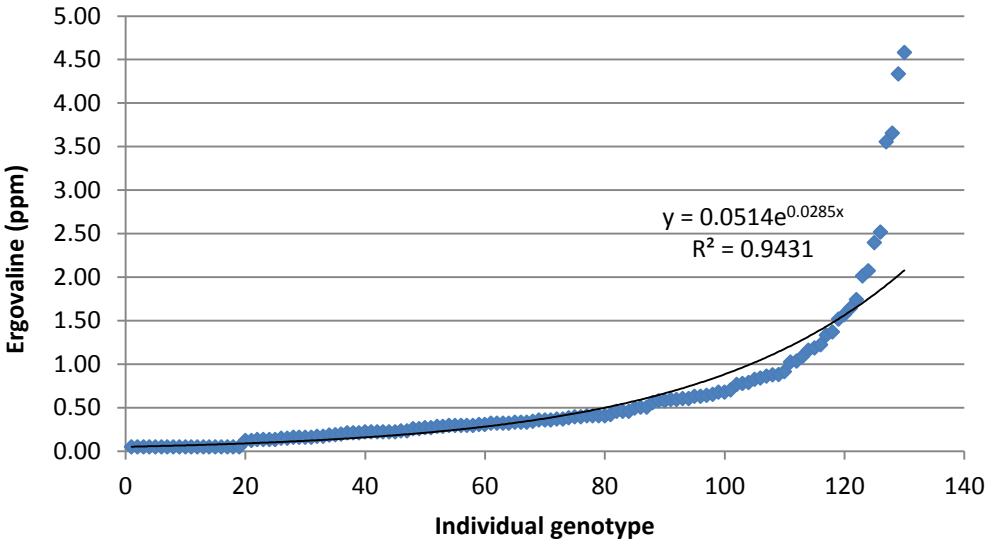

Figure 3.3 Ergovaline concentration (ppm) of the 130 individual genotypes in the second selection cycle of C1 AR5 population.

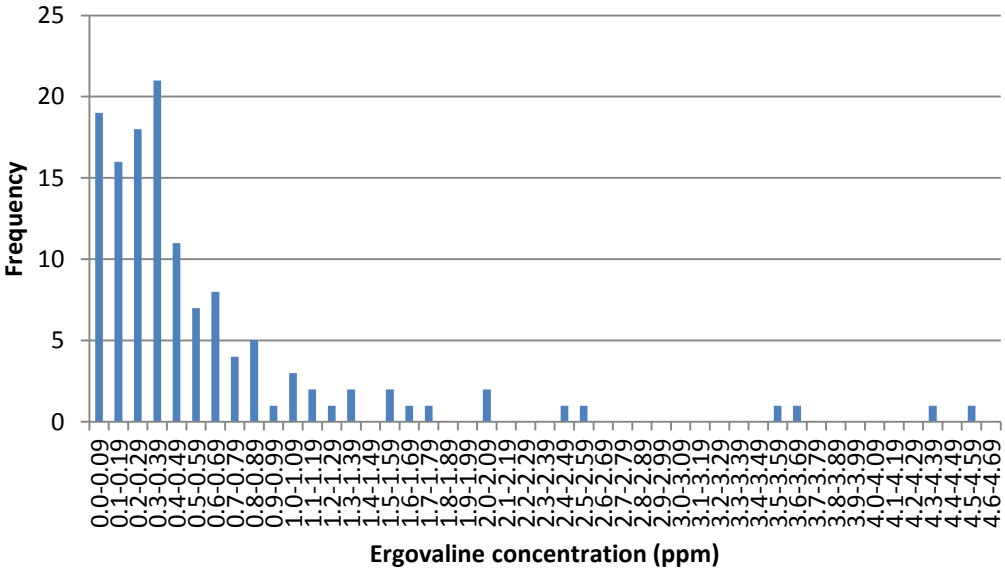

Figure 3.4 Distribution frequencies of the individual genotypes in the second selection cycle (C1) for ergovaline concentration. Ranges of 0.1 ppm are depicted.

3.4 Discussion

Both population cycles expressed similar distribution patterns but also a very large variance of ergovaline production across all the individual genotypes within the AR5 populations. The increase in

C2 showed over a 90-fold (0.05 ppm to 4.53 ppm) range in ergovaline levels. This is not surprising given it has been reported across a number of previous experiments at other locations and in different background populations. For example, Latch (1994) reported a 10-fold variation in ergovaline concentration of only 19 perennial ryegrass genotypes from one population infected with the same WT endophyte. What is surprising in the current experiment is the dramatic increase and variation in comparison to the previous studies reported by Latch (1994). However it could be argued that the population of 19 genotypes may not have the variability as the 180 and 130 genotypes analysed for secondary metabolites in the C1 and C2 populations respectively in this study. Furthermore, the selection pressure from the C1 (40 out of 180) and C2 (39 out of 130) populations would be considered very modest selection pressure.

It is very difficult to interpret and explain the differences in actual concentrations reported between the summer of 2009-10 and 2011-12, and why the C2 had some individual genotypes (eight outliers) that expressed considerably higher levels (< 1.8 ppm) than the previous C1 (Figure 3.3). The observed variability between the C1 and C2 could include large environmental effects in terms of preceding rainfall and temperature, herbage removal and nutrient status, growth phase of the plant, consequence of potential undetected stresses (i.e. insect predation) and perhaps in-field sampling techniques.

The question of how much variation there is within a plant, not just between plants within a population, needs to be examined as a potential source of data discrimination. Mace and Baker (2012) suggested that this has not been addressed and the knowledge of variation within and between plants may enable efficient sampling for breeding for low (or high) alkaloid expression. Mace and Baker (2012) used a WT strain of endophyte to study the inter-plant variation within the same population as it produces three of the four classes of endophyte alkaloid they wanted to target. These workers found that generally inter-plant variation accounted for the majority of variation, with the exception of the ergot alkaloid chanoclavine. The inter-plant variability for the ergot alkaloids chanoclavine and ergovaline was found to be 36.5 and 68.0%, whereas within-plant variation was 63.5 and 32.0% respectively. In the indole diterpene biosynthetic pathway, variability was generally attributed to inter-plant variation, with the exception of paxilline. It was highlighted by Mace and Baker (2012) that although only two alkaloids in the ergot alkaloid pathway were examined, the chanoclavine and ergovaline relationship were very different as they represent both the start and end product of the pathway and further work is warranted on the intermediate alkaloids.

Mace and Baker (2012) concluded that when sampling for secondary metabolites, it is important to take representative samples not just at the population level, but also at the plant level too. This concept was not appreciated at the time of initial samplings in the current study. Nevertheless, the

protocols applied in the current study aimed at a high number of tillers sampled (40 from each genotype) to ensure representative samples of each individual plant. Mace and Baker (2012) randomly sampled 6 tillers from each plant to achieve the 50 mg of material required for alkaloid extractions. Expression of alkaloid from each individual tiller was used to evaluate intra-plant variation. This was not analysed to this detail in the current experiment because it was not counted for in the hypothesis. However, 40 tillers were sampled from each individual genotype which should account for most of the intra-plant variation to provide relatively accurate secondary metabolite concentrations. Quantification of how many tillers are actually required to account for the inter-plant variation has not been determined in any reported studies

Selecting low producing ergovaline endophyte-genotype combinations is important to continue to reduce the mean of the population. However, when determining critical thresholds in a pasture sward situation, this may not necessarily represent accurately to what may occur in a field situation. Therefore, when determining the final cultivar, it must be done on a row or plot situation, rather than an individual genotype situation to show typical field situations.

Selecting those individual genotypes with the higher ergovaline levels (i.e. > 1.8 ppm) to recombine and evaluate would have provided some evidence towards estimated heritability that has been shown previously in tall fescue. Although greater genetic gain is achieved when selecting for lower levels rather than high levels of ergovaline (Adcock *et al.* 1997). Unfortunately this selection programme was to select reduced levels with an objective to produce a commercially viable cultivar; therefore the high ergovaline plants were discarded from the single plant field nursery. If these high ergovaline producing genotypes from the initial base cycle had been selected, recombined and reselected simultaneously to the low population, it would be hypothesised those populations would have expressed significantly high levels of ergovaline in the field experiment. For example, the 20 genotypes that produced greater than 1.0 ppm ergovaline (Figure 3.1) would have expressed significantly higher levels of ergovaline in the first and second cycle progenies than the current experiments C1 and C2. More selection could have been made to increase this concentration as it has already been shown on multiple occasions that this is a highly heritable trait (Adcock *et al.* 1997, Easton *et al.* 2002). However the effect of recurrent selection for higher ergovaline may have on the secondary metabolites is unknown. Although this was not originally developed to form part of the study, it would have provided an interesting treatment to evaluate for the selection effect on the other ergot alkaloid pathway intermediaries as discussed in the proceeding chapters. Although with little commercial relevance, perhaps any further work in alkaloid selections on intermediate pathway analysis should include a divergent treatment from the intended outcome.

Selection for higher ergovaline levels in various endophyte strains would not be desirable in commercial forage grass development programmes given the associated animal grazing implications. This is with exception to other alkaloids which are not known to cause any toxicosis to grazing animals e.g. lolines or peramine. It is however interesting to recognise that other applications for this activity does exist in the turf seed industry, specifically for amenity situations. Pennell *et al.* (2010) described the development and selection of the 'AR601' strain tall fescue associated endophyte and 'AR95' strain into a turf type perennial ryegrass. Endophyte-infected plants underwent a similar recurrent selection process to exploit increased levels of high alkaloid concentration, specifically the lolines (> 1100 ppm) and ergovaline (> 3.4 ppm). The outcome of the AR601 and AR95 strains in a turf cultivar were to induce a condition known as 'post digestion feedback', essentially a feeding deterrent in insectivorous birds such as Canada Geese (*Branta Canadensis*) and starling (*Sturnus vulgaris*). The aim was to reduce bird strike around airports and other amenity situations while maintaining insect resistance with greater sward integrity (Pennell and Rolston 2013). These unique cultivar-endophyte associations were recently commercialised successfully and released under the trademark name Avanex™.

Chapter 4

Analysis of secondary metabolites

4.1 Introduction

It is unclear whether the other intermediate secondary metabolites in the ergot alkaloid pathway are under independent or associated control in a host perennial ryegrass-AR5 endophyte association. Remnant seed from each population (Base, first cycle – C1, and second cycle – C2) was subsequently used in a field experiment to determine what affect that two recurrent selection cycles for the end-product concentration of ergovaline has on the expression of the other ergot alkaloids in the biosynthetic pathway. Furthermore there is limited evidence the effect recurrent selection for reduced ergovaline has on peramine concentrations in AR5-ryegrass associations.

Investigation was also warranted on whether environmental stresses, such as moisture and nitrogen deficit, may have on the intermediate biosynthetic pathway alkaloids or peramine. A fully replicated field experiment was established in south west Victoria to determine what effect indirect recurrent selection for reduced ergovaline had on the three populations (Base, C1 and C2) intermediate ergot alkaloids and peramine concentrations. A fourth 'Cnil' (endophyte-free) treatment was also generated and included as a control plot so that any agronomic differences in-field due to endophyte presence could be determined. A split-split plot trial design allowed for analysis in two nitrogen treatments (plus nitrogen and no nitrogen) within two separate environments (dryland and irrigation).

4.2 Materials and methods

4.2.1 Field experiment

The field experiment was sown on the 25th May 2012 at the PGG Wrightson Seeds Research Station, Leigh Creek, central-west Victoria (-37°56'S, 143°95'E). The soil type consisted of a deep red clay-loam (Krasnozem) derived from basalt rock. The mean average annual rainfall is 705 mm, with an annual mean daily temperature of maximum 16.8°C and minimum 7.7°C (Bureau of Meteorology).

The experimental site was previously sown to narrow-leaf lupins (*Lupinus angustifolius*) as a green-manure crop and fully incorporated in spring 2011 and subsequently summer fallowed with minimal tillage. Prior to sowing the site was sprayed with Roundup® PowerMax (glyphosate a.i. 480 g/L) at a rate of 1.5 L/ha, rotary-hoed and final preparation with a fine-point maxi-till cultivator. The trial was sown using single row plots with a three-point linkage, three-row single cone seeder. A starting fertiliser was applied at sowing a uniform application rate of 230 kg/ha, which consisted of 80%

sulphate of ammonia; 20% Potash 5 in 1 (42.3 kg nitrogen (N); 3.4 kg phosphorus (P); 3.7 kg potassium (K); 49.3 kg sulphur (S). Broad-leaf weeds such as wild radish (*Raphanus raphanistrum*) and wire-weed (*Polygonum aviculare*) were chemically controlled post-sowing with broadleaf selective herbicides MCPA 500® (MCPA a.i. 500g/L) and Kamba 500® (Dicamba a.i. 500 g/L) with rates of 1.5 L/ha and 0.5 L/ha respectively. The annual grass weed winter grass (*Poa annua*) was controlled post-sowing with the selective herbicide Tramat® 500 (a.i. ethofumesate 500 g/L) at a rate of 3 L/ha.

4.2.2 Experimental design

The field trial consisted of a split-split plot design with five replicates. Each individual plot consisted of a 2.2 m long single row, with row spacing of 40 cm. The first split block consisted of two environments, one with treatment for irrigation and the other dryland, spatially separated by an area of 8 m to avoid any water diffusion from the irrigation to the dryland block. The second split-block consisted of the plus nitrogen (N+) and minus nitrogen (N-) treatment within both environments. Two border rows of tetraploid perennial ryegrass buffer plots (nil endophyte) were sown to minimise any edge effect. The field trial design is presented in Appendix A.

Irrigation was applied through an overhead sprinkler system, applying 50 mm water each week from the beginning of November 2012, through until post-sampling. This maintained the irrigation block at field capacity. The dryland block received little rainfall with the exception of February 2013 with 76.8 mm. Growth was subsequently limited as a result.

The field trial was harvested using a disc-blade mower to simulate grazing and all biomass was removed down to a residual height of 40 mm. Each simulated grazing event occurred on a 3 to 4 week rotation during the spring-summer period, out to 6 weeks during the winter months. Timing of harvest was determined by leaf-stage and harvested at the 2.5 – 3 leaf stage as per best practice management for rotational grazing perennial ryegrass (Fulkerson and Donaghy 2001). Nitrogen was applied to the N+ treatment blocks by hand spreading Urea (45% N) following every harvest at an equivalent rate of 100 kg/ha Urea.

4.2.3 Generation of the nil population

The nil population (endophyte-free) was created from a subset of seed from the (C2) population. A random sample was drawn from C2 following storage in a freezer (-20°C) and heat treated to eliminate viable endophyte. 100 g of seed was placed in a porous paper bag and the opening folded down to loosely seal and positioned on a stainless steel separation screen. A glycerol-water mix was prepared (Glycerol B.P) at a 75:25 ratio (150 mL glycerol: 25 mL water) and poured into the base of a large plastic Tupperware container. A separation screen was placed inside the plastic container,

fixed above the glycerol-water mixture and the lid tightly sealed. The container was then subjected to 28 days in a drying oven at 47°C.

4.2.4 Determining endophyte frequency

All four ryegrass treatments were tested by 'grow-out' for infection with viable endophyte prior to sowing the field trial. 100 seeds from each treatment (Base, C1, C2 and Cnil) were individually grown out in seed raising mix in the greenhouse at PGG Wrightson Seeds Leigh Creek research station during March to April 2012. Each tiller from the 100 plants was sampled at 8 weeks post-sowing to determine endophyte presence using a tissue print immuno-blot assay as described by Hahn *et al.* (2003).

Sampling to detect endophyte frequency in-field occurred in November 2013 by sampling at ground level every plot and immuno-blotting to determine in-field frequency. Sampling for secondary metabolite concentration occurred on the 5th May 2013. 100 tillers from each plot were harvested just above the growing point with a scalpel in each replicate. The tillers were immediately placed inside a porous paper bag, stapled and then transferred into a container of dry-ice (-78.5°C) in the field to be immediately frozen. These samples were then stored in dry-ice and transported for freeze drying 1 day following harvest.

4.2.5 High Performance Liquid Chromatography (HPLC)

Ergovaline was detected using High-Performance Liquid Chromatography (HPLC) and conducted at AgResearch analytical laboratory, AgResearch Grasslands, Palmerston North (New Zealand). Ergovaline was extracted from plant material using a method modified from Baldauf *et al*. (2011). A sample of lyophilized and ground grass tissue (50 mg) was extracted for 1 hour with 1 mL of the prepared extraction solvent (50% methanol with 0.54 mg/mL ergotamine tartrate as internal standard). The sample was then centrifuged (8000 G^{1}, 5 min), and a 500 ml aliquot of the supernatant transferred to an HPLC vial for analysis. The limit of quantification of this technique was 0.1µg/g.

4.2.6 Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-MS/MS)

Peramine and the ergot alkaloids (chanoclavine, lysergal alanine, ergine) were detected using Liquid Chromatography – Electrospray Ionization Mass Spectrometry (LC-MS/MS) conducted at AgResearch analytical labs, AgResearch analytical laboratory, AgResearch Grasslands, Palmerston North (New Zealand). Peramine and the ergot alkaloids were extracted from plant material using the method as described by Rasmussen *et al.* (2012). A sample of 50 g ground freeze dried material was extracted with 1 mL of isopropanol: water (1:1 v/v) extraction solution. The sample was then centrifuged (8000

G[1], 5 min) for 1 hour and transferred to HPLC vial. Mass Spectrometry set to operate in positive ESI method with capillary at 275°C, the probe voltage at 5 kV and N_2 as carrier gas.

4.2.7 Statistical analysis

The secondary metabolite data was analysed using a REML analysis of variance (ANOVA) using the split-split plot function in GENSTAT 13 (GENSTAT Committee 2011).

4.3 Results

4.3.1 Weather observations

The summer of 2012-13 at the experimental site was particularly hotter and drier than the long-term average for this location. With the exception of a very high out-of-season rainfall event in February 2013 (76.8 mm), biomass production of the treatments in the block was limited. The mean monthly rainfall and monthly minimum and maximum temperatures for the experimental site can be seen in Table 4.1 and Table 4.2.

Table 4.1 Monthly mean rainfall (mm) for the experimental site 2012-13.

	2012	2013	*Mean*
January	27	2	48
February	65	77	48
March	75	24	50
April	45	17	65
May	74	57	80
June	125	67	84
July	114	99	84
August	108	100	94
September	55	90	88
October	42	78	81
November	32	68	68
December	41	31	59
Annual	802	710	849

Table 4.2 Monthly mean maximum and minimum temperatures (°C) for the experimental site 2012-13.

	Monthly mean maximum temperature (°C)			Monthly mean minimum temperature (°C)		
	2012	2013	*Mean*	2012	2013	*Mean*
January	26.6	27.3	25.1	12.5	10.8	10.9
February	25.7	27.7	25.1	12.4	12.8	11.5
March	20.5	24.4	22.2	9.8	11.9	10.0
April	19.0	19.2	17.7	6.9	7.2	7.5
May	13.6	14.6	13.6	4.6	4.8	5.7
June	10.8	11.7	10.8	3.8	3.2	4.0
July	10.9	11.4	10.1	3.5	3.7	3.2
August	11.1	12.3	11.4	3.2	4.2	3.7
September	14.6	16.0	13.9	4.7	5.9	4.8
October	17.2	16.3	16.6	5.5	5.7	6.2
November	21.6	18.4	19.6	8.4	7.8	7.8
December	23.6	23.7	22.6	10.0	8.8	9.4
Annual	17.9	18.6	17.4	7.1	7.2	7.1

4.3.2 Endophyte frequency

The immuno-blot results determined that all 3 infected treatments with AR5 had above 90% infection frequency. One hundred tillers for each strain were sampled and balanced across all replicates. Table 4.3 shows the highest was the C1 population with 100% in both the grow-out test and field test and lowest observed in the C2 with 94% field infection frequency. The 0% result in the grow-out shows the heat-treatment procedure to eliminate endophyte was successful, with an unexplained 3 % low infection frequency observed in the field sample. This may possibly be due to error in the development and interpretation of the immune-blot or in-field sample error.

Table 4.3 Endophyte percentage (%) infection frequency for the grow-out test and in-field sample for the four population treatments.

Population	% Infection Frequency	
	Grow-out	Field
Base	100	98
C1	100	100
C2	95	94
Cnil	0	3

4.3.3 Secondary metabolite concentrations

LC-MS/MS did not detect any concentrations of the intermediate ergot alkoids agrocalvine, lysegrol or lysergic acid in any of the population cycles within the AR5 strain of endophyte. There were detectable levels of chanoclavine, but the concentrations were very low across all environments and treatments which were just below the limit of quantitation. The levels therefore of chanoclavine presented should be treated with caution and used only as indicative rather than definitive results. Concentration values of ergine, lysergol alanine and peramine were above the limits of quantification, as well as ergovaline which were all determined by HPLC-fluorescence.

The expression of ergovaline increased from the Base population significantly (P<0.05) to the C1, and then decreased significantly (P<0.05) in the C2 population across all environments and nitrogen treatments (Table 4.4). The concentration of lysergyl alanine mirrored this rise and fall pattern from the Base to C1 to C2 population, with a significant (P<0.05) increase from the Base to C1, then a slight and non-significant (P>0.05) decrease by 0.05 ppm to the C2 population. None of the other secondary metabolites reflected this pattern as the means of chanoclavine, ergine and peramine increased across all environments and treatments.

Ergine concentration showed a significant difference (P<0.05) between the Base and both the C1 and C2 population, which nearly doubled from Base (7.85 ppm) to C2 (14.5 ppm) (Table 4.4). There was only a small non-significant (P>0.05) difference between C1 and C2 of 0.56 ppm. Chanoclavine also increased significantly (P<0.05) from the Base to C1, but only a very slight increase between C1 and C2 which was not significant (P>0.05). However the levels were very low of chanoclavine in comparison to the other intermediary ergot alkaloids. Peramine concentration significantly increased (P<0.05) over each population cycle across all environments and nitrogen treatments, with the greater rise observed from the Base to C1.

Table 4.4 Means of endophytic secondary metabolites (ppm) measured in three cycles of perennial ryegrass, evaluated across all nitrogen and irrigation treatments

	Chanoclavine ppm	Ergine ppm	Lysergyl Alanine ppm	Ergovaline ppm	Peramine ppm
Base	0.25^{b}	7.85^{b}	1.49^{b}	0.89^{b}	9.52^{c}
C1	0.33^{a}	13.94^{a}	2.02^{a}	1.12^{a}	12.24^{b}
C2	0.35^{a}	14.50^{a}	1.97^{ab}	0.91^{b}	13.67^{a}
lsd (0.05)	*0.04*	*1.40*	*0.26*	*0.18*	*1.20*

4.3.4 Chanoclavine

Although the detected values of chanoclavine concentration were very low, there were some significant differences observed within environment and nitrogen treatment (Table 4.5). It must be stressed that these results are more indicative, rather than definitive, as these were just below the limits of quantification for LC-MS/MS. The dryland environment expressed significantly greater ($P<$ 0.05) concentrations of Chanoclavine than the irrigation environment (Table 4.6) in both plus and minus nitrogen treatments and was almost double from the Base to the C2 population. In the dryland environment with no nitrogen treatment, chanoclavine increased significantly ($P<0.05$) between the Base and C2 population. Significant increases ($P<0.05$) in chanoclavine were measured in the irrigation environment with plus and minus nitrogen treatments from Base to C1, however in both treatments chanoclavine declined by 0.03 ppm from C1 to C2.

Table 4.5 Means of chanoclavine (ppm) within environment (dryland and irrigation) and plus nitrogen treatment (N+) and minus nitrogen treatment (N-)

	Dryland		Irrigation	
	N-	N+	N-	N+
Base	0.30[b]	0.36[a]	0.15[b]	0.18[b]
C1	0.36[ab]	0.37[a]	0.27[a]	0.32[a]
C2	0.43[a]	0.44[a]	0.24[a]	0.29[a]
lsd (0.05)	*0.08*	*n.s*	*0.08*	*0.10*

Table 4.6 Means of chanoclavine (ppm) within each nitrogen treatment (nitrogen plus (+) and nitrogen minus (-)) within the dryland and irrigation environment

	Nitrogen (+)			Nitrogen (-)		
	Base	Cycle 1	Cycle 2	Base	Cycle 1	Cycle 2
Dryland	0.359[a]	0.368[a]	0.438[a]	0.299[a]	0.362[a]	0.43[a]
Irrigation	0.177[b]	0.319[a]	0.291[b]	0.151[b]	0.266[b]	0.241[b]
lsd (0.05)	*0.08*	*0.08*	*0.08*	*0.10*	*0.10*	*0.10*

4.3.5 Lysergyl Alanine

The concentrations of lysergyl alanine across the selection cycles generally increased from the Base to C1, and then decreased from C1 to C2 (Table 4.7). The exception to this was the dryland environment minus nitrogen treatment. In this particular treatment, the increase from the base to

C1 was significant (*P*<0.05), but there was no significant difference (*P*>0.05) between C1 and C2 with a slight increase of 0.14 ppm. The minus nitrogen treatment under irrigation also showed a significant increase (*P* 0.05) in the Base population to C1, but this decreased only slightly non-significantly (*P*>0.05) from the C1 to C2 population. The application of nitrogen did not result in any significant difference (*P*>0.05) in the concentration of lysergyl alanine in either environment. Under both nitrogen treatments, the concentrations of lysergyl alanine were significantly greater under a dryland stress environment than irrigation in all three recurrent populations (Table 4.8).

Table 4.7 Means of lysergyl alanine (ppm) within environment (dryland and irrigation) and plus nitrogen treatment (N+) and minus nitrogen treatment (N-)

	Dryland		Irrigation	
	N-	N+	N-	N+
Base	2.11[b]	2.14[a]	0.82[b]	0.90[a]
C1	2.94[a]	2.73[a]	1.11[a]	1.30[a]
C2	3.08[a]	2.56[a]	1.05[a]	1.14[a]
lsd (0.05)	*0.60*	*n.s*	*0.16*	*n.s*

Table 4.8 Means of lysergyl alanine (ppm) within each nitrogen treatment (nitrogen plus (+) and nitrogen minus (-)) within the dryland and irrigation environment

	Nitrogen (+)			Nitrogen (-)		
	Base	C1	C2	Base	C1	C2
Dryland	2.14[a]	2.73[a]	2.60[a]	2.11[a]	2.94[a]	3.08[a]
Irrigation	0.90[b]	1.31[b]	1.14[b]	0.82[b]	1.11[b]	1.05[b]
lsd (0.05)	*0.60*	*0.60*	*0.60*	*0.60*	*0.60*	*0.60*

4.3.6 Ergine

The concentrations of ergine detected were higher than both chanoclavine and lysergyl alanine, with the lowest being 4.97 ppm recorded in the Base population minus nitrogen irrigation treatment, and highest up to 19.08 ppm detected in C2 of the dryland minus nitrogen treatment (Table 4.9). In the dryland environment minus nitrogen, the concentration of ergine increased significantly (*P*<0.05) in each selection cycle, which resulted in the concentration almost doubling from 9.86 ppm in the Base to 19.09 ppm in C2. With the addition of the nitrogen treatment under the dryland environment, the significant increase (*P*<0.05) from the Base to C1 population of 7.47 ppm was in a similar magnitude to the nitrogen minus treatment (6.63 ppm).

No significant differences in the nitrogen plus treatment were observed in the irrigation environment, especially given the considerable increase in concentration from the Base to C1 population of 5.69 ppm. There was only a minor decrease (0.04 ppm) from the C1 to C2 population. The minus nitrogen treatment under irrigation did however show a significant increase (P<0.05) from the Base to C1 population of 4.55 ppm, although this was less than the increase seen in the nitrogen plus treatment. The C1 to C2 population decreased in concentration but was not significant (P>0.05).

Ergine concentration in the dryland environment was significantly higher (P<0.05) than the irrigation environment across each nitrogen treatment and across in selection cycle populations (Table 4.10). The addition of nitrogen slightly increased the concentration of ergine in each of the selection cycles in both environments but were not significant (P<0.05), with the exception of the C2 population in the dryland environment.

Table 4.9 Means of ergine (ppm) within environment (dryland and irrigation) and plus nitrogen treatment (N+) and minus nitrogen treatment (N-)

	Dryland		Irrigation	
	N-	N+	N-	N+
Base	9.86^{c}	10.32^{b}	4.97^{b}	6.26^{a}
C1	16.49^{b}	17.79^{a}	9.52^{a}	11.95^{a}
C2	19.08^{a}	18.63^{a}	8.35^{a}	11.91^{a}
lsd (0.05)	*2.00*	*4.00*	*2.00*	*n.s.*

Table 4.10 Means of ergine (ppm) within each nitrogen treatment (nitrogen plus (+) and nitrogen minus (-)) within the dryland and irrigation environment

	Nitrogen (+)			Nitrogen (-)		
	Base	C1	C2	Base	C1	C2
Dryland	10.32^{a}	17.79^{a}	18.63^{a}	9.86^{a}	16.49^{a}	19.08^{a}
Irrigation	6.26^{b}	11.95^{b}	11.91^{b}	4.97^{b}	9.52^{b}	8.35^{b}
lsd (0.05)	*4.00*	*4.00*	*4.00*	*2.80*	*2.80*	*2.80*

4.3.7 Ergovaline

There is a significant difference (P<0.05) amongst the means from the Base population to C2 across all irrigation and nitrogen treatments (Table 4.11). Table 4.4 showed overall there is an increase from Bas to C1 and then a decrease from C1 to C2. Table 4.11 shows that the patterns holds for all treatment combinations but is not significant (P>0.05) when examined separately as no significant

differences (*P*>0.05) in ergovaline concentration was detected between any of the population cycles, or within environments or between treatments (Table 4.11). In each environment across all treatments, generally ergovaline increased from the Base to C1, then decreased from C1 to C2 populations. The nitrogen treatment in each population and both environments showed a slight increase in concentration in the dryland, and greater increase in the irrigation environment. The only exception being the C2 population where the nitrogen plus treatment had a lower ergovaline concentration, which was similar to the situation with ergine. The concentration of ergovaline was higher in the dryland environment than irrigation in each population cycle in both nitrogen treatments (Table 4.12).

Table 4.11 Means of ergovaline (ppm) within environment (dryland and irrigation) and plus nitrogen treatment (N+) and minus nitrogen treatment (N-)

	Dryland		Irrigation	
	N-	N+	N-	N+
Base	1.03[a]	1.10[a]	0.55[a]	0.86[a]
C1	1.25[a]	1.30[a]	0.77[a]	1.15[a]
C2	1.14[a]	0.99[a]	0.66[a]	0.87[a]
lsd (0.05)	*n.s.*	*n.s.*	*n.s.*	*n.s.*

Table 4.12 Means of ergovaline (ppm) within each nitrogen treatment (nitrogen plus (+) and nitrogen minus (-)) within the dryland and irrigation environment

	Nitrogen (+)			Nitrogen (-)		
	Base	C1	C2	Base	C1	C2
Dryland	1.10[a]	1.30[a]	0.99[a]	1.03[a]	1.25[a]	1.14[a]
Irrigation	0.86[a]	1.15[a]	0.87[a]	0.55[a]	0.77[a]	0.66[a]
lsd (0.05)	*n.s*	*n.s*	*n.s*	*n.s*	*n.s*	*n.s*

4.3.8 Peramine

Generally peramine concentration increased in all selection cycles across environment and within nitrogen treatments (Table 4.13). There was a significant increase (*P*<0.05) from the Base to C1 population in the dryland minus nitrogen treatment, but from C1 to C2 this was only a minor (0.8 ppm) increase. The plus nitrogen treatment in the dryland environment also increased. The Base to C1 and C1 to C2 were both non-significant (*P*>0.05), however from Base to C2 was significant. Under irrigation, the minus nitrogen treatment expressed a similar pattern of concentrations to the dryland

environment, with a significant increase (P<0.05) between the Base and C1, but only a very minor increase from the C1 to C2 population. On the other hand, each selection cycle significantly increased (P<0.05) in the plus nitrogen irrigation environment. The peramine concentration in the dryland environment was significantly higher (P<0.05) than the irrigation environment, in both nitrogen treatments and each selection cycles (Table 4.14).

Table 4.13 Means of peramine (ppm) within environment (dryland and irrigation) and plus nitrogen treatment (N+) and minus nitrogen treatment (N-)

	Dryland		Irrigation	
	N-	N+	N-	N+
Base	10.3[b]	11.6[b]	8.5[a]	7.7[c]
C1	15.0[a]	13.8[ab]	10.3[a]	9.9[b]
C2	15.8[a]	17.0[a]	10.6[a]	11.4[a]
lsd (0.05)	*2.0*	*4.0*	*n.s.*	*1.4*

Table 4.14 Means of peramine (ppm) within each nitrogen treatment (nitrogen plus (+) and nitrogen minus (-)) within the dryland and irrigation environment

	Nitrogen (+)			Nitrogen (-)		
	Base	C1	C2	Base	C1	C2
Dryland	11.6[a]	13.8[a]	17.0[a]	10.3[a]	15.0[a]	15.8[a]
Irrigation	7.7[b]	10.0[b]	11.4[b]	8.5[a]	10.3[b]	10.6[b]
lsd (0.05)	*2.2*	*2.2*	*2.2*	*n.s.*	*2.0*	*2.0*

4.3.9 Percentage of ergot alkaloid pathway components

Selection for reduced ergovaline in this AR5 population appears to have reduced ergovaline and lysergyl alanine and increased ergine when the ergot alkaloid components are expressed as a percentage of the total biosynthetic pathway. This pattern has occurred across all environments and nitrogen treatments. Although in the irrigation minus nitrogen treatment, the trend is reversed for ergine and lysergyl alanine in the C1 to C2 population. For example, Table 4.15 shows each selection cycle mean concentration of ergot alkaloid expressed as a percentage of the total pathway across all treatments (Table 4.15a) and within each environment and treatment (Table 4.15b, c, d and e). The expression of chanoclavine is very small relative to the other ergot alkaloids (<3%) and its contribution to the differences is difficult to interpret and should be treated with caution.

Ergine expression is a very high contributor to the total percentage of the pathway, with the Base population increasing from 74.90% to 81.78% in the C2 across all treatments. The proportion of percentage increase in the ergine component was relatively consistent across all environments, with a slight trend to be higher in the minus nitrogen treatments. For example, between the Base and C2 populations, there was a 6.26% increase in the dryland environment minus nitrogen (Table 4.15b); 8.22% in dryland plus nitrogen (Table 4.15c); 4.49% in irrigation minus nitrogen (Table 4.15d); and 7.47% in irrigation plus nitrogen.

The decrease in both ergovaline and lysergyl alanine were proportional to each other, with approximately a 3% decrease in each one across both environments and within nitrogen treatment. The total contribution to the pathway was consistent across all treatments (Table 4.15a). One exception to this was when lysergyl alanine percentage increased very slightly (0.68%) from C1 to C2 against the trend of reduction in the irrigation minus nitrogen treatment (Table 4.15d). Interestingly, this was also the same population and environment treatment that ergine very slightly decreased against the general trend of increasing proportions. This reflects the actual mean concentrations (ppm) of both these ergot alkaloids in Tables 4.7 and 4.11, but both were determined to be non-significant changes ($P>0.05$).

Table 4.15 Each alkaloid component as a percentage of the total ergot alkaloid pathway for the AR5 strain: (a) across all treatments; (b) dryland minus nitrogen; (c) dryland plus nitrogen; (d) irrigation minus nitrogen; and (e) irrigation plus nitrogen.

(a)	Across all treatments: % of Pathway			
	Chanoclavine	Ergine	Lysergyl Alanine	Ergovaline
Base	2.39	74.90	14.22	8.49
C1	1.90	80.07	11.60	6.43
C2	1.97	81.78	11.11	5.13

(b)	Dryland -N: % of Pathway			
	Chanoclavine	Ergine	Lysergyl Alanine	Ergovaline
Base	2.26	74.14	15.86	7.74
C1	1.71	78.37	13.97	5.94
C2	1.81	80.40	12.98	4.80

(c)	Dryland +N: % of Pathway			
	Chanoclavine	Ergine	Lysergyl Alanine	Ergovaline
Base	2.59	74.14	15.37	7.90
C1	1.67	80.17	12.30	5.86
C2	1.95	82.36	11.32	4.38

(d)	Irrigation -N: % of Pathway			
	Chanoclavine	Ergine	Lysergyl Alanine	Ergovaline
Base	2.31	76.58	12.63	8.47
C1	2.31	81.58	9.51	6.60
C2	2.33	81.07	10.19	6.41

(e)	Irrigation: +N: % of Pathway			
	Chanoclavine	Ergine	Lysergyl Alanine	Ergovaline
Base	2.20	76.34	10.98	10.49
C1	2.17	81.18	8.83	7.81
C2	2.04	83.81	8.02	6.12

4.4 Discussion

Recurrent selection has successfully maintained high endophyte frequency in each selection cycle (Table 4.3). As each individual genotype was sampled for ergovaline concentration, they were initially immuno-blotted to ensure the plant was infected prior to any chemical analysis and subsequent genetic recombination, as endophyte is only maternally inherited in seed and is absent in pollen. It is critical to retain high endophyte transmission in a breeding programme as endophyte frequency is a key indicator of performance of the grass plants in grazed pastures (Hume and Sewell 2014). The high frequency of endophyte-infected pastures and observation of increased frequency over time, all indicate that endophyte-infected plants are better adapted than endophyte-free plants to stresses over the lifetime of the pasture. Although it will remain a high priority to ensure tiller and

seed frequency infection remain very high in this novel endophyte-host association, small failures to colonise do occur (Welty *et al.* 1994). This could explain the 5% and 6% reduction observed in the C2 from the C1 in both the grow-out test and in-field samples respectively.

As expected, the perennial ryegrass-AR5 strain expressed ergovaline and peramine, as well as the individual intermediates in the ergot alkaloid biosynthetic pathway including chanoclavine, lysergyl alanine and ergine. None of the indole-diterpenes (i.e. lolitrem B) or other ergot alkaloids agroclavine, lysergol or lysergic acid intermediates was detected in any of the population cycles. This is consistent with the expected AR5 alkaloid profile (W.J. Mace, *pers. comm*). The concentrations of chanoclavine in the selection cycles were below the limits of detection for LC-MS/MS, therefore these results need to be considered with caution as they are more indicative rather than definitive.

Recurrent selection for reduced levels of ergovaline should show the C2 population having a lower concentration than the C1 and in turn, the C1 having a lower concentration than the Base population, regardless of treatment. This was not evident at all from the results presented in Table 4.4. This does not support the initial hypothesis of deceasing concentrations through the recurrent selection cycles. In fact, the opposite of this occurred in the Base to C1 selection cycle, where ergovaline expressed a small significant (P>0.05) increase from 0.89 to 1.12 ppm. There were no other significant increases or differences detected between the recurrent selection populations or across all environments and treatments, which was also unexpected. Further discussion of the possible contributing factors is discussed in Chapter 6. One possible explanation is the accuracy of the analytical analysis; however, ergovaline concentration was conducted through HPLC-fluorescence, which is considered the gold-standard for secondary metabolite analysis. The results are unlikely to be confounded by any analytical error.

Concentrations of lysergyl alanine across the selection cycles generally increased from the Base to C1, and then decreased from C1 to C2 (Table 4.4). The exception to this was the dryland treatment minus nitrogen. This reflected a similar trend to ergovaline which also observed an initial increase in concentration and then subsequent decrease. The concentrations of ergine detected were higher than both chanoclavine and lysergyl alanine, with the lowest being 4.97 ppm recorded in the Base population minus nitrogen irrigation treatment, and highest up to 19.08 ppm detected in C2 of the dryland minus nitrogen treatment (Table 4.9). Ergine generally increased under each environment and nitrogen treatment across each population cycle, with the exception of the C1 to C2 populations under irrigation in which each of the nitrogen treatments decreased, however this was not significant (P>0.05).

When all the ergot alkaloid concentrations are expressed as percentage components of the biosynthetic pathway (Table 4.15), selection for reduced ergovaline in the host-AR5 population has

reduced ergovaline and lysergyl alanine and increased ergine. When applying recurrent selection for reduced ergovaline, it appears there is a biochemical shunt in production from ergovaline towards ergine, rather than a down regulation or block of the whole biosynthetic pathway. The catalysts driving this shunt and an alternative pathway are proposed in Chapter 6. According to Panaccione (2005), the observed accumulation of these intermediates may result from differences in concentration and/or activities of the relevant enzymes, or partitioning of intermediates from downstream enzymes. The results here clearly support the hypothesis that the ergot alkaloid pathway is inefficient and certain intermediates do not flow rapidly through to the end pathway product ergovaline, but accumulate and as observed in this study, they accumulate as the simple amide ergine. These data support that of Panaccione (2005) who indicated that the pathway is not just a collection of enzymes operating at different rates, but rather it is closely regulated, indicating that accumulation of particular intermediates (ergine) is controlled and not random.

Peramine concentration increased with each recurrent selection cycle significantly across all environments and treatments (Table 4.4). There were significant (P<0.05) increases observed within each environment and nitrogen treatment (Table 4.13), with the exception of the irrigation minus nitrogen treatment. It appears that direct selection for reduced ergovaline indirectly increased peramine concentration significantly at each cycle across all environments and treatments. The greatest increase was observed between the Base and C1 population (2.72 ppm), which was double compared to the increase between the C1 to C2 population (1.43 ppm), both of which were significant (P<0.05). Peramine expression in perennial ryegrass has been previously shown to be a highly heritable trait (Easton *et al.* 2002), similar to that of ergovaline with additive heritable elements being major factors in the host control. Interestingly the inconsistency in ergovaline concentration following recurrent selection does not correlate at all with the significant (P<0.05) increase in peramine concentration. This indicates that peramine in this host-AR5 population are independently regulated, rather than under any associated genetic control with ergovaline.

Environment had a significant effect on alkaloid concentration. The levels of the ergot alkaloids chanoclavine, lysergyl alanine and ergine, as well as peramine showed a significant (P<0.05) increase in concentration under water deficit (dryland treatment). The exceptions being peramine Base population minus nitrogen treatment and chanoclavine in the C1 population plus nitrogen treatment. Of particular interest is that under water deficit, the nitrogen plus treatments had greater concentrations of ergovaline and some of the other intermediate ergot alkaloids. This effect was more prominent in the Base to C1 cycle, but inconsistent in the C1 to C2. This greater level of alkaloid concentration in the dryland nitrogen plus treatments may be better explained through a plant physiological response (see Chapter 6).

Chapter 5

Effect of recurrent selection on mycelial density

5.1 Introduction

There is at least some degree of correlation between the growth of endophyte throughout the plant and secondary metabolite production in perennial ryegrass (Ball *et al.* 1995; Easton *et al.* 2002; Faville *et al.* 2007) and tall fescue (Adcock *et al.* 1997). This fungal colonisation of the plant is often referred to as mycelium density or hyphal mass. This trait has also been determined to be highly heritable (Easton *et al.* 2002; Norriss *et al.* 2007) and by envirnoment (Stewart 1986). As the relationship appears intrinsictly linked, increased density coinciding with seasonal production may result in undesirable animal toxicosis effects. There is no evidence whether selection for reduced ergovaline influences the mycelial density in a ryegrass-AR5 association. Freeze dried material from the Base, C1 and C2 populations were analysed through enzyme-linked immunosorbent assay (ELISA) to determine what influence recurrent selection, environement or nitrogen treatment has on mycelial mass.

5.2 Material and methods

All freeze dried samples used in the metabolite analysis (see Chapter 4.3.3) were sent to AgResearch, Hamilton (New Zealand) for ELISA analysis to determine endophyte quantity in the three populations across all envirnonments and treatments. The semi-quantitative ELISA methodology applied here is desribed in detail by Faville *et al.* (2007). Breifly, extractions were made from 2 replications of all populations submitted and ELISA was analysed based on the following immunoreagents: Antibody (Sapu F2 anti-*N.lolii*); reference standard *N.lolii* preparation prepared by O.Ball (L Briggs, *pers. comms).* The ELISA plates were coated with *N.lolii* antigen and 1% BSA was used as a blocking agent. The presence of endophyte in the sample extracts was indicated by inhibition of specific antibody binding to coating antigen which was determined using anti-rabbit-horseradish peroxidise (HRP) conjugate. Data was presented as immunoreactive equivalents in µg/g and statistically analysed using REML analysis of variance (ANOVA) using the split-split plot function in GENSTAT 13 (GENSTAT Committee 2011).

5.3 Results

No significant differences (P>0.05) were detected in mycelial density between any of the selection cycles, across environments or within treatments (Table 5.1). Furthermore, no significant differences (P>0.05) were observed between the means of the dryland against the irrigation environment (Table

5.2). There is however slight trends which reflected some previous data observed in the intermediate ergot alkaloid concentrations. Table 5.1 shows a general increase in mycelial density across each selection cycle in each environment and nitrogen treatment, with the exception of the irrigation minus nitrogen treatment and dryland minus nitrogen treatment where the mycelial density deceased from the C1 to C2 by 0.06 and 0.25 µg/g respectively. There was a small change in the amount of mycelial density in the Base to C2 in the dryland plus and minus nitrogen, and irrigation minus nitrogen treatments, increasing by 0.50, 0.34 and 0.53 µg/g respectively. The irrigation plus nitrogen treatment on the other hand was observed to increase (not significant, P>0.05) by almost double compared to the other three treatments, with a 0.93 µg/g increase in mycelial mass. Interestingly the largest proportion of this increase (0.82 µg/g) was from the C1 to C2 population.

Table 5.1 Means of mycelium density (µg/g) ELISA within environment (dryland and irrigation) and plus nitrogen treatment (N+) and minus nitrogen treatment (N-)

	Dryland		Irrigation	
	N-	N+	N-	N+
Base	3.23[a]	3.47[a]	3.54[a]	4.25[a]
C1	3.79[a]	3.69[a]	4.31[a]	4.36[a]
C2	3.73[a]	3.81[a]	4.07[a]	5.18[a]
lsd (0.05)	*n.s*	*n.s*	*n.s*	*n.s*

Table 5.2 Means of mycelium density (µg/g) ELISA within each nitrogen treatment (nitrogen plus (+) and nitrogen minus (-)) within the dryland and irrigation environment

	Nitrogen (+)			Nitrogen (-)		
	B	C1	C2	B	C1	C2
Dryland	3.42[a]	3.59[a]	4.06[a]	3.30[a]	3.97[a]	3.82[a]
Irrigation	4.29[a]	4.46[a]	4.93[a]	3.46[a]	4.13[a]	3.98[a]
lsd (0.05)	*n.s*	*n.s*	*n.s*	*n.s*	*n.s*	*n.s*

5.4 Discussion

There appears to be no effect of recurrent selection for reduced ergovaline on mycelial density in this perennial ryegrass-AR5 association. No significant differences (*P*>0.05) were detected between any selections across all environments and treatments (Table 5.1). Nevertheless, some trends were observed. There is a general increase in mycelial density (µg/g) in each cycle of selection, both within

environment and within nitrogen treatment. This is with the exception of the C1 to C2 in both the nitrogen minus treatments across environments. There is a consistent increase in mycelial density between the two environments, with the irrigation treatment greater than the dryland treatment in all selection cycles (Table 5.2). The disparity between the two environments mycelial density appears to be slightly greater in the nitrogen plus treatment (average 0.87 μg/g) than without nitrogen (average 0.16 μg/g). One possible explanation is this was a function of greater biomass production of the irrigation environment plus nitrogen treatment, over the dryland environment minus nitrogen treatments. This observation is supported by Fletcher (1986) who showed evidence that increased rainfall does enhance growth of the endophyte within the host-plant. Furthermore, di Menna and Waller (1986) also showed evidence that summer moisture deficit can reduce density counts.

Based on this evidence it could be argued that the host-genetic control for mycelial mass is independent to the ergot alkaloid pathway regulation. As mycelial density was not affected, there is further evidence that there is tight genetic control of the ergot alkaloid pathway independent of mycelial mass. This result opposed the hypothesis that a reduction in ergovaline may have been associated or contributed to a decrease in endophyte density, as this is influenced by genetic background provided by the host plant. This was suggested because it is known that direct selection for increased mycelial density is highly heritable (Adcock *et al.* 1997; Easton *et al.* 2002); therefore direct selection for reduced mycelial density would probably subsequently decrease secondary metabolite concentrations as well. This would be a direct function of alkaloid expression and subsequent seasonal production being strongly associated with the growth of endophyte within the plant (Easton *et al.* 2002), rather than any recurrent selection influence on mycelium density.

Previous studies in perennial ryegrass have shown that mycelial density accounted for 19% (Reed *et al.* 2011b) and 41% (Easton *et al.* 2002) of the variation in ergovaline concentration. Both authors concluded this was a function of mycelial density. Furthermore, Ball *et al.* (1995) showed that ergovaline concentration in spring was highly correlated with mycelium density, but not the annual means. Although not consistent with the current study's findings, it must be highlighted that the field experiment in the current study was only sampled during the autumn period and not spring. Ergovaline variation cannot be explained by mycelial density in the current study and no consistent relationship between mycelial mass and ergot alkaloid concentration was observed. This outcome is supported by studies in WT tall fescue by Hiatt and Hill (1997) who also found no correlation. These authors further suggested that selection for plants with low alkaloid concentration (eg. ergovaline) resulted in little, or no, antagonistic effects on the endophyte-host association expression of mycelial mass.

As a significant increase in peramine concentration as the percentage component of ergovaline is reduced (see Chapter 4.3.9), there is also a small trend for increased mycelial density in each selection cycle which coincides with increased peramine. Although this was not found to be significant (P>0.05), a marker development experiment by Faville *et al.* (2007) found that peramine concentration was partly determined by mycelial mass due to a large-effect of a Quantitative Trait Loci (QTL) coinciding with a major mycelial mass QTL. Although the current study's ELISA results do not definitively support this, it could be argued that there might be some confounding circumstances with smaller population size of why this association between mycelial density and peramine is not expressed as strongly.

It could be advantageous to have higher endophyte colonisation for particular strains of novel endophyte to impart greater alkaloid production of the non-toxic alkaloids. There is potential to exploit this relationship in perennial ryegrass (Norriss *et al.* 2007). If this was a breeding objective or target, there would need to be some consideration on how to incorporate into a selection programme with other secondary metabolite traits of interest. Although the current study did not show any significant differences between environments, there is a trend that irrigation and nitrogen may impart greater densities. Therefore, a breeder wouldn't want to confound these conditions when making selection decisions. Furthermore the environment and conditions to cycle or run evaluation experiments need careful consideration as changes in plant development, seasonal conditions, rainfall and temperature can all influence mycelium density (Fletcher 1986; di Menna and Waller 1986; Ball *et al.* 1995). Greater understanding would also be required for the effect of mycelial density on other secondary metabolites. Even though it appears there is little, or no antagonistic effects on mycelial density from recurrent selection of ergovaline in this host-endophyte association, this may not be the case in other alkaloid classes or host-strain associations.

Chapter 6

General Discussion

6.1 Recurrent selection for reduced levels of ergovaline

A key objective of this study was to generate a low ergovaline producing perennial ryegrass-endophyte cultivar by applying recurrent selection to an AR5 population for reduced expression of ergovaline. Regardless of environment or treatment, the initial increase and subsequent decrease of ergovaline concentration in each recurrent population was not expected. Each population cycle was anticipated to have a concentration lower than the previous cycle. For example, second cycle (C2) lower than first cycle (C1) and the C1 lower than the Base population. In fact the opposite of this occurred in the Base to C1 selection cycle, where ergovaline expressed a significant (P<0.05) increase from 0.89 to 1.12 ppm across all environments and treatments. Selection for a lower ergovaline producing host-genotype in perennial ryegrass and tall fescue has previously shown to be highly heritable in wild-type (WT) endophyte strains (Roylance *et al.* 1994; Adcock *et al.* 1997; Easton *et al.* 2002). Unfortunately these data in the current study for ergovaline concentration showed inconsistency. Whilst ergovaline concentration subsequently decreased again from the C1 to C2 (1.12 to 0.91 ppm) showing that further reselection was successful, this was non-significant (P>0.05) and still not below the initial Base population concentration.

The initial increase and subsequent decrease pattern was reflected across both environments and within nitrogen treatments. Because no significance was detected it is hard to make any definitive conclusions, although the actual trend is of interest. However, when looking at ergovaline concentration as a percentage component of the whole ergot alkaloid biosynthetic pathway (Table 4.15), ergovaline concentration did reduce at each recurrent selection cycle suggesting that other secondary ergot alkaloid metabolites have influenced this trend. This increase and subsequent decrease pattern reported in ergovaline concentration was also observed in the intermediate metabolites chanoclavine and lysergyl alanine. This showed a significant increase (P<0.05) from the Base to C1 population, followed again by a non-significant (P>0.05) decline across all environments and treatments, with the exception of chanoclavine which remained constant.

6.2 Effect of recurrent selection on ergot alkaloid pathway intermediate concentrations

As expected the AR5 alkaloid profile expressed ergovaline and peramine, as well as the individual intermediates in the ergot alkaloid biosynthetic pathway: chanoclavine (although just below the limit of detection); lysergyl alanine; and ergine. No agroclavine, lysergol or lysergic acid intermediates

were detected in any of the population cycles. Lolitrem B was not detected which is consistent with the expected AR5 chemical profile. The majority of animal toxicosis work and associated roles of intermediate ergot alkaloids, such as those reported by Reed and Mace (2013), has been with common toxic WT endophyte and not the AR5-ryegrass association in the current study. There are differences in the expression of the various pathway intermediates between these two endophyte alkaloid profiles and these differences in expression are not due to environmental or nitrogen treatment. However, variations in the level of concentrations may possibly be influenced by environment. For example, Reed and Mace (2013) presented mean ergot alkaloid concentrations present in the ergovaline biosynthetic pathway for the naturalised WT endophyte strain, from Victorian (Australia) and New Zealand field trials. Their results showed that WT chanoclavine (although also below the critical threshold for detection), ergine, lysergol alanine and ergovaline were detected, but additionally lysergic acid was also detected (below <0.2 ppm).

The actual mean concentrations of some of the intermediate ergot alkaloids appear considerably lower in the WT-ryegrass association in Reed and Mace's (2013) experiment, than the current study's AR5 C2 population. It must be stressed that these two experiments were conducted in different years, locations and with two distinct ryegrass-endophyte associations. There is very little information available on production of the intermediate ergot alkaloids under field conditions; therefore the study by Reed and Mace (2013) provided some insight into potential expression concentrations of pathway intermediates. The largest discrepancy between the WT and AR5 associations relates to ergine. The mean concentration across all environment and treatments in the AR5 C2 population was as high as 14.50 ppm, whereas in Reed and Mace's (2013) study, the WT strain only produced a mean ergine concentration of 0.90 ppm. Furthermore, ergine concentration In Reed and Mace (2013) WT when expressed as a percentage component of the total pathway was a far less than the AR5 C2 ergine component, being 26.47% in the WT compared to 83.81% in the AR5. Lysergyl alanine was double the concentration in the AR5 compared to the WT, but accounted for a greater concentration component of 33.22% in the WT, compared to only 8.02% in the AR5. Mean ergovaline concentration was relatively comparable between both strains. However it must be noted that both these studies discussed differed in many ways, not just the endophyte strain involved.

In Reed and Mace's (2013) experiment, environmental influence certainly had a significant effect between both Victoria and New Zealand, with significant increases in concentration shown in the New Zealand samples for lysergyl alanine, ergine, ergovaline and chanoclavine (plus peramine). These authors concluded that the ergovaline:lolitrem B ratio in WT strains is expected to be greater than that reported in overseas studies (such as New Zealand (NZ) and Oregon, United States of America (USA)) and appears likely to be more important than other metabolites in explaining the

different expression of perennial ryegrass toxicosis between the regions. It is thought the relatively high solar radiation and temperature expected in Victoria compared with NZ will result in more heat stress in ruminants and aggravate the well documented effects of the vaso-constricting ergot alkaloids in WT-infected ryegrass pastures on animal physiology (Reed and Mace 2013).

6.3 Effect of recurrent selection on ergine

It appears that when applying recurrent selection for reduced ergovaline there is a biochemical shunt in production from ergovaline towards ergine, rather than a down-regulation of the whole biosynthetic pathway. Given the low levels of chanoclavine as an early precursor in the pathway, and that any early intermediate changes in expression or concentration of these secondary metabolites is difficult to determine in this current study, the regulation of ergot alkaloid production appears to be modified relatively late in the pathway. The step that this occurs is most likely at the lysergyl peptide synthetase complex, before production of the simple amides ergine and lysergyl alanine, or ergopeptide ergovaline. A review by Panaccione (2005) highlights several fungi that produce alternative products away from their ergot alkaloid pathway. These accumulating intermediates via short shunt pathways off of the presumed primary or main pathway are generally distinct in form and function from the end product. A number of examples of these intermediary pathway shunts are highlighted, but most notably is one that led to the production of 6,7-secolysergine in some *Neotyphodium* spp. endophytes (Panaccione *et al.* 2003). This particular clavine was presumed to derive from chanoclavine, or another intermediate prior to chanoclavine in the pathway. The significance of this is that its concentration increases in *Neotyphodium* sp. Lp1 when one regulatory gene is knocked out, whereas the other intermediates are maintained near their control concentrations.

The assembly of the ergopeptines (i.e. ergovaline) is catalysed by a multi-functional peptide synthase complex named lysergyl peptide synthase (Walzel *et al.* 1997). Panaccione *et al.* (2003) described this peptide synthase as different to other eukaryotic peptide synthases because it is made up of two separate polypeptides (Lysergyl peptide synthetase 1 and 2 – LPS1 and LPS2) rather than all activities being encoded on a single polypeptide. The product of this multifunctional enzyme complex is the lysergyl 'peptide lactam', illustrated in Figure 2.4 (Chapter 2). According to a number of biochemical studies cited by Panaccione *et al.* (2003), the peptide lactam intermediate is thought to spontaneously cyclize to the final ergopeptine products. In Panaccione's *et al.* (2003) experiment, a mutant endophytic fungus from perennial ryegrass was developed with a targeted gene knockout in the LPS1-encoding gene *lpsA* which eliminated ergovaline. Investigating the impact on other intermediaries in the ergot alkaloid pathway discovered that: a) two simple lysergic acid amides, ergine and lysergyl-alanine, were also eliminated by the knockout; and b) the production of these

simple amides depended on the activity or products of lysergyl ergovaline-associated peptide synthetase. Therefore, the regulation of ergot alkaloid production is modified in response to the relatively late block in the pathway. Panaccione *et al.* (2003) explained that biosynthesis of these simple amides as direct products of the lysergyl peptide synthetase complex would not be expected, as non-ribosomally synthesiszed peptides are bound to enzymes and require specific catalysts to be released. These workers alternatively suggested a more plausible explanation that these simple amides are metabolite products of the lysergyl peptide synthetase complex. This study provides further supporting evidence to the hypothesis that inefficiency in the ergot alkaloid biosynthetic pathway is controlled rather than random enzymes and genes operating without coordination.

Given the previous work by Panaccione and colleagues, a possible hypothesis is proposed that the LPS1 and LPS2 are operating independently to each other and are both under tight host-ryegrass genetic control. As each cycle to select genotypes expressing low levels of ergovaline, indirect selection is occurring for genotypes expressing lysergyl peptide synthetase complexes that are shunting either LPS1 or LPS2, and possibly encoding for greater production of the simple amide ergine. Lysergyl alanine on the other hand seems intrinsically linked to the production of ergovaline and could be under similar genetic host control to this ergopeptide. Direct selection on ergovaline in this host-endophyte association has not eliminated this ergopeptine, but has reduced it, similarly with lysergyl alanine. The catalyst or genes involved for this diversion to ergine is unknown and determining what these are is beyond the scope of this current study.

There does not seem to be a block in this ergot alkaloid pathway as each selection cycle still expresses both the detectable simple amides and ergovaline. It is also important to remember that this study was completed on the AR5 strain, whereas Panaccione et al. (2003) undertook this work on a WT strain mutant ryegrass host-endophyte association. Whilst there are some differences in the expression of the ergot alkaloid profiles, comparisons arguably can be drawn between the functioning of the downstream biochemical pathway intermediates. An alternative pathway is proposed in Figure 6.1 to illustrate the AR5 biosynthetic pathway shunt and this effect of the peptide lactam regulation under recurrent selection. This proposed pathway suggests there is further work required to determine what genes are involved in the diversion to ergine from peptide lactam, away from ergovaline and/or lysergyl alanine.

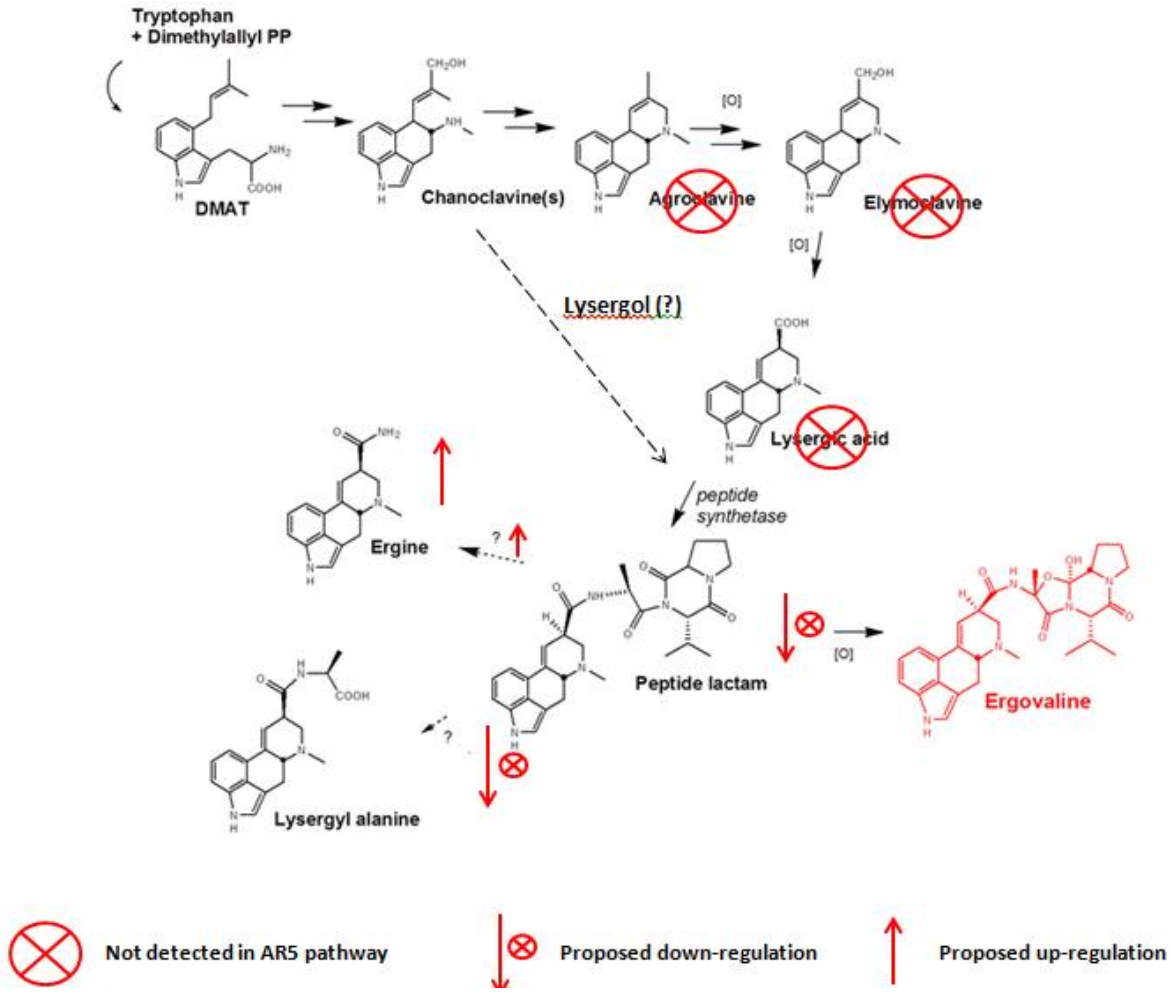

Figure 6.1 Proposed alternative pathway production for AR5 following recurrent selection for reduced ergovaline. Adapted from Panaccione *et al*. (2003).

There are several other fungi that have some diversification of ergot alkaloid profiles within individual producers caused by shunt pathways. For example, in clavicipitaceous fungi, the alkaloid festuclavine appears to be a minor shunt product of the ergot pathway, whereas most of the preceding intermediates are typically cyclised into agroclavine (Panaccione 2005). On the other hand, festuclavine is a critical accumulating intermediate in the main pathways of *Aspergillus fumigatus* and *Claviceps africana* to production of fumigaclvine C. Furthermore, clavine products setoclavine and penniclavine/isopenniclavine, have been reported from *Neotphyodium*-grass plant associations from sclerotia of certain *Claviceps* isolates (Flieger *et al.* 1997). Panaccione's (2005) explained that the peroxidases can oxidise agroclavine and elymoclavine to setoclavine, and penniclavine respectively, indicating again that pathways in some ergot alkaloid producers contains shunts along which intermediates may be diverted to alternative products.

It is not clear whether any evidence exists of biosynthetic pathway shunts in other species where ergine has been reported at high levels. For example, ergine was reported to be up to 180 ppm in

drunken horse grass (*Achnatherum inebrians*) with *N.gansuense* associated endophyte which is reported toxic against grazing livestock (Zhang *et al.* 2011). Furthermore, ergine has also been found in high concentrations (10 - 20 ppm reported) in sleepygrass (*Stipa robusta)*, several varieties of morning glories (*Calystegia* spp.) and Hawaiian baby woodrose (*Argyreia nervosa*) (Chao and Der Marderosian 1973; Petroski *et al.* 1992).

6.4 Host endophyte assoication and control for ergine

Ergine has been shown to have the highest concentration of all the ergot alkaloids, and makes up the majority percentage component of the ergot alkaloid pathway in this AR5 association. The highest mean concentration was reported in the C2 population in the dryland environment without nitrogen (19.08 ppm). In fact, there was a substantial differentiation (significantly $P<0.05$) in environment effect, with significantly ($P<0.05$) greater concentrations expressed in the dryland compared to the irrigation treatment. In some cases this was almost two-fold.

There is evidence to suggest that there is strong host genetic control in the perennial ryegrass populations over the expression of the intermediates ergot alkaloids, particularly for the increase of ergine. This questions how much of this selection was genetic and how much was epigenetic related (where any potentially stable and heritable change in gene expression occurs without a change in DNA sequence). Both general combining ability (GCA) and specific combining ability (SCA) effects have shown evidence of host genetic control for accumulation of endophyte-derived alkaloids and that the interaction between the two parents was indicated through a residual SCA for ergovaline (Easton *et al.* 2002). This was either achieved through dominance (rather than additive heritability) or epistasis affects. The fluctuations observed in this study maybe epigenetic in nature rather than any specific combinatorial change. This could explain cycle differences and the subtle shifts in the metabolite flux.

There could be a number of other possible explanations for this tight genetic control from the host plant on ergine production. Previous studies in expression analysis have shown that genes for the ergot alkaloid pathway are preferentially expressed *in planta*, suggesting that plant-specific signalling is required for expression of these pathways (Young *et al.* 2006). Given that endophytes have evolved over many thousands of years (Young *et al.* 2015), they have developed numerous mechanisms for survival which tend to be those considered undesirable in high performing and well managed pasture swards. For example, the protection of their host plant from predation by producing secondary compounds which are toxic to invertebrate pests and mammals (i.e. lolitrem B and ergot alkaloids). When selection interferes with the biosynthetic pathway as shown in the current study, it could possibly make these plant-endophyte associations less fit in nature, which is not reflective of a typically well managed pasture sward. One practical example where this has

occurred is the novel AR1 strain host-endophyte association and its reduction in efficacy to ASW when infected into different genotypes (Fletcher *et al.* 2006).

As synthesis of the ergopeptines is catalysed by a multi-functional peptide synthase complex and production is shunted towards ergine, this could potentially be a compensatory effect of a reduction in the end product ergovaline. The response may be a form of substitution to maintain a high alkaloid expression to confer high insect resistance. As the hypothesis in this study suggested, inefficiency has been selected for in this particular host-AR5 biosynthetic pathway because a diverse profile of the ergot alkaloids must provide an advantage to the host-endophyte association. If this is the case, then it has fulfilled two predictions that: a) the pathway has been regulated by host-control to produce the observed profile (i.e. higher percentage component of ergine) as opposed to being a collection of enzymes operating at randomly uncoordinated rates; and b) alternative end products or accumulating intermediates should have bioactivities that differ from those of the ultimate end product (Panaccione 2005). A greater understanding of these bioactivities of ergine is required.

The potential for ergine in the associated host plant to confer greater resistances to specific biotic (and abiotic) stress events is required to understand whether this would be an advantageous strategy to the host-endophyte association in agricultural grazing systems. When attempting to breed new cultivars which alter this specific host-endophyte association, the triggers in the biosynthetic pathway need to be better understood. Nevertheless, the understanding of ergot alkaloid biosynthesis has greatly increased recently through genomics and dissection and manipulation of the biochemical pathway (Young *et al.* 2015).

Indirectly increasing ergine concentration may not be a desirable outcome in a commercial ryegrass-endophyte breeding programme. Numerous researchers (Hill *et al.* 2001, Gadberry *et al.* 2003) have previously suggested that these intermediates may have a significant role in animal toxicosis independent of ergovaline. Although limited studies in the literature have shown that ergine may work synergistically with ergovaline to cause full expression of toxicity affects in grazing animals, suggesting that reducing (or eliminating) ergovaline is still an important target for selection. For example, in the lamb feeding experiment Gadberry *et al.* (2003) suggested that even though feed sources showed a low ergovaline concentration, the toxicity effect was due to a greater ergine concentration, with only a 1.26 ppm concentration in ryegrass. Although this study was conducted with seed, it could be argued that there is a gap in knowledge on the content of ergine in ryegrass seed relative to what is identified in forage. Ergovaline on the other hand is known to contain higher concentrations in seed rather than herbage in both ryegrass and tall fescue (Roylance *et al.* 1994). However, it has also been shown that even at high concentrations (up to 20 ppm) ergine had no feeding deterrent ability to adult African Black Beetle *(Heteronychus arator*) in comparison to the

ergopeptines, such as ergovaline even at much lower concentration (5 ppm) (Ball *et al.* 1997). Clearly ergine has as an accumulating intermediate has a different bioactivity from ergovaline given it is not as effective feeding deterrent as ergovaline; it must therefore have another role that is not well recognised.

Another concern for higher concentrations of the intermediate ergot alkaoids is the evidence that absorption through the gastric tissues of ruminants (i.e. cattle and sheep) favours the simple lysergol amide alkaloids over the ergopeptines to cross digestive barriers (Hill 2005). However, recently researchers described that ergovaline is still thought to be the most physiologically active ergot alkaloid produced by *Neotyphodium* spp. (predominantly *N.coenophialum*) and demonstrated that it is approximately 1,000 times more potent and 5 times more efficacious in causing vasoconstriction than lysergic acid (Klotz *et al.* 2006; Klotz *et al.* 2007). Although well beyond the scope of this study, the hypothesis presented by Hill (2005) is supported by the evidence presented in this study that there is a strong requirement for more understanding on the toxicity effects of ergine, and any other non-ergovaline bioactive ergot alkaloids under animal grazing systems, and what synergies exist between ergovaline and other intermediates in the biosynthetic pathway. It could be reasonable to suggest that more emphasis could be placed on discovery or selection for higher concentrations of the early pathway intermediates (i.e. chanoclavine) that could maintain or enhance specific tolerances to biotic and abiotic stress, yet have no animal toxicity effects.

6.5 Effect of recurrent selection on peramine

Direct selection for reduced ergovaline indirectly increased peramine concentration significantly at each cycle across all environments and treatments. The greater increase was observed between the Base and C1 population (2.72 ppm), which was double compared to the increase between the C1 to C2 population (1.43 ppm), both of which were significant ($P<0.05$). Although ergovaline concentration increased from the Base to C1, peramine also increased but did not subsequently decrease as observed with ergovaline from the C1 to C2. This suggests there is some inconsistency in what genetic control is regulating this and independent control of both ergovaline and peramine production. Similar to the trend observed in ergovaline, albeit non-significant ($P>0.05$), the peramine concentration was significantly greater ($P<0.05$) in each population cycle under the dryland environment compared to the irrigation environment. There was little consistency on the effect that nitrogen application had on peramine concentration across all environments. This supports findings by Lane *et al.* (1997b) that peramine concentration was not affected by N treatment.

This increase in peramine concentration has also previously been shown to be a highly heritable trait. For example, Easton *et al.* (2002) showed high heritability for infection intensity and alkaloid concentration traits for peramine. Those additive heritable elements are also major factors in the

genetic control of ergovaline, which appear to be exercised over peramine concentration. The inconsistency in the current experiments ergovaline concentration questions why the peramine concentration significantly increased. Ball *et al.* (2005) and Easton *et al.* (2002) had also previously shown a high positive correlation with seasonal mycelial density and peramine concentration in a WT perennial ryegrass. However, no detection of any significant increases in mycelial density across the populations in these data with the AR5 ryegrass association supports that these alkaloids are more independently regulated rather than any associated control. Faville *et al.* (2007) on the other hand identified loci in perennial ryegrass genome that influenced three traits, including mycelial mass, ergovaline level and peramine level with three Quantitative Trait Loci (QTL) identified for each trait. These workers found that the largest-effect QTL for peramine level (18% variation) coincided with a major mycelial mass QTL, which they implied peramine concentration is partly determined by mycelial mass. This was in both a WT and AR6 ergovaline producing grass-endophyte associations.

Roylance *et al.* (1994) supported this in WT-infected tall fescue and found that endophyte derived alkaloids are independently regulated and controlled by both the host plant and endophyte. However, those workers subsequently suggested that selection and breeding for low ergovaline concentration is unlikely to indirectly effect peramine concentration, which is not consistent with the current experiments findings in the AR5-ryegrass association, as selection for low ergovaline has indirectly increased permaine concentration.

As peramine has no known detrimental effects on animal health (Fletcher 2012), it is desirable to maintain or increase concentrations of this alkaloid in any endophyte-ryegrass association due to its strong pest resistant properties (Popay and Latch 1993). This activity as a pest feeding deterrent can greatly enhance the competiveness of a cultivar infected with an endophyte strain that produces it. For example, peramine has been shown to be a powerful feeding deterrent to adult Argentine stem weevil (ASW, *Listronotus bonariensis*) (Rowan *et al.* 1990; Popay and Wyatt 1995). To achieve strong resistance to ASW, it is estimated that a high concentration between 15 – 20 ppm is maintained (Popay and Wyatt 1995). However, as results have shown in this current experiment between the Base, C1 and C2 populations, grass host genotype can influence the concentration expression of peramine, which may have consequential differences in the levels of resistance to ASW. For example, Popay *et al.* (2003) found a ryegrass cultivar effect where tetraploid and hybrid ryegrass (*Lolium boucheanum*) cultivars sustained more damage than diploids and perennials. The differences were linked to feeding damage due to different concentrations of peramine in the leaf lamina. Furthermore, a number of commercial AR1 strains infected into different host-cultivar associations have failed to support effective peramine production which could have significant impact on host plant survival (Fletcher *et al.* 2006). This current study is limited to only having the AR5-association in a tetraploid cultivar. It does reinforce the importance of having the presence of other alkaloids to

impart protection against other potential invertebrate pests when expression of alkaloids is altered through selection.

6.6 Effect of environment and nitrogren treatment

Large environmental effects were observed on the ergot alkaloid concentrations between the irrigation and dryland environments as well as within the plus nitrogen (N+) and minus nitrogen (N-) treatments. Ergovaline concentration under water deficit (dryland) was consistently higher than the irrigation environment and generally higher in the N+ compared to the N- under irrigation. Although there was no statistical significance (P<0.05), there was a general trend. This was consistent with previous work by Lane *et al.* (1997b) who showed water deficit and addition of nitrogen was associated with increased ergovaline concentration in a WT strain. There seems to be no reports in the literature what effect water deficit and nitrogen has on the intermediate ergot alkaloids in the biosynthetic pathway in the reselected populations. In the current experiment, these data show an effect of environment on ergine concentration in nearly all populations (Base, C1 and C2) with the dryland environment significantly greater (P<0.05) than irrigation, and generally greater concentrations in N+ under dryland. The most prominent increases in concentration occurred in the C1, with mostly non-significant (P>0.05) increases in the C2 for the dryland N+, and irrigation N- and N+. This response was also observed in lysergyl alanine, with the concentration under dryland significantly higher (P<0.05) than irrigation, although there is only some significant differences (P<0.05) between the selection cycles in the N- and none in the N+ within the environments.

Intermediate ergot alkaloid concentration appears to be greater under water deficit. This was expected given the response of the end-product in the biosynthetic pathway ergovaline to dryland environments and that drought (water deficit) is commonly understood to enhance alkaloid concentrations in endophyte-infected grasses (Lane *et al.* 2000; Hahn *et al.* 2008). However Reed *et al.* (2011a) found higher concentrations were associated with good pasture growing conditions and long summer drought stress, similar to what the dryland treatment was exposed to in the current experiment, but this subsequently resulted in lower concentrations. This is not consistent with the current findings within the two environments.

Water deficit would have triggered stress response signals, perhaps even confounded by a greater presence of insects, which unfortunately were not surveyed in the field for this study. Even though it has been previously emphasised that the levels of chanoclavine were below the limits of detection and should be treated with caution, there is a clear trend and significant difference observed (P<0.05) with the dryland treatment having higher concentrations of chanoclavine than irrigation in the N+ (significant in the Base and C1 population) and N- for all 3 populations. The higher concentrations later in the biosynthetic pathway could likely be a function of greater production of

the early ergot alkaloid pathway intermediates. Hahn *et al.* (2008) concluded that levels of ergovaline altered in response to the levels of abiotic stress that was specific for each host-endophyte association. However, Hahn *et al.* (2008) also reported no differences in steady state levels of transcripts (signalling) from genes known to be required for synthesis of the alkaloids were observed in response to water deficit.

The intriguing result is that under water deficit, the N+ treatments showed greater concentrations of ergovaline and some of the other intermediates. This was more prominent in the Base to C1 cycle, but inconsistent in the C1 to C2. Given that there were no significant differences ($P>0.05$) found in mycelial density for any environment or nitrogen treatment to account for this variation, this greater level of secondary metabolite found in the dryland N+ treatment may be better explained through a plant physiological response. In light of this, it is assumed there must have been enough precipitation (e.g. rainfall, dew etc.) of some form to ensure the applied nitrogen fertiliser became available for plant uptake in the dryland N+ treatment. Tisdale and Nelson (1975) and Gomm (1979) demonstrated that pasture plants grown in saturated soils were low in nitrate, even at high levels of nitrogen. These waterlogged soils tended to be more anaerobic, which inhibited conversion of ammonia to nitrate for uptake (as soil nitrate). Nitrate is the principal form of nitrogen available to plants from the soil, under normal circumstances (Wright and Davison 1964). As nitrogen was applied in the form of sulphate of ammonia (SOA) in this current experiment, the saturated soils maintained at field capacity under irrigation may have caused this affect. Another possible explanation for higher concentrations of ergot alkaloid in the dryland N+ treatments is that plants with infected with an associated symbiotic endophyte under drought stress may extract water better than non-symbiotic plants through the function of better root growth, as observed by Belesky *et al.* (1989) in tall fescue. Yet, this still does not explain why secondary metabolite concentrations are higher given there was no significant increase in hyphae mass. A hypothesis is proposed that this could simply be a function of higher nitrate availability and subsequent uptake in the dryland N+ treatment, directly resulting in higher synthesis of ergot alkaloids.

Peramine concentration was significantly affected by environment, with a higher concentration in each population cycle in the dryland in both N+ and N- treatments, with significant ($P<0.05$) increases in N+ under both environments. The response of peramine under water deficit in this study reflected that of ergovaline response (Hahn *et al.* 2008). The greatest increase and higher concentrations reported in the dryland N+ (17.0 ppm in C2) and the dryland N- treatment. This is inconsistent with work by in tall fescue by Faeth *et al.* (2002) where peramine levels did not differ among soil moisture treatments (plus and minus) in long-term field experiments. There is also inconsistency in reports by both Lane *et al.* (1996) and Faeth *et al.* (2002) who found that peramine concentration was not affected by N application. Unfortunately the application protocols used in the current experiment

are atypical, which may have further resulted in other confounding stress responses not examined here.

6.7 Development of a commercial cultivar

One final objective of this study was to produce a commercially viable cultivar for potential release to the Australian market. This was achieved through the development of a terminal C2 population, designated the breeding code 'KLp701 AR5', for future evaluation in the PGG Wrightson Seeds perennial ryegrass breeding programme. This has been largely achieved due to a number of factors. Firstly, high endophyte-infection frequency at 95% (C2 population) is a key indicator for the agronomic performance of grass plants in grazed pastures (Hume and Barker 2005). This is also important for nucleus seed multiplication going forward that high endophyte-infection frequency is maintained in breeders and pre-nucleus seed stocks. Secondly, ergovaline (when expressed as a percentage of the pathway across all treatments) is reduced and the concentration of peramine significantly increased beyond 15 ppm across all treatments and environments. Furthermore, the AR5 strain does not produce the indole-diterpene alkaloid lolitrem B and therefore will not cause perennial ryegrass staggers, and the reduced levels of ergovaline in 'KLp701 AR5' should also result in a lower risk of PRGT. 'KLp701 AR5' should provide some confidence to farmers that the reduced ergovaline AR5 strain is a significant improvement on the old WT endophyte strain. Given the large body of evidence provided in the literature on the negative effects of lolitrem B, high ergovaline and the potential synergistic effects due to the presence of both alkaloids, WT-infected perennial ryegrass should not be sown when establishing new ryegrass pastures in Australia.

6.8 Limitations of the experiment

In light of the current result suggesting a possible shunt of the ergot alkaloid biosynthetic pathway away from ergovaline to ergine, it would have been of great interest to explore further whether this was a true shunt switch. Starting with a base population with higher concentrations of ergovaline would have perhaps allowed for some greater selection intensity and to identify what the proportion of the biosynthetic pathway would have ergine occupied in early cycles. Recurrent selection for high ergovaline levels, rather than low, as an alternative population which would have had no commercial reality (with the exception of a turf amenity product). However, it could have offered some further insight into the heritability and effects on the simple lysergol amides, ergine and lysergyl alanine, and whether this relationship is inversely related with ergovaline. Further interest may have been to run similar populations through cycles of selection with emphasis on higher ergine concentrations and investigate this effect in reverse on the end-product ergovaline. For example, once ergovaline concentration is below the limit of detection, can more pressure be placed on increasing ergine

concentration or other early intermediates (if ergine was shown not to have severe toxicity issues and higher synergistic effect with ergovaline) and completely eliminating ergovaline?

There is strong evidence suggesting that host genetic differences at the ploidy level may considerably impact the behaviour of the symbiosis in terms of secondary metabolite expression and production. Developing a genetically identical diploid and tetraploid AR5 population (through use of the chemical colchicine) to apply recurrent selection concurrently and evaluated under the same treatments would have provided more evidence to test if this was a true shunt switch of the biosynthetic pathway. Anecdotal evidence from previous commercial field experiments by PGG Wrightson Seeds (Australia) Ltd have indicated that some diploid perennial ryegrasses infected with ergot alkaloid producing endophytes contain higher levels of ergovaline than genetically similar tetraploid cultivars. Further experimentation would have provided strong evidence to substantiate this and higher initial concentrations to select on could help prove this flux and host-genetic control of all other intermediate precursor alkaloids.

Although the environments simulated *in situ* with irrigation and nitrogen application were well controlled, a number of other tests and measures could have been employed to attempt to account for further variation. For example, nitrogen flux around the N+ treatment under dryland environment could have been better explained if tissue N tests, both soil N test at 0 – 10 cm and 10 - 60 cm for deep N soil testing were reported. Actual biomass production (dry matter production in kilograms per hectare) directly harvested from each plot may have provided more evidence of the host-endophyte effect between the population cycles. Further to this, harvest of root mass may have provided evidence for greater uptake of nitrogen and endophyte symbiosis in each population cycle that could not be observed phenotypically above ground. This would have had to have been a destructive harvest which was not set out in the early experimental protocols.

Insect surveying at the experimental site to determine the presence or absence of key invertebrate pests such as root aphid, ASW, black beetle and pasture mealey bug may have explained some potential stress responses during the summer-autumn period. For example, the absence of any change in mycelial mass as a response to selection may have been triggered if there was an increased level of biotic stress. Because alkaloid concentrations vary considerably throughout the year due to a multitude of contributing factors (Reed *et al.* 2011a, 2011b; Moate *et al.* 2012), only one point in time was chosen to sample these populations for the intermediate pathways. This sample time in the current study was chosen given the knowledge to capture the high expression of the secondary metabolites. It is not known whether this could be a confounding factor in the expression of intermediate alkaloids which may also vary in different seasons and times of the year.

One major and perhaps most consequential limitation in further understanding of the biosynthetic intermediate pathway in *N.loli* is that recurrent selection for reduced ergovaline was on only conducted in one tetraploid genetic pool infected with the AR5 strain. There are a number of other commercial endophytes available that produce the end-product ergovaline and would have likely produced variation in expression of the intermediates. For example, Tian *et al.* (2013) found significant variation due to different endophyte and host genotypes and those differences in both endophyte and host genotypes contribute to host-endophyte performance in a complex interactive manner. Future work would include study on naturalised WT-host associations, freely available in a wide range of background genetic ecotypes and cultivars. Secondly inclusions of other host-endophyte associations selected for reduced levels of ergovaline which would include the strain 'NEA2' (van Zijll de Jong *et al.* 2008). Inclusion of these host-endophyte populations, at both the diploid and tetraploid level with initially high ergovaline levels in newly generated base populations, may have provided evidence into the variation of biosynthetic intermediates between different strains, further insight into the pathway shunt and if this recurrent selection can be successfully applied more widely to other endophyte-grass associations.

Any future studies of recurrent selection for low ergovaline should consider investigating both diploid and tetraploid populations in identical genetic background, infected with other ergovaline producing endophytes, to better understand this host-genetic control in different host-endophyte associations. There should be further work in understanding both the agronomic advantages that intermediates such as ergine may confer to the association and quantify what animal toxicity affects these may impart in grazing systems.

Chapter 7

Conclusions

1) The host-AR5 population expressed the expected profile of secondary metabolites, including the ergot alkaloid intermediates chanoclavine, lysergyl alanine, ergine and ergovaline, as well as peramine. No lolitrem B was detected.

2) The expected decrease in ergovaline concentration in the C1 to C2 population cycle was consistent with the hypothesis. The initial increase in ergovaline concentration from the Base to C1 was not expected.

3) When ergot alkaloid concentrations are expressed as percentage components of the biosynthetic pathway, it shows direct selection for reduced ergovaline in the host-AR5 population reduced both ergovaline and lysergyl alanine and simultaneously increased ergine.

4) It appears that when applying recurrent selection for reduced ergovaline there may be a biochemical shunt in the pathway from ergovaline towards ergine, rather than a down-regulation of the whole pathway. This has provided evidence to suggest that these intermediate alkaloids are under independent host-control.

5) The results here support the hypothesis that the ergot alkaloid pathway is inefficient and intermediates do not flow rapidly through to the end pathway product ergovaline, rather they accumulate in this host-endophyte association as ergine.

6) Direct selection for reduced ergovaline appears to have indirectly increased peramine concentration significantly across all environments and treatments.

7) Large environmental effects were observed on the ergot alkaloid concentrations between the irrigation and dryland environment as well as within the nitrogen treatments. Intermediate ergot alkaloid concentration appears to be greater under water deficit.

8) Peramine concentration was significantly affected by environment, with higher concentrations in each population cycle in the dryland environment within both nitrogen treatments.

9) There appears to be no effect of recurrent selection for reduced ergovaline on mycelial density in this perennial ryegrass-AR5 association.

10) A major limitation to the study in understanding this biochemical shunt is that only one host-endophyte association was studied. Further knowledge of this host-genetic control in different

host-endophyte associations and polyploid germplasm, as well as the implications of ergine (and other intermediates) in grazing animal toxicosis and potential to confer agronomy advantage, will allow for better selection targets and cultivar development.

11) A terminal population has been successfully developed for future field evaluation in a commercial breeding programme.

Appendix A

Field trial design

	REP 1		REP 2		REP 3		REP 4		REP 5	
Irrigation (I+)	N+	N-	N-	N+	N1	N+	N-	N+	N-	N+
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	B	C1	Cnil	B	C2	Cnil	B	C1	C1	C2
	C1	B	C2	Cnil	B	C1	C2	Cnil	C2	B
	Cnil	C2	B	C1	Cnil	B	C1	C2	Cnil	C1
	C2	Cnill	C1	C2	C1	C2	Cnil	B	B	Cnil
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*

Dryland (I-)	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	B	C1	Cnil	C1	B	C2	Cnil	B	B	Cnil
	Cnil	Cnil	C2	B	C1	B	C1	C2	Cnil	B
	C1	C2	B	C2	Cnil	Cnil	B	C1	C2	C1
	C2	B	C1	Cnill	C2	C1	C2	Cnil	C1	C2
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	Buffer	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*	*Buffer*
	N-	N+	N+	N-	N-	N+	N+	N-	N+	N-
	REP 1		REP 2		REP 3		REP 4		REP 5	

Figure A.1 Field trial split-split plot design, with Irrigation (I+) and Dryland (I-) as the first split for environment, and the second split block for Nitrogen (N+) and minus nitrogen (N-) for the four treatments: Base population (B); Cycle 1 minus endophyte (Cnil); Selection Cycle 1 (C1); and Selection Cycle 2 (C2).

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