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Epoxy-janthitrems, effects of temperature on *in planta* expression and their bioactivity against porina larvae

A thesis submitted in partial fulfilment
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Abstract

Ryegrass infected with the endophyte AR37 is of significant value to New Zealand’s pastoral sector. It provides superior protection against a range of ryegrass pest insects, and added an estimated $42 million dollars to New Zealand’s economy between 2007 and 2011. Epoxy-janthitrem alkaloids, produced by AR37, are thought to provide this bioactivity, but limited experiments investigating this have been conducted to date. A porina larval bioassay was undertaken to investigate the bioactivity of epoxy-janthitrem I, the major epoxy-janthitrem compound expressed by AR37. The alkaloid was found to be a feeding deterrent at all three concentrations tested (1, 2.5 and 5 μg/g wet weight), with stronger deterrence observed at the two higher concentrations. Some evidence of toxicity of epoxy-janthitrem I was also identified; future work to resolve this is discussed.

Temperature and ryegrass cultivar are two factors known to affect the concentration of alkaloids in ryegrass. How these factors affect epoxy-janthitrem concentrations in AR37-infetced ryegrass has never before been examined. Perennial and Italian ryegrass were grown in high (20°C) and low (7°C) temperature controlled environment rooms for 10-12 weeks, ryegrass was then freeze dried, ground and incorporated in a semi-synthetic diet which was fed to porina larvae to examine how these variations in alkaloid concentration would affect larvae. AR37-infected ryegrass grown at high temperature was found to contain higher concentrations of epoxy-janthitrem, which when fed to porina larvae had a strong anti-feedant effect and reduced larval survival. In comparison, AR37-infected ryegrass grown at low temperature had a small anti-feedant effect on larvae and only when in perennial ryegrass. Epoxy-janthitrem concentrations were slightly affected by plant cultivar and concentrations were identified to be higher in the pseudostems when compared to the leaves of ryegrass plants.

Findings from this thesis improve on the understanding of the bioactivity of epoxy-janthitrem and explain reports from colder areas of the country, that AR37 is not able to adequately control porina populations. Further research should clarify the limitations of the AR37 endophyte in cooler regions by first identifying whether these results are replicated in the field and then by identifying the temperature ranges the AR37 endophyte may not be as effective in.

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Finally to my parents I thank you for all that you have done for me and for your support and understanding throughout my years at University. Words cannot describe how grateful I am.

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Chapter 1: Introduction

Fungal endophytes have been recognised as an important part of New Zealand's pastoral sector since the early 1980s when it was discovered that the wild type endophyte (*Epichloë festucae* var. *lolii*) produced alkaloids (nitrogen containing compounds) which were beneficial to ryegrass, as these alkaloids provided ryegrass with protection against pest insects. However, around the same time it was also identified that the wild type endophyte also produced alkaloids which were detrimental to the health of grazing livestock. Endophytes that produced only beneficial alkaloids were then sort from grasslands overseas and a select few have since been introduced and are now used on New Zealand farms to improve production. The current endophyte strains in New Zealand contribute around $200 million dollars to the economy each year (Johnson *et al.*, 2013). It is therefore very important to continue to study these endophytes and the relationships between ryegrass, endophytes, the alkaloids they produce and pest insects.

This thesis will focus on an introduced strain of endophyte available in ryegrass in New Zealand called AR37 (a strain of *Epichloë festucae* var. *lolii*). The first part of this thesis will examine whether epoxy-janthitrem I, the major epoxy-janthitrem alkaloid expressed by AR37, is toxic to porina larvae (*Wiseana* spp.). This information will improve on the understanding of the bioactivity provided by the epoxy-janthitrems and how this alkaloid affects porina larval populations in the field. The second part of this thesis will explore how temperature and plant genotype affect epoxy-janthitrem concentrations in AR37-infected ryegrass and examine what affect any variation in concentration may have on growth and survival of porina larvae. Information obtained from this study may then be used to inform farmers of any limitations of the AR37 endophyte in the field.

1.1 *Epichloë* endophytes

1.1.1 Classification of *Epichloë* endophytes

Fungal endophytes in agricultural grasses are from the tribe Balanseiaë which is located within the family Clavicipitaceae. There are 7 genera within this tribe; *Atkinsonella, Balansia, Balansiopsis, Echinodothis, Myriogenospora, Parepichloë* and *Epichloë* (White, 1994; White & Reddy, 1998). The fungal endophytes of the *Epichloë* genus infect grasses in the family Poaceae. Within the *Epichloë* genus there are sexual endophytes (telemorphs) and asexual endophytes (anamorphs). The anamorphs of *Epichloë* have previously been classified in a separate genus called *Neotyphodium* (formally *Acremonium*). However, a recent paper Leuchtmann *et al.* (2014) has proposed a realignment of this group to improve the understanding of the relationships between these endophytes and the features they share. They propose that the majority of species previously separately classified in either *Epichloë* (telemorph) or *Neotyphodium* (anamorph) now be classified under the same genus, *Epichloë*. Forty three taxa are now accepted into this genus. Of specific importance for this thesis is the name change of *Neotyphodium lolii* (the wild type endophyte found naturally occurring in ryegrass in New Zealand) to *Epichloë festucae* var. *lolii*.

1.1.2 Growth, life cycle and transmission of *Epichloë* endophytes

Endophyte hyphae grow within ryegrass leaves as the leaves grow, when a leaf stops growing the hyphae will stop growing. Growth is usually parallel to the leaf axis and the hyphae unbranched (Christensen *et al.*, 2002). Endophytes grow through intercellular spaces of the hosts cells (Clay, 1989) (Figure 1.1) and hyphae will only colonise the above ground tissues of the plant such as the leaf sheath, leaf blades and the inflorescence.

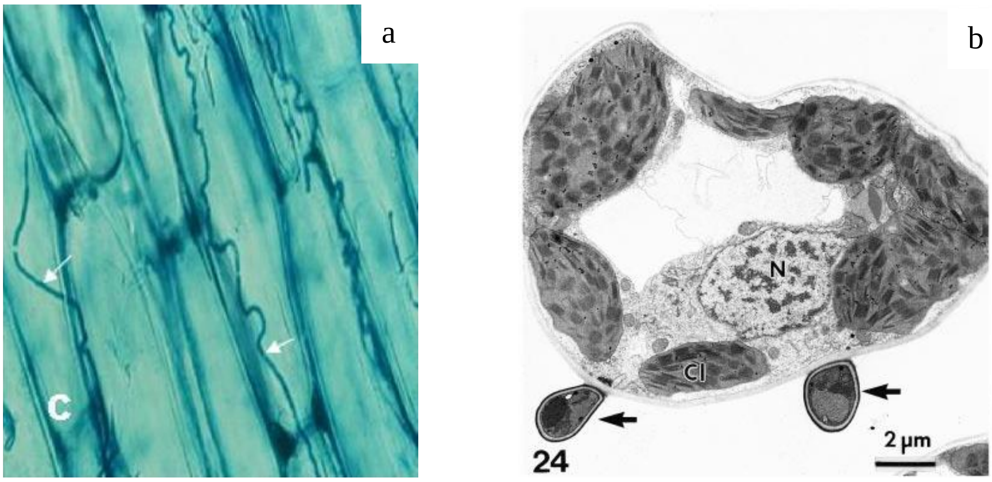

Figure 1.1: (a) Hyphae of an Epichloë endophyte growing through the intercellular spaces of the hosts cells (Kuldau & Bacon, 2008) (b) Two hyphae of *E. Festucae* var. *lolii* attached to a mesophyll cell (Christensen, *et al.*, 2002)

Asexual *Epichloë* endophytes that infect agricultural grasses exclusively reproduce asexually. Unlike sexual endophytes asexual endophytes do not have an external form and so remain within the plant for their entire lifecycle. Reproduction of asexual endophytes (Figure 1.2) occurs via the seed of the host plant. Endophytes are not found within ryegrass pollen. The endophyte grows up into the inflorescence of its host and is then transmitted, in the seed, directly from the host to its offspring (Philipson & Christey, 1986). The endophyte will then grow with the developing coleoptile (Philipson & Christey, 1986). Aseuxal endophytes are therefore completely dependent on their host for survival and transmission.

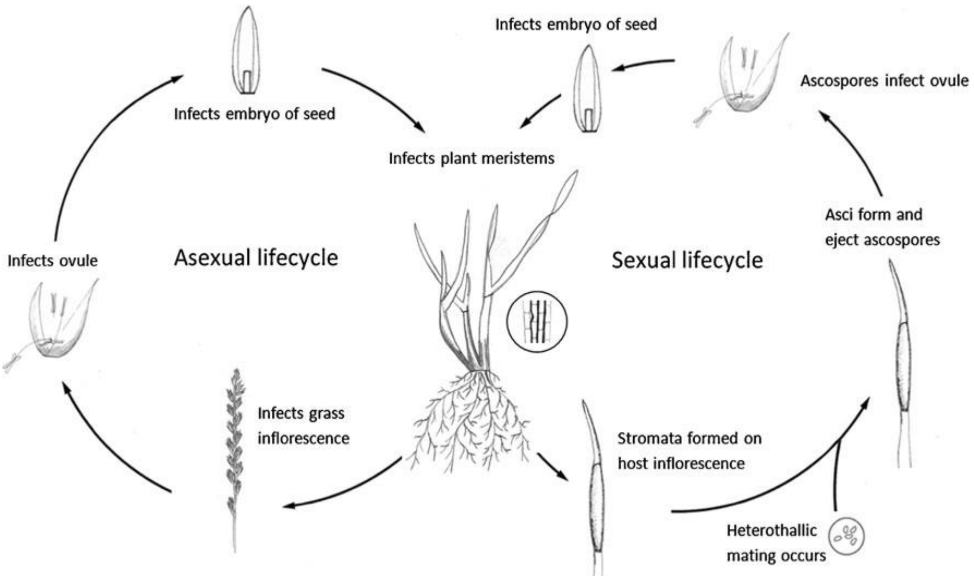

Figure 1.2: Lifecycles of *Epichloë* endophytes(Johnson, *et al.*, 2013)

1.1.3 Relationship between ryegrass and *Epichloë* endophytes

Grasses and their *Epichloë* endophytes have co-evolved over time, although there is an ongoing debate over the nature of the relationship between endophytes and their host (Saikkonen *et al.*, 1998; Saikkonen *et al.*, 2010). The relationship between agricultural grasses and asexual *Epichloë* endophytes is thought to be defensive mutualistic, as first proposed by Clay 1988, and involves both organisms benefiting from the relationship. The endophyte gains shelter, nutrients and a means of transmission from its host (Saikkonen *et al.*, 2004). In return the plant gains increased protection from biotic stresses including insects (Prestidge *et al.*, 1982; Ball & Prestidge, 1992; Pennell *et al.*, 2005; Popay *et al.*, 2012), mammalian herbivores (Fletcher & Sutherland, 2009), pathogens (Pańka *et al.*, 2013) and nematodes (Eerens *et al.*, 1997; Bacetty *et al.*, 2009) as well as increased tolerance to abiotic stresses such as drought (Ravel *et al.*, 1997) and nutrient stress (Ravel, *et al.*, 1997).

1.1.4 History of *Epichloë* anamorphs in New Zealand and a brief outline of their effect on mammalian and insect herbivores.

Ryegrass staggers has been observed in New Zealand since the early 1900s but the link between endophyte occurrence and staggers was not discovered until 1981 (Fletcher & Harvey, 1981). Ryegrass staggers is a neurological impairment of livestock. The symptoms of ryegrass staggers range in severity from small spasms after movement to severe spasms which leave the animal unable to move (Cunningham & Hartley, 1959). Ryegrass staggers itself does not kill animals but it can lead to deaths caused by accidents (Cunningham & Hartley, 1959). Staggers can also effect live weight gain in sheep, with studies showing that sheep grazing infected pastures have a reduced weight gain just prior to the onset of symptoms (Fletcher, 1982, 1983). The condition will affect sheep (Fletcher & Sutherland, 2009), cattle, horses, alpaca, deer and has been reported in white rhinoceri (*Ceratotherium simum*) at Auckland Zoo (Bluett *et al.*, 2004). Wild type endophytes (strains of *E. festucae* var. *lolii)* found naturally occurring in ryegrass in New Zealand cause ryegrass staggers as they produce the tremogenic mycotoxin lolitrem B (Gallagher *et al.*, 1981; Gallagher *et al.*, 1982).

Due to the detrimental effect of the wild type endophyte on livestock there was a push for it to be removed from ryegrass. However, it was subsequently found that ryegrass without endophyte was susceptible to attack by the Argentine stem weevil (ASW) (*Listronotus bonariensis*), a major pasture pest in New Zealand (Prestidge, *et al.*, 1982; Mortimer & di Menna, 1983). It has since been identified that resistance to ASW is provided by the production of a second alkaloid called peramine, by the wild type endophyte (Rowan *et al.*, 1990).

Latter studies have also found that the wild type endophyte improves resistance from the pasture mealybug (*Balanococcus poae*) (Pennell, *et al.*, 2005), reduces feeding of the adult black beetle (*Heteronychus arator*) (Ball & Prestidge, 1992; Popay & Baltus, 2001) and may have some effect on the root aphid (*Aploneura lentisci*) (Popay *et al.*, 2004; Popay & Gerard, 2007).

A third alkaloid produced by the wild type endophyte is ergovaline. Although this alkaloid is thought to provide ryegrass with resistance to adult black beetle (Ball *et al.*, 1997a) it has also been found to be detrimental to grazing livestock, as it causes heat stress (Fletcher, 1993a).

There was therefore a need to identify new strains of endophytes which contained the necessary alkaloids to deter pest insects but did not contain the alkaloids, lolitrem B and ergovaline, which are toxic to mammalian grazers. These are known as 'novel' strains of endophyte. Novel endophytes are obtained by screening endophytes from other countries to identify those which might meet the above requirements. These endophytes are then inoculated into our grasses and go through stages of testing before they are eventually sold to farmers. Several 'novel' strains of *Epichloë festucae* var. *lolii* have since been introduced and made available for use on New Zealand farms.

In the early 1990s Endosafe became the first 'novel' endophyte introduced in to New Zealand (Milne, 2007). Endosafe was advantageous as it did not contain the alkaloid lolitrem B, which causes ryegrass staggers, but still contained peramine to protect against certain insects. However, Endosafe did contain ergovaline, and this alkaloid was produced in higher concentrations than found in the wild type endophyte (Davies *et al.*, 1993). As a result sheep grazed on pasture containing Endosafe had reduced weight gain, higher body temperatures and increased respiration rates (Fletcher, 1993a, 1993b; Fletcher & Easton, 1997). Due to the negative effects on animals this endophyte is no longer sold in perennial ryegrass (Easton *et al.*, 2001). Other 'novel' strains were then sought that did not contain either lolitrem B or ergovaline.

This led to the introduction of the 'novel' strain AR1 in 2001. AR1 does not cause ryegrass staggers or heat stress (Fletcher, 1999) and does not contain lolitrem B or ergovaline. But, it does produce peramine. Studies have shown that ryegrass infected with AR1 is effective against Argentine stem weevil (Popay *et al.*, 1999) and reduces populations of the pasture mealybug (Pennell, *et al.*, 2005). This led to a rapid uptake of AR1 after it was commercialized (Milne, 2007). However, AR1 does not provide the high level of protection that the wild type endophyte provides against black beetle (Popay & Baltus, 2001). Black beetle is a significant pasture pest in northern areas of New Zealand so it is important to have an endophyte that provides adequate control in these areas. In addition AR1 does not appear to have an effect on root aphid (*Aploneura lentisci*) (Pennell, *et al.*, 2005) and has even been shown in some cases to be more susceptible to this insect (Popay, *et al.*, 2004).

The endophyte AR37 was commercialized in 2007. This endophyte does not contain lolitrem B, ergovaline or peramine but it was soon discovered to provide superior protection against a wide range of important pasture pests including Argentine stem weevil (Popay & Wyatt, 1995), porina (Jensen & Popay, 2004; Finch *et al.*, 2010), black beetle (Ball *et al.*, 1994), root aphid (Popay, *et al.*, 2004; Popay & Gerard, 2007) and the pasture mealybug (Pennell, *et al.*, 2005). The only known alkaloids to be produced by AR37 are the epoxy-janthitrems (Tapper & Lane, 2004), a group of five compounds highly unstable outside of the plant. To date the only study which has investigated whether the epoxy-janthitrems are involved in providing the pest resistance of AR37 is Finch, *et al.* (2010). In this study pure epoxy-janthitrem I, was incorporated into a semi-synthetic diet and fed to porina larvae in a bioassay over seven days. Epoxy-janthitrem I was shown to reduce consumption and growth of porina larvae. Further research is required to gain a greater understanding of the bioactivity of the epoxy-janthitrems, by determining whether this alkaloid is deterrent to porina larvae over periods longer than seven days and identifying whether this alkaloid is also toxic to larvae.

As well as the anti-insect properties AR37 also provides agronomic benefits to its host ryegrass, and has been shown to increase ryegrass yield and tiller density (Hume *et al.*, 2007; Popay & Hume, 2011).

AR37 does cause ryegrass staggers. However, because the staggers is not caused by the alkaloid lolitrem B, as in the wild type endophyte, the effects are not as severe and outbreaks are less frequent (Fletcher & Sutherland, 2009). In addition, body temperature and respiration rate are not negatively affected by AR37 (Fletcher & Sutherland, 2009).

Since AR37 was commercialized it has had a strong uptake, and is now available in eleven cultivars (Caradus *et al.*, 2013). The widespread re-grazing of AR37 between 2007 and 2011 is estimated to have added NZ$42 million dollars to New Zealand’s pastoral sector (Caradus, *et al.*, 2013).
Thus, AR37 is a good option as an endophyte in New Zealand, especially in areas which are under high insect pest pressure, and is of important value for New Zealand’s agricultural sector as it reduces pasture loss which increases production.

1.2 Alkaloids

An alkaloid is a chemical compound that contains nitrogen. Alkaloids occur naturally and are produced by organisms such as fungi, plants, animals and bacteria. Four main classes of alkaloids are produced by *Epichloë* endophytes; indole diterpenes (e.g. lolitrems and epoxy-janthitrems), ergot alkaloids, peramine and lolines. All but the lolines are found in different strains of *E. festucae* var. *lolii*. The chemical structures of some of these alkaloids are shown in Figure 1.3.

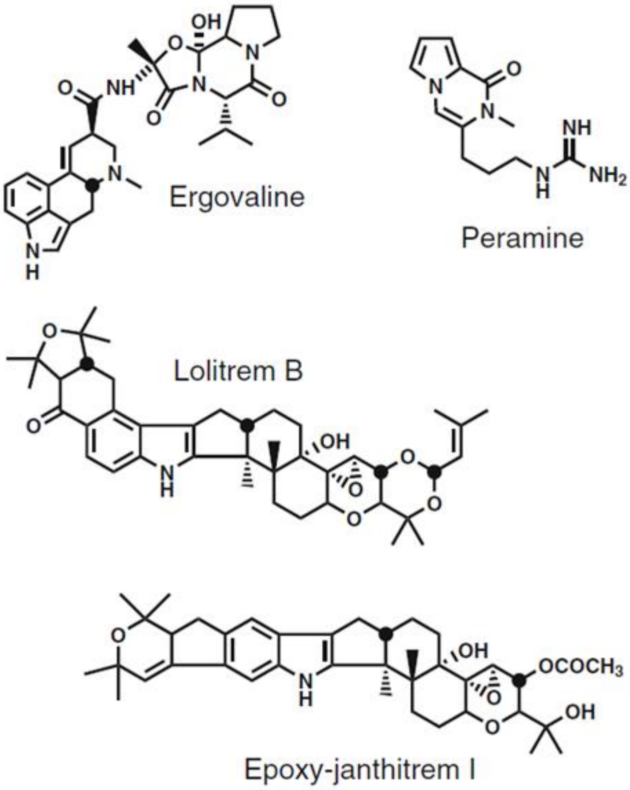

Figure 1.3: Chemical structures of alkaloids produced by *Epichloë* anamorphs. Modified from (Johnson, *et al.*, 2013)

Different *Epichloë* endophytes and different strains of *E. festucae* var. *lolii* will produce different alkaloids and different combinations and concentrations of these alkaloids (Table 1.1).

Table 1.1: Alkaloids produced by a selection of *E. festucae* var. *lolii* strains in ryegrass in New Zealand

Endophyte strain	Fungal species	Alkaloids
Wild Type	*E. festucae* var. *lolii*	Lolitrem Ergovaline Peramine
Endosafe	*E. festucae* var. *lolii*	Ergovaline Peramine
AR1	*E. festucae* var. *lolii*	Peramine
AR37	*E. festucae* var. *lolii*	Epoxy-janthitrem

1.2.1 Indole diterpenes

The epoxy-janthitrems, produced by the endophyte AR37, are within the indole diterpene class of alkaloids. The epoxy-janthitrems are a group of five compounds which are highly unstable and will readily degrade in light, heat and certain solvents, which makes working with these alkaloids difficult. The four main epoxy-janthitrem compounds are shown in Figure 1.4.

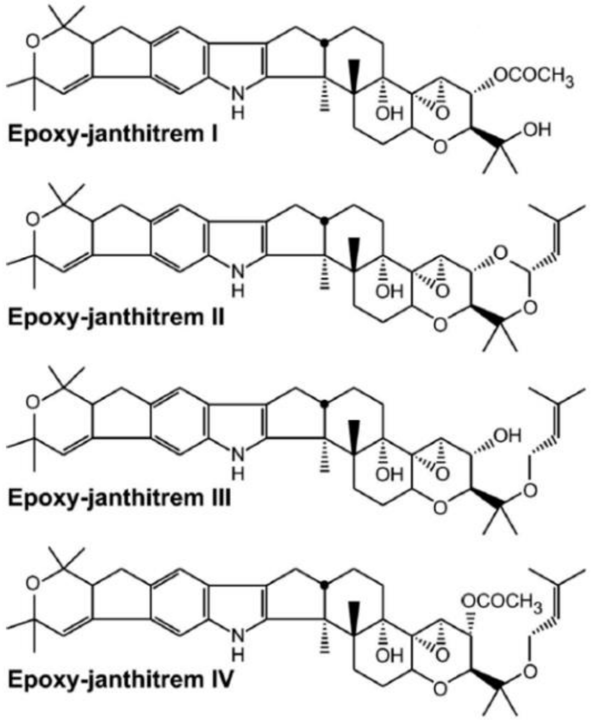

Figure 1.4: Chemical structures of epoxy-janthitrems I- IV, epoxy-janthitrem triol is not shown (Finch, *et al.*, 2010)

Little research has been conducted on the epoxy-janthitrems in comparison to other significant alkaloids expressed by ryegrass endophytes. However, the alkaloid lolitrem B and the epoxy-janthitrems are both within the indole diterpene class of alkaloids. Therefore, research on lolitrem B may give us some clues about the epoxy-janthitrems.

The highest concentrations of lolitrem B are found within the seed of the plant. Ball *et al.* (1997b) reported that lolitrem B concentrations were three times higher in the seed of the plant when compared with other plant tissues. Lolitrem B levels are also high in outer leaf sheaths and dead leaves of vegetative tillers (Ball, *et al.*, 1997b), and occur in higher concentrations in the pseudostems than the leaves (di Menna *et al.*, 1992). As lolitrem B and the epoxy-janthitrems are both lipophilic compounds (Munday-Finch & Garthwaite, 1999), which will affect the way thse alklaoids are distributed throughout the plant, a similar distribution pattern may be expected for the epoxy-janthitrems.

Alkaloid concentrations within the plant can be affected by a number of abiotic and biotic factors. Understanding how these factors increase or decrease alkaloid concentrations within the plant is important as ryegrass with low alkaloid concentrations can be more susceptible to insect attack. Two important factors which are known to affect alkaloid concentrations are temperature and plant genotype. What affect these factors have on epoxy-janthitrem concentrations in ryegrass is currently unknown.

Studies examining lolitrem B have shown that alkaloid concentrations change throughout the seasons. A study by Ball *et al.* (1995) inspected lolitrem B concentrations in ryegrass plants each month for a year. They found that concentrations were lowest during winter and spring and were higher in summer and autumn, when temperatures are higher and plants are likely under some degree of moisture stress (Figure 1.5).

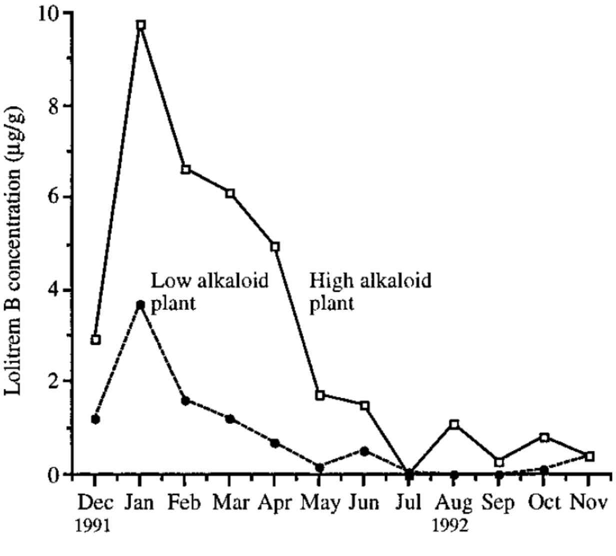

Figure 1.5: Seasonal changes in the concentration of lolitrem B in basal components of perennial ryegrass plants (Ball, *et al.*, 1995)

The effect of temperature on lolitrem B concentrations has also been studied in pot trials. Eerens *et al.* (1998) grew wild type infected ryegrass at low temperature

(15°C night and 25°C day) and high temperature (25°C night 35°C day). Temperature did not significantly affect lolitrem B concentrations. However, other alkaloids such as ergovaline were significantly increased at higher temperature.

Ryegrass cultivar and individual plant genotype are important biotic factors which can influence alkaloid concentrations within ryegrass. An experiment by Spiering *et al.* (2005) looked at three genotypes of perennial ryegrass infected with *E. festucae* var. *lolii.* The three genotypes were found to have varying levels of both endophyte mycelial mass and alkaloid concentrations (lolitrem B, ergovaline and peremine), with alkaloid concentration and endophyte mycelial mass only weakly correlated in this experiment.

Gaining an understanding of how temperature and plant cultivar may affect epoxy-janthitrem concentrations in ryegrass infected with the endophyte AR37 is important as the epoxy-janthitrems are suspected to provide AR37-infected ryegrass with increased pest resistance. Therefore it is important to identify whether abiotic and biotic factors cause concentrations in infected ryegrass to decrease to levels that maybe ineffective against insects, to identify any limitations of the AR37 endophyte in the field.

1.3 Perennial ryegrass (Lolium perenne)

1.3.1 Classification/ history in New Zealand

Lolium perenne, commonly referred to as perennial ryegrass, is a naturally diploid cool season grass from the family Poaceae. Perennial ryegrass originates from Europe, North Africa and temperate Asia and was introduced to New Zealand by English settlers in 1880 (Minnee, 2011). It is now widely used throughout New Zealand as a pasture species (Charlton & Stewart, 1999) as it is; quick to establish, produces good yields of palatable and quality herbage and is capable of withstanding grazing (Young *et al.*, 2013). It can also be planted in mixture with white clover and other pasture species (Charlton & Stewart, 1999). A number of cultivars have since been selected for their differing flowering times and ploidy level and are available to New Zealand farmers.

1.4 Italian ryegrass (*Lolium multiflorum*)

1.4.1 Classification/ history in New Zealand

Lolium multiflorum, commonly known as Italian ryegrass, is another cool season grass from the family Poaceae. Italian ryegrass is native to Europe and was introduced to New Zealand as a pasture species. Italian ryegrass is not as widely used as perennial ryegrass as it does not persist as well (Thom & Prestidge, 1996). However, it does have good growth through the winter months (Thom & Prestidge, 1996) and is often used between crops (Charlton & Stewart, 1999).

1.5 Porina (*Wiseana* spp.)

1.5.1 Porina classification

Porina (*Wiseana spp.* Table 1.2) are a group of closely related moths endemic to New Zealand (Barlow *et al.*, 1986). Seven species of *Wiseana* have been described first using morphological characteristics of the adult (Dugdale, 1994) and later using phylogenetic analyses (Brown *et al.*, 1999a, 1999b; Brown *et al.*, 2000).

There are no morphological characteristics which allow larva of this genus to be distinguished from one another. Using morphological features to distinguish between moth species in this genus is also difficult, especially for female moths. Species identification of the females is only achieved via dissection and expert examination of the female genitalia (Dugdale, 1994).

The seven identified species are *W. copularis, W. cervinata, W. umbraculata, W. signata, W. fuliginea, W. jocosa,* and *W. mimica* (Dugdale, 1994). Species from this genus are distributed from Northland to Stewart Island and can also be found on Chatham Island (Dugdale, 1994). *W. umbraculata* is the most widely distributed species in the genus, followed by *W. cervinata* (Dugdale, 1994).

The habitat of these 7 species varies. *W. jocosa* is located in forest margins and clearings; *W.umbraculata* inhabits swampy margins and *W. signata* is found in freely draining soils (Dugdale, 1994). The remaining 4 species; *W. cervinata*, *W. fuliginea*, *W. mimica* and likely *W. copularis* have thrived with the increase in farmland in recent decades, feeding on introduced grass species and clover plants (Dugdale, 1994), causing severe damage to pastures.

Table 1.2: Scientific classification of porina (*Wiseana* spp.)

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Family	Hepialidae
Genus	Wiseana

1.5.2 Porina lifecycle

Porina go through one life cycle each year. The lifecycle begins in October/ November when the adult moths emerge from their pupa, although some moths can emerge as late as Feburary (*W. copularis*). On emergence, which occurs in the hour after sunset (Pottinger, 1968), the adults will immediately try to find a mate. Adult moths will only live for up to four days (Dick, 1945) so it is important they mate soon after emergence. Female moths will produce a sex pheromone by fanning their wings (Allan & Wang, 2001). When males downwind of the famale detect this pheromone they will fly to the female and mate (Allan & Wang, 2001). Once the female is mated she will begin oviposition (Allan & Wang, 2001) and is capable of depositing a considerable number of eggs amongst the pasture. A study by Allan and Wang (2001) which observed the mating behaviour of *W. copularis* adults in a wind tunnel found females were producing between 450 and 1750 eggs once mated. Usually these eggs will be laid near the burrow from which the female emerged (Dick, 1945). The adult moths themselves are not destructive to pasture due to their short lifespan and the fact they do not have mouth parts capable of feeding (Dugdale, 1994). It is the larvae which inflict the damage to pastures.

Porina eggs (Figure 1.6) are small, globular, and turn black in colour within 24 hours (Dugdale, 1994). The duration of egg development is very much dependent on temperature, but this stage usually last 30- 42 days (French & Pearson, 1979). Once the young larva emerges from its egg it will remain on the soil surface for four to six weeks (Barlow, *et al.*, 1986), during this time the larva produces a webbing it can reside under. When the larva is old enough it is able to burrow down into the soil, eventually creating vertical tunnels up to 20cm deep (Barlow, *et al.*, 1986). These burrows are then lined with a silk webbing (Dugdale, 1994).

Figure 1.6: Porina eggs from a single moth on top of a moist piece of filter paper within a Petri dish

Porina larvae (Figure 1.7) are nocturnal, emerging from their burrows at night to feed. When a larva emerges from its tunnel it largely feeds within an 8cm radius around its burrow entrance (Harris, 1969). Larvae will feed on ryegrass, clover and cocksfoot plants that are available up to 5cm off the soil surface (Harris, 1969). An experiment by Harris (1969), which observed feeding of porina, indicated that feeding on ryegrass predominantly occurred via the larva severing tillers at the base. Also observed in the experiment were whole leaf blades that had been pulled into the burrow while still attached to the plant.

Figure 1.7: Young porina larva

Larval feeding is most destructive to pastures during the autumn and winter months, with the maximum feeding occuring during September as larvae begin to accumulate fat for pupation (French & Pearson, 1981). During September French & Pearson (1981) found that larvae were consuming an average of 0.0340 grams of green matter/day of white clover. Due to their long larval period, porina can grow up to 7cm long and weigh 1g (Barlow, *et al.*, 1986).

Porina larvae will pupate in September/ October, forming their pupa within the larval burrow. The pupa is able to move within the burrow and in the later stages is able to move up to the burrow entrance to allow the moth to emerge (Dugdale, 1994). The life cycle will then begin again (Figure 1.8).

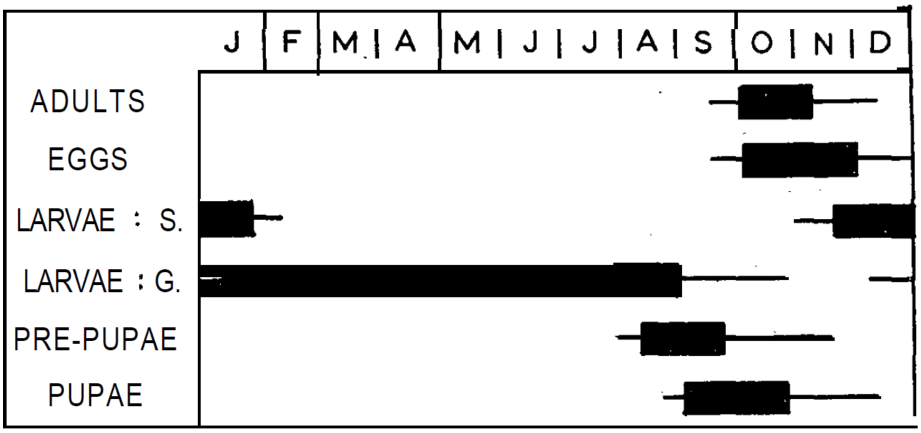

Figure 1.8: *Wiseana cervinata* life cycle (Pottinger, 1968). Larvae: S represents the stage when larvae are surface dwelling and Larvae: G when the larvae are able to burrow underground.

1.5.3 Methods of control

Porina are significant pasture pests and are capable of occurring in very large densities so it is important to try to control their numbers in a farming system to reduce damage. There are a few methods of control that farmers can implement to reduce porina populations on farms.

Insecticides can be applied, either when young larvae are living on the surface or after they have burrowed. Mobstocking is another viable option to avoid having to apply insecticides to pasture. A study by Stewart and Ferguson (1992) has shown multiple heavy mobstocking was able to reduce larval density by 69%, when young larvae where on the surface.

Farmers also have the option of sowing with endophyte infected ryegrass. The novel endophyte AR37 has been shown to provide ryegrass with protection against porina larvae (Jensen & Popay, 2004; Finch, *et al.*, 2010; Popay, *et al.*, 2012). This protection can be seen in Figure 1.9. Here two ryegrass plants, one infected with AR37 and the other endophyte-free, were planted in between two glass slat boards with two porina larvae, within days larvae had consumed the entire endophyte-free plant. The wild type endophyte may also provide some protection against porina larvae but AR1 has no effect on larvae (Jensen & Popay, 2004).

Figure 1.9: Two ryegrass plants, one infected with AR37 and the other with Nil endophyte planted between two glass slat boards with two porina larvae.

1.6 Previous research of AR37 on porina larvae

Jensen and Popay (2004) looked at the effect of AR37 on porina larvae in pot trials and choice and no-choice bioassays. AR37 was shown to affect the growth and development of porina larvae, and reduce survival in the pot trials. In the no-choice bioassay larvae fed AR37-infected ryegrass consumed a similar amount to larvae fed Nil endophyte ryegrass. However, in the choice bioassay porina larvae preferred Nil endophyte ryegrass when given the choice between Nil and AR37, indicating some deterrence by AR37 on porina larvae. Whether AR37 is also toxic to larvae was not able to be determined.

Popay, *et al.* (2012) looked at the effect of AR37 on porina larvae in two field trials to see if the results from the earlier lab trials were replicated in the field. The first trial involved Italian ryegrass infected with AR1, AR37 or Nil. Although only low numbers of porina were found in AR1 and Nil plots this result was significantly different to AR37 plots where no porina larvae where found. The second trial involved a number of tetraploid and diploid plants infected with either AR37, NEA2 (mix of *Epichloë festucae* var. *lolii* strains), AR1 or a low wild type infection. Porina populations were lower in the AR37 treatments compared to all other treatments. A lower density of larvae was also observed in two of the diploid AR37-infected cultivars when compared to the tetraploid AR37 cultivar. Indicating diploid cultivars may be more resistant to larvae.

Finch, *et al.* (2010) looked at whether the deterrent effects of AR37 on porina larvae were caused by the epoxy-janthitrems. In this bioassay epoxy-janthitrem I, the main epoxy-janthitrem compound produced by AR37, was added to a semi-synthetic insect diet and feed to porina larvae daily for seven days. This alkaloid was found to reduce both the feeding and growth of porina larvae. Whether larvae are deterred over a longer period of time or whether the alkaloid was also toxic to larvae could not be determined.

1.7 Thesis aims

The aims of this thesis are to fill gaps in the literature by: (a) investigating the toxicity of epoxy-janthitrem I to porina larvae; (b) exploring what affect temperature and plant cultivar have on epoxy-janthitrem concentrations in ryegrass and how these variations in concentration affect feeding, growth and survival of porina larvae.

AR37-infected ryegrass has been shown to have some deterrence from porina larvae when compared to Nil endophyte ryegrass (Jensen & Popay, 2004) and the epoxy-janthitrem I alkaloid, produced by AR37, has been shown to reduce feeding and growth of larvae over 7 days (Finch, *et al.*, 2010). The first section of this thesis will investigate whether epoxy-janthitrem I is deterrent to porina larvae over an extended period of time and determine whether this alkaloid is toxic to larvae. To explore this, epoxy-janthitrem I will be incorporated into a semi-synthetic diet and fed to porina larvae for 3-4 weeks. Feeding, growth and survival of larvae in the alkaloid treatments will then be compared to that of larvae fed on equivalent Nil control diets and a starvation control.

Popay, *et al.* (2012) has shown through field trials in Canterbury that AR37 provides resistance to ryegrass from porina larvae in the field. However, there have been reports, both from the South Island and the North Island, that AR37 is not able to provide adequate control of larvae. These reports are generally from colder areas where there were late flying moths (Popay & Ferguson unpublished information). The second part of this thesis will investigate these reports by exploring what affect temperature and plant cultivar have on epoxy-janthitrem concentrations in ryegrass. To investigate this, two AR37-infected ryegrass cultivars will be grown in a pot trial in one of two controlled environment rooms, one set at high temperature (20°C) and the other low temperature (7°C). Ryegrass from the pot trial will then be harvested, freeze-dried and ground and the epoxy-janthitrem concentrations determined. Ground plant material will then be incorporated into a semi-synthetic diet to be fed to porina larvae. Feeding, growth and survival of larvae in AR37-infected treatments will be compared to Nil endophyte treatments. Information from this experiment may then be able to explain why AR37-infected ryegrass in these colder areas does not seem to provide adequate resistance to porina.

The hypotheses for this thesis are:

- Epoxy-janthitrem I, produced by the endophyte AR37, is not only deterrent to porina larvae but is also toxic;
- (a) Ryegrass cultivars will differ in epoxy-janthitrem concentration and (b) epoxy-janthitrem concentrations will be higher when ryegrass is grown at high temperature and that both these variations in concentration will affect feeding, growth and survival of porina larvae.

Chapter 2: General Materials and Methods

Materials and methods described in this chapter are the methods which are in common to both experiments in this thesis.

2.1 Porina (*Wiseana* spp.)

2.1.1 Collection of adult porina

Porina moths were collected by Colin Ferguson (AgResearch). Adults were caught at night at Allanton, near Mosgiel, in the South Island using an incandescent light as an attractant. One of the moths was collected on the 27th of November and thirty nine on the 5th of December 2013.

2.1.2 Identification of female moths

Female moths were identified by Colin Ferguson by examination of the bursa copulatrix (Dugdale, 1994). Twenty eight of the moths were identified as *Wiseana cervinata* and three as *Wiseana copularis*. The eight remaining moths were not identified.

2.1.3 Egg sterilization

Moths were held in 60mL specimen vials at room temperature overnight, to allow the 40 female moths to lay their eggs. Eggs were sent to AgResearch Ruakura on the 9th of December.

Porina eggs were surface sterilized on the 10th of December (Figure 2.1) following methods outlined in Carpenter (1983) using a copper oxychloride solution (0.5mg of copper oxychloride powder and 500mL of water). A piece of fine nylon cloth was placed on top of a glass beaker. The eggs from a single moth were poured onto the mesh and 10mL of copper oxychloride solution applied (using a 5mL pipette). Eggs were left to sit for 2 minutes before they were washed with 20mL of Milli- Q water. Care was taken to ensure all eggs were rinsed thoroughly.

Following sterilization the eggs were placed on a moist piece of filter paper (LabServ qualitative filter paper, 90mm) within a Petri dish (9cm). The Petri dish was sealed in parafilm and placed into a labelled 50ml plastic container with a lid (length 175 mm, width 120, depth 70). Containers were kept in a controlled environment room set at 18°C until larvae hatched.

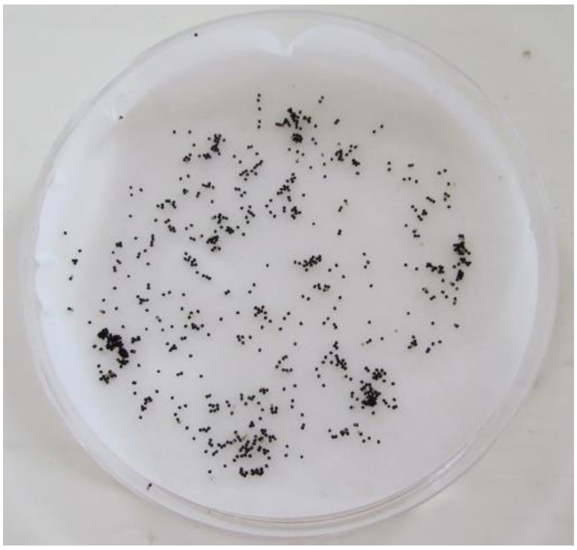

Figure 2.1: Surface sterilized porina eggs laid out on a moist piece of filter paper within a Petri dish to hatch

2.1.4 Hatching of larvae

The majority of larvae hatched on the 24th of December, after 13 days in the controlled environment room. Once larvae hatched they were moved into labelled plastic containers quarter filled with bark (Yates Bloom Décor Bark fine sized chip, some containers were later toped up with Flourish Decoration Bark fine sized chip). Approximately 40 larvae were added to each container (Figure 2.2) along with 20 small chunks of semi-synthetic diet (refer to section 2.1.5) evenly spread over the bark (Popay, 2001). Eggs that were unhatched by the afternoon of the 24th were left in their Petri dishes, with the lids removed, and placed into a container of bark with semi-synthetic diet. Larvae from different adult moths were always kept separated.

Porina larvae were originally reared in a 15°C controlled environment (CE) room (16 hour light: 8 hour dark regime), but were transferred to a 7°C CE room (12 hours light: 12 hours dark) to slow larval growth.

Figure 2.2: Hatched larvae transferred into a bark filled takeaway container. Some larvae are circled in red

2.1.5 Semi-synthetic diet

A semi-synthetic diet was made according to methods outlined in Popay (2001) and fed to porina weekly. The ingredients were; 100g white clover, 100g carrot, 500mL Milli-Q water (purified water), 16g yeast, 12g agar, 1g methyl 4-hydroxybenzoate, 0.5g sorbic acid, 1.6g ascorbic acid, and 1mL of 10% formaldehyde.

White clover was harvested from pots maintained in an AgResearch screenhouse. Flowers, dead leaf material and thick stems were not included. Carrot was cut into small chunks and put into a blender (Waring commercial blender, model HGB2WTG4) along with 200mL of Milli-Q water. Handfuls of clover and then the yeast were added to the blender as it was mixing and blended to a fine mush.

Agar, methyl 4-hydroxbenzoate, sorbic acid, and 300mL of Milli-Q water were added to a glass beaker (900ml) and mixed. The beaker was warmed on high heat in a microwave for approximately 2.5-3 minutes, with regular mixing, until the agar began to boil (indicated by the level of agar ‘rising’). A thermometer was added to the beaker and the contents occasionally stirred until it cooled to 70°C. Ascorbic acid and formaldehyde were then added to the beaker followed by the carrot/ clover mix. The diet mix was transferred into 14-16 Petri dishes (9cm) and left to set. This process was completed quickly before the agar began to set. Diet was stored at 4°C.

2.1.6 Maintenance of porina larvae

After hatching the porina colony was maintained for up to 8 months. Maintenance of the colony required weekly diet changes (Figure 2.3). When larvae were young they were often attached to the diet so they were removed from the diet individually using a fine paint brush. As larvae grew they were distributed between additional containers to reduce their density and fresh bark chips (Yates Bloom Décor Bark fine sized chip) were supplied as required.

Figure 2.3: Process if changing over semi-synthetic diet in larval containers

2.2 Insect bioassays

2.2.1 Bioassay setup

Plastic specimen containers (for the epoxy-janthitrem I bioassays- LabServ, 70mL, polystyrene; for the ryegrass temperature trial bioassay- LabServ, 150mL, polystyrene) were labelled and sorted by treatment into polystyrene trays (Figure 2.4). Containers were approximately ¾ filled with bark (Yates Bloom Décor Bark fine sized chip).

Figure 2.4: Bioassay specimen containers within the polystyrene boxes they were kept in throughout the trial.

The day before the beginning of the insect trials the colony larvae were sorted through and selected larvae were placed into individually labelled 30mL containers. Containers where kept in a polystyrene box with a layer of damp paper towels, to maintain humidity, overnight at 15°C.

2.2.2 Weighing and sorting of porina larvae

After larvae were held overnight without food they were weighed individually (Metler Toledo analytical balance XS204, readability 0.1mg) and their weights recorded (Figure 2.5). Care was taken to remove any large pieces of soil or frass from the larvae before they were weighed.

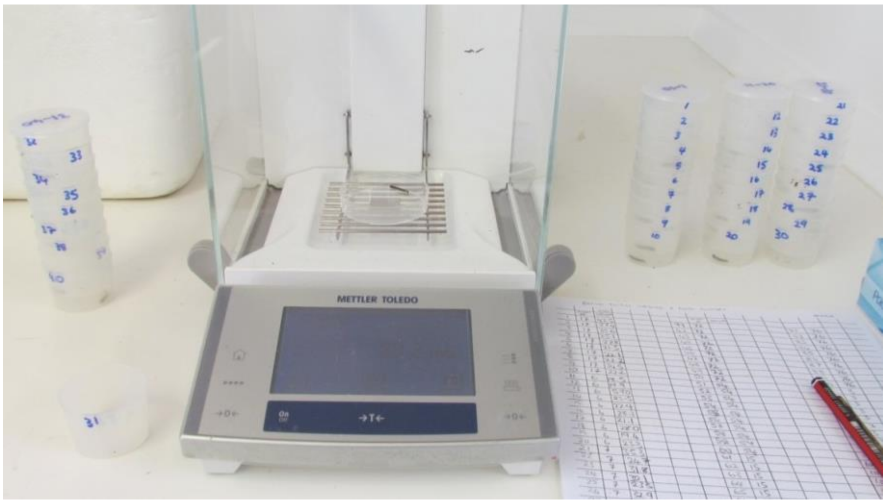

Figure 2.5: Process of weighing porina larvae

Porina were sorted by weight and allocated to a treatment and replicate. Larvae were allocated so that larvae in the same replicate across all treatments were of similar weight. The seven larvae in each replicate were randomly assigned to a treatment to ensure all of the smallest larvae within each replicate were not all in the same treatment.

The correct larvae were then added to the correct specimen containers ¾ filled with bark (Figure 2.6).

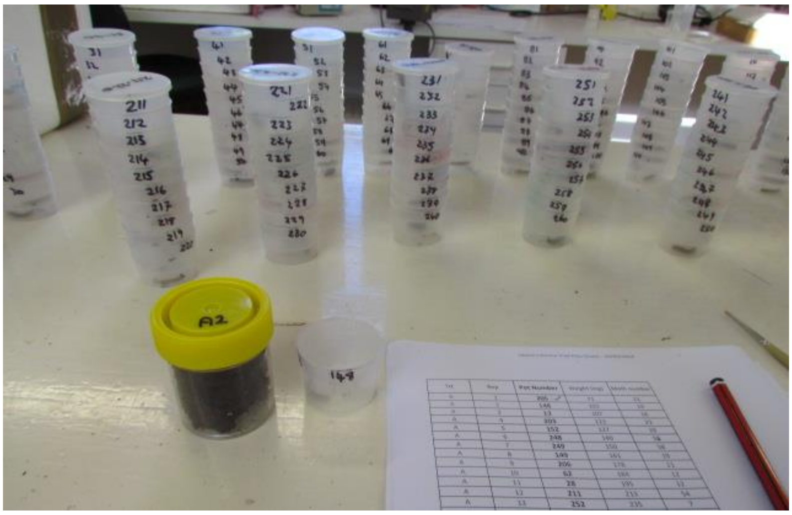

Figure 2.6: Larvae transferred into their assigned specimen container

2.2.3 Weighing of diet and diet changes

Discs of the different treatment diets were cut using a cork borer (12- 15mm) and then weighed, the weight recorded, and the diet added to the correct specimen container. Diets in Petri dishes were kept under aluminium foil and specimen containers were covered with cloth to exclude light.

A fresh batch of diet was made weekly and diet in the specimen containers was changed twice weekly. This was necessary due to degradation of epoxy-janthitrems and mould growing on the diet over time.

The diet changing process involved removing and weighing remnant diet from each specimen container to determine consumption of each larva. Porina frass and small pieces of bark were removed from the diet before it was weighed. Porina can move their diet through the container so the surface layer of bark, and the sides and base of the container were examined for pieces of diet.

2.2.4 Larval survival

Larvae were recorded as alive, moribund, or dead. A larva was recorded as alive if it exhibited significant movement. Larvae that had very limited movement, e.g. small leg twitches, were recorded as moribund to indicate they were not healthy. Larvae were recorded as dead if they did not move or twitch when gently touched with a paint brush. Without exception, larvae recorded as moribund did not recover and were usually dead within 24 hours. Dead larvae were removed from their containers, weighed and discarded. The diet was retained in each specimen container until it was weighed at the next change of diet.

2.2.5 Bioassay conditions

The insect bioassays were conducted in a controlled environment room set at 15°C. Black plastic polythene was placed over the polystyrene trays, holding the specimen containers, to exclude light and the lights directly above the experiment were switched off. These conditions were necessary as epoxy-janthitrem in the diet will degrade in light.

2.3 HPLC analysis of semi-synthetic diet and herbage

Epoxy-janthitrem concentrations within semi-synthetic diet samples and herbage were determined using high performance liquid chromatography (HPLC).

HPLC is an analytical chemistry technique used to separate components of an extract using a chromatography column. Components are then detected using a detector, for the epoxy-janthitrems a fluorescence detector is used, and quantified by comparison with a reference standard or a direct standard.

A chromatogram produced from an AR37-infected ryegrass herbage sample is shown in Figure 2.7.

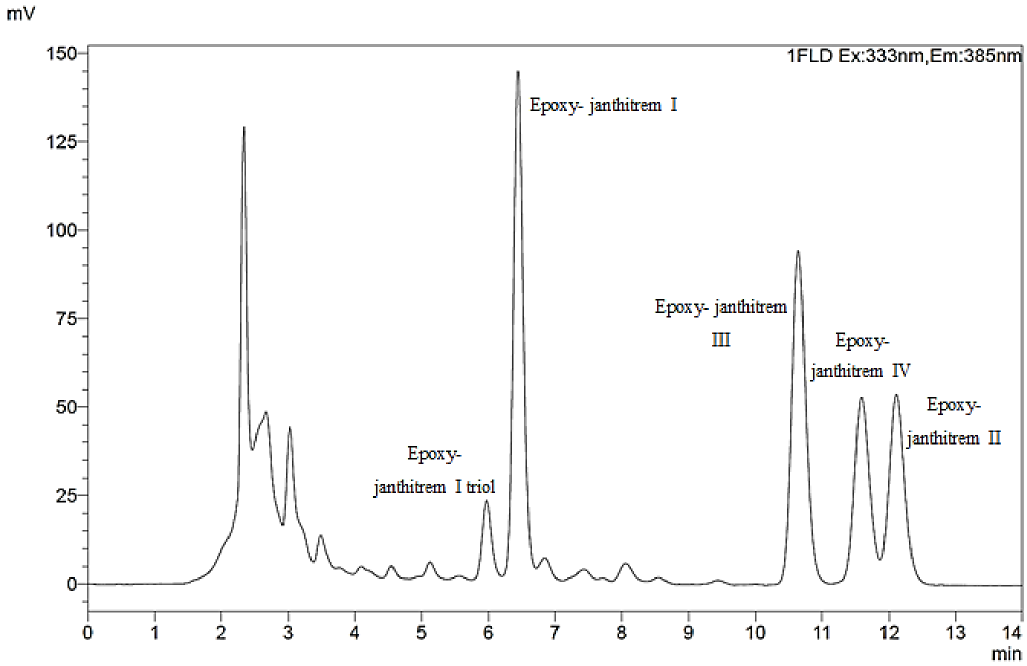

Figure 2.7: Chromatogram of the five epoxy-janthitrem alkaloids obtained from AR37-infected ryegrass. For column and detector settings see 2.3.1.2

2.3.1 HPLC equipment

2.3.1.1 Epoxy-janthitrem I bioassay

The column used was a 4.6250mm ODS C18 column (Phenomenex, Torrance CA, USA) with an eluent of water-acetonitrile (1:19, 1mL/min). Eluting compounds were detected with an Agilent Series 1100 fluorescence detector (excitation at 333nm, emission detection at 385nm).

2.3.1.2 Ryegrass temperature trial bioassay

The column used was a 4.6 x 250mm ODS C18 column (Phenomenex, Torrance CA, USA) with an eluent of water-acetonitrile (1:19, 1mL/min). Eluting compounds were detected with a Shimadzu LC-20AD Prominence Liquid Chromatograph and a Shimadzu RF-10AXL Fluorescence Detector (excitation at 333nm, emission detection at 385nm).

2.3.2 Extraction of porina semi-synthetic diets and herbage samples for HPLC analysis

50± 5mg of the semi-synthetic diet sample or 20± 2mg of the plant material was weighed into an eppendorf tube (Eppendorf safe-lock tube, 2mL). Duplicates were prepared for each sample.

Samples were extracted by adding 1mL of 80% acetone and leaving the samples on a rotating wheel for one hour (Labnet international, inc. mini labroller™). Samples were then spun on a centrifuge for one minute at 12000rpm (Eppendorf Centrifuge, 5418).

The supernatant was transferred from the eppendorf tube into an auto sampler vial using a pasteur pipette. 5µL of each sample was either manually injected (epoxy-janthitrem I bioassay) or automatically injected (ryegrass temperature trial bioassay) into the HPLC machine. A chromatogram was produced and the epoxy-janthitrem concentration calculated from the known peaks.

Semi-synthetic diet samples were analysed on the day (Epoxy-janthitrem I bioassay) or the day after (ryegrass temperature trial bioassay) the diets were changed. Samples of fresh diet (diet to be added to the specimen containers) were analysed to ensure that the correct epoxy-janthitrem levels were in the diets and the diets were homogenous. Remnant diets (pieces of diet remaining after exposure to larvae for 3-4 days) were analysed to calculate epoxy-janthitrem degradation.

2.4 Chemicals: supplier and grade

- Acetone: LabServ, redistilled through a small cyclone evaporator
- Methanol: Fisher Scientific, HPLC grade
- Petroleum spirit: LabServ, 40-60°C, analytical reagent grade
- Sodium chloride: BDH, AnalaR®
- Toluene: Fisher Scientific, HPLC grade
- Water: Millipore Milli-Q grade

Chapter 3: Bioactivity of Epoxy-janthitrem I to Porina Larvae

3.1 Introduction

The endophyte AR37 provides superior protection to ryegrass from a number of ryegrass pest insects including porina larvae (*Wiseana* spp.) (Johnson, *et al.*, 2013). Porina are a group of seven closely related moth species endemic to New Zealand (Barlow, *et al.*, 1986; Dugdale, 1994). Nocturnal larvae live beneath the soil surface in silk lined tunnels, emerging at night to feed on ryegrass plants by severing tillers off at the base of the plant and dragging them back into the tunnel (Harris, 1969).

In a study by Jensen and Popay (2004) growth, development and survival of porina larvae were reduced by AR37 in pot trials and in a choice bioassay larvae were shown to prefer Nil endophyte ryegrass over AR37-infected ryegrass, indicating AR37 was deterrent to larvae.

The only alkaloids known to be produced by AR37 are the epoxy-janthitrems, a group of five compounds. These alkaloids are difficult to work with as they are highly unstable and readily degrade when exposed to light, heat and certain solvents. The epoxy-janthitrems are thought to be involved in the bioactivity of AR37. In a 7 day bioassay, Finch, *et al.* (2010) showed that pure epoxy-janthitrem I reduced growth and feeding of porina larvae. Whether epoxy-janthitrem I has a deterrent effect on larvae over an extended period of time and whether it is toxic to larvae was not determined.

The aim of this study was to investigate the effect of epoxy-janthitrem I on porina growth and survival to determine whether epoxy-janthitrem I is toxic to porina larvae. Toxicity is defined as a reduction in growth and survival of larvae, above that which can be attributed to starvation. It is important to determine toxicity to improve understanding of how AR37 and the epoxy-janthitrems effect porina populations in the field. If the epoxy-janthitrems are only deterrent to larvae,

larvae may search for alternative food sources within the farm. For example porina may be deterred and feed on white clover which is often planted in conjunction with ryegrass in a paddock. In comparison, if the epoxy-janthitrems are also toxic, survival of porina in the population would be reduced. The hypothesis tested was that epoxy-janthitrem I, produced by the AR37 endophyte, is not only deterrent to porina larvae but is also toxic.

3.2 Materials and methods

An AR37 ‘whole seed’ extract was prepared from perennial ryegrass seed and purified to gain a fraction that contained epoxy-janthitrem I. Three concentrations of this fraction along with equivalent Nil controls, produced by purification of a Nil ‘whole seed’ extract, were incorporated into a semi-synthetic diet and fed to porina larvae in an insect bioassay. Feeding, survival and growth on epoxy-janthitrem treatments were compared with Nil treatments and a starvation treatment to determine how epoxy- janthitrem I affects growth and survival of larvae. Nil treatments were also included to eliminate any effects of the matrix or the purification process on larvae as the epoxy-janthitrem fraction tested here was not pure epoxy-janthitrem I.

3.2.1 Experimental design

The epoxy-janthitrem I bioassay was performed twice, first with 9 week old larvae and then with 21 week old larvae. The insect bioassays contained seven treatment groups (Table 3.1) each with 15 replicates. Each replicate was a single porina larva (105 larvae). The treatments were three concentrations of epoxy-janthitrem I (1, 2.5, 5µg/g) plus three equivalent Nil controls and a starvation control.

Table 3.1: Epoxy-janthitrem I insect bioassay treatments.

Treatment number	Treatment name	Epoxy-janthitrem I extract wet weight concentration (µg/ g)
A	Epoxy-janthitrem I low concentration	1
B	Epoxy-janthitrem I medium concentration	2.5
C	Epoxy-janthitrem I high concentration	5
D	Nil extract low concentration	
E	Nil extract medium concentration	
F	Nil extract high concentration	
G	Starvation control	

3.2.2 Extraction and purification of epoxy-janthitrem I

Epoxy-janthitrem extraction and purification was performed by Dr Sarah Finch (AgResearch, Ruakura). An outline of the methods used to extract the AR37 whole seed and the purification steps to obtain epoxy-janthitrem I are shown in Figure 3.1. The epoxy-janthitrem I extract to be tested was not pure epoxy-janthitrem I, this extract also contained a small amount of epoxy-janthitrem triol. Analogous extraction and purification methods were used to obtain Nil equivalent samples from Nil endophyte ryegrass seed. Sufficient quantities were produced from the extraction and purification to allow for two bioassays. Samples were stored in a freezer until required.

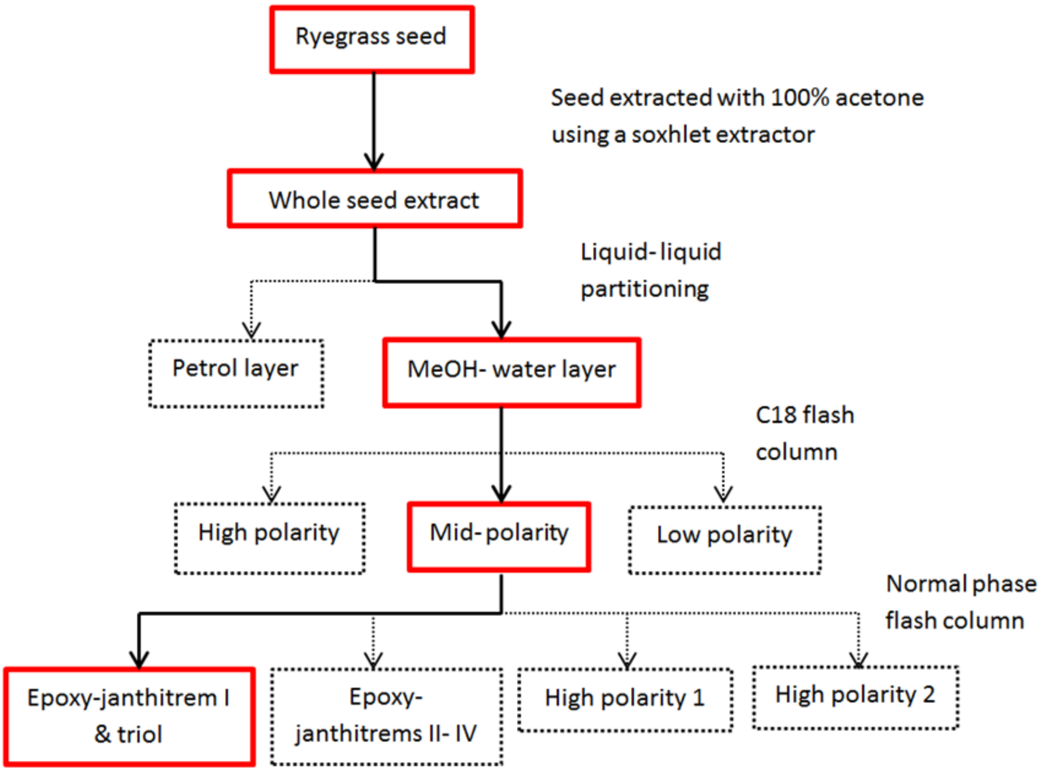

Figure 3.1: Steps involved in the extraction and purification of the epoxy-janthitrem I extract (which also contained a small amount of epoxy-janthitrem triol) and the Nil equivalent controls to be tested in the bioassay. Extracts which were retained from each purification step are highlighted.

3.2.2.1 Extraction of ryegrass seed

Seventy grams of AR37 and Nil endophyte perennial ryegrass seed (cv 'Extreme') was ground (UDY Cyclone sample mill) and packed into separate cellulose extraction thimbles (Labserv, 41 by 123mm, 1mm thick). Thimbles were placed into a soxhlet extractor with approximately 400mL of acetone (100%) and left to extract for 7 hours, separately. Tin foil was wrapped around the soxhlet to exclude light and prevent degradation of the epoxy-janthitrems. The extract collected was filtered and dried using a rotary evaporator (Buchi rotary evaporator).

3.2.2.2 Liquid- liquid partitioning

The two extracts (AR37 and Nil) were each dissolved in 150mL of MeOH and added to a separating funnel along with 12.5mL of H_2O and 0.5mL of NaCl. The MeOH-H_2O extract was then partitioned with petroleum spirit (40- 60°C 3x 100mL). Two layers were formed, a petrol layer which contained the fats from the extract and an MeOH- H_2O layer which contained the epoxy-janthitrems.
The MeOH- H_2O layer was retained and dried using a rotary evaporator.

3.2.2.3 C18 flash column

A C18 flash column was packed with LiChroprep® silica (Merck Millipore, RP-18, 40-63µm).The column was flushed twice with 50mL of acetone and then equilibrated with 50mL of 75% MeOH-H_2O.

The epoxy-janthitrem and Nil equivalent extracts from the liquid-liquid partition were dissolved, using a sonicator, in 10mL of MeOH. To the extract 3.3mL of H_2O was added and the extract applied to the C18 flash column. The column was eluted with 25mL of 75% MeOH-H_2O, 150mL of 90% MeOH-H_20 and 100mL of 100% acetone. The 90% MeOH-H_20 fraction (mid-polarity) was analysed by HPLC to confirm that the epoxy-janthitrems were present in this fraction. The fraction was then retained and dried down on a rotary evaporator.

3.2.2.4 Normal phase flash column

A normal phase flash column (21 x 2cm) containing silica gel (Merck, art. 9385) packed as a slurry with 90% toluene-acetone was prepared. The mid-polarity fraction, from the C18 flash column, was re-dissolved in 10mL of 90% toluene-acetone and applied to the column.

The column was eluted with 100mL of 90% toluene-acetone and the extract collected in a conical flask. The column was then eluted with 600mL of 85% toluene-acetone and the extracts collected in test tubes (12 per row, 14mL). Test tubes were stored on ice and in the dark.

The test tubes collected from the AR37 extract were analysed for epoxy-janthitrems by HPLC. Test tubes A7-B9 were found to contain epoxy-janthitrem I and epoxy-janthitrem triol. These test tubes were combined and dried down to form the epoxy-janthitrem I fraction used in the porina bioassays. As the same methods were used to extract and purify the Nil endophyte extract, test tubes A7-B9 from the normal phase flash column using the Nil extract were also dried down to form the Nil extract equivalents for the porina bioassays.

3.2.2.5 Preparation of sample aliquots

The dried fractions from the normal phase flash column were re-dissolved in 50mL of methanol. The AR37 extract was analysed by HPLC to determine the quantities of epoxy-janthitrem I and epoxy-janthitrem triol within the fraction. The fraction was found to contain a total of 2439µg of epoxy-janthitrems, 1948µg of this was epoxy-janthitrem I (80% of the total) and 491µg was epoxy-janthitrem triol (20% of the total).

For preparation of 20g of semi-synthetic diet/treatment/week, aliquots of 25, 62.5 and 125µg which is equivalent to 513, 1281, 2563µL of the 50mL epoxy-janthitrem fraction, were produced to give the required concentrations of 1, 2.5 and 5µg/g required in the epoxy-janthitrem treatment diets. Equal aliquots were obtained from the Nil extract fraction to create the Nil equivalent treatments for the bioassay. Fractions were transferred into glass vials (4mL) and dried under a stream of nitrogen before the vials were put onto a freeze drier for 15 minutes.

Aliquots from the same AR37 and Nil fractions, produced for the first bioassay, were prepared for the second bioassay (Experiment 2). Before the beginning of Experiment 2 epoxy-janthitrem concentrations in the fraction were re-analysed and aliquots for AR37 and Nil samples produced using similar methods as those above. Vials were stored in a freezer (-80°C).

3.2.3 Insect bioassay

Experiment 1 was run for 24 days and Experiment 2 for 28 days.

Porina larvae for the first experiment were 9 weeks old and weighed between 19 and 42mg. Larvae in the second experiment were 21 weeks old and weighed between 70 and 270mg. Healthy larvae (170 larvae for Exp. 1 and 273 larvae for Exp. 2) were selected from progeny of 13-14 parent moths, that had been reared separately, to increase genetic variation amongst larvae in the experiment.

Porina were sorted by weight and allocated to a treatment and replicate (see 2.2.2). Larvae were allocated so that larvae in the same replicate across all treatments were of similar weight. Larvae in Experiment 1 were not sorted by size in each replicate before they were assigned to treatments, so the smallest larvae of each replicate were in treatment A and the largest in treatment G. In Experiment 2 the seven larvae in each replicate were randomly assigned to a treatment to ensure all of the smallest larvae within each replicate were not all in the same treatment.

Individual larvae were then placed into their assigned specimen vials ¾ filled with bark (see 2.2.1 and 2.2.2).

3.2.3.1 Diet preparation

The semi-synthetic diet used in this bioassay was produced using the same ingredients and methods as the diet the larvae were reared on (see 2.1.5) except the anti-fungals (methyl 4-hydroxybenzoate, sorbic acid, ascorbic acid and formaldehyde) were not included. This is because previous work had shown that anti-fungals could accelerate epoxy-janthitrem degradation. Half a batch of the diet described in 2.1.5 was produced when making diet for Experiment 1 (using half the amount of each diet ingredient). A full batch was produced for Experiment 2, as larvae were larger and consumed more diet per week.

An additional step was also added to the bioassay diet method. The carrot/ clover mix was warmed on med-low (Sharp Carousel) for one minute, mixed and stirred, and then warmed for a further minute before it was incorporated into the agar.

This step was added to prevent the agar from cooling and setting until the treatments could be incorporated. Diet was kept in a water bath to maintain temperature while individual batches of diet were weighed out.
While the diet was prepared, vials containing the treatments (extracts) to be added to individual batches of diet were removed from the freezer and warmed to room temperature. To each vial 400µL of DMSO was added, using an HPLC syringe. The vials were then placed into a sonicator to aid dissolution of the extracts.

Six labelled (A-G) glass beakers (75-150mL) were warmed on a warming plate (Heidolph, MR3002C, aprox. 125°C). Twenty grams of diet in Experiment 1 and Twenty five grams in Experiment 2 were weighed out into each beaker. Each extract (400µL) was added to one of the beakers, using an HPLC syringe, and stirred thoroughly to ensure the diet was homogenous. Each diet was poured into a correspondingly labelled Petri dish (9cm) and smoothed flat using a spatula. Diets were put inside a closed chilli bin, until plugs were removed to feed to porina, Petri dishes were then sealed with Parafilm and kept refrigerated at 4°C. The diet making process was timed carefully to ensure all treatments were made before the agar set.

Fresh diets were made weekly and diet in the insect containers changed twice a week. Diets were weighed in and out of the specimen containers on day 4 and day 7 of each week to determine how much diet each larva was consuming (see 2.2.3). Larvae were examined daily for survival (see 2.2.4). Data were recorded on spreadsheets with care taken to ensure accuracy.

Epoxy-janthitrem concentrations in fresh diets and remnant diets (diets fed on by larvae) at each diet change were determined by HPLC (see 2.3).

3.2.4 Statistical analyses

Data on larval diet consumption, mortality and growth were collected during the bioassay and analysed using Microsoft Excel 2010 and GenStat 16th edition.

3.2.4.1 Larval feeding analysis

Larval feeding analysis was based on amount of diet eaten per week.

Data from both experiments were transformed by log (to base 10). For Experiment 1 a general ANOVA was performed on weeks 1 and 2 consumption data and an unbalanced ANOVA performed on week 3 consumption data as mortality of larvae reduced the number of replicates in some treatments. Initial weight was used as a covariate in these analyses. Replicate could not be used as a blocking variable in Experiment 1 as larvae in each replicate were not blocked by weight.

In Experiment 2 a general ANOVA was performed on consumption data for weeks 1 and 2 data and an unbalanced ANOVA on weeks 3 and 4. Replicate was used as a blocking variable.

An unbalanced ANOVA was applied for some consumption data due to larval mortality over time. When larvae died they could not contribute to diet eaten data, resulting in an unbalanced design. Treatments were compared using Fisher’s least significant difference post hoc tests, conducted at the 5% confidence level.

3.2.4.2 Mortality analysis

Mortality data were obtained by recording larval survival daily.

Cumulative mortality in the epoxy-janthitrem treatments and the starvation control treatment were compared using a Kaplan-Meier survival analysis. Experiment 2 was blocked by replicate. Significant differences were compared in the Kaplan-Meier survival analysis using a log rank t-test.

3.2.4.3 Larval growth analysis- Experiment 1

Larval growth was determined by weighing larvae at the beginning and the end of the experiment. Larvae that died during the experiment were also weighed and their weights recorded.

Larval growth data (final larval weight – initial larval weight) were analysed and data checked for normality and homogeneity. As a result a REML linear mixed model analysis with an experimental factor which grouped the epoxy-janthitrem I and starvation treatments separately to the Nil treatments was applied. This analysis was chosen so that large differences in variance between the Nil treatments and the epoxy-janthitrem and starvation treatments could be taken into account. Data were not transformed and not blocked by replicate. Treatments were compared using Fisher’s least significant difference post hoc tests, conducted at the 5% confidence level.

3.2.4.4 Larval growth analysis- Experiment 2

Larval growth was analysed with a general ANOVA with replicate as a blocking variable. A REML analysis was not chosen as there were not large differences in variance between Nil and epoxy-janthitrem treatments. Treatments were compared using Fisher’s least significant difference post hoc tests, conducted at the 5% confidence level.

3.3 Experiment 1: Results

3.3.1 Larval feeding

Larvae in the treatments fed diet containing epoxy-janthitrem I consumed significantly less ($P<0.05$) diet each week than their Nil equivalents (Figure 3.2). Larvae in the low, medium and high epoxy-janthitrem treatments consumed on average 35, 60 and 71mg less diet per week than their Nil equivalents, respectively.

In the first week, the amount of diet consumed by larvae in the low epoxy-janthitrem treatment was significantly greater ($P<0.05$) than the amount consumed in the medium and high concentrations. No significant differences were found between the three epoxy-janthitrem concentrations in weeks 2 and 3.

Overall, diet consumed by larvae in the Nil treatments was higher in the first week than the second or third. The amount of diet consumed was similar in each of the Nil treatments in weeks 1 and 3. However, in week 2 more ($P<0.05$) diet was consumed by larvae in the Nil high concentration treatment (an average of 93mg) than that in the medium (61mg) and low Nil (60mg) treatments.

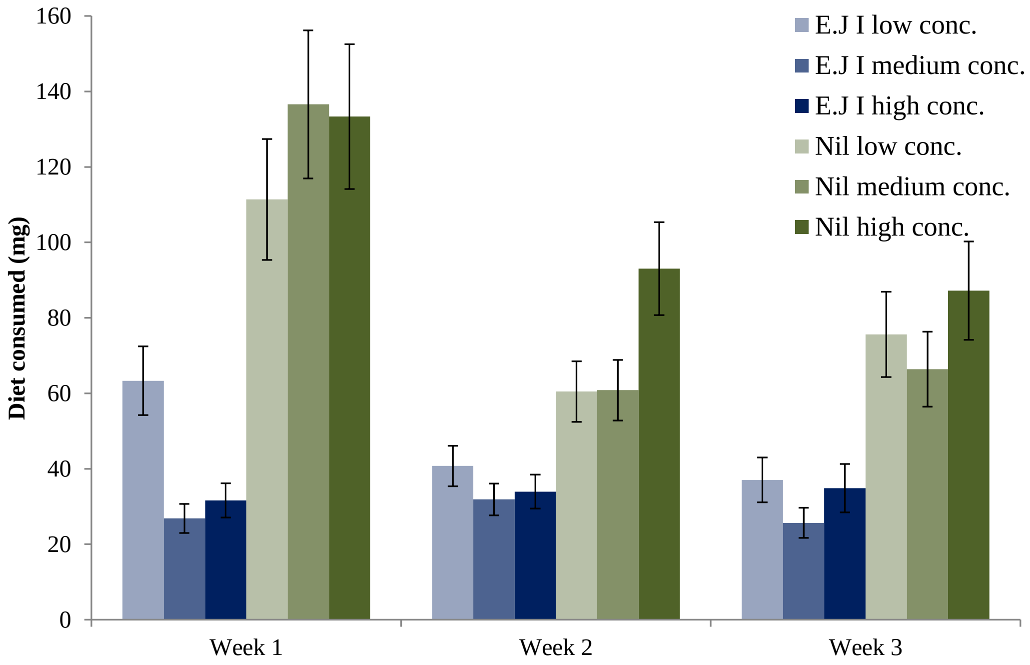

Figure 3.2: Average diet consumed (mg) by porina larvae in the three epoxy-janthitrem I (E.J I) concentration treatments and equivalent Nil treatments in each week of the trial for Experiment 1 (+/- SEM)

3.3.2 Larval growth analysis

Larval growth in the Nil treatments was significantly ($P<0.05$) higher than growth in the epoxy-janthitrem and starvation treatments. No significant differences in growth were found between the three epoxy-janthitrem treatments or between the epoxy-janthitrem treatments and the starvation control.

Larvae that died in the Nil treatments lost between 6.7 and 12.1mg compared to larvae that lived which gained between 12.2 and 19.4mg (Table 3.2).

Larvae the died in the epoxy-janthitrem treatments lost weight (between -11.4 and -12.4mg) but were still consuming between 5.4 and 7.2mg of diet per day. Larvae that died in the Nil treatments also lost weight (between -6.7 and -12.1mg) and consumed 7.3- 11.8mg of diet per day.

Table 3.2: Mean weight change (final weight of larvae at death or weight of live larvae at the end of the trial minus the initial larval weight) and mean consumption per day (total larval consumption divided by number of days the larva lived) of larvae in Experiment 1. The experiment was run for 24 days. N represents the number of larvae used to calculate the means

	Larvae that died				**Larvae that lived**		
Treatment	**Mean weight change (mg)**	**Mean consumption (mg/ day)**	**N**		**Mean weight change (mg)**	**Mean consumption (mg/ day)**	**N**
EJI low concentration	-11.4	7.2	13		-2.1	8.5	2
EJI medium concentration	-11.7	5.4	15		-	-	-
EJI high concentration	-12.4	6.1	15		-	-	-
Nil extract low concentration	-12.1	7.3	2		12.2	16.7	13
Nil extract medium concentration	-6.7	11.8	7		17.6	16.3	8
Nil extract high concentration	-10.3	9.7	3 (weight change = 2)		19.4	20.3	12
Starvation control	-11.9	-	-		-	-	-

3.3.1 Mortality analysis

Some larval mortality occurred in the Nil treatments, in particular the Nil medium concentration where mortality was 47% at the end of the trial (Figure 3.3). However, mortality in the Nil treatments was significantly ($P<0.05$) less than all three epoxy-janthitrem treatments and the starvation control.

Larval mortality in both the medium and high epoxy-janthitrem treatments and in the starvation control was 100% by day 22. However, differences in mortality between the epoxy-janthitrem treatments and the starvation control were observed. In the medium epoxy-janthitrem treatment over a third of the larval mortality occurred on the 19th day with six larval deaths. Similarly in the high epoxy-janthitrem treatment 5 larvae died on the 17th day. In comparison the decline in larval numbers in the starvation treatment was more gradual, with 1 or 2 larvae dying at a time, there was one exception where 3 larvae died in a 24 hour period.

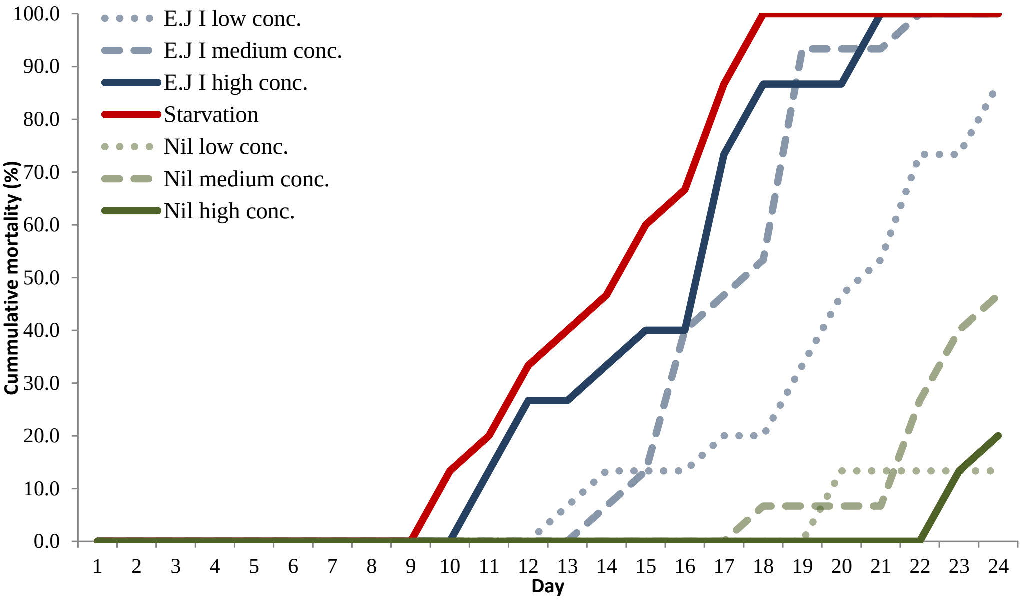

Figure 3.3: Cumulative percent mortality of porina larvae in the seven treatments in Experiment 1

A Kaplan-Meier survival analysis was performed and differences between treatments compared using t-tests. No significant differences ($P>0.05$) in cumulative mortality were found between the three Nil treatments (Table 3.3). Mortality was significantly less ($P<0.05$) in the low epoxy-janthitrem treatment when compared to the two higher epoxy-janthitrem concentration treatments and the starvation control. No significant difference was found between the high epoxy-janthitrem treatment and the medium epoxy-janthitrem treatment or the high epoxy-janthitrem treatment and the starvation control. However, there was a significant difference ($P<0.05$) in mortality between the medium epoxy-janthitrem treatment and the starvation control.

Table 3.3: P-values for the Kaplan-Meier t-tests between the epoxy-janthitrem I (E.J. I), Nil and starvation treatments in Experiment 1. Differences which were not significant (P>0.05) are highlighted in red

Starvation							*
Nil High conc.						*	<0.001
Nil Medium conc.					*	0.091	<0.001
Nil low conc.				*	0.07	0.697	<0.001
E.J. I high conc.			*	<0.001	<0.001	<0.001	0.125
E.J. I medium conc.		*	0.165	<0.001	<0.001	<0.001	0.001
E.J. I low conc.	*	0.003	<0.001	<0.001	0.006	<0.001	<0.001
	E.J. I low conc.	E.J. I medium conc.	E.J. I high conc.	Nil low conc.	Nil Medium conc.	Nil High conc.	Starvation

3.3.2 HPLC results

Epoxy-janthitrem concentrations in the diet fed to porina (fresh diet and diet from fridge) were all close to the concentrations planned; 1, 2.5 and 5 µg/g. Results from the remnant diet show that these concentrations were not substantially degraded while diet plugs were exposed to porina larvae (Table 3.4).

Table 3.4: Epoxy-janthitrem (EJ) concentrations in fresh diet, remnant diet 1 (fresh diet fed on by larvae for 3 days), diet from fridge (diet made 3 days prior and stored in a fridge) and remnant diet 2 (diet stored in fridge for 3 days then fed to larvae for 4 days) for Experiment 1

Epoxy-janthitrem I low concentration

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
25/02/2014	1.10	28/02/2014	1.03	28/02/2014	1.01	4/03/2014	1.13
4/03/2014	1.03	7/03/2014	0.98	7/03/2014	0.93	11/03/2014	1.00
11/03/2014	1.09	14/03/2014	1.00	14/03/2014	1.01	18/03/2014	0.97
18/03/2014	0.91	21/03/2014	0.99				

Epoxy-janthitrem I medium concentration

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)
25/02/2014	2.33	28/02/2014	2.60	28/02/2014	2.55	4/03/2014	2.36
4/03/2014	2.11	7/03/2014	2.28	7/03/2014	2.61	11/03/2014	2.54
11/03/2014	2.20	14/03/2014	2.18	14/03/2014	2.55	18/03/2014	2.30
18/03/2014	2.34	21/03/2014	2.57				

Epoxy-janthitrem I high concentration

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)
25/02/2014	4.58	28/02/2014	5.33	28/02/2014	5.23	4/03/2014	4.75
4/03/2014	4.76	7/03/2014	4.97	7/03/2014	5.10	11/03/2014	4.82
11/03/2014	4.96	14/03/2014	4.89	14/03/2014	4.80	18/03/2014	4.55
18/03/2014	5.34	21/03/2014	4.97				

3.4 Experiment 2: Results

3.4.1 Larval feeding

Larvae in the Nil control treatments consumed significantly more ($P<0.05$) diet each week than larvae fed diet containing epoxy-janthitrem I, at all three concentrations (Figure 3.4). Larvae in the Nil treatments would often consume all of their diet between diet changes, some even when fed in excess of 1300mg a week.

Significant differences ($P<0.05$) in larval feeding were found between the three epoxy-janthitrem treatments. In all 4 weeks significantly more ($P<0.05$) diet was consumed by larvae in the low epoxy-janthitrem treatment than by larvae in the medium and high epoxy-janthitrem treatments. The amount of diet consumed in the medium concentration was also significantly greater than diet consumed in the high concentration in week 2 but not in the other weeks.

There were no significant differences ($P>0.05$) in diet consumed between larvae in the three Nil control treatments in the first 3 weeks of the experiment. In week 4 however, porina fed the low concentration Nil diet consumed significantly more ($P<0.05$) diet than larvae fed the medium concentration.

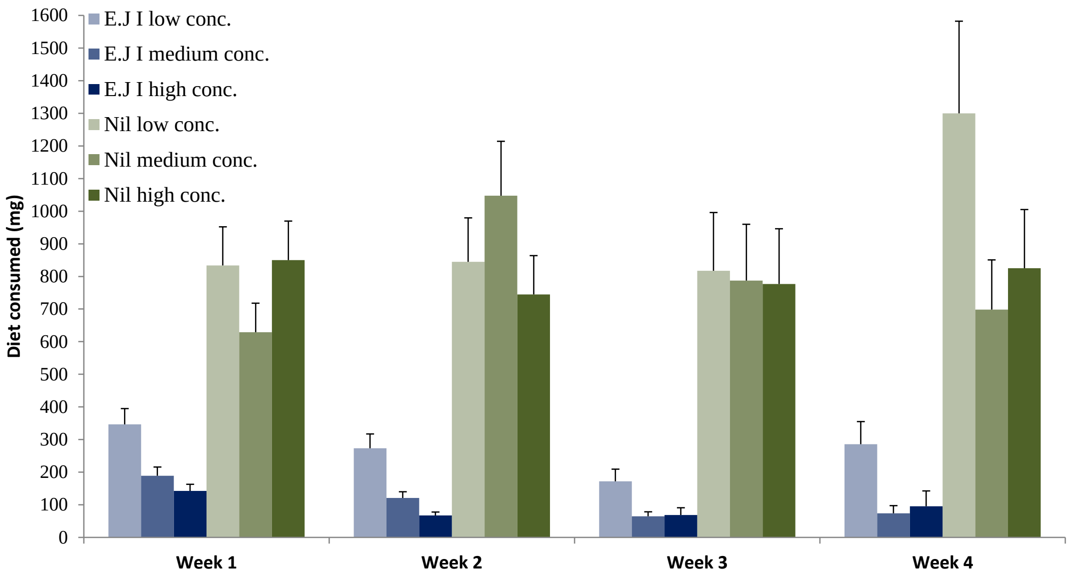

Figure 3.4: Average diet consumed (mg) by porina larvae in the three epoxy-janthitrem I (E.J I) concentration treatments and equivalent Nil control treatments for Experiment 2 (+SEM)

Differences in the amount of frass and webbing produced were observed. In general, larvae in the epoxy-janthitrem treatments produced less webbing than those in the Nil treatments. There was also often dark / bark coloured frass in the epoxy-janthitrem treatments, as opposed to the green / semi-synthetic diet coloured frass usually observed. Figure 3.5 displays differences in webbing and frass produced between Nil and epoxy-janthitrem treatments.

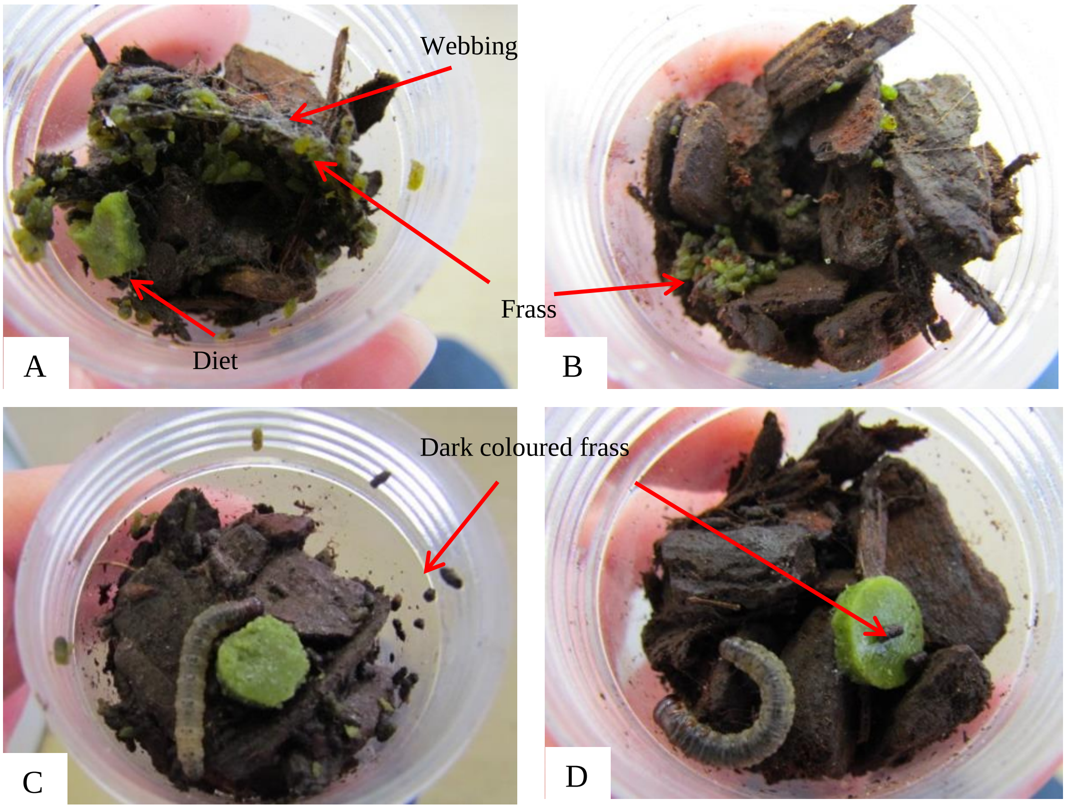

Figure 3.5: Photographs taken looking down on the bioassay specimen containers before a diet change in Experiment 2. Photographs A and B are from Nil endophyte treatments and C and D from epoxy-janthitrem I treatments

3.4.2 Larval growth analysis

Larval growth (weight change) was significantly ($P<0.05$) higher in the Nil treatments when compared to the epoxy-janthitrem treatments and starvation control. There was no difference in growth between Nil treatments.

Growth in the low epoxy-janthitrem treatment was significantly higher ($P<0.05$) than in the medium and high epoxy-janthitrem treatments and starvation control. There was no significant difference in growth between the medium epoxy-janthitrem treatment, high epoxy-janthitrem treatment and starvation control.

Mean weight change in the medium and high epoxy-janthitrem treatments was negative for both larvae that died and larvae that survived to the end of the experiment (Table 3.5). In comparison, larvae that lived to the end of the experiment in the low epoxy-janthitrem concentration gained weight (65.8mg), although this was a comparatively small weight gain when compared to the average gained by larvae in Nil treatments (203.4mg). Larvae in the Nil treatments consumed substantially more diet per day (137-149mg) than larvae that died (15-28mg) and larvae that lived (26-60mg) in the epoxy-janthitrem treatments.

Table 3.5: Mean weight change (final weight of larvae at death or of live larvae at the end of the trial minus the initial larval weight) and mean consumption per day (total larval consumption divided by number of days the larva lived) of larvae in Experiment 2. The experiment was run for 28 days. N represents the number of larvae used to calculate the means

	Larvae that died			**Larvae that lived**		
Treatment	**Mean weight change (mg)**	**Mean consumption (mg / day)**	**N**	**Mean weight change (mg)**	**Mean consumption (mg / day)**	**N**
EJI low concentration	-54.3	28	6	65.8	60	9
EJI medium concentration	-69.2	19	13	-36	29	2
EJI high concentration	-59	15	13	-50.5	26	2
Nil extract low concentration	-	-	-	215.9	149	15
Nil extract medium concentration	-	-	-	190.3	147	15
Nil extract hihg concentration	-	-	-	204.1	137	15
Starvation control	-71.2	-	15	-	-	-

3.4.3 Mortality analysis

There were no larval deaths in the Nil control treatments throughout the experiment. The low concentration treatment had the lowest mortality at the end of the experiment (40%), with 6 larval deaths (Figure 3.6). Half of these deaths occurred in the final 3 days of the experiment. In both the medium and high epoxy-janthitrem treatments mortality at the end of the trial was 87%. Almost half of the mortality in the high epoxy-janthitrem treatment occurred on the 14th day when seven larvae died. This is in contrast to the starvation treatment in which larvae gradually died off from the ninth day of the trial. The final larva survived in the starvation treatment without diet for 25 days.

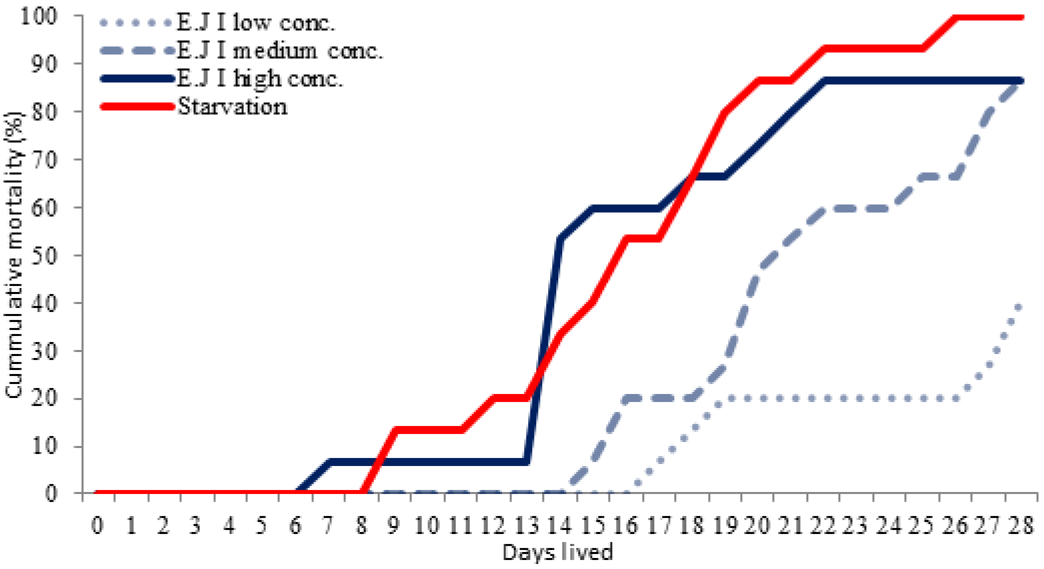

Figure 3.6: Cumulative percent mortality of porina larvae in the three epoxy-janthitrem I (E.J I) concentration treatments and the starvation treatment in experiment 2. No larval deaths occurred in the three Nil treatments.

Results of the Kaplan-Meier survival analysis showed a significant difference ($P<0.05$) in mortality between each pair of treatments compared, except for between the high and the medium epoxy-janthitrem treatment, and the high epoxy-janthitrem treatment and starvation control (Table 3.6). Highly significant differences ($P<0.001$) were found between the low epoxy- janthitrem treatment and the high epoxy-janthitrem and starvation control treatments.

Table 3.6: P-values for the Kaplan-Meier t-tests between the three epoxy-janthitrem I (EJI) treatments and the starvation control for Experiment 2. Differences which were not significant (P>0.05) are highlighted in red. Comparisons with Nil controls were not included as no larval deaths occurred.

Starvation				*
EJI High Conc.			*	0.496
EJI medium conc.		*	0.119	0.002
EJI low conc.	*	0.005	<0.001	<0.001
	EJI low conc.	EJI medium conc.	EJI high conc.	Starvation

3.4.4 HPLC results

Epoxy-janthitrem concentrations show that the concentrations in diet fed to porina (fresh diet and diet from fridge) were all close to the concentrations expected; 1, 2.5 and 5 µg/g. Results from the remnant diet show that these concentrations were not substantially degraded while diet plugs were exposed to porina larvae (Table 3.7).

Table 3.7: Epoxy-janthitrem concentrations in fresh diet, remnant diet 1 (fresh diet fed on by larvae for 3 days), diet from fridge (diet made 3 days prior and stored in a fridge) and remnant diet 2 (diet stored in fridge for 3 days then fed to larvae for 4 days) for Experiment 2.

Epoxy-janthitrem I low concentration

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)
19/05/2014	1.01	22/05/2014	0.89	22/05/2014	0.99	26/05/2014	0.94
26/05/2014	1.01	29/05/2014	0.95	29/05/2014	1.04	2/06/2014	1.00
2/06/2014	1.06	5/06/2014	1.09	5/06/2014	Not analysed	9/06/2014	1.00
9/06/2014	1.15	12/06/2014	1.05	12/06/2014	1.07	16/06/2014	1.06

Epoxy-janthitrem I medium concentration

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)
19/05/2014	2.605	22/05/2014	2.23	22/05/2014	2.44	26/05/2014	2.33
26/05/2014	2.445	29/05/2014	2.56	29/05/2014	2.59	2/06/2014	2.47
2/06/2014	2.715	5/06/2014	3.38	5/06/2014	3.07	9/06/2014	2.18
9/06/2014	2.26	12/06/2014	2.50	12/06/2014	2.42	16/06/2014	2.63

Epoxy-janthitrem I high concentration

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)	Date	EJ concentration (µg/g)
19/05/2014	5.58	22/05/2014	5.03	22/05/2014	5.26	26/05/2014	4.98
26/05/2014	4.95	29/05/2014	5.28	29/05/2014	5.38	2/06/2014	4.83
2/06/2014	5.215	5/06/2014	5.16	5/06/2014	4.72	9/06/2014	4.72
9/06/2014	5.075	12/06/2014	5.43	12/06/2014	5.46	16/06/2014	5.40

3.5 Discussion

Epoxy-janthitrem I, produced by the endophyte AR37, was found to deter feeding of porina larvae in both insect bioassays. Larvae were deterred from feeding at all three epoxy-janthitrem I concentrations tested (1, 2.5, 5µg/g wet weight), with larvae in these treatments consuming significantly less diet than their Nil equivalents. The anti-feedant effect was stronger at the two higher concentrations than the low concentration, with larvae in the low concentration treatment consuming significantly more diet than the higher concentrations in week 1 of Experiment 1 and all 4 weeks in Experiment 2.

This result agrees with that of previous studies. Jensen and Popay (2004) found porina larvae were deterred from feeding on AR37 ryegrass when given the choice between AR37-infected ryegrass and Nil endophyte ryegrass. Furthermore, Finch, *et al.* (2010) tested the effects of pure epoxy-janthitrem I at concentrations of 1, 2.5, 5 and 10µg/g on porina larvae in a bioassay over 7 days. Results from Finch, *et al.* (2010) were similar in finding that epoxy-janthitrem I had a deterrent effect on porina larvae, and that this affect increased with higher concentrations of epoxy-janthitrem I.

The aim of this bioassay was to determine if epoxy-janthitrem I is toxic to porina larvae. Toxicity is defined as a compound which reduces the growth and survival of larvae, above that which can be attributed to starvation. Results from these bioassays do not provide definitive evidence for toxicity of epoxy-janthitrem I. Growth and survival were significantly reduced in the epoxy-janthitrem treatments when compared to their equivalent Nil controls, in both experiments. However, when compared to the starvation treatments neither growth nor survival were significantly reduced, indicating that the primary effect of epoxy-janthitrem I may be as a strong feeding deterrent to larvae resulting in larvae dying due to starvation.

However, there is some evidence of toxicity in the patterns of larval mortality (cumulative mortality) over time. In the medium epoxy-janthitrem treatment in the first bioassay over a third of larval mortality occurred on the 19th day, with six larvae dying. Similarly in the high epoxy-janthitrem treatments, in Experiment 1 five larvae died on the 17th day and in Experiment 2 nearly half of the larvae died on the 14th day. This pattern was not observed in the starvation treatment, in either experiment, where a more gradual decline in larval numbers occurred, with larval numbers decreasing by 1 or 2 larvae at a time, with one exception in the first experiment where 3 larvae died within 24hrs. Several larval deaths occurring within a 24 hour period, as was found in the medium and high epoxy-janthitrem treatments, could indicate toxicity of epoxy-janthitrem I because if larvae in the epoxy-janthitrem treatments were dying due to starvation caused by strong deterrence of the alkaloid you would expect to see a gradual, but slower, decline in larval numbers similar to that which occurred in the starvation treatments.

Additionally if the negative effects on growth and survival in the epoxy-janthitrem treatments were due to starvation you would expect that larvae would have consumed little or no diet. Results show that larvae that died in the epoxy-janthitrem treatments were consuming diet, although the amount consumed may not have been sufficient to sustain them. Further experiments are required to determine the minimum amount of diet required to sustain a larva. With this data it would be possible to determine if larvae in the epoxy-janthitrem treatments were consuming enough diet to survive. If larvae were consuming enough diet it could be concluded larvae were dying as a result of toxicity of epoxy-janthitrem I, rather than starvation.

It is important to determine if epoxy-janthitrem I is not only deterrent to larvae but also toxic to understand how AR37 affects porina larvae in the field, to improve pasture management. If AR37 is only strongly deterrent to larvae, this may cause larvae to feed heavily on white clover which is often sown in conjunction with ryegrass in paddocks. Deterrence may also cause larvae to accumulate in paddocks containing Nil or less effective endophytes, increasing the damage caused to these paddocks. An alkaloid which is not only deterrent but also toxic is ideal for minimising pasture damage, as larval survival is reduced.

The extract tested in this bioassay was not pure epoxy-janthitrem I so it cannot be ruled out that there was not another unknown bioactive in the extract which was responsible for the deterrent effects. However, this extract has been through a number of purification steps to get to this point and pure epoxy-janthitrem I has previously been shown to reduce growth and feeding of larvae, over a period of 7 days, at the same concentrations tested in this thesis (Finch, *et al.*, 2010).

Diet consumption was calculated by subtracting the weight of remnant diet from the initial weight of the diet plug. Thus, some of the weight lost by the diet could have been as a result of moisture loss, during the 3 to 4 days the diet plug was on the bark chips, rather than consumption by larvae. Steps were taken to estimate moisture loss, but due to variability in plug size over time and differences in moisture content of different batches of diet, moisture loss was over-estimated and so was not included in the results. Not all of the weight lost in epoxy-janthitrem diet treatments could be attributed to moisture loss, as feeding marks on diet discs were observed during the bioassay.

Differences in diet consumption and survival were noted between larvae fed Nil diets in Experiment 1 and Experiment 2. In Experiment 1 diet consumption was low after the first week and larval mortality in the Nil equivalent medium concentration was 40% at the end of the trial. Porina larvae in a similar experiment run at the same time also performed poorly on Nil diets with very high mortality occurring. The cause was not able to be determined but was suspected to be the type of bark used. A different brand of bark was used in the second experiment and larvae performed well on the Nil diets with no larval deaths occurring.

A study by French and Pearson (1981) examined the average amount of white clover consumed by larvae in each month from March to September. Assuming larvae in the March sampling were of similar weight to larvae in Experiment 1 (as experiment 1 was conducted in March) of this study and larvae in the June sampling were similar in weight to Experiment 2, consumption per day can be compared. French and Pearson (1981) found larvae in March consumed 6.8mgGM/day, similar to the amount of diet consumed by larvae that died in the epoxy-janthitrem treatments. Larvae that lived in the Nil treatments in this experiment consumed almost three times as much diet. In the June sampling in year one French and Pearson (1981) found larvae consumed 11.3mg of green matter (GM)/day and 15.6mgGM/day in year two, well below the 137-149mg of semi-synthetic diet/day consumed in the Nil treatments in this experiment and less than the amount consumed by larvae that died in the low (19mg/day) and medium (28mg/day) epoxy-janthitrem treatments. As the larvae in this experiment were reared from eggs with ample diet it is possible they were larger in size and thus could consume more diet than larvae from the field in French and Pearson (1981). However, consumption in French and Pearson (1981) in September when larvae are at their heaviest, as they accumulate fat for pupation, was 34mgGM/day in year one, still well below the consumption in Experiment 2 of this study. Due to the differences in diet and methods used to collect data, consumption in these two studies cannot be directly compared however this information does show that larvae were feeding well on the semi-synthetic diet in this experiment.

This thesis and Finch, *et al.* (2010) are the only published studies to look at the effect of the epoxy-janthitrems on insects, and only porina larvae have been tested. What effect the epoxy-janthitrems have on other ryegrass pest insects in New Zealand such as the Argentine stem weevil, black beetle, root aphid or pasture mealybug has yet to be studied. However, the effects of other endophyte alkaloids on pest insects have been examined; some of these studies which have looked at feeding deterrence and alkaloid toxicity are discussed below. Ball, *et al.* (1997a) incorporated a number of alkaloids including ergovaline, lolitrem B, peramine and loline into artificial diet to determine whether these alkaloids deter adult black beetle (*Heteronychus arator*) in choice bioassays. Ergovaline at 5µg/g and 10µg/g significantly reduced feeding when compared to the control. However, lolitrem B, peramine and loline did not significantly deter feeding. Peramine has however, been shown to deter feeding of the Argentine stem weevil (adults and larvae) (Rowan, *et al.*, 1990) and lolines have been shown to have an anti-feedent effect on grass grub (Popay & Lane, 2000) and a toxic effect on porina, the large milkweed bug (Yates *et al.*, 1989), and the Argentine stem weevil (Jensen *et al.*, 2009). In a no-choice bioassay Popay and Lane (2000) tested four concentrations of lolines (0, 100, 250, 500 and 1000µg/g) on porina larvae. At 250µg/g lolines had anti-feedant effect on larvae and at 500µg/g and 1000µg/g were toxic to larvae, although no starvation control was included in this experiment.

Information obtained from this study can be used to increase understanding of how epoxy-janthitrem affects porina larvae and how the AR37 endophyte may affect porina populations in the field. Results suggest that if larvae are exposed to diet containing epoxy-janthitrem with no alternative they will die due either to starvation or toxicity of the alkaloid. This information could be used to inform farmers on how best to control porina populations in the field, by ensuring there is no supply of ryegrass uninfected with AR37, or alternative food present such as clover or cocksfoot to potentially sustain populations.

As discussed above, further experiments are required to determine whether epoxy-janthitrem I is toxic to porina larvae, and to resolve this how the maintenance level of diet required to sustain a larva could be determined. This information could then be used to determine whether larvae in these bioassays were starving to death or whether they were acquiring adequate diet and dying due to toxicity of epoxy-janthitrem I. Another way to examine this could be to design a bioassay in which larvae were fed diet containing epoxy-janthitrem for a number of days and Nil diet for a few days, to ensure larvae were acquiring enough diet so that they do not starve to death. Any larval deaths could then be attributed to toxicity of epoxy-janthitrem I. A third way could be to incorporate concentrations lower than 1µg/g into diet. At 1µg/g epoxy-janthitrem I is deterrent to larvae, whereas, at lower concentrations epoxy-janthitrems may not have as large a deterrent effect on larvae allowing larvae to consume enough diet to prevent them from starving to death so that any deaths could then be attributed to toxicity. This experiment would need to be run over two months as a low concentration is likely to take longer to have a toxic effect on larvae.

Further experiments could also look at synergistic effects between the 5 epoxy-janthitrem compounds present in AR37. The extract in this bioassay contained epoxy-janthitrem I and a small amount of epoxy-janthitrem triol, further studies could combine this extract with combinations of other semi-pure epoxy-janthitrem compounds to determine whether epoxy-janthitrem I acts synergistically with other compounds to cause more adverse effects on larvae.

Chapter 4: Epoxy-janthitrem Concentrations in AR37 are Decreased at Low Temperature Resulting in Reduced Bioactivity Against Porina Larvae

4.1 Introduction

Porina (*Wiseana* spp.) are a group of moth species endemic to New Zealand. The larvae of many of these species are a pest of cultivated grasses (Dugdale, 1994). Larvae are nocturnal and emerge at night, from vertical burrows created beneath the soil (Barlow, *et al.*, 1986), to feed on ryegrass plants (Harris, 1969). It has been demonstrated in pot trials, choice bioassays and field trials that the endophyte AR37 is able to provide infected ryegrass with resistance to porina.

In a pot trial Jensen and Popay (2004) found AR37-infected ryegrass plants reduced the growth and survival of porina larvae. In choice bioassays AR37-infected ryegrass has been shown to deter feeding of larvae when given the choice between AR37 and Nil endophyte ryegrass (Jensen & Popay, 2004) and in field trials, carried out in Canterbury, lower populations of porina larvae were found on AR37-infected ryegrass when compared to Nil, AR1 and a low wild type infected ryegrass (Popay*, et al.*, 2012).

The only known alkaloids to be produced by AR37 are the epoxy-janthitrems (Tapper & Lane, 2004), a group of five compounds. The resistance provided by AR37 to porina is likely to be due to the epoxy-janthitrems. Pure epoxy-janthitrem I has previously been shown to reduce growth and consumption of porina larvae in a bioassay over seven days (Finch, *et al.*, 2010). However, there have been reports, usually from colder areas, in both the North Island and South Island, that AR37 is not providing adequate control over porina larvae (Popay & Ferguson, unpublished information). It is hypothesised that when grown at colder temperatures AR37 does not produce high enough concentrations of epoxy-janthitrem to adequately control porina populations. What effect temperature has on epoxy-janthitrem concentration is not known; although temperature has been tested on the other significant endophyte alkaloids lolitrem B, peramine and ergovaline in Eerens, *et al.* (1998) and Lane *et al.* (1997). Of these alkaloids only ergovaline was identified to be significantly affected by temperature and only in Eerens, *et al.* (1998). The lowest temperature tested in these studies was 15°C, so it is not known what effect cooler temperatures have on the concentrations of these alkaloids. However, alkaloid concentrations are known to be effected by season, and have been shown to be lower during the winter when temperatures are cooler (Ball, *et al.*, 1995).

Concentrations of other endophyte alkaloids are known to be affected by the genotype of the host plant (Spiering, *et al.*, 2005). In field trials Popay, *et al.* (2012) also found differences in porina larval density between AR37-infected ryegrass cultivars. This may indicate that epoxy-janthitrem concentrations could be affected by plant genotype and cultivar. It is important to identify which plant cultivars may affect epoxy-janthitrem concentrations to understand limitations of the AR37 endophyte, as plant cultivars which result in low concentrations may be more susceptible to insect attack.

The distribution of an alkaloid within a plant varies depending on the alkaloid, the genotype of the host plant and the tissue type (Spiering, *et al.*, 2005). The distribution pattern of the epoxy-janthitrems is not known. However, it is likely that the epoxy-janthitrems are distributed in a similar manner to lolitrem B, as these alkaloids are both within the indole-diterpene class of alkaloids and are both lipophilic compounds, which will affect the way they are distributed throughout the plant. Lolitrem B concentrations are very high in the seed and are higher in the pseudostems in comparison to the leaves (di Menna, *et al.*, 1992; Ball, *et al.*, 1997b). Given this information pseudostems and leaves were examined separately in this study to determine whether any differences in concentration between these two tissues affects feeding, growth and survival of porina larvae.

Two experiments, a ryegrass pot trial and a porina larval bioassay, were designed to investigate the effect of temperature on epoxy-janthitrem concentrations in AR37-infected ryegrass. The hypotheses tested were that (a) ryegrass cultivars would differ in epoxy-janthitrem concentration and (b) epoxy-janthitrem concentrations would be higher when ryegrass is grown at high temperature and that both of these variations in concentration would affect feeding, growth and survival of porina larvae.

4.2 Materials and methods

Perennial and Italian ryegrass plants were grown in one of two controlled environment (CE) rooms, one set at a warm temperature (20°C) and the other a cold temperature (7°C). After 10-12 weeks ryegrass plants were harvested, freeze dried and ground and the epoxy-janthitrem concentrations within them determined by HPLC. Ground plant material from the pot trial was then incorporated into a semi-synthetic diet and fed to porina larvae in an insect bioassay over three weeks to determine what effect the differing epoxy-janthitrem concentrations in the ryegrass plants had on consumption, growth and survival of porina larvae.

4.2.1 Ryegrass pot trial

The ryegrass pot trial contained 8 treatment groups with 20 replicate plants (Table 4.1).

4.2.1.1 Establishment of ryegrass from seed

Diploid perennial ryegrass (cv Samson) and diploid Italian ryegrass (cv PG255) plants were grown from AR37-infected or Nil endophyte seed. Seeds were placed on top of a moist piece of filter paper within a petri dish (9 cm) and left to germinate. After five days 100 germinated seedlings each of; Samson AR37, PG255 AR37, Samson Nil and PG255 Nil were sown into trays (perennial ryegrass seedlings were sown into two forestry trays (50 cells (5cm/ 5cm) per tray (52cm/ 26.6/ 12)); and Italian ryegrass seedlings into two polystyrene trays (length 54.5cm, width 33.5, depth 11) filled with potting mix (Daltons commercial) and left to establish in a glasshouse. Plants were handwatered and trimmed regularly to maintain strong growth.

4.2.1.2 Immunoblotting

After seven and a half weeks AR37 and Nil endophyte ryegrass plants were tested for endophyte infection using a tissue print immunoassay technique (Gwinn *et al.*, 1991; Hahn *et al.*, 2003).

One tiller per plant (400 plants) was cut horizontally within 0.5cm of the base of the plant, where the highest endophyte mycelial mass occurs. Dead sheath was removed and the moist cut surface pressed onto a nitrocellulose membrane (Protran BA 85 0.45µm). Immunoblots were sent to AgResearch Grasslands for development. Methods for immunoblot development can be found in Simpson *et al.* (2012).

4.2.1.3 Cloning of plants

Thirty plants of each of the treatments were selected from the forestry and polystyrene trays (120 plants). Plants were selected based on immunoblot results and tiller numbers (eight or more tillers). Each plant was split in two (cloned), to create two plants with the same genetic makeup, cut to a height of 5cm and planted into individual pots (12.5cm by 10cm) ¾ full with potting mix (Daltons commercial) (240 plants). Clones were appropriately labelled with endophyte status, cultivar, and either 20°C or 7℃ and left to establish in a screenhouse. Plants were watered and trimmed regularly and fertilized (1.8g/L thrive and 1.3g/L urea) as required. Plants were left to establish for 16 weeks.

4.2.1.4 Treatments

There were 8 treatment combinations with 20 replicates (160 plants) in the ryegrass pot trial. The treatments were perennial (cv Samson) and Italian (cv PG255) ryegrass infected with endophyte (AR37) or without endophyte (Nil), grown at high (20℃) or low (7℃) temperature (Table 4.1).

Table 4.1: Ryegrass pot trial treatment list

Cultivar	Endophyte	Temperature
Perennial	AR37	High
Perennial	AR37	Low
Italian	AR37	High
Italian	AR37	Low
Perennial	Nil	High
Perennial	Nil	Low
Italian	Nil	High
Italian	Nil	Low

4.2.1.5 Randomised block design

Eight days before plants were transferred to CE rooms they were trimmed to a height of 5cm. Twenty plants per treatment (8 treatments, 160 plants) were then transferred into two CE rooms (Figure 4.1), one set at high (20°C) temperature and the other low (7°C) temperature and both with a 12hr light: 12hr dark cycle. Plants were arranged following a randomised block design which was identical in the two CE rooms, with the two clones of each plant in separate CE rooms, so that plants between the two rooms were genetically identical. Humidity was not controlled. One CE room was used for each temperature; it was not possible to have multiple CE rooms for each temperature. Plants were maintained in the CE rooms with regular watering. After 5 weeks plants in both CE rooms were trimmed to 10cm and fertilized (75mL).

Figure 4.1: Potted ryegrass plants arranged in a CE room

4.2.1.6 Harvesting of plants

After 10 weeks in CE rooms plant material was harvested by replicate over a period of two weeks. Plants were harvested by cutting all tillers off at the very base of each plant; care was taken to ensure that the meristem was not removed from the sample. Dead tillers, sheath and leaves were removed from the sample and live pseudostems and leaves separated. Material was placed into sealed and labelled airtight bags and frozen at -20°C.

4.2.1.7 Preparation of plant material

Ryegrass samples were freeze dried (W.G.G Cuddon LTD, Blenheim New Zealand) for three to four days. Freeze dried grass samples were then warmed to room temperature and ground (IKA-A10 blade mill, IKA®-WERKE, Staufen, Germany) to a fine powder. Ground samples were kept out of the direct light to avoid degradation of epoxy-janthitrem. Ground samples were stored in airtight bags and refrozen at -20°C.

4.2.1.8 HPLC analysis of epoxy- janthitrem concentration

Epoxy-janthitrem concentrations in pseudostem and leaf samples from AR37-infected ryegrass plants in replicates one to five were determined separately by HPLC (see 2.3). Epoxy-janthitrems were quantified by comparison with a BNI standard.

4.2.2 Insect bioassay

The insect bioassay contained 16 treatments with 12 replicate larvae (192 larvae) tested against each treatment. The treatments were the leaves and pseudostems of plants in each of the eight treatments from the ryegrass pot trial (Table 4.2); perennial (Samson) ryegrass infected with AR37 or with Nil endophyte and Italian (PG255) ryegrass infected with AR37 or Nil endophyte grown at high (20°C) or low (7°C).

Table 4.2: Insect bioassay treatment list

Cultivar	Leaves/ Pseudostems	Endophyte	Temperature ryegrass was grown at
Perennial	L	AR37	Low
Perennial	PS	AR37	Low
Italian	L	AR37	Low
Italian	PS	AR37	Low
Perennial	L	AR37	High
Perennial	PS	AR37	High
Italian	L	AR37	High
Italian	PS	AR37	High
Perennial	L	Nil	Low
Perennial	PS	Nil	Low
Italian	L	Nil	Low
Italian	PS	Nil	Low
Perennial	L	Nil	High
Perennial	PS	Nil	High
Italian	L	Nil	High
Italian	PS	Nil	High

4.2.2.1 Porina larvae

Porina larvae (249 larvae) were selected from 27 parent moths. Larvae were 32 weeks old and weighed between 226 and 692mg. Porina were sorted by weight and allocated to a treatment and replicate (see 2.2.2) (192 larvae). Larvae were allocated so that larvae in the same replicate across all treatments were of similar weight. The sixteen larvae in each replicate were randomly assigned to a treatment to ensure an even spread of larval sizes across treatments. Individual larvae were then placed into their assigned specimen containers ¾ filled with bark (see 2.2.1 and 2.2.2).

4.2.2.2 Diet preparation

To obtain a representative ryegrass sample of each treatment to be tested on porina larvae an approximately equal amount of freeze dried and ground plant material from each plant in the treatment (20 plants per treatment) was combined and mixed thoroughly. Three grams of plant material was then incorporated with 27g of semi-synthetic diet for each treatment and fed to porina larvae over three weeks.

The semi-synthetic diet ingredients were 500g of carrot, 1000ml of Milli-Q water and 18g of agar. Carrot was blended (Waring Commercial Blender, model HGB2WTG4) with Milli-Q water and then strained to obtain 750mL of carrot juice. Carrot juice and agar were mixed in a glass beaker (900mL). The beaker and its contents were then warmed in a microwave, with occasional stirring, until the agar began to boil (indicated by the level of agar 'rising' in the beaker). The diet was separated into two beakers which were submerged in water baths, to prevent agar cooling and setting. Diet was left to cool to 70°C before it was weighed out into individual beakers.

Sixteen labelled (A-P) glass beakers (75-150mL) were warmed on a warming plate (Heidolph, MR3002C, aprox. 125°C). Twenty seven grams of the diet was weighed out (Satorius weighing technology, TE3102S, Geottingen, Germany) into each beaker, one at a time. One of the ryegrass samples (3g) was added to each beaker and stirred thoroughly to ensure the mix was homogenous. Each diet was poured into a labelled Petri dish (9cm) and smoothed flat using a spatula. Diets were put inside a closed cooler until discs were taken and weighed for the porina, and were then refrigerated at 4°C. The diet making process was well timed to ensure all treatments were made before the agar set.

Fresh diets were made weekly and diet in the insect containers changed twice a week. Diets were weighed in and out of the specimen containers on day 4 and day 7 of each week to determine how much diet each larva was consuming (see 2.2.3). Larvae were examined at each diet change for survival (see 2.2.4). Epoxy-janthitrem concentrations in fresh diets and remnant diets at each diet change were determined by HPLC (see 2.3).

4.2.3 Statistical analyses

Data on larval diet consumption, mortality and growth were collected during the bioassay and analysed using Microsoft Excel 2010 and/or 2007 and GenStat 16th edition.

4.2.3.1 Insect bioassay: Diet consumption

Consumption data was based on average diet consumed by individual larvae per day calculated for the 3 weeks of the trial. A REML linear mixed model analysis with an experimental factor which grouped the AR37 Italian and perennial high temperature treatments (four treatments) separately to all other treatments was applied. This analysis was chosen to take into account the large differences in variance in consumption data; variance in AR37 high temperature treatments was small in comparison to all other treatments. Data were not transformed and replicate was used as a blocking variable. Treatments were compared using Fisher's least significant difference post hoc tests, conducted at the 5% confidence level. This analysis was performed on average diet consumed per day, calculations of diet consumed per day could not take into account that nine larvae died before the end of the experiment.

4.2.3.2 Insect bioassay: Larval growth

Larval growth was determined by weighing larvae at the beginning and end of the experiment. Larvae that died during the experiment were weighed at the next diet change. A general ANOVA was performed on growth data and data checked for normality and homogeneity. Data were not transformed and replicate was used as a blocking variable. Differences between treatments were compared using Fisher's least significant difference post hoc tests, conducted at the 5% confidence level.

4.2.3.3 Insect bioassay: Larval mortality

Larval mortality data was obtained by checking the survival of larvae at each diet change (twice weekly) and at the end of the trial. Percent mortality and cumulative mortality in each treatment was calculated.

4.3 Results: Ryegrass pot trial

4.3.1 Epoxy-janthitrem concentrations

The epoxy-janthitrem concentrations in each AR37-infected plant in replicates one to five were determined by HPLC. Epoxy-janthitrem concentrations in plants grown at high temperature were 9 to 32 times higher than concentrations in plants grown at low temperature (Table 4.3). Concentration differences between high and low temperature were significant ($P<0.05$), except for between the Italian AR37 high temperature leaves and the Perennial and Italian AR37 low temperature pseudostems.

Concentrations were higher in the pseudostems than the leaves at both temperatures; with low temperature pseudostems containing concentrations 15 times higher than in the leaves, and high temperature pseudostems containing concentrations four times higher than the leaves.

Epoxy-janthitrem concentrations and the effect of temperature on concentrations were similar ($P>0.05$) for the two cultivars (perennial and Italian ryegrass).

Table 4.3: Mean epoxy-janthitrem concentrations (ppm) and the range for leaves and pseudostems of individual plants kept at two temperatures (high (20°C) and low (7°C)) in replicates one to five. Note in treatments with four replicates, two plants were combined. SEM (+/-)

Sample	Mean	Range	N	SEM
Perennial AR37 high leaves	34.0	22.5- 45.0	5	7.175
Perennial AR37 high pseudostems	136.5	100.6- 184.8	5	28.78
Perennial AR37 low leaves	0.8	0.2- 3.1	4	0.1986
Perennial AR37 low pseudostem	13.9	9.5- 16.1	4	3.272
Italian AR37 high leaves	25.1	13.1- 43.1	4	5.909
Italian AR37 high pseudostems	130.3	108.1- 170.3	4	30.74
Italian AR37 low leaves	1.0	0.8- 1.9	5	0.2156
Italian AR37 low pseudostems	14.3	10.5- 22.0	5	3.006

4.4 Results: Porina bioassay

4.4.1 Growth and consumption

Endophyte infection (AR37 or Nil), temperature (high 20°C or low 7°C) and plant part (pseudostems or leaves) had highly significant (P<0.001) effects on larval diet consumption and growth. Cultivar (perennial (Samson) or Italian (PG255)) did not have a significant effect on larvae unless it was within an interaction.

The two main effects in this experiment were infection and temperature. Multiple interactions were significant for larval consumption and larval growth (Table 4.4). The significant interactions which relate to the aims and hypotheses of this study are presented.

Table 4.4: Significant (P<0.05) main effects and interactions for consumption and growth data

Consumption Interactions	P-value
Infection	<0.001
Temperature	<0.001
Plant part	<0.001
Infection.Temperature	<0.001
Infection.Cultivar	0.005
Temperature.Plant Part	0.006
Infection.temperature.Cultivar	0.033
Infection.Cultivar.Plant Part	0.006

Growth Interactions	P-value
Infection	<.001
Temperature	<.001
Plantpart	<.001
Infection.Temperature	<.001
Infection.Cultivar	0.006
Infection.Plantpart	0.002
Temperature.Plant part	<.001
Infection.Temperature.Cultivar	0.002
Infection.Cultivar.Plantpart	0.022
Infection.Temperature.Plantpart	0.022

4.4.1.1 Infection x Temperature

The endophyte infection x temperature interaction was highly significant (P<0.001) for consumption and growth data. Larvae fed endophyte (AR37) infected ryegrass grown at high temperature consumed significantly (P<0.05) less diet and gained significantly less weight than larvae fed AR37-infected ryegrass grown at low temperature and larvae fed Nil plants from both high and low temperature (Figure 4.2).

There was no difference (P>0.05) in consumption between larvae consuming AR37-infected ryegrass grown at low temperature and larvae consuming Nil ryegrass grown at low temperature. However, larvae fed Nil low temperature plant material (177mg) gained significantly (P<0.05) more weight than larvae fed AR37 low temperature plant material (141mg).

No significant difference (P>0.05) in consumption was found between Nil treatments grown at low temperature and Nil treatments grown at high temperature. However, larvae fed Nil low temperature plant material gained significantly more weight than larvae fed Nil high temperature treatments (177mg cf. 129mg respectively P<0.05).

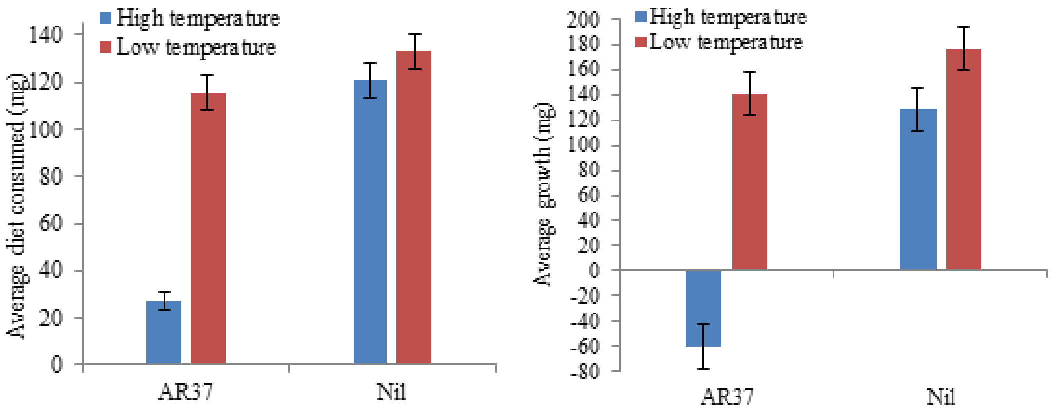

Figure 4.2: Comparison of average diet consumed (mg) (+/- SE) and average weight change (mg) (+/- SED) within the infection (AR37-infected or Nil endophyte ryegrass) x temperature interaction (high (20°C) or low (7°C) temperature)

4.4.1.2 Infection x Temperature x Cultivar

For both consumption and growth the three way interaction between endophyte infection, temperature and cultivar was significant. There was no significant difference (P>0.05) in consumption between AR37 and Nil low temperature treatments in the infection x temperature interaction. However, when cultivar was taken into account significantly more (P<0.05) diet was consumed and larvae gained more weight (P<0.05) when fed Nil compared with those fed AR37 perennial ryegrass grown at low temperature (Figure 4.3), but no such effect was found for Italian ryegrass.

There was no significant difference (P>0.05) in larval consumption or growth between cultivars either infected with AR37 or endophyte free when these were grown at high temperatures.

In AR37-infected ryegrass treatments grown at low temperature there was no significant difference (P>0.05) in larval consumption between cultivars. However, larvae in Italian ryegrass treatments (169mg) gained significantly more weight than larvae in perennial ryegrass treatments (112mg).

In endophyte free ryegrass treatments grown at low temperature; larvae in perennial ryegrass treatments consumed more diet (P<0.05) and gained more (P<0.05) weight than larvae in Italian ryegrass treatments.

When comparing Nil treatments grown at high and low temperatures, there were no significant differences (P>0.05) in consumption or growth for Italian ryegrass. In comparison, significantly more (P<0.05) diet was consumed and larval growth was higher in Nil low temperature Samson than Nil high temperature Samson treatments.

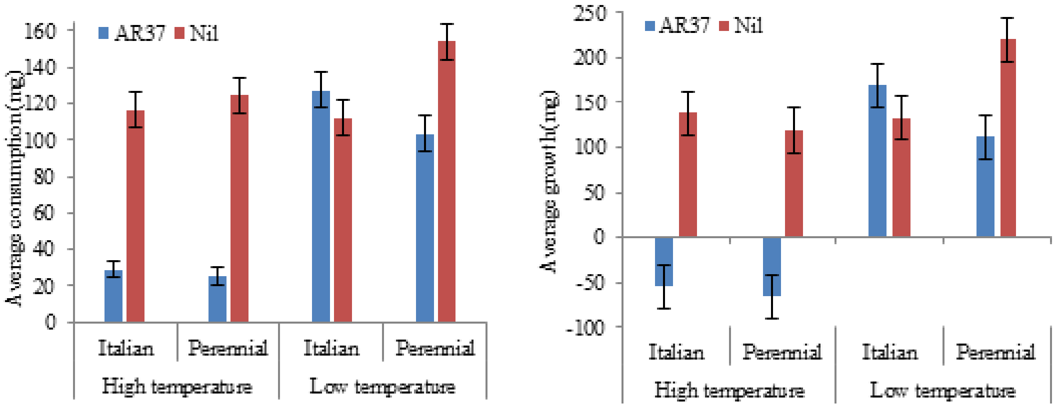

Figure 4.3:Comparison of average diet consumed (mg) (+/- SE) and average growth (mg) (+/- SED) within the infection (AR37-infected or Nil endophyte ryegrass) x temperature (high (20°C) or low (7°C) temperature) x cultivar (Perennial or Italian ryegrass) interaction

4.4.1.3 Infection x Temperature x Plant part

Consumption was not affected by an infection x temperature x plant part interaction. However, growth of larvae was affected by this interaction (Figure 4.4). There was a significant (P>0.05) difference in growth between plant part in AR37-infected treatments grown at high temperature. Larvae fed AR37-infected pseudostems from plants grown at high temperature (growth=-100mg) lost significantly more weight than larvae fed the leaves of these plants (-22mg). In comparison larvae fed AR37-infected ryegrass grown at low temperature gained weight; with larvae fed leaves (growth=195mg) gaining more (P<0.05) weight than larvae fed pseudostems (87mg).There was no significant difference (P>0.05) in growth between larvae fed AR37-infected ryegrass grown at low temperature and larvae fed Nil endophyte ryegrass grown at low temperature, for both pseudostems and leaves.

A significant (P<0.05) difference in growth between leaves and pseudostems was found within Nil treatments. For Nil treatments grown at high temperature significantly (P<0.05) more weight was gained by larvae fed pseudostems (growth=157mg) than leaves (100mg) (Figure 4.4). In comparison, for Nil treatments grown at low temperature significantly (P<0.05) more weight was gained by larvae fed leaves (growth=220mg) than pseudostems (134mg).

When comparing larval growth in Nil endophyte treatments grown at high and low temperature no significant (P>0.05) difference was found for pseudostems. In comparison, larave fed leaves grown at low temperature (growth=219mg) gained significantly (P<0.05) more weight than larvae fed leaves grown at high temperature (100mg).

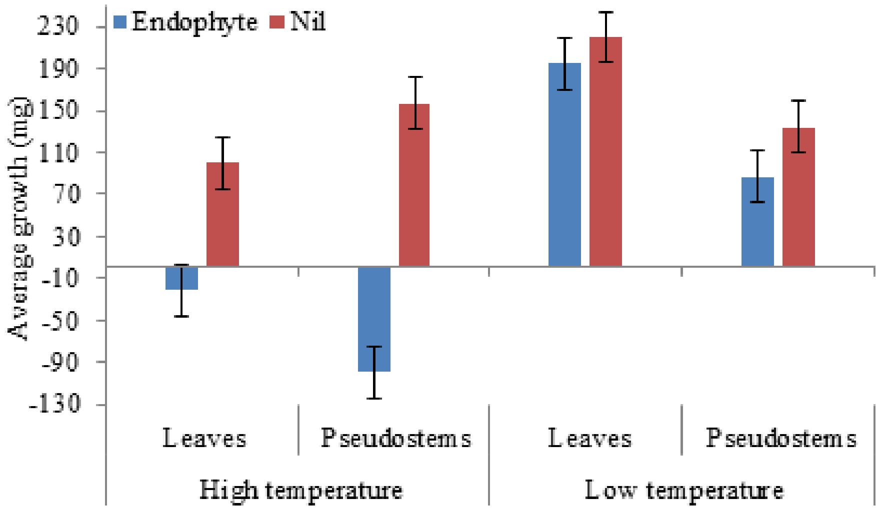

Figure 4.4: Comparison of average growth (mg) within the infection (AR37-infected or Nil endophyte ryegrass) x temperature (high (20°C) or low (7°C) temperature) x plant part (leaves or pseudostems) interaction. +/- SED

4.4.1.4 Infection x Cultivar x Plant part

The three way interaction between infection, cultivar and plant part was significant for both consumption and growth data. When cultivar is compared in this interaction larvae fed Nil perennial pseudostems consumed significantly more diet and gained significantly more weight than larvae fed Nil Italian pseudostems (Figure 4.5).

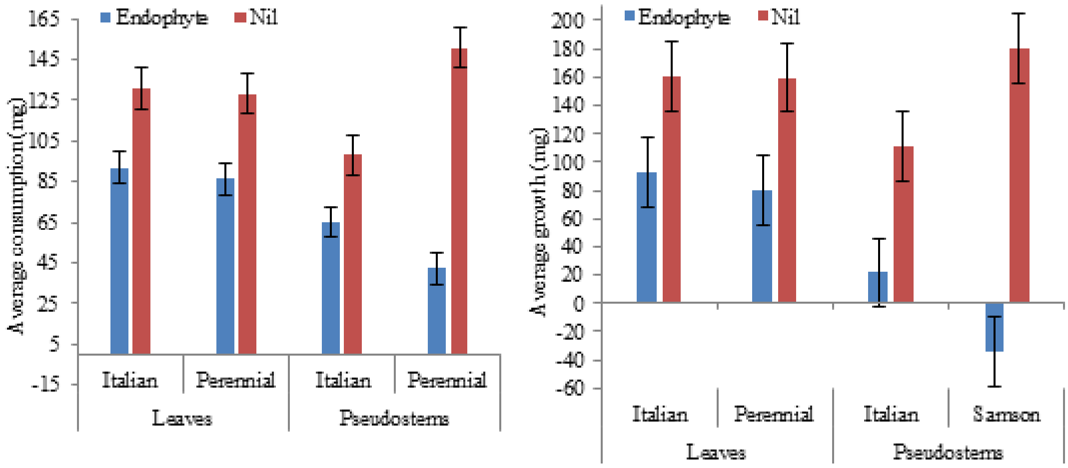

Figure 4.5: Comparison of average consumption (mg) (+/- SE) and average growth (mg) (+/- SED) within the infection (AR37-infected or Nil endophyte ryegrass) x cultivar (Perennial or Italian ryegrass) x plant part (leaves or pseudostems) interaction

4.4.2 Mortality

Mortality in this experiment was low overall, except for larvae fed AR37-infected ryegrass pseudostems grown at high temperature. Two larvae died in Nil treatments and two larvae in AR37-infected ryegrass treatments grown at low temperature. In contrast, ten larval deaths occurred in the AR37-infected ryegrass treatments grown at high temperature. One larva died in each of the two leaf treatments. The highest deaths occurred in the pseudostem treatments (Figure 4.6), with five larvae dying in the perennial ryegrass high temperature treatment (mortality=42%) and three larvae in the Italian ryegrass high temperature treatment (25%).

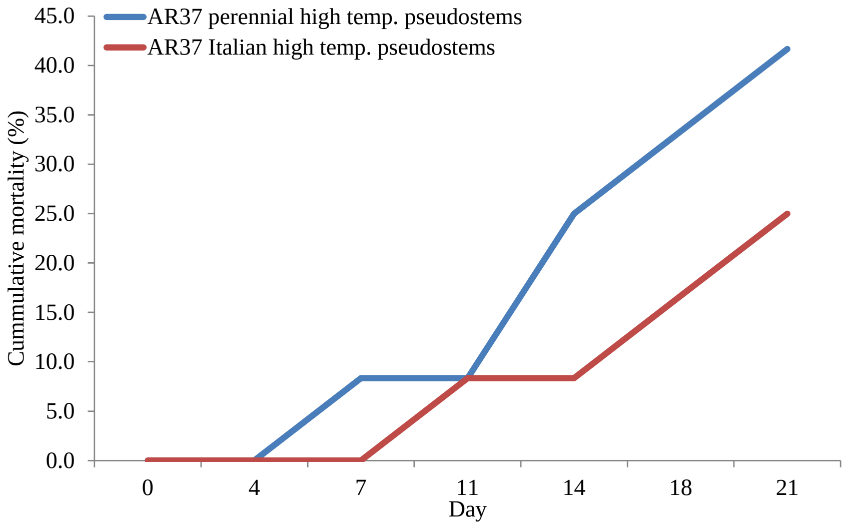

Figure 4.6: Cumulative mortality (%) of larvae in the AR37-infected perennial ryegrass high temperature treatment against larvae in the AR37-infected Italian ryegrass high temperature treatment.

4.4.3 HPLC diet results

Epoxy-janthitrem concentrations in the diet (fresh diet and diet from fridge) show that perennial ryegrass contained higher epoxy-janthitrem concentrations than Italian ryegrass, when grown at both temperatures. Results from the remnant diet show that these concentrations were not substantially degraded while diet plugs were exposed to porina larvae (Table 4.5).

Table 4.5: Epoxy-janthitrem concentrations in fresh diet, remnant diet 1 (fresh diet fed on by larvae for 4 days), diet from fridge (diet made 4 days prior and stored in a fridge) and remnant diet 2 (diet stored in fridge for 4 days then fed to larvae for 3 days)

AR37 perennial low temperature leaves

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	0.10	11/08/2014	0.08	11/08/2014	0.09	14/08/2014	-
14/08/2014	0.10	18/08/2014	0.08	18/08/2014	0.09	21/08/2014	0.07
21/08/2014	0.09	25/08/2014	0.09	25/08/2014	0.09	27/08/2014	0.09

AR37 perennial low temperature pseudostems

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	1.63	11/08/2014	1.08	11/08/2014	1.41	14/08/2014	-
14/08/2014	1.65	18/08/2014	1.34	18/08/2014	1.44	21/08/2014	1.20
21/08/2014	1.59	25/08/2014	1.32	25/08/2014	1.46	27/08/2014	1.39

AR37 Italian low temperature leaves

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	0.07	11/08/2014	0.07	11/08/2014	0.07	14/08/2014	-
14/08/2014	0.07	18/08/2014	0.06	18/08/2014	0.07	21/08/2014	0.06
21/08/2014	0.10	25/08/2014	0.06	25/08/2014	0.06	27/08/2014	0.07

AR37 Italian low temperature pseudostems

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	0.88	11/08/2014	0.44	11/08/2014	0.77	14/08/2014	-
14/08/2014	0.86	18/08/2014	0.71	18/08/2014	0.77	21/08/2014	0.56
21/08/2014	0.82	25/08/2014	0.77	25/08/2014	0.74	27/08/2014	0.81

AR37 perennial high temperature leaves

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	3.60	11/08/2014	3.32	11/08/2014	3.45	14/08/2014	-
14/08/2014	3.93	18/08/2014	3.67	18/08/2014	3.66	21/08/2014	3.31
21/08/2014	3.81	25/08/2014	3.49	25/08/2014	3.40	27/08/2014	3.57

AR37 perennial high temperature pseudostems

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	13.98	11/08/2014	10.28	11/08/2014	12.30	14/08/2014	-
14/08/2014	14.18	18/08/2014	12.88	18/08/2014	11.28	21/08/2014	12.67
21/08/2014	12.89	25/08/2014	11.83	25/08/2014	12.60	27/08/2014	13.16

AR37 Italian high temperature leaves

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	2.40	11/08/2014	1.96	11/08/2014	2.12	14/08/2014	2.06
14/08/2014	2.33	18/08/2014	-	18/08/2014	2.00	21/08/2014	2.10
21/08/2014	2.27	25/08/2014	2.25	25/08/2014	1.87	27/08/2014	1.90

AR37 Italian high temperature pseudostems

Fresh diet		Remnant diet 1		Diet from fridge		Remnant diet 2	
Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g	Date	EJ concentration µg/g
7/08/2014	11.31	11/08/2014	10.23	11/08/2014	10.22	14/08/2014	10.40
14/08/2014	11.09	18/08/2014	9.86	18/08/2014	10.00	21/08/2014	10.48
21/08/2014	11.02	25/08/2014	11.12	25/08/2014	9.95	27/08/2014	10.47

4.5 Discussion

The hypothesis that higher temperatures would increase epoxy-janthitrem concentrations in ryegrass was supported by the data. When AR37-infected ryegrass was grown at high temperature epoxy-janthitrem concentrations were increased, resulting in a strong anti-feedant effect on porina larvae that lead to reduced weight gains. When plants were grown at low temperature epoxy-janthitrem concentrations were considerably decreased, Italian ryegrass had no negative affect on larvae when compared to the Nil equivalent, but perennial ryegrass grass did have a small anti-feedant effect which lead to a reduced weight gain. Results from this study suggest AR37 grown in cooler areas of the country would contain insufficient concentrations of epoxy-janthitrems to adequately protect ryegrass from porina larvae. Cultivar did not have a significant effect on epoxy-janthitrem concentration in ryegrass plants initially sampled. However, when material from all plant replicates were combined and a representative sample added to semi-synthetic diet to be fed to larvae, epoxy-janthitrem concentrations in diets containing perennial ryegrass were slightly higher than in Italian ryegrass.

AR37-infected ryegrass pseudostems grown at high temperature, which contained the highest epoxy-janthitrem concentrations, reduced larval survival. A reduction in survival could indicate toxicity of the alkaloid. Toxicity is defined as a compound which reduces growth and survival of larvae above that which can be attributed to starvation. Toxicity was investigated in the epoxy-janthitrem I bioassay in chapter 3 of this thesis, by comparing growth and survival of porina larvae to a starvation treatment. However, it could not be determined whether larvae were dying due to toxicity of the alkaloid or due to starvation caused by the strong anti-feedant effect of the alkaloid. Therefore it is not known whether larvae in the high temperature treatments in this trial died due to toxicity or starvation. Mortality in the perennial ryegrass treatment was higher (42%) than mortality in the Italian ryegrass treatment (25%). This difference is likely due to the higher epoxy-janthitrem concentrations in the perennial ryegrass treatment. Despite the higher epoxy-janthitrem concentration in perennial ryegrass, there were no significant differences in larval consumption or growth between the two cultivars when AR37 plants were grown at high temperature. This could indicate that when epoxy-janthitrem concentrations reach a certain level the anti-feedant effect cannot get any strong.

When AR37-infected ryegrass grown at low temperature was fed to porina larvae, Italian ryegrass had no effect but perennial ryegrass did have a small anti-feedant effect. The anti-feedant effect was not as strong as in the high temperature treatments as growth and consumption were not decreased to the same extent and larval survival was not affected. Epoxy-janthitrem concentrations were higher in perennial than Italian ryegrass treatments, with perennial pseudostems containing concentrations twice as high as those in Italian pseudostems. This is likely enough to explain why AR37-infected perennial ryegrass grown at low temperature had an effect on larvae but Italian did not. The differences in effect on larvae may also have been compounded by different ratios of the five epoxy-janthitrem compounds in the two cultivars, particularly if certain compounds, or combinations of compounds, are more bioactive than others. Ratio differences were found between pseudostem samples grown at low temperature. Perennial ryegrass was found to contain higher concentrations of epoxy-janthitrem III than epoxy-janthitrem IV. The opposite was found in Italian ryegrass samples. The difference in bioactivity between the two cultivars may also be due to another unknown alkaloid, which may be produced in higher concentrations when in perennial ryegrass.

In comparison, when endophyte free ryegrass was grown at low temperature larvae fed perennial ryegrass consumed significantly more diet and grew significantly larger than larvae fed Italian ryegrass. This may indicate that when Nil infected ryegrass is grown at low temperature perennial ryegrass is more palatable and/or nutritious to larvae than Italian ryegrass. Alternatively, this result could indicate that perennial ryegrass does not contain enough nutrition for larvae. Larvae must then consume more diet in order to gain enough nutrition. Nitrogen is an important part of an insect’s diet, equal amounts of fertilizer were added to the plants in this experiment but nitrogen levels within plants were not determined. It is possible that perennial ryegrass could have contained lower nitrogen levels, resulting in larvae consuming more to gain adequate nitrogen.

The AR37-infected ryegrass tested on larvae in this trial would have contained other unknown alkaloids and it is possible that these compounds could have contributed to the deterrent effects observed. However, as similar deterrence was seen in the epoxy-janthitrem I bioassay in chapter 3 of this thesis and Finch, *et al.* (2010) has previously identified that pure epoxy-janthitrem I has a deterrent effect on porina larvae, it is likely that the negative effects caused to larvae in this trial were largely due to the epoxy-janthitrems.

This study was the first to examine what effect temperature has on epoxy-janthitrem concentrations in ryegrass. Studies show that alkaloids are not always significantly affected by temperature and results can be variable (Lane, *et al.*, 1997; Eerens, *et al.*, 1998). The effects of temperature on lolitrem B concentrations have previously been examined. As both of these alkaloids are in the indole-diterpene class of alkaloids it was suggested in the first chapter of this thesis that effects of temperature may be similar for the two alkaloids. In a pot trial Eerens, *et al.* (1998) examined the effect of temperature on lolitrem B concentrations in wild type infected ryegrass grown at low (15°C night and 25°C day) or high (25°C night and 35°C day) temperature. This study found lolitrem B concentrations were slightly higher at low temperature, but the difference was not significant. The temperatures examined in Eerens, *et al.* (1998) were high, it is not known whether lolitrem B is reduced in a similar way by cold temperatures, as epoxy-janthitrem was found to be in this study. However, in field trials lolitrem B concentrations have been found to be higher during summer and autumn and lower during winter, when temperatures are cooler (di Menna, *et al.*, 1992; Ball, *et al.*, 1995). Although, it is likely that in the field other factors such as moisture stress, grazing and insect pressure are also impacting on alkaloid concentrations.

The light regime in this trial, 12 hours light: 12 hours dark, may have affected alkaloid concentrations, especially for ryegrass grown at low temperature (7°C), as these conditions are unlikely to occur in the field. Exposing ryegrass to conditions which are not natural or that ryegrass is not adapted to, such as cooler temperatures, will reduce growth of both the ryegrass and the endophyte and as a result alkaloid levels in the plant will be decreased.

The mechanisms by which temperature affected alkaloid levels in this study are not known. Temperature could have affected the amount of hyphae in ryegrass plants (di Menna & Waller, 1986; di Menna, *et al.*, 1992), resulting in a decrease/ increase in alkaloid level in the plant. Temperature may also have directly affected alkaloid concentrations by effecting alkaloid biosynthesis rates.

In this study ryegrass plants in the high temperature CE room were watered with 150–300mL of water, as required, in an attempt to standardise the amount of water received by individual plants. However, towards the end of the trial some of the plants became wilted. It is suspected this could be due to water stress or the lack of fresh circulating air in the CE room, as plants were grown in the rooms for 10-12 weeks. What effect moisture stress has on epoxy-janthitrem concentrations is unknown. However, moisture stress has been shown to significantly affect concentrations of other alkaloids (Lane, *et al.*, 1997) and the moisture x temperature interaction has been shown to be highly significant for some alkaloids (Eerens, *et al.*, 1998). Similar patterns have also been reported in field trials where alkaloid levels were found to fluctuate with seasonal temperature and water availability (di Menna, *et al.*, 1992; Ball, *et al.*, 1995). Therefore, it cannot be ruled out that in this trial some of the variation in epoxy-janthitrem concentration at high temperature was due to another factor such as moisture stress.

No significant difference in epoxy-janthitrem concentration was found between perennial and Italian ryegrass plants in the first 5 plant replicates initially sampled for epoxy-janthitrem analysis. However, when all plant replicates (20 plants) were combined and a representative sample taken and incorporated into diets to feed to porina larvae, concentrations were found to be higher in perennial ryegrass diets (average concentration in fresh diet, leaves=0.1µg/g, pseudostem=1.62µg/g) than in Italian ryegrass (leaves=0.08µg/g, pseudostem=0.85µg/g). The ryegrass plants initially sampled were all from one area of the CE room, it is possible that these differences in concentration are due to climatic differences within the CE room.
Epoxy-janthitrem concentrations did also vary between individual ryegrass plants tested. Indicating that the epoxy-janthitrems, like other alkaloids (Spiering, *et al.*, 2005) are affected by genotype of the host plant. Whether alkaloid levels are altered in certain genotypes because the endophyte level has been altered or

whether genotype has an effect on alkaloid level is not entirely clear. Plant genotype may influence alkaloid level by affecting alkaloid biosynthesis rates or alkaloid metabolism and degradation rates (Spiering, *et al.*, 2005).

There is no published information comparing epoxy-janthitrem concentrations in leaves and pseudostems of AR37-infected ryegrass plants. In this study, epoxy-janthitrem concentrations were found to be higher in the pseudostems than the leaves at both temperatures. This relationship is not uncommon. Lolitrem B, which like the epoxy-janthitrems is within the indole diterpene class of alkaloids, also contains higher concentrations in the pseudostem than the leaves (di Menna, *et al.*, 1992; Davies, *et al.*, 1993; Keogh *et al.*, 1996; Ball, *et al.*, 1997b). Alkaloids such as lolitrem B and the epoxy-janthitrems are lipophilic compounds and are not easily translocated around the plant (Ball *et al.*, 1993; Munday-Finch & Garthwaite, 1999; Spiering, *et al.*, 2005) thus distribution tends to be similar to that of the endophyte, which is generally higher in the pseudostem and lower in the leaves (Musgrave, 1984; Musgrave & Fletcher, 1984; Keogh & Tapper, 1993). However, this relationship may not be true in all cases as Spiering, *et al.* (2005) found only a weak correlation between endophyte and alkaloid levels.

Having higher alkaloid concentrations in the pseudostem of the plant than the leaves is advantageous for the host ryegrass and the endophyte, which depends on its host for survival and transmission. This is because the meristem, the tissue containing undifferentiated cells and were growth occurs, is located at the base of the ryegrass plant. If an insect damages the meristem of a ryegrass tiller the tiller will die. It is therefore advantageous to have high alkaloid concentrations in the pseudostem to protect the meristem from damage. Insect damage to the leaves of ryegrass plants is not as harmful, as ryegrass is adapted to animal grazing. Although, severe damage would have negative impacts over time. Feeding on leaves however is not advantageous for farming as the more leaf material insects consume, the less is available for consumption by livestock, which would result in a decrease in productivity. Therefore, from a farming prospective it is advantageous to have adequate alkaloid concentrations throughout the ryegrass plant, to protect against tiller death and to protect leaves.

The results from this study can be used to inform farmers of the limitations of the AR37 endophyte when grown at low temperatures in controlling porina larvae, and possibly other pest insects, so that farmers can implement additional control methods such as mobstocking (Stewart & Ferguson, 1992) or applying insecticides (Ferguson, 2000) to reduce pasture loss caused by porina.

This trial was a pot trial undertaken in controlled environments, where plants were regularly watered and fertilized and exposed to light/ dark ratios of 12hours light: 12 hours dark, caution must therefore be applied when interpreting results from this trial and applying them to the field. Also, in the field ryegrass will be exposed to additional abiotic and biotic stresses which may also impact on the epoxy-janthitrem concentrations and consequently will affect the level of resistance.

To follow on from this study field trials should be conducted to identify how temperature affects epoxy-janthitrem concentration in the field. AR37-infected ryegrass tillers from a range of locations representing different climatic conditions could be sampled and the epoxy-janthitrem concentrations determined. Concentrations could then be related with climatic conditions and results from this thesis and Finch, *et al.* (2010) to determine whether levels found are high enough to be effective against porina larvae. Popay, *et al.* (2012) has previously looked at the effect of AR37 on porina populations in the field. In this study porina populations were found to be lower on AR37 treatments when compared to ryegrass infected with the endophytes AR1, wild type or NEA2. This trial was in Canterbury and sampling was in July. Mean monthly temperatures in Canterbury during July are around 7°C, the same temperature AR37 was found not to be effective against porina in this study. This result does not necessarily indicate that results from this thesis would not be replicated in cooler regions in the field, as this could be explained by differences in moisture, lighting and other abiotic and biotic pressures which influence alkaloid levels in the field.

If these results are backed up by field trials it will be important that more research is conducted to further clarify the limitations of the AR37 endophyte in cooler environments, by identifying the temperature range at which the alkaloid will be effective in. Identifying the length of time ryegrass needs to be exposed to cooler temperatures for epoxy-janthitrem concentrations to decrease to ineffective levels is also important. For example will concentrations decrease over a day or will it take weeks of exposure to cooler temperatures before alkaloid concentrations decrease.

The next step could be to identify existing ryegrass cultivars or identifying plant genotypes, from which a new breeding line could be produced, which produce higher epoxy-janthitrem concentrations at low temperature. The existing cultivar or new breeding line may then be recommended for use by farmers in cooler regions. Alternatively, if another novel endophyte is found that also produces the epoxy-janthitrems, this new strain could be tested in cooler temperatures to identify whether it produces adequate concentrations to deter porina larvae.

In the field ryegrass exposed to high temperatures are also likely to be under some moisture stress. Future experiments could examine the role of moisture stress and the moisture stress x temperature interaction in affecting epoxy-janthitrem concentrations in ryegrass, to improve understanding of how these abiotic factors may interact to affect epoxy-janthitrem concentrations.

Chapter 5: Conclusion

The aims of this thesis were to investigate the toxicity of epoxy-janthitrem I to porina larvae and to explore what effect temperature and plant cultivar have on epoxy-janthitrem concentrations in ryegrass and how these variations in concentration affect feeding, growth and survival of porina larvae.

Epoxy-janthitrem I was found to have an anti-feedant effect on porina larvae at all three concentrations tested (1, 2.5, 5µg/g of diet, wet weight), with deterrent effects stronger at the two higher concentrations. Some evidence of toxicity of epoxy-janthitrem I was identified, but further work is required to resolve this. By growing ryegrass at two different temperatures an understanding of the effect of temperature on epoxy-janthitrem concentrations was achieved which may explain reports from the field that AR37-infected ryegrass is not able to adequately control porina in cooler regions. Epoxy-janthitrem concentrations in ryegrass were found to be greatly increased when plants were grown at high temperature (20°C) compared with those grown at low temperature (7°C). When AR37-infected ryegrass grown at high temperature was fed to porina larvae it was found to have a strong anti-feedant effect and reduced larval survival. In contrast, AR37-infected ryegrass grown at low temperature, containing low concentrations of epoxy-janthitrems, had a small anti-feedant effect on larvae when in perennial ryegrass, and had no effect on larvae when in Italian ryegrass. Plant cultivar did have a small effect on epoxy-janthitrem concentration; at both temperatures perennial ryegrass contained higher concentrations than Italian ryegrass. Epoxy-janthitrem concentrations differed amongst individual plant genotypes and concentrations were shown to be higher in the pseudostems than the leaves of AR37-infected ryegrass plants.

The anti-feedant effects observed in the two experiments in this thesis were consistent. In both trials the higher concentrations, 2.5 and 5µg/g of diet in the epoxy-janthitrem I bioassay and to 2.3- 4 µg/g and 11- 14.2µg/g in the ryegrass temperature trial bioassay, had strong anti-feedant effects on larvae, which resulted in reduced larval growth and survival. However, whether this was due to toxicity of the alkaloid or starvation due to the strong anti-feedant effect could not be determined. In both trials the lower concentrations, 1µg/g in the epoxy-janthitrem I bioassay and 0.1µg/g and 0.82- 1.65µg/g in the ryegrass temperature trial, had weaker anti-feedant effects on larvae. In the ryegrass temperature trial concentrations in Italian ryegrass were below 1µg/g and were not effective against larvae, 1µg/g (wet weight in semi-synthetic diet) was effective against larvae in the epoxy-janthitrem I bioassay. This could indicate that epoxy-janthitrem concentrations need to be above 1µg/g to be effective.

Results from this thesis can be used to improve performance and identify the limitations of the AR37 endophyte in the field. Bioactivity results can be used to help control porina populations on farms severely affected by porina, by advising farmers to reduce alternative food sources such as clover, cocksfoot and Nil endophyte ryegrass on farms. These alternative food sources can sustain porina populations in the field. The evidence presented in this thesis has shown that if only AR37-infected ryegrass is available, and epoxy-janthitrem concentrations in AR37 are sufficient, porina larvae will die either due to toxicity of the alkaloid or starvation as the alkaloid has a strong anti-feedant effect.

This thesis also identifies a potential limitation of the AR37 endophyte when AR37-infected ryegrass is grown at cooler temperatures, which has important implications for farmers. Although, caution must be applied when relating results in this pot trial to the field, farmers should be advised that AR37 may not produce sufficient concentrations of epoxy-janthitrem when grown at lower temperatures. This means that AR37 ryegrass is probably more vulnerable to pasture damage caused by porina larvae, and possibly other pest insects, when grown at cooler temperatures (<7°C) and alternative control methods such as applying insecticides (Ferguson, 2000) or mobstocking (Stewart & Ferguson, 1992) should be implemented to prevent pasture loss.

Research on the epoxy-janthitrems, their effect on pest insects and how abiotic and biotic factors influence their concentrations is very limited in comparison to the other significant alkaloids produced by pastoral endophytes. There are therefore many recommendations for future research that can be made. These have been discussed in detail in previous chapters, and the most important are highlighted below.

Bioactivity results indicated that epoxy-janthitrem I may be toxic to porina larvae, but it could not be determined whether larvae were dying because of toxicity of the alkaloid or because of starvation due to the strong anti-feedant effect of the alkaloid. Further experiments to resolve this will be important to fully understand the bioactivity of the epoxy-janthitrems and how they affect porina populations in the field. If the epoxy-janthitrems are only deterrent to porina larvae, larvae may search for alternative food sources such as clover, cocksfoot or ryegrass infected with a less effective endophyte. If the alkaloid is also toxic survival in the population will be reduced. To resolve this an experiment could be designed to determine the minimum amount of diet 9 and 21 week old porina larvae require to survive. This information could then be related to results of the experiments in this thesis to identify if larvae were consuming an adequate amount of diet to prevent starvation. If larvae are found to have consumed enough diet in this thesis the mortality identified could be attributed to toxicity of the alkaloid. Alternatively a bioassay could be designed in which larvae are fed semi-synthetic diet containing epoxy-janthitrem for 5 days of the week and Nil endophyte diet for 2 days. Providing larvae with Nil diet would ensure larvae are receiving enough diet to prevent starvation meaning that any deaths may be attributed to toxicity of the alkaloid.

AR37 is known to affect other pasture pests including; Argentine stem weevil (Popay & Wyatt, 1995), black beetle (Ball*, et al.*, 1994), root aphid (Popay*, et al.*, 2004; Popay & Gerard, 2007) and pasture mealybug (Pennell, *et al.*, 2005), but so far work has been focused on porina larvae. Further studies should work to establish whether the epoxy-janthitrems are responsible for the anti-feedant effects observed on other insects.

Determining whether the epoxy-janthitrems have an effect on Argentine stem weevil larvae and black beetle adults can be explored using similar bioassays to those presented in this thesis, where epoxy-janthitrem is extracted from ryegrass seed and incorporated into a semi-synthetic diet and fed to insects in a bioassay to compare consumption, growth and survival of insects. Alison Popay and Sarah Finch (AgResearch, Ruakura) are currently exploring what effect the epoxy-janthitrems have on these two insects and identifying whether any unknown bioactives in AR37 may be affecting these insects.

Root aphids and the pasture mealybug feed by sucking sap from ryegrass roots. Therefore a different method would be required to test the effects of the epoxy-janthitrems on these insects. The epoxy-janthitrems are not likely to be responsible for the deterrent effects of AR37 on root aphid or pasture mealybug as only low concentrations have been found in the roots (from Popay & Gerard 2007, Popay unpublished data) and the epoxy-janthitrems are not hydrophilic compounds, meaning they are not likely to be transported within the phloem of tillers or roots in ryegrass plants. However, to test whether the epoxy-janthitrems do have an effect on these insects, cotton swabs could be soaked in solutions containing epoxy-janthitrem, allowing the swabs to absorb the liquid (Johnson *et al.*, 1985; Yates, *et al.*, 1989). Epoxy-janthitrem or Nil equivalent swabs could then be placed into containers with individual aphids or mealybugs. Aphid nymph counts and survival could be monitored to determine whether the epoxy-janthitrems affect these insects.

Further research is required to clarify the limitations of the AR37 endophyte in controlling porina populations in cooler regions of New Zealand. To follow on from the pot trials in this thesis, field trials should be conducted to identify how temperature affects epoxy-janthitrem concentrations in the field. Initially ryegrass tillers from paddocks known to be infected with AR37 could be sampled from different locations, representing a range of climatic conditions. Ryegrass samples could then be freeze dried and ground and the epoxy-janthitrem concentrations determined by HPLC. Concentrations could then be related to climatic data. Ryegrass plants could also be planted in replicated pots placed on a farm in an area where AR37 has been reported to be ineffective against porina larvae. Ryegrass tillers from the pots could be sampled monthly and epoxy-janthitrem concentrations determined to identify seasonal effects on epoxy-janthitrem concentrations and identifying if concentrations are reduced to low levels.

If epoxy-janthitrem concentrations are found to be reduced in the field further work will be required to clarify the limitations of the endophyte. It would be important to identify the temperature ranges the AR37 endophyte may not be effective in and identify the length of time ryegrass must be exposed to these temperatures to cause epoxy-janthitrem concentrations to decrease to ineffective levels. To test this plants could be grown in controlled environment rooms and tillers sampled for epoxy-janthitrem analysis at time zero, and each week for 2 months to monitor the changing alkaloid levels.

Further studies could also be undertaken to identify existing ryegrass cultivars, or plant genotypes from which a new breeding line could be produced, which produce higher epoxy-janthitrem concentrations at low temperature. The existing cultivar or new breeding line may then be recommended for use by farmers in cooler regions. Alternatively, if another novel endophyte is found that also produces the epoxy-janthitrems, this new strain could be tested in cooler temperatures to identify whether it produces adequate concentrations to deter porina larvae.

Results from this thesis have contributed to an understanding of the bioactivity of the epoxy-janthitrems and identified limitations of the AR37 endophyte when grown in cooler regions of the country. It was also shown there was no major difference in expression of the alkaloid epoxy-janthitrem by two of the major ryegrass species; Perennial and Italian ryegrass. These results can be used to inform farmers and improve the performance of AR37 in the field.

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