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The biological control of the red clover casebearer in New Zealand

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy
at
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by
Joel Anthony Faulkner

Lincoln University

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Abstract of a thesis submitted in partial fulfilment of the
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Biocontrol of insect pests represents an important aspect of sustainable crop management that has potential to reduce the current reliance on chemical control measures. However, often the potential of all forms of biocontrol is undermined in contemporary productive systems and their resulting landscapes.

In New Zealand seed three species of clover feeding moths of the *Coleophora* genus have seriously hindered red and white clover seed production during the last 100 years in New Zealand. From the 1920s until the 1960s two species of white clover casebearer (*Coleophora mayrella* and *C. alcyonipennella*) caused varying levels of seed yield reduction in white clover seed crops. During the 1960s efforts were made by government science agencies to establish parasitoid wasps of these species. This programme was highly successful, resulting in the total suppression of clover casebearer seed pests in New Zealand, until the arrival of the red clover casebearer (*C. deauratella*) during 2016. This pest quickly spread throughout the country's clover growing regions, leading to yield reductions.

This thesis explores the arthropod community of red clover seed crops under a main theme of 'the biocontrol of the red clover casebearer'. The experiments employed are varied, although typically with a major aim being exploring the function of species within the red clover seed crop ecosystem. Three species of parasitoid wasp were able to successfully use *C. deauratella* as a host; *Bracon variegator*, *Pteromalus puparum* and *Eupelmus (Macroneura) messene*. The red clover casebearer was clearly undergoing remission as a pest and only two damaging populations of this pest could be located. The predominant parasitoid, *B. variegator*, had a field parasitism incidence of up to 75% on *C. deauratella*, but despite this these two populations of the pest persisted.

Over the course of these experiments it was noted that a fungal growth was leading to mortality of *C. deauratella* larvae. From infected *C. deauratella* cases a potentially entomopathogenic strain of *Fusarium pseudograminearum* was isolated and identified through PCR sequencing.

It is still uncertain how *C. deauratella* is being regulated in New Zealand red clover seed crops. The role of parasitoid wasps, a potential fungal pathogen and other unknown biocontrol agents is yet to be determined.

Keywords: Agricultural entomology, Agro-ecology, Arthropod community, Biocontrol, *Bracon variegator*, *Coleophora deauratella*, Community ecology, Parasitism, Entomopathogenic, Internally feeding insect pest, Natural enemies, Red clover, Seed pest, Seed production, Sustainable production, New Zealand

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'If hard work pays, show me rich donkey' - Unknown

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Chapter 1

Introduction and literature review

Biological control of crop pests and insect pest issues facing red clover seed production in New Zealand

1.1 Introduction: The current mode of agricultural production and the need for sustainable production

Crops and grazed grasslands are a dominant part of terrestrial landscapes in that they comprise about 38% of the world's ice-free land area (FAO 2022). Historically, much of this extent has been driven by the growing and developing global human population (Foley et al 2007; Zhang et al 2008). However, in recent years there has been continual growth in the global rate of malnourishment despite increasing net global food production (FAO 2022).

Currently around 4 billion tonnes of food is produced each year globally, of which around one third is wasted (Al-Obadi et al 2022). Indeed, this total volume of food is enough to feed the predicted global population of 10 billion people by 2050, but more factors than simply the total volume of production define the ability of people to access adequate and healthy food sources (Allee et al 2012; UN 2023b; FAO 2022). Therefore, the issue is better framed as food insecurity rather than food shortages due to insufficient production (Misslehorn et al 2012; Long et al 2020; Clapp et al 2022).

This argument shows that the growing global population itself cannot be used to justify the continued spread of 'conventional agriculture' because increased production within all other current parameters will clearly not solve world hunger issues (Sage 2013; Sage 2014; Dou et al 2016); some of which are political (e.g. Ethiopia) or the ravages of climate change (e.g. Pakistan) (Misslehorn et al 2012). Furthermore, continued expansion of the 'conventional system' will provide diminishing returns in cost and labour along with increased environmental damage due to productive limits being reached in critical resources such as soil health (Pellegrini and Fernandez 2018).

Thus, there are many reasons for food insecurity, which include environmental/ecosystem instability, low production, politics and poverty (Hasegawa et al 2018; Clapp et al 2022; FAO 2022). More widely, a contributing factor to this issue is the global distribution network which supplies some but not all people with a sufficient amount and variety of food (Holt-Gimenez et al 2012; Allee et al 2021).

Farming landscapes have rapidly changed over the last 200 years due to processes and developments such as mechanisation, the green revolution and urbanisation; however, the fastest pace of change

has been seen in the years between the end of the Second World War and today (Sassenrath et al 2008; Quigley et al 2019). Despite this rapid change, today around 80% of farms in the world are still small (under 2ha) family run operations (Lowder et al 2016; Cohn et al 2017). Whilst there is debate around the relative yields of conventional and sustainable forms of agriculture, much of this 'critique' is driven by overly 'contained' arguments; the cases against sustainable forms of production typically fail to recognise ecological processes, long-term productivity, food wastage and food allocation issues: for example biofuels, and U.S.A animal feedlot systems (Holt-Gimenez et al 2012). This is why modern sustainable approaches go beyond both conventional and historical agriculture paradigms in an effort to foster the sustainable intensification of production (Pretty 2018; Melchoir and Newig 2021).

The development of modern agriculture and its resulting yield increases has been accompanied with a consistent increase in pesticide and fertiliser application which have become core components of the system, often referred to as 'conventional agriculture' (Sharma et al 2019; Yao et al 2020). Despite this intensification of agricultural production, there continues to be a deficiency in conclusive data on safe levels of agri-chemical use and economic return of applications (Ecobichon 2001; Lechenet et al 2014; LaCanne and Lundgren 2018; Zhang et al 2018; Jepson et al 2020).

The development and adoption of conventional agricultural practices can be attributed to a combination of factors such as economic imperatives, the drive for increased production, limited information on environmental impacts, and the need for farmers to remain competitive in a rapidly changing world (Saltiel et al 1994; Lechenet 2014). Profit as a measure of farm viability is not related to wider goals of environmental sustainability and long-term productivity. Furthermore, its pursuit can harm these ends (Saltiel et al 1994; Siebrecht 2020). Nonetheless, financial stability is important to the people who will be responsible for implementing sustainable agriculture practices (Siebrecht 2020). Therefore, rural community and farmer viability must be of high priority when trying to change agriculture practices. In addition, highlighting the enhanced profitability of certain sustainable practices can be beneficial to their adoption (Rodriguez et al 2009; Pingali 2012 Lechenet et al 2014; LaCanne and Lundgren 2018).

It is unequivocal that climate change is increasing the intensity and frequency of extreme weather events globally and that therefore agriculture must adapt to these changes (Calzadilla et al 2013; UN 2023). The impacts of climate change on agricultural production are large and complex, including things such as increased pest pressure, drought, flooding and disrupted plant ripening cycles (Raza et al 2018; Gomez-Zavaglia et al 2020).

Farmers are aware of these impacts, but they often find it is difficult to implement change (Gezie et al 2019; Hasan and Kumar 2019; Mitter et al 2019).

1.1.1 Pest issues associated with these developments

The increased unpredictability of weather that comes with climate change is very detrimental to farming as it brings high potential to reduce the yields and success of crops (Kukul et al 2018). These changes in weather also make pest issues harder to anticipate and control because of factors such as increased crop susceptibility to pests, reduced biocontrol efficacy, reduced efficacy of other control methods, biosecurity failure and environmental conditions which favour certain pests, allowing populations to reach atypically destructive levels (Taylor et al 2018).

Insect populations are affected by many complex factors. Some of these are described as density dependent mechanisms, which help explain the population trends of species over time (Lehmann et al 2020). These density dependent mechanisms can be influenced by climate change and lead to unexpected pest outcomes (Than et al 2020). Density dependent mechanisms are things such as increased disease outbreak with increased population density, consuming vital resource faster than it is replenished, increased efficacy of natural enemies and limitations in development rate due to external conditions (Walter et al 2018).

Research on the cotton boll worm (*Helicoverpa amigera* (Hubner 1808) Lepidoptera: Noctuidae) found that its density dependent population limiting mechanisms were weakened with increased temperature, likely due to factors such as an increased crop growing season, leading to an increased abundance of the pest's food supply (Ouyang et al 2014). For many pest species already adapted to conventional agriculture landscapes, any increased crop growth caused by warmer temperatures will likely increase the carrying capacity of key pests due to the weakening of their density dependent mechanisms (Ouyang et al 2014; Huang and Hao 2020).

Climate change related pest-impacts are already becoming clear in growing systems around the world. A study that analysed 50 years of data comprised of 200 tea tortrix moth (*Homona coffearia* (Neitner 1861) Lepidoptera: Tortricidae) outbreaks in Japan found that tea tortrix population cycles were dependent on spring and autumn temperatures, with outbreaks amplifying when spring temperatures exceeded a threshold of 15°C (Nelson et al 2013). Furthermore, the model developed in this study showed that increased temperatures would reduce the stability of tea tortrix population cycles, increasing the likelihood of outbreaks (Nelson et al 2013).

The Inter-Governmental Panel on Climate Change (IPCC) estimates that the primary sector is responsible for 22% of global greenhouse gas emissions; however, the total emissions of the primary sector are on a downward trend because of an increase in global afforestation within productive systems (IPCC 2022). It must also be noted that greenhouse gas emissions are not the only impact of agriculture, of which all require a diverse range of solutions. In addition, the potential carbon sink

represented by proper soil management is substantial and should be prioritised (Tudi et al 2021; Yang et al 2022).

1.1.2 Responses to these issues

The increase of afforestation of agricultural land is broadly positive but it is not often composed of plant species beneficial to agricultural or even native ecosystems (Markou et al 2020). While afforestation of agricultural land could be a substantial part of climate mitigation strategies with a potential to capture 4.9Gt CO₂/year by 2050 (Fitton et al 2019), it is important that it is implemented in an informed ecological manner to avoid worsening food security issues as well as to ensure that maximum ecosystem services are realised (Gillespie and Wratten 2012; Macdonald et al 2018)

In New Zealand, afforestation programmes on agricultural land have been of mixed benefit. Initially shelterbelts and remnant patches of native vegetation were very common in New Zealand farming landscapes. However, these have been reduced over time through productivity campaigns and the adoption of centre pivot irrigation to support intensive dairy operations in parts of the country (Norton et al 2020; Pannell 2021).

Currently afforestation is on the rise again within New Zealand's agricultural landscapes and there is a government programme to plant native riparian species around waterways (Monaghan et al 2021). However, the bulk of afforestation in New Zealand (and globally) has been in carbon credit-based planting, often featuring exotic conifer species that can have adverse impacts on native ecosystems and agricultural land. Although erosion prevention has been cited as an additional benefit from planting exotic species, the same benefit can be acquired with native species that do not cause ecological harm and may also never be felled (Norton et al 2020; West et al 2020; Edwards et al 2020).

Carbon farming programmes often oversimplify the complexity of environmental and social issues in the rural sector (West et al 2020). An analysis of a carbon farming programme in Calabria, Italy found this to be the case, highlighting that afforestation programmes need to take more than just carbon price and timber harvesting into account to have a positive environmental and social impact (West et al 2020). The key issue to be highlighted in this is that there is an ability and will to convert agricultural land into 'non-agricultural' plant species, but such intention is not often informed by biocontrol science or part of a cohesive landscape-wide plan. Any programme that aims to increase 'non-agricultural' plant species on agricultural land should develop systems that can offer support for beneficial and benign insect species as well as mitigate other impacts of agricultural activity (West et al 2020).

Being able to show that land converted away from directly productive crop species can provide ecosystem services largely in the form of pollination and biocontrol can help farmers understand the benefits of, and be more interested in, the adoption of ‘non-productive’ land cover (Brancalion and Holl 2020). Furthermore, the aesthetic improvements (wellbeing, native birds etc.) and nutrient capture can promote the adoption of these plantings (Staddon et al 2021).

Despite numerous negative impacts, the use of agri-applications has been justified through the levels of food production they allow. Nonetheless, in order to ‘sustainably’ use these tools, improvement in their application must always be sought (Singh et al 2020; Lykogianni et al 2021). There has fundamentally been a lack of action on this issue by agrichemical companies historically and even now. Although, agrichemical companies have become associated with a substantial portion of global sustainable agriculture research, sustainability related research is still a small fraction of the money invested in the development and maintenance of agricultural chemicals (Clapp et al 2022). The use of agrichemicals currently remains necessary (through dependence), but their continued use must be based on a sustainable framework at the very least (Howden et al 2007). As well, there must be efforts that are more cohesive across the board with a stronger will of all parties to prioritise the development of sustainable practices (Pretty et al 2018).

1.1.3 Prospects for sustainable agriculture

Modern agro-ecological theory recognises the importance of sustainable agriculture also being high yielding; this is often described as the ‘sustainable intensification of agriculture’ (LaCanne and Lundgren 2018). This term does not denote a specific type of farming system such as organic farming, but describes farming that is scientifically informed and uses the full suite of modern and ecological tools to produce high yields with the lowest possible impact (Xie et al 2019).

Regenerative agriculture is one articulation of the sustainable intensification of agriculture that is becoming increasingly popular globally. This approach can be highly effective, and appears to be particularly good at capturing the interest of farmers today (LaCanne and Lundgren 2018).

These approaches to agriculture do not imply an endpoint in farm management, but they become an underlying ideology that should influence the overall reasons for using certain practices and the direction of long-term farm planning (Mahon et al 2018). Again, any success of implementing sustainable intensification programmes over large areas relies on intense cooperation between all parties (Grewer and Rodriguez 2019).

Another widely employed ‘sustainability’ orientated management strategy is integrated pest management (IPM). This approach to agricultural management aims to reduce the environmental impacts of agriculture by minimising pesticide use through improvements to the specificity and

timing of applications as well as by enhancing ecosystem services (Dara 2019; Baker et al 2020; Deguine et al 2021). However, IPM does not appear to be an adequate adjustment of the mode of agriculture production to avoid most environmental impacts, as well as falling short of sustainability targets (Deguine et al 2021). Whilst any improvement to agrochemical application is ultimately good in terms of reducing the environmental impact of that application, this concept fails to address the structural issues that may be leading to the need for the application in the first place. In comparison, regenerative agriculture approaches aim to build up natural resources and ecosystem services on farms to reduce agrichemical dependence over time (Pretty et al 2005; LaCanne and Lundgren 2018).

1.1.4 Legislation, research programmes and funding

Legislation is one way that sustainable agriculture can be fostered, and providing it is based around coherent sustainable goals, it can be effective (Niles et al 2000; Olesen and Bindi 2002). However, government policy is typically slow to respond to environmental issues in general (Eskander et al 2021). For many reasons the implementation of sustainability-focused legislation is mainly a reactive move and in itself is usually discontinuous (Cornell 2014). The will to implement sustainable practices and develop a sustainable mode of food production must be present in all parties and actors, along with a serious understanding of the reasons for such changes being important (Banik 2022).

Researchers often play a role in finding solutions, but too often this role is under tight time and financial restraints (McDonald et al 2019). The goal of international collaboration and assistance in this area must be to aid the development of biocontrol systems and fund the sustainability of local farmers and practitioners in carrying out this work (Pretty et al 2018; Dumont et al 2021).

There are also many government-funded services that can assist in the transition to sustainable farming practices. One example is government funded crop scouters that provide farmers with pest information and advice, along with gathering data about pest issues across growing regions to prepare responses to their impacts. Such a service is valuable in that it helps crop manager's decision-making rather than the imposition of punitive fines for incorrect pesticide use (Kusnandar et al 2019).

One such programme is the myGAP (my good agricultural practice) initiative in Malaysia which aims to inform and guide farmers towards more sustainable practice that improves the profitability and stability of production. A review of myGAP framework in Malaysia compared myGAP certified farmers from this programme to farmers that were uncertified. It was found that myGAP certified farmers used much less pesticide annually than uncertified farmers and were 32% more likely to adopt non-synthetic pest control measures (Amekawa et al 2017)

A positive approach by governments and other large bodies will help to build a culture of food production that thinks in terms of production into the future and the constant betterment of agricultural land as a key resource (Rose et al 2019). This will depend though on the types of programmes and initiatives chosen (Pineiro et al 2020). That said, it is unclear how cohesively all the large bodies from governments to agrichemical companies will be able to work together, or even agree on broad approaches and goals towards sustainable agriculture.

1.1.5 International organisations

There are intergovernmental groups that can fund and influence sustainable goals and the adoption of sustainable practices (Carmagnac and Carbone 2018). Funding and resource provision towards these goals is especially needed by developing countries where recognised inequalities in accessing all resources, often due to historic colonial modes of production, have led to less developed and often less sustainable agriculture practices. Developing sustainable agriculture globally requires investment beyond just reduced environmental impact; it is also required in education and access to all resources and freedoms that humans need (Altieri 2004; Carmagnac and Carbone 2018; Bopp et al 2019). Because issues like climate change are inherently global, the sustainable development of agriculture must be a global effort, with those that can always assisting those without. There is much disparity between capabilities and knowledge of sustainable practices around the world (Kaul et al 2022). This causes farmers in some countries to lack the information they need, and has led to a reduction of cultural and locally specific knowledge of farming (Ecobichon 2001; Erikson et al 2021).

International bodies should make it a core responsibility to foster sustainable practice through funding, education and advocacy (Carmagnac and Carbone 2018). It is their responsibility to promote and assist in the implementation of sustainable practice due to their financial resources, industry standing and ability to have a wide impact (Pretty et al 2020). The more communication and transparency there is between parties in their goals and motives, the smoother the development of sustainable practices will be (Piso et al 2016; Baker et al 2020).

One example of this is the UN Sustainable development Goals (SDGs) which were adopted by all UN members in 2015. The UN SDGs are described as a 'shared blueprint' of 17 goals for sustainable global development (UN 2023a). Whilst these goals cover core aspects of prosperity such as elimination of poverty, elimination of hunger and universal access to education, the 2023 UN report on SDGs found progress towards all 17 goals to be moderately to severely off track (UN 2023b).

1.1.6 Conveying these messages to farmers and relevant communities

There is some discontent among parties of sustainability movements, especially within agriculture. This leads to resistance to sustainable rhetoric in general. Thus, it is important to frame the development of sustainable agriculture as the improvement of farms and protection of the land resource for future generations.

A review of sustainable agriculture messaging has found that to be successful. Messaging from large bodies should be transparent in that sustainable practices do not always equate to reducing fossil fuel use/carbon emissions (Seddon et al 2021). There must also be cognisance of cultural and ecological rights of indigenous peoples and local communities. It should be clear what the benefits to ecosystems and people are. Messaging that fails to regard these points tends to fail in its aims due to poor reception and audience comprehension or disinterest (Seddon et al 2021).

1.1.7 Sustainable agricultural practice and pesticide use

Generally, the increased use of pesticides led to an increased return on investment until c. 2000s, but since then the cost/benefit ratio of increased pesticide use has consistently decreased (Popp et al 2013; Delcour et al 2015; Zhang et al 2018; Beltran-Pena et al 2020).

One way pesticides can limit yield is by causing mortality of insect pollinators. In bee pollination dependent crops, pollination success can be an equally or even a more important yield determining factor than insecticide application rate. Furthermore, enhanced bee pollination often provides better returns on investment than increased pesticide use for farmers (Catarino et al 2019). Farmers recognise this and consider pollination services to crops when planning insecticide applications. This often means that farmers use products that are less harmful to pollinators and apply them at times when pollinators are not likely to be active (Kambrekar 2020).

The use of agri-applications can also be harmful to biocontrol agents and thus reduce their effectiveness (LaCanne and Lundgren 2018). The impact of pesticides on biocontrol agents is so strong in fact, that pesticide use often significantly reduces the potential benefit of other agro-ecological practices (Stark et al 2007).

A study in Australia investigated predation rates of cotton bollworm egg-baits in 53 fields with semi natural field margins under insecticide and no insecticide regimes over two years. Within fields sprayed with insecticide there was no detectable benefit from semi natural field margins, whereas the opposite was true for unsprayed fields (Gagic et al 2019). The reduction of agrichemical applications is essential to enhancing biocontrol to a point where it can be employed as a major

component of crop protection strategy (Wratten et al 2013; Gagic et al 2019; Gonzales-Chang et al 2021).

1.1.8 Pest invasions and mitigating their impacts

Pest invasions of new localities remain poorly understood and almost never prepared for, even though they are increasingly common (Venette and Hutchinson 2020). It is impossible to predict pest invasions and outbreaks, but it is possible to develop agroecosystems that are more resilient and resistant to pest outbreaks regardless of the invading pest species (Holt-Gimenez et al 2002; LaCanne and Lundgren 2018). Governments and industry bodies are funding pest monitoring and containment strategies to help ensure responses are as effective as possible (Venette and Hutchinson 2020).

Although still a very new practice, important biocontrol agents, provided they pass safety testing, can be established in advance of an invasion, or at least a culture can be established with correct documentation for its release. This gives nations the economic benefit from immediate responses to pest incursions such that the cost of maintenance and importation of biocontrol agents is quickly justified (Caron et al 2021).

An example of this is the pre-emptive culturing of *Trissolcus japonicus* (Ashmead 1904) (Hymenoptera: Scelionidae) for release in New Zealand in the event that the brown marmorated stinkbug (*Halyomorpha halys* (Hal 1855) Hemiptera: Pentatomidae) establishes. This pest has had serious economic impacts in other countries and has been identified as having a high potential for economic damage in New Zealand, along with it having been intercepted at the border numerous times in recent years (Caron et al 2021).

T. japonicus was assessed for host range specificity and potential to establish as a parasitoid of native Pentatomidae species. It was found to parasitise seven out of the nine tested Pentatomids but it also retained a host preference for brown marmorated stinkbug and the habitats it occurs in (Charles et al 2019). Because of this and the low likelihood of substantial *T. japonicus* populations being able to establish in the habitats where native Pentatomidae occur, this application was approved. However, it has been stipulated that *T. japonicus* must remain under strict quarantine and is only to be released in a controlled manner in response to the establishment of the brown marmorated stinkbug in New Zealand (Caron et al 2021).

1.1.9 Improving biocontrol science

Most attempts at the introduction of insect biocontrol agents of insect pests have been failures, with an average global success rate in establishment of around 30% and a satisfactory control rate being achieved only in 10% of attempts (Cock et al 2016). Some of the causes for failure have been overcome, but new challenges to biocontrol programmes constantly emerge. However, over time there has been a reduction in the attempts at introductions but an increase in the rate of all measures of success (Cock et al 2016).

Historically one issue has been the approximate nature of biocontrol programmes. Often a selection of biocontrol agents were sent to a destination, often many died during transportation, then attempts at rearing were made, and those agents that emerged were released into the field. Today these simplistic approaches still exist, often in countries with less funding, but there now are modern and more effective methods (Cameron et al 1993; Cock et al 2016). These more modern approaches incorporate a range of technologies and researchers from relevant fields with the aim of maximising the programmes chance of success (Brodeur et al 2018; Schulz et al 2021).

An example of a modern biocontrol programme has been the introduction of *Tamarixia radiata* (Waterson 1922) (Hymenoptera: Eulophidae) against the Asian citrus psyllid (*Diphorina citri* (Kuwayama 1908) (Hemiptera: Liviidae) in California. Pakistan was identified as an area within the psyllid's native range that was a reasonable environmental match for Californian citrus orchards (Hoddle 2021). To increase the chance of success great care was taken when selecting parasitoids from populations that would have genetics that favoured them in California on Californian citrus types, as well as on the genetic isolates of the citrus psyllid populations present there (Milosavljevic et al 2019).

Once the *T. radiata* lineages had been imported to the US, intensive breeding began in California. Specific genetic lines were developed and constantly crossbred to reduce the chance of the captive populations evolving laboratory fitness. Finally, care was taken in the release of parasitoids to ensure that the local climate and host provisions were suitable (Milosavljevic 2018).

During the field assessment phase of the programme, it was noticed that introduced ant species were causing significant interference with *T. radiata*, and so further work was conducted to control these pest ant species (Kistner et al 2017).

This type of thorough informed approach to the introduction of biocontrol agents enhances the effectiveness and chance of success of biocontrol agents and is crucial to developing the level of effective biocontrol needed to aid farmers.

1.1.10 Conclusions

Sustainability initiatives and aims of better practice cannot be applied in a top-down manner. It is important that government legislation, funding and subsidies are themselves always adapted and informed by all relevant parties. Thus, implementation change must be driven by farmers and aided by wider resources instead of vice versa (Barratt et al 2018; Melchoir and Newig 2021).

Biocontrol can be very effective and provides enormous levels of economic benefit to agriculture and other aspects of life (Cullen et al 2008). The total value of ecosystem services and non-productive habitat cannot be put into economic terms because their value is absolute, meaning these resources and processes are essential to supporting all life on earth. However, there is most definitely relevance in highlighting cost savings/enhanced profitability of biological provisions when compared to their agrichemical counterparts.

There is no reason biocontrol cannot be developed into a major aspect of agricultural pest control in the future. Providing this type of research is funded and real sustainable change occurs within agricultural landscapes, biocontrol will be a viable and enhanceable resource for farmers to draw pest control from and further alleviate pesticide use (Cullen et al 2008; Lundgren and Fausti 2015; Wratten et al 2018).

Therefore, the development and implementation of biocontrol is essential to any form of sustainable agricultural production (Cock et al 2016; Pretty 2018; Araj et al 2019). As this literature review has clearly demonstrated, the development of sustainable agriculture is critical to a sustainable global system. These sustainable agriculture systems must use technology and science to improve management practices as well as incorporating a major ecological component that carries through to the philosophy and long-term planning of agriculture (Lundgren and Fausti 2015; LaCanne and Lundgren 2018)

1.2 Literature review: Biocontrol in New Zealand

New Zealand is a world leader in biocontrol, and it has always engaged in a high level of novel programmes, which has affected success rates (Cameron et al 1993). The motives for pursuing biocontrol agents and the methods of implementation do differ between each agricultural sector but overall, the progress of biocontrol in New Zealand resulted from a cohesive effort partaken by all relevant parties (Barratt 2009).

1.2.1 The history of biocontrol in New Zealand

From 1870-1990, 375 importations of 341 insect species into New Zealand were carried out for the purpose of biocontrol. Of these species, 242 were released and 75 successfully established (Cameron et al 1993). Efforts in New Zealand over this time were less successful compared to those globally, with an establishment rate of around 30% for imported biocontrol agents to New Zealand compared to a global average of around 40% (this may have been affected by the high degree of novelty in New Zealand biocontrol programmes) (Cameron et al 1993).

During the 1900s the New Zealand Department of Scientific and Industrial Research (DSIR) (active 1926-1992) and the Ministry of Agriculture and Forestry (MAF) conducted most biocontrol science. The work carried out during this time has had many long-term positive impacts on pest issues and in turn, the economy (Longworth 1987).

1.2.2 Refining biocontrol in New Zealand

Some major developments in biocontrol programmes during the 1990s were host range testing, climate matching, genetic testing of biocontrol agents, improvements to release programmes and shipment methods (Cameron et al 1993).

The first shifts towards modern biocontrol in New Zealand occurred during this time, with a major trend being the move from generalist to specialist biocontrol agents; 80% of biocontrol agents imported before 1940 were generalist predators whereas by the 1990s 70% of imported agents were specialist parasitoids (Longworth 1987; Cameron et al 1993; Barratt et al 2010).

1.2.3 Pre-release screening of biocontrol agent host range

An example of a biocontrol programme that was not concerned with host specificity was the 1930s releases of the parasitoids, *Pteromalus puparum* (L.) Hymenoptera: Pteromalidae) and *Cotesia glomerata* (L.) Hymenoptera: Braconidae), for the control of the cabbage white butterfly (*Pieris rapae* (L. 1758) Lepidoptera: Pieridae) in brassica crops. The lack of host specificity in both

parasitoids was known but not considered an issue. Neither of these parasitoids were successful in their target pest suppression and quickly began attacking a wide range of insects across ecosystems. A particular issue with *P. puparum* is that it often competes with other parasitoids for hosts, and because it has a large brood of small internal parasites it readily outcompetes single-egg laying parasitoids.

The 1939 biocontrol programme for the diamondback moth (*Plutella xylostella* (L. 1758) Lepidoptera: Plutellidae) was the first programme to employ pre-release studies in New Zealand. From this point on it became more common to conduct some prior investigation and seek ideal biocontrol agents that would not have unintended consequences (Muggeridge 1943; Cameron et al 1993).

In 1984, it became a legal requirement to screen imported individuals for diseases and parasites before release (Longworth 1987). It is now also a requirement that a prospective biocontrol agent be screened to assess its potential to negatively affect native species. Taken into consideration are how the biocontrol agent develops on native hosts, the relative fitness of these offspring and if the biocontrol agent is likely to encounter these native species in native ecosystems (Longworth 1987).

1.2.4 Climate and biocontrol programme quality

Some attention has always been given to the local climate where a biocontrol agent will be introduced, and attempts have frequently been made to source biocontrol agents from a suitable native climate. However, during the early 1900s introductions of biocontrol agents usually featured species that were readily available. Often for New Zealand, this meant biocontrol agents operating on the same crops in Australia, which affected establishment rates (Cameron et al 1993). Climate matching is a particularly challenging aspect for biocontrol programmes in New Zealand due to the often-localised conditions across the extended altitudes, latitudes and environments where pest problems occur.

The quality of a biocontrol release can be an important factor in determining the chances of the species successful establishment. While it is true that releases of a small number of individuals with limited genetic diversity can be completely successful, analysis of release attempts in New Zealand shows that biocontrol agent releases of over 1000 individuals have a 70% success rate compared to less than 30% for releases of less than 1000 individuals (Cameron et al 1993).

The duration and intensity of an importation and release programme is also important. Biocontrol programmes that lasted for two or more seasons and had four or more importations with at least one release containing around 1000 individuals of one species, had a success rate of 67% compared to 58% for smaller programmes (Cameron et al 1993).

Over time, there was an increase in the quality and duration of biocontrol agent release programmes in New Zealand. This was both due to refinement of the science and the intensification of oversight ensuring that programmes were more thoroughly executed.

1.2.5 Government restructuring

In the early 1990s, the DSIR along with other government departments were disestablished and replaced by seven Crown owned Research Institutes with the stated goal of 'carrying out scientific research for the benefit of New Zealand'. The two major differences between the DSIR and the new Crown Research institutes were that the new institutes were smaller, meaning they were given more specific portfolio briefs, and the institutes had to be financially viable operating along commercial lines (Davenport and Bibby 2007).

This has impacted many aspects of how science is conducted in New Zealand; it has led to the conduct of programmes of more limited scope due to funding constraints, competition and the demands of commercial funders (Davenport and Bibby 2007).

Other major changes to how biocontrol science operates in New Zealand over this time included the adoption of the Hazardous Substances and the New Organisms legislation act 1996 (HSNO act 1996), and the formation of the Environmental Protection Agency of New Zealand (EPA) in 2011 which oversees all importations of new Organisms under the HSNO act 1996 (Gerard and Barratt 2021).

One implication of the HSNO act of 1996 is that any organism not present or recorded as present in New Zealand before 1998 is considered a new organism and so approval from the EPA must be acquired for the importation, field testing or rearing of any new species. This has developed into a rigorous process of justification for importations and pre-release testing (Barratt et al 2000).

This framework requires the applicants to meet a range of conditions and ensures that establishing the new organism is of little risk to native organisms, the environment, public health, societal goods or other aspects of cultural significance to Māori (Barratt et al 2010).

Due to the importance of biocontrol for food production and the meeting of sustainability goals, it has been essential that this process is stringent in its assessment yet accommodating of the need for exploration of new biocontrol opportunities (Barratt and Moeed 2005). Based on the process

outlined above, prospective biocontrol agents are assessed qualitatively, and the EPA must decline any agent where the perceived cost outweighs the perceived benefit. However, it is always clearly articulated why certain agents pass or fail this testing and the EPA is constantly working to improve its process based on domestic and international research (Ehlers et al 2020).

1.2.6 Post release assessments

There have been in-depth studies conducted on biocontrol agents following their release in New Zealand (Shields et al 2022a). This type of information can help researchers choose optimum times, locations, densities and frequencies of biocontrol agent releases to increase the chance of successful outcomes (Ehlers et al 2020). While there is some very thorough research in this area, much more is needed (Goldson et al 2015).

1.2.7 Issues with the rearing and release of biocontrol agents

The rearing of biocontrol agents often poses challenges, and frequently the quality of releases is severely reduced due to poor rearing outcomes. It is unclear exactly what factors of rearing affect establishment success, but it is clear that releases of a high number of healthy and genetically diverse individuals have a greater chance of success (Cameron et al 1993; Shea and Possingham 2001; Paynter et al 2016). However, it should be noted that biocontrol agents imported into New Zealand for the control of weeds that had been successful elsewhere in the world were also 100% successful in their establishment in New Zealand, regardless of the quality of the release (Fowler et al 2010).

This shows that biocontrol agents that can successfully establish and persist in modified landscapes are sometimes able to do so in all modified landscapes with suitable conditions (Goldson et al 2020). However, those species that have not already been selected through these programmes are of unknown suitability to modified landscapes outside of their native range (Julien et al 2007; Paynter et al 2016).

These difficulties of biocontrol agent establishment are exemplified by the potato tuber moth (*Phthorimaea operculella* (Zeller 1873) Lepidoptera: Gelechiidae) parasitoid programme, where the importation of 580 000 individuals of 17 species in 60 consignments over 22 years eventually resulted in the establishment of one species, *Apanteles subandinus* (Blanchard 1947) (Hymenoptera: Braconidae) (Cameron et al 1993).

The persistence of researchers with introductions of different strains of a species can also be essential to achieving establishment after initial failures, as was the case with *Aphidius ervi* (Haliday 1834) (Hymenoptera: Braconidae) where widespread establishment was achieved only after the

release of five strains over five years (Cameron and Walker 1988). The level of prior research and understanding of biocontrol was clearly insufficient at the time of the first releases to achieve the desired goals in many cases. However, there still remains a lack of in-depth genetic science and application of advanced technologies (Hoddle 2006).

1.2.8 Examples of biocontrol programmes conducted in New Zealand

The following section will explore some examples of insect biocontrol programmes as undertaken by various sectors of the New Zealand primary industry. In general, the success of a biocontrol programme will be deemed as the rate of successful establishment and at least some level of desired function. Failure of a biocontrol programme can also be explored for its root causes and amended with appropriate research (Debach 1964; Cameron et al 1993).

1.2.9 Orchards and the development of a world leading IPM system

Within the orchard industry in New Zealand there has long been effort applied to developing IPM growing strategies (Collyer and van Geldermalsen 1974). This has led to the importation of numerous biocontrol agents that provide pest control services of varying degrees, which have allowed growers to reduce pesticide use over time (Wearing et al 1993; Walker et al 2017). This has been possible due to factors such as the highly centralised structure of the New Zealand horticulture industry, grower willingness to participate, clear articulation of the need to reduce pesticide use and thorough science underpinning alternative management systems (Suckling et al 2004).

The following section will discuss some of the important insect biocontrol agents incorporated into orchard IPM systems. Some of these species have been purposefully imported during the development of the IPM programme and others were already present in New Zealand and benefited from these changes to management practices.

Orchards are very long term/permanent, which is beneficial to the establishment of reliable biocontrol programmes due to the more stable land cover they represent. Whereas crops are in the ground for one-three years and move around the landscape, with phases between crops and during crop establishment providing few resources to maintain biocontrol agents.

1.2.10 Woolly apple aphid

Aphelinus mali (Haldenman 1851) (Hymenoptera: Aphelinidae) was introduced from multiple collections in the USA and released in New Zealand from 1921-1924 for the biocontrol of the woolly apple aphid (*Eriosoma lanigerum* (Hausmann 1802) Hemiptera: Aphididae) in orchards. It quickly became established and widespread (Cottier 1935; DSIR files, Auckland, in Shaw and Walker 1996). Initially *A. mali* successfully controlled the woolly apple aphid but pressures from this pest increased

over time in commercial orchards sprayed with broad spectrum insecticides and it had become an issue again by the 1960s (Collyer and van Geldermalsen 1975; Walker et al 1989; Wearing et al 2010).

With the adoption of IPM practices in orchard production, *A. mali* once again provides substantial biocontrol services. Studies in the 1990s found that after four years of IPM spray practices (in particular an avoidance of broad-spectrum insecticides) *A. mali* was able to regulate the woolly apple aphid below damaging levels in orchards (Shaw and Walker 1996).

1.2.11 Mealy Bugs

Mealy bugs (Hemiptera: Pseudococcidae) of various species have always been pests of concern in New Zealand orchards (Lo et al 2021). The first biocontrol programme against mealy bugs in New Zealand was against the polyphagous Australian cottony cushion scale (*Icerya purchasi* (Maskell 1878) Hemiptera: Pseudococcidae) in 1888 with the introduction of a species of parasitoid fly (*Cryptochaetum iceryae* (Williston 1888) Diptera: Cryptochetidae) from its native range (Cameron et al 1993). Over the following decades, further predator and parasitoid biocontrol agents were released in continued attempts to control the cottony cushion scale. Today it is partially controlled by these biocontrol agents, but insecticide sprays are still a major part of this pest's management strategy (Cameron et al 1993).

Subsequent research has found three main species of mealy bug, *Pseudococcus longispinus*, *P. calceolariae* and *P. affinis* make up 99% of sampled individuals in New Zealand pip fruit orchards. These mealybug species were found to be attacked by 14 species of natural enemy, only two of which had been deliberately introduced into New Zealand specifically for their control (Charles et al 2008). Under normal conditions these biocontrol agents provide substantial control of mealybugs, but there can still be localised outbreaks which could be prevented with further introductions of new biocontrol agents or inundative releases of biocontrol agents that are already present (Charles et al 2008).

1.2.12 Codling moth

One of the first biocontrol agents approved for release within New Zealand by the EPA was the parasitoid wasp *Mastrus ridens* (Horstmann 2009) (Hymenoptera: Ichneumonidae) imported from Argentina for control of the codling moth (*Cydia pomonella* (L. 1758) Lepidoptera: Tortricidae) in pip fruit orchards and adjacent land (Sandanayaka et al 2012).

The justification for the release of this parasitoid was based on its promising ability to support the IPM system being developed in New Zealand pip fruit orchards. This IPM system is very effective against the codling moth locally, but the codling moth is highly abundant outside of orchards,

meaning constant reinvasion is a serious issue for growers during the growing season (Charles et al 2019a).

The potential for *M. ridens* as a suitable biocontrol agent of the codling moth was inferred from a successful release programme in California and associated pre-importation native range host research conducted in New Zealand (Charles and Sandanayaka 2013).

Due to an intensive release programme running from 2011-2015, *M. ridens* has been the most successful parasitoid of codling moth in New Zealand to date. Research into the establishment and parasitism rates of *M. ridens* from 2012-2017 showed maximum parasitism rates of 40-80% in all growing regions (Waikato, Gisborne, Hawke's Bay, Nelson and Central Otago) (Charles et al 2019b). Variation in parasitism rates by *M. ridens* may have in part been due to competition by other parasitoid species, but often it is also due to other unknown factors. The minimum rate for parasitism at release sites with *M. ridens* present was 0% (Charles et al 2019b). *M. ridens* presence has only achieved partial success in the suppression of codling moth and reduction in other management requirements across New Zealand (Charles et al 2019b). This work highlights the need for more in-depth research into agroecosystems and interactions between biocontrol agents in New Zealand.

1.2.13 Some pasture and crop pests and their biocontrol programmes in New Zealand

Programmes in this section cover biocontrol agents that control pests of productive plant species grown as pasture, arable and vegetable crops as well as seed crops. Whilst usually there was a main industry sector behind each biocontrol agent's introduction, the benefits of pest control are experienced across all types of farming that have crop or pest species in common.

1.2.14 Cereal aphids

Aphid parasitoids, largely of the *Aphidius* genus (Hymenoptera: Braconidae), are one of the most longstanding and frequently introduced types of biocontrol agent worldwide. There are many cases of success in the establishment of aphid parasitoids in crops and pastures in New Zealand.

In more recent decades there have been extensive host range testing and parasitoid surveys which have established that exotic aphid parasitoids (including those released before host range testing was mandated) pose little to no threat to native aphid species (Cameron et al 2013; Bulman et al 2021).

The following section explores some cases of aphid biocontrol by parasitoids in New Zealand crops and pastures.

Aphidius eadyi (Stary 1980 Hymenoptera: Braconidae) was introduced into New Zealand for the biocontrol of the pea aphid (*Acyrtosiphon pisum* (Harris 1776) Hemiptera: Aphididae) on lucerne (*Medicago sativa* L.). From 1977 to 1981, specimens were released across New Zealand from various shipments of a Moroccan strain that was widespread in California. It had become established in all lucerne growing areas by 1987, and quickly achieved parasitism rates of 30-40% which were associated with declining pea aphid populations, making it a successful release programme (Cameron and Walker 1989). Further research has shown it also contributes to the biocontrol of the blue green lucerne aphid (Cameron et al 1993).

Aphidius ervi (Haliday 1834) (Hymenoptera: Braconidae) was released for the biocontrol of the blue green lucerne aphid (*Acyrtosiphon kondoi* Shinji 1938) and the pea aphid. Via California, strains from Australia and the UK were released into New Zealand from 1977 to 1981. *A. ervi* provides partial control services against these pests in a range of crops including the target crop lucerne (Cameron et al 1993).

Aphidius rhopalosiphi (de Stefani-Perez 1902) (Hymenoptera: Braconidae) was released for the biocontrol of the rose-grain aphid (*Metopolophium dirhodum* (Walker 1849) Hemiptera: Aphididae) on cereals. It was introduced from England and France and released from 1985 to 1987. This parasitoid was frequently recovered from most cereal-growing areas in subsequent establishment surveys during the 1987 growing season (Stufkens and Farrell 1989). This programme was considered to be successful in its aims. Grundy (1990) estimated that *A. rhopalosiphi* provided annual benefits of between NZ\$0.3 and \$5 million per year. *A. rhopalosiphi* also readily parasitises another cereal aphid pest found in New Zealand, *Rhopalosiphum padi* (L. 1758) (Hemiptera: Aphididae) (Cameron et al 1993).

1.2.15 Weevils in pastures and crops

Three weevil pests (*Sitona discoideus* (Gyllenhal 1834), *Sitona obsoletus* (Gmelin 1790) and *Listronotus bonariensis* (Kuschel 1955) (Coleoptera: Curculionidae)) have been highly damaging forage pests in New Zealand, whilst causing very few issues within their respective native ranges (Goldson et al 2020). All three species have been subject to very successful biocontrol programmes that were based on host native range searches for natural enemies and pre-release testing. The outcomes of these programmes provide interesting insights into the issues associated with farming systems and biocontrol within New Zealand. The review by Goldson et al. (2020) will be used here to explore these unique weevil pest issues in New Zealand.

Listronotus bonariensis: it is unclear when this pest established in New Zealand (O et al 2022), but it established readily and extensively in pasture systems causing major damage to the grasses it feeds

on. Prior to the establishment of biocontrol, *L. bonariensis* reached densities of up to 720 larvae/m² and caused NZD \$200m worth of damage annually (Ferguson et al 2019). These larval densities are much higher than those reached in its native ecosystems in South America where it poses no serious threat to pastures. Its parasitoid, *Microtonus hyperodae* (Loan (Hymenoptera: Braconidae)) was imported from South America where local parasitism rates were usually low at around 5%. In New Zealand, it was very successful in the suppression of the target pest species, until after around 14 generations, damage by *Listronotus bonariensis* was noted to be increasing (Popay et al 2011). Investigations into the causes found a world first case of a target pest evolving resistance to an imported biocontrol agent. It was determined this was due to the weevil being able to quickly evolve enhanced parasitoid avoidance behaviours that the asexual parasitoid has not been able to respond to (Tomasetto et al 2017; Shields et al 2022a; Shields et al 2022b).

Before the release of *M. hyperodae* for the control of the Argentine stem weevil, this parasitoid underwent rigorous host range testing by AgResearch. It was found to be able to parasitise a few native weevil species as a host, contrary to it only being known as an obligate parasitoid. However, the relative fitness of the offspring when developed in native species tended to be lower, and it was very unlikely that *M. hyperodae* would encounter these weevils from cooler environments in the wild (Goldson et al 1997).

Sitona obsoletus: this weevil was first identified in the North Island of New Zealand in 1994 and rapidly spread, causing extensive losses to clover productivity. Soon after establishment it reached densities of 1291-1400 larvae/m², far higher than in its native range of the UK where it reaches densities of 30 larvae/m². The larval density and survival rate of *S. obseletus* increased linearly with available white clover root nodules. This, along with the lack of competition and predators that can access the root feeding, are proposed as mechanisms for it being able to rapidly reach high larval densities. It caused at least NZ\$650 million of economic damage annually to New Zealand clover production (Goldson et al 2020).

In 2006 an Irish strain of the parasitoid *Microtonus aethiopoides* (Loan) (Hymenoptera: Braconidae) was imported into New Zealand and it quickly suppressed *S. obsoletus* below damaging levels. In Ireland, parasitism rates never exceeded 8% but it proved to be very effective in New Zealand where it prevents NZ\$338-431 million of economic damage annually (Basse et al 2015).

Sitona discoideus: First detected in Hawkes Bay in 1974, it quickly spread to any areas where lucerne was cultivated. Similar to *Sitona obsoletus*, this weevil found little to no competition or control pressures within its root nodule niche and reached extremely high densities of 5000 larvae/m² (Goldson et al 1988). Dry matter yield reductions of 46% were recorded in lighter free-draining soils

with low available nitrogen. It is likely that the reduced density of root nodules in areas with higher soil available nitrogen limited larval densities of *Sitona discoideus* (Goldson et al 1985)

A strain of *Microtonus aethiopoides* was collected from Morocco. This is the only parasitoid of the three introduced weevil species for which a sexually reproducing population could be located. In Morocco the parasitism rate was never found to exceed 31%, however, following its establishment, it has been completely successful in the suppression of the target pest species in New Zealand (Barlow and Goldson 1993).

1.2.16 Biocontrol on other crops in New Zealand

There is a long history of biocontrol programmes in crops in New Zealand. Initially this largely centred on introducing internationally well-known biocontrol agents of serious lepidopteran pests.

In tomato and brassica crops, the establishment of parasitoids of the cotton bollworm, cabbage white butterfly and diamondback moth necessitated the development of management practices that have decreased the reliance on insecticidal pest control (Cameron et al 1993).

1.2.17 Cabbage white butterfly

Continual crop damage from the cabbage white butterfly led to the introduction of a further parasitoid species to improve biocontrol efficacy. In 1993 *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae), was introduced from Adelaide, South Australia (Cameron et al 1995). It was released in nine regions and quickly established in eight of these, ranging from Northland to Southland. Since then, the average geographic spread of *C. rubecula* has averaged 2 km per year (Cameron and Walker 2002).

Post establishment surveys have recorded parasitism rates of the cabbage white butterfly by *C. rubecula*, of between 48 and 97%, with the lower rates tending to be sampled within commercial crops. Overall, this programme was partially successful in improving parasitism rates of the cabbage white butterfly. In addition, this work also found that *P. puparum* interfered with *C. rubecula*, which may have affected biocontrol outcomes (Cameron and Walker 2002).

1.2.18 Northern armyworm

The northern armyworm (*Mythimna separata* (Walker 1865) (Lepidoptera: Noctuidae) has continued to be a major pest of maize crops in New Zealand despite numerous partially successful biocontrol programmes carried out against it. One of the most effective biocontrol agents introduced against this pest is the larval parasitoid *Apanteles ruficus* (Haliday) (Hymenoptera: Braconidae).

This parasitoid was introduced from Pakistan in the early 1970s and following its establishment across New Zealand there has been an increase in average parasitism of the northern armyworm from 50-55% to around 80% (Longworth 1987). *A. ruficrus* is considered to have been successful in reducing the level of armyworm damage in crops, due to its added level of parasitism to the pre-existing biocontrol assemblage (Hill 1988). Overall, this programme has been successful in its aim to reduce the reliance maize growers have on insecticide sprays to control this pest.

1.2.19 Tomato potato psyllid

In 2016 the EPA approved the importation and release of *Tamarixia triozae* (Burks 1943) (Hymenoptera: Eulophidae) into New Zealand, as part of an effort to provide growers with sustainable tools to control the tomato potato psyllid (*Bactericera cockerelli* (Sulc 1909) Hemiptera: Triozidae). In 2017, following microbe screening and genetic assessment 2000 individual wasps were dispersed to Plant and Food Research Ltd and Bioforce Ltd rearing facilities for multiplication and release (Davidson et al 2018).

Subsequent establishment surveys found *T. triozae* to be a very poor disperser and it generally has not established more than a few tens of meters from its release sites (Davidson et al 2018).

It is also noted that the field parasitism rate is low, especially in the South Island. This difference is likely due to the colder South Island temperatures slowing development of *T. triozae*. Currently this programme has only been partially successful, but there is research being conducted on its potential as a greenhouse augmentatively released biocontrol agent. Within glasshouses experiments have shown *T. triozae* to be a partially successful biocontrol agent of tomato potato psyllid providing it is supplied with alternative food sources such as buckwheat flowers (Veronesi et al 2020; Veronesi et al 2022).

1.3 Literature review of Issues relating to red clover seed crop management in New Zealand

Seed production makes up a small proportion of the total agriculture any country engages in.

Because of this, there is less economic incentive to invest in biocontrol programmes specific to seed production. Biocontrol programmes are more likely to be funded by other agricultural sectors facing pest issues. Furthermore, it is very uncommon for a pest to only be an issue within seed crops, which also decreases the likelihood of seed production specific biocontrol being undertaken.

What this means is that biocontrol programmes against insect seed pests specifically within seed crops are rare, because most biocontrol work is carried out on major forms of production and control within seed crops is a secondary benefit.

The only successful New Zealand introduction of insect agents specifically for the biocontrol of a seed pest is that for white clover casebearers. The following section will explore seed production in New Zealand with a focus on clover and the biology of *Coleophora* spp. (Lepidoptera: Coleophoridae) species within seed crops. This will lay out the groundwork for exploring insect biocontrol of insects in seed crops, specifically for *Coleophora* spp. that attack clover seed crops (Cameron et al 1993).

1.3.1 Herbage seed production in New Zealand

Overall, four species; perennial ryegrass (*Lolium perenne* (L.)), Italian ryegrass (*Lolium multiflorum* (Lam.)), cocksfoot (*Dactylis glomerata* (L.)) and white clover (*Trifolium repens* (L.)), account for more than 98% of the herbage seed grown in New Zealand (Pyke et al 2004). Red clover is the second most widely grown forage legume seed crop in New Zealand with 554 ha certified for seed production, although this is just a fraction of white clover seed production which has 6,637 ha certified for seed production (Hampton et al 2012). However, less economically important seed crops still matter as part of a wider system that can supply growers with the whole range of seeds they need.

Ninety-five percent of herbage seed production in New Zealand is certified, which ensures it is of consistently high genetic quality and fit for the international market (Pyke et al 2004; Chynoweth et al 2015). However, to continue to increase high quality seed production and supply markets into the future, the New Zealand seed industry must commit to meeting growers needs to ensure increased profitability (Hampton et al 2012). Part of this is a commitment to research that aims to benefit seed growers and address problems they are facing; examples of this are the development of endophytes and plant growth regulators that increase seed production (Lee et al 2012), as well as the pest management aims of this Study.

1.3.2 Red clover management

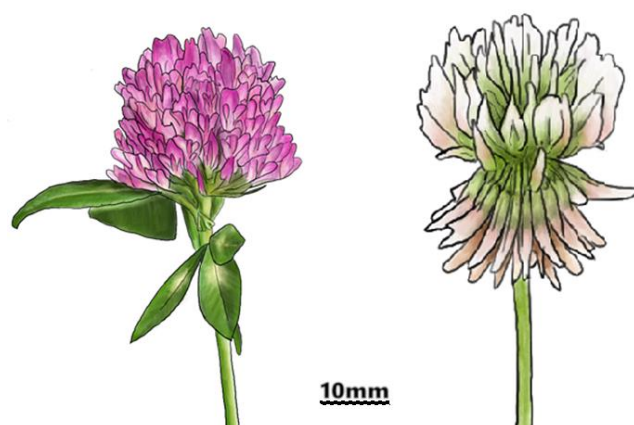


Figure 1.1: Comparison of a red clover (left) and white clover (right) flowerhead (Drawing by Katie McIvor).

Red clover seed crop management practices are often derived from those used for white clover, with adjustment for phenological differences between the crops. The major difference between red and white clover that affects management practices is that white clover is a creeping stoloniferous species, whereas red clover is an upright tap-rooted species (Black and Laidlaw 2009). This leads to different grazing/topping strategies and timing which, in turn, affect peak flowering times (Black and Laidlaw 2009). These different forms also lead to different types of crop habitat that may influence habitat suitability for certain invertebrate species. In addition, the flowerhead of red clover plants has very densely packed florets, compared the more open white clover flowerhead (Figure 1.1).

Insect pests impact seed crops by limiting seed yield or directly damaging the formed or developing seed (Lundin et al 2017). However, there has been little research on insect species specifically within red clover seed crops and how they might influence yields, or their economic importance, especially within New Zealand. Therefore, pest species and their potential for damage are usually inferred from extensive studies within white clover seed production systems (Hardwick and Barton 2019; Rolston pers. com. 2021).

1.3.3 Arthropods in red clover

There is a paucity of work analysing the pest and beneficial arthropod assemblages present within red clover in New Zealand, apart from one industry study (Hardwick and Barton 2019). Schroeder (1998) had previously recognised this as an area needing further work during his studies in white clover production systems in New Zealand.

As part of preliminary work associated with another commercial project funded by the Foundation for Arable Research, the author spent the summer of 2019/2020 surveying red clover seed crops in

the Methven area, New Zealand (-43°S, 171°E). Results from this survey allowed preliminary comparison with the earlier white clover based work by Schroeder and Clifford (1995). The following section outlines some important arthropods found in red clover crops in New Zealand.

1.3.4 Beneficial arthropod species present

There are a range of beneficial arthropod species present in red clover crops, many being generalist predators that attack a range of important crop pests. It is difficult to determine specific deliveries of biocontrol services by generalist predator species, but it is assumed that these predators provide some level of control services that complement other pest control methods.

The pacific damsel bug (*Nabis kinbergii* (Reuter 1872) Hemiptera: Nabidae) is a generalist predator. It emerges in spring but does not build up substantial populations until late summer/early autumn. *N. kinbergii* is known to feed on important red clover pests such as aphids and the Australian crop mirid (Siddique 1985). *N. kinbergii* uses ambush-hunting techniques and is a strong disperser, being found in most landscapes in New Zealand (Siddique 1985; Schroder 1998)

The Tasman brown lacewing (*Micromus tasmaniae* (Walker 1860) Neuroptera: Hemerbiidae) is a largely aphidophagous species of *Micromus* (Figure 1.2). It is native to New Zealand and very common in most settings including red clover crops (Hardwick and Barton 2019; preliminary surveys, 2020). Adults emerge in early spring and breeding is continuous throughout the season. Numbers build up steadily and because of its short life cycle, populations can quickly capitalise on new resource during summer. However, *M. tasmaniae* has a low-temperature threshold, which means late frosts can slow population growth (Islam and Chapman 2001).



Figure 1.2: Tasman brown lacewing larvae feeding on aphids within a red clover flowerhead.

The eleven-spotted ladybird (*Coccinella undecimpunctata* (L.) Coleoptera: Coccinellidae) is an introduced voracious European generalist predator (Fagan et al 2009; Cameron et al 2013). The females emerge and begin laying eggs in spring. There are at least two generations per year, with the first normally coinciding with spring aphid surges (Figure 1.3) (Fagan et al 2009). Other ladybird species are present but less common in red clover seed crops (Hardwick and Barton 2019). These include the two-spotted ladybird, the five-spotted ladybird, the seven-spotted ladybird, nine-spotted ladybird (Figure 1.4) and the harlequin ladybird, which often predate on other ladybird species.



Figure 1.3: Ladybird larvae on a red clover amongst red clover florets.



Figure 1.4: 9-spotted ladybird on a red clover plant.

The super-family Chalcidoidea (Hymenoptera), most commonly the family Eurytomidae, are present in red clover seed crops. This is a diverse super-family of wasps that includes parasitoids, predators and even plant or seed feeding pests (DiGiulio 1997). Another Chalcid family present is the

Pteromalidae, members of which are largely parasitoids, with some being recorded as important parasitoids of clover weevils in Europe (Lundin et al 2012; Faraone et al 2017).

Aphidius colemani (Viereck 1912) (Hymenoptera: Braconidae) is a very prevalent aphid specialist parasitoid whose host range includes the green peach aphid (*Myzus persicae*) which can carry various leaf mosaic and roller viruses (Grasswitz and Reese 1998; Khatri 2017). *A. colemani*'s hyper-parasitoid *Alloxysta victrix* (Westwood 1833) (Figitidae: Hymenoptera), is also common (Grasswitz and Reese 1998).

Other parasitoid wasps that were introduced to control pests on other crops also provide control services within red clover crops such as the clover root weevil parasitoid (*Microtonus* spp.) (Goldson et al 2020).

Hoverflies (Diptera: Syrphidae) can occur in high numbers across agricultural landscapes. The larvae are generalist predators that favour aphids, and the adults provide pollination services (Figure 1.5). The two most important Syrphid species in New Zealand agricultural landscapes are *Melanostoma fasciatum* (Macquart 1850) and *Melangyna novaezelandiae* (Macquart 185) (Syrphidae: Diptera) both of which are native (Wratten et al 1995).



Figure 1.5: Large hoverfly on a red clover flowerhead.

The European harvestman (*Phalangium opilio* (L.) Phalangidae: Opiliones) is a very effective generalist predator under the right conditions but often does not occur in fields under a heavy mechanical disturbance (Madsen et al 2004). *P. opilio* (Figure 1.6), is one of the four most common predators in white clover crops with a gut content containing an average of 70% aphid material (Schroeder and Clifford 1995).



Figure 1.6: European harvestman on a red clover plant.

Various species of spider (Araneae) from multiple genera are also common predator species within red clover seed crops in New Zealand. Araneae are important biological control agents, especially within a wider 'healthy' biological control system. In addition, honeybees and Bumblebees (*Bombus terrestris* (L.) (Hymenoptera: Apidae) are major pollinators of red clover seed crops, but longer tongued *Bombus* spp. (Figure 1.7), are the most effective pollinators of red clover flowers (McLachlan 2000; Madsen et al 2004).



Figure 1.7: Bumble bee visiting a red clover flowerhead.

1.3.5 Pest species present

There are multiple potential insect pests of red clover in New Zealand, although this is always dependent on local ecological conditions. The red clover casebearer, *Thrips*, aphids and mirid bugs are the most prevalent species present and are thought to be the most economically important (in the absence of other pest outbreaks) (Schroeder and Clifford 1995; Chynoweth et al 2018).

Various aphid spp. (including but not limited to, the blue green lucerne aphid, the pea aphid (*Acyrthosiphon pisum* (Harris)), the dock aphid (*Aulacorthum solani* (Kaltenbach)), the black bean colonising aphid (*Aphis craccivora* (Koch)) and the green peach aphid (*Myzus persicae* (Sulzer) (Aphididae: Hemiptera)), feed on red clover crops, and they tend to peak in abundance in late spring/early summer (Lowe 1974; Kelly pers. com. 2020).

The blue green lucerne aphid was first recorded in New Zealand in the 1970s (Cameron and Walker 1988). It is the most important aphid pest identified in white clover and is likely to have a similar potential for damage in red clover seed crops (Rolston pers. com. 2020). This aphid has a uniform distribution across fields and completes dispersal flights in autumn and spring (Cox and Dale 1977). Infestations tend to peak in late spring and ongoing population growth may be constrained by growing pressure from natural enemies as the season progresses (Schroeder and Clifford 1995).

Additionally, two spp. of *Thrips* (Thysanoptera) are known to feed on red clover (Figure 1.8); red clover thrips (*Haplothrips niger* (Osborn 1883) Thysanoptera: Phlaeothripidae) and occasionally onion thrips (*Thrips tabaci* (Lindeman 1889) Thysanoptera: Thripidae) (Foundation for Arable Research 2020; Kelly pers. com. 2020). These species of *Thrips* are of increasing concern and have a high potential to limit red clover seed yields if outbreaks occur (Rolston pers. com. 2020).



Figure 1.8: A *Thrips* spp. amongst red clover florets (Circled).

The Australian crop mirid (*Sidnia kinbergi* (Stal 1859) Hemiptera: Miridae) is a small polyphagous insect found in New Zealand and Australia. Although, it is the most common mirid pest in New Zealand, it is currently thought to be of minor importance, despite its abundance in red clover seed crops (Schroeder 1998; Foundation for Arable Research 2020). *S. kinbergi* (Figure 1.9), begins appearing in crops in mid-November and declines in numbers through March and April. Mirids are concentrated in the first 5m from the crop boundary (Schroeder 1998).



Figure 1.9: An Australian crop mirid on a red clove plant.

The potato mirid *Closterotomus norvegicus* (Gmeli 1790) (Hemiptera: Miridae) was first recorded in New Zealand in 1949 (Figure 1.10) (Eyles 1999). This European species occurs in high nymph densities within 5m of paddock margins from mid-November and slowly spreads deeper into the field as nymphs mature, but then become virtually absent in crops from mid-March (Schroeder and Clifford 1995; Eyles 1999). The preferred feeding site for *C. norvegicus* in white clover is the developing flowers that contain a rich concentration of sugars and nutrients (Schroeder and Clifford 1995). This pest was considered to have the potential to cause economic losses of up to NZ\$740 per ha in white clover (Schroeder and Clifford 1995).



Figure 1.10: A potato mirid on a red clover flowerhead.

The clover root weevil (*Sitona obsoletus*) can cause serious losses both of seed and vegetation because the adults feed on leaves and stems while the grub feeds on the plant roots and nodules. Red clover seed production benefits from the substantially successful biocontrol programme carried out against this pest in pastures (Barratt et al 1996).

1.3.6 *Coleophora* in New Zealand

Coleophora (Coleophoridae: Lepidoptera) is a large genus of ‘casebearing’ lepidopterans which makes up the bulk of the Coleophoridae family. They are typically herbivores of the leaves, seed and flowers of plants. Five species of this genus are present in New Zealand, three of which are pests of red and white clover (Pearson 1975) (Table 1.1).

Table 1.1: *Coleophora* spp. introduced into New Zealand.

Name	Hosts	New Zealand distribution of moth	Origin of the species
<i>Coleophora deuratella</i> (Lienig and Zeller 1846)	Red clover, zig zag clover (<i>Trifolium medium</i> L.)	Throughout New Zealand (Chynoweth et al 2018)	Europe and Western Asia (Chynoweth et al 2018)
<i>Coleophora alcyonipennella</i> (Kollar 1832)	White clover	Throughout New Zealand (Pearson 1989)	Eurasia (Pearson 1989)
<i>Coleophora mayrella</i> (Huber 1813)	White clover, Strawberry clover (<i>Trifolium fragiferum</i> L.)	Throughout New Zealand (Pearson 1989)	Eurasia (Pearson 1989)
<i>Coleophora versurella</i> (Zeller 1849) (Quinoa moth)	<i>Chenopodium</i> spp. (amaranth or fathen) and <i>Hieracium</i> spp.	Throughout New Zealand (Syrett and Smith 1998)	Eurasia (Frias et al 1996)
<i>Coleophora striatipennella</i> (Nylander 1848)	<i>Sterallia</i> spp. (Chickweed) and <i>Conium maculatum</i> L. (hemlock)	Throughout New Zealand (Hoare 2001)	Eurasia (Min-young and Parker 2009)

1.3.7 Introductions of *Coleophora* into New Zealand

A *Coleophora* spp. was first recorded in 1922 following the unexpected collection of *C. mayrella* in Governors Bay, near Christchurch by a local resident. The identity of this moth was not confirmed until 1926 when more specimens of the same species were collected from the grounds of the Canterbury Museum and sent to entomologist Stuart Lindsay (Lindsay 1927). At the time, Lindsay noted that this was probably a recent introduction and that the moth had the potential to become a pest of clover production; he doubted any of its natural enemies were present in New Zealand (Lindsay 1927). In 1930, this species was also recorded from Whangarei suggesting that it had since spread across New Zealand (Patterson 1930).

C. alcyonipennella was not identified as a separate species attacking clover crops in New Zealand until 1955, but based on mass flight observations made in earlier research it is very probable that this pest was present in New Zealand decades prior to this date. This is based on Hamilton (1944) describing activity that suggested the presence of both a univoltine and multivoltine species feeding on white clover seed crops in New Zealand (Hamilton 1944; Pearson 1975).

The third *Coleophora* pest species, the red clover casebearer (*C. deauratella*) was not detected in New Zealand until 2015 in Auckland. In the summer of 2016 outbreaks occurred across seed-producing regions in New Zealand and a subsequent nationwide pheromone survey detected this pest in all localities sampled (South Island: Invercargill-Canterbury-Marlborough; North Island: Wairarapa) (Chynoweth et al 2018).

These outbreaks persisted until 2018 before receding across New Zealand. Over this period, serious seed yield losses were recorded, especially in third and fourth year red clover stands (Chynoweth et al 2018). Significantly, similar patterns of seed yield loss were reported in Canada during red clover casebearer outbreaks (Mori et al 2014; Hardwick and Barton 2019).

1.3.8 Research into the white clover casebearer species in New Zealand

Pearson (1975) completed detailed work on populations of *C. mayrella* and *C. alcyonipennella* in Lincoln, Canterbury, New Zealand. His aims were firstly, to examine the feasibility of conducting population dynamics studies on *C. mayrella* and *C. alcyonipennella*, and secondly, to devise collection and extraction methods for these studies.

Species within this genus feed on closely related plants and those feeding on clover are particularly difficult to discern as they share close taxonomic affinity and evolutionary pressures. The species require dissection to be differentiated and they share many similarities ecologically and phenologically. This has presented on-going challenges in linking field observational studies to specific species and accordingly much of this work had to be carried out within laboratories (Pearson 1975).

In Canada collected specimens of red clover casebearer attacking red clover crops were misidentified as a *C. mayrella* for 30 years, due to their very similar morphology (Evenden et al 2010).

Pearson (1975) attempted to sample overwintering population numbers, but this again proved impossible due to the range of locations the larvae overwintered in, and the distance they crawled to find such sites. Various traps were also used to try to assess the emerging adult populations in spring, but again, all proved unsuccessful (Pearson 1975).

The mobility of the fourth instar larvae also means that a substantial proportion of the population is not in the flowerheads at any given point in time, making absolute population assessment impossible. In general, in-depth investigations into these pests found many difficulties associated with such cryptic and usually concealed habits (Pearson 1975).

Importantly however, this work did produce results on the timing of population sampling. Due to the rapid development of these moths, a specific sampling interval of one week allowed an accurate assessment of population trends of both *Coleophora* spp. (Pearson 1975).

Much of the observational work carried out by Pearson (1975), documenting clover casebearer behaviour, is applicable to the red clover casebearer, as recent studies of this pest have shown many similarities between these closely related species (Landry 1991).

1.3.9 Adult and reproduction biology of the red clover casebearer



Figure 1.11: A fourth instar red clover casebearer larvae within a red clover flowerhead.



Figure 1.12: Three fourth instar red clover casebearer larvae within a red clover flowerhead.



Figure 1.13: A third instar red clover casebearer larvae within a red clover flowerhead, notice the less developed case compared to Figure 1.10.



Figure 1.14: A fourth instar red clover casebearer larvae with its head and legs revealed.

The red clover casebearer is univoltine and is typically observed in flight from mid-spring until mid-summer, with significantly higher abundances around older than younger clover stands (Chynoweth et al 2018). The species relies on chemical cues from red clover to detect its host plant and flits close to the ground, travelling up the pheromone stream to find new red clover plants to colonise (Evenden et al 2010; Walenta et al 2018).

The pattern within the Coleophoridae, as with most lepidopterans, is that the more intimately larval development is with a host plant (internal feeders) the narrower its host range, with external feeders (leaf and other exposed tissue feeders) having wider host ranges. The clover casebearers all complete most of their development concealed within the clover florets, so they rely on the specific conditions and resources available within their preferred host plant species.

1.3.10 Larval development

Female moths lay eggs on the calyx (petals) of red clover florets; typically, they select flowerheads with few or no declining florets (Walenta et al 2018). After just over a week in favourable conditions the eggs hatch and the larvae burrow into the base of the florets to access developing seeds. The larvae complete the first instar development and ecdysis within this first seed they burrow into (Landry 1991; Walenta et al 2018).

The second instar then consumes the remainder of the first seed and begins consuming the other seeds within the original floret or pod. The third instar consumes any remaining seed within the original floret before boring out of the floret and into the ovules of adjacent florets (Walenta et al 2018). The third instar generally stays within this original flowerhead and consumes 3-5 seeds before completing its third ecdysis. These first three instars are very rarely observed in the field, as they remain entirely concealed within the flowerhead (Walenta et al 2018).

1.3.11 Fourth instar

The fourth instar larvae continue to feed on seeds in the same floret they have been developing inside and begin case construction, after which, they become mobile (Landry 1991). All species of clover casebearers construct their cases by using their silk to stick together the margins of dried petals that are still attached to the flower stem, before cutting this 'petal tube' off at the base near the calyx and fusing it around other 'petal tubes' they have constructed (in a sort of cigar like fashion) (Figure 1.13) (Pearson 1975). This case is then reinforced with silk to ensure protection through the insect's development and diapause (Walenta et al 2018).

The features of the case are a round feeding hole from which the front of the larva emerges to feed and move around (Figure 1.13), and a distal end that is made of three trilobite flaps that can be opened for removing frass and other debris (Pearson 1975).

The later instar cased larvae are very mobile and venture out of the initial flowerhead once food supplies have been exhausted. This life stage moves around by the normal lepidopteran locomotion (whilst keeping its rear third to half in the case to drag it in tow when on a surface (Figure 1.13). It also uses its silk to create 'zip lines' between plants by which it abseils across to reach new food sources (Pearson 1975; Landry 1991; Walenta et al 2018).

When a fourth instar larvae is feeding on a developing seed it will burrow down in between the florets leaving the back half of its case visible but firmly wedged between the petals (Figures 1.10-.12). Then it begins to feed on all the developing seeds it can reach by extending its body while remaining attached inside the case (Pearson 1975).

Once a larva has satisfied its food requirement it begins to prepare the case for diapause, by further reinforcing its walls with silk and closing the distal end with extra flaps constructed out of the same petal (what was the frass exit tube). The proximal end remains open as the larva migrates to a suitable over winter site (Pearson 1975; Walenta et al 2018).

This is normally observed as cased larvae dropping down from the florets and crawling along the ground looking for things such as stems and other stubble, cracks in the earth, fence posts and tree trunks to over winter within/on (Chynoweth et al 2018). Once they have found a suitable site, they seal off the proximal end of the case with silk and enter diapause. This usually starts during March in New Zealand (Pearson 1975). The larvae then emerge as adult moths November-December of the following spring/summer (Chynoweth et al 2018).

1.3.12 Control options for casebearers

Because these pests are concealed within flower parts for most of their life cycle, and the population peaks occur during crop flowering, chemical control is not a favoured long-term solution. The other issue caused by this concealed life cycle is that unless the adult moths are monitored in the spring outbreaks, the larvae are generally undetectable until the fourth instar larval stage (which causes the bulk of seed damage) is common in the field. By this time, it is generally too late to avoid most of the damage this pest causes to a seed crop (Mori and Evenden 2014; Chynoweth et al 2018).

1.3.13 Pesticides

Populations of red clover casebearer build up in the red clover seed crop through the three years that they are typically harvested, and this accumulation can be reduced/slowed by spraying chlorpyrifos (See Table 1.2), in the year that fourth instar larvae are first noticed. However, this does not offer reliable protection from sudden or heavy infestations (Chynoweth et al 2018).

Currently chemical control is viewed as necessary in the event of outbreaks of the red clover casebearer, but there are no products specifically registered for red clover casebearer in New Zealand. Tau-fluvalinate and chlorpyrifos are registered for white clover casebearer control in New Zealand and these insecticides are used by farmers in the absence of specific red clover casebearer registered products.

Chynoweth et al (2018) investigated the efficacy of common insecticide sprays during the first season of the outbreak (Table 1.2).

Table 1.2: Pesticide treatments and red clover casebearer mortality rate (%)

Treatment	Chemical Group	Mortality rate 48h after application
Water	-	20% (+/- 6%)
Water and adjuvant	-	42% (+/- 7%)
Cyantraniliprole	Ryanoid	75% (+/- 6%)
Tau-fluvalinate	Synthetic pyrethroid	96% (+/- 3%)
Lambda cyhalothrin	Synthetic pyrethroid	100%
Chlorpyrifos	Organophosphate	100%

From Chynoweth et al (2018): '50 mL/ha Mavrik® Aquaflo (a.i. 240 g/L tau-fluvalinate, Adama, 250 mL/ha Lorsban™ 50 EC (a.i.). 500 g/L chlorpyrifos. Dow Agrosiences, 40 mL/ha Karate® Zeon (a.i. 250 g/L lambda cyhalothrin. Syngenta or 150 mL/ha Exeril® (a.i. 100 g/L cyantraniliprole, DuPont with an adjuvant 25 mL Spreadwett 1000/100 L of water (a.i. alkoxylated alcohols, SST NZ Ltd) as required. water or water + adjuvant for control treatments.'

Chynoweth et al (2018) found that tau-fluvalinate, chlorpyrifos or lambda cyhalothrin achieved high moth mortality within 4.5h, and near 100% mortality by 48h after foliar application. Cyantraniliprole had a poor mortality rate at 11% and 75% mortality after 24 and 48h respectively (Table 1.2).

In New Zealand, growers have had to employ insecticides during these outbreaks to protect seed yields, but even so large seed yield losses of up to 80% have still been recorded. Farmers have been using between one and four applications of sprays of the organo-phosphate or synthetic-pyrethroid to red clover following outbreaks of red clover casebearer (Chynoweth et al 2018).

1.3.14 Mating disruption and pheromone trapping

Pheromone-baited traps are widely used in IPM systems as the chemicals contained are very specific to the target species and will normally only disrupt the one target pest; these traps are generally used for mating disruption and monitoring (Mori and Evenden 2014). Pheromone monitoring of adults helps inform red clover casebearer control actions, as the concealed larval stages are often not identified in time to prevent serious yield loss.

Pheromone baited traps have been successfully used in New Zealand where the red clover casebearer pheromone developed for Canadian and New Zealand research was used to assess the distribution of this pest in New Zealand following its discovery (Chynoweth et al 2018).

In Canada, both pheromone population monitoring and mating disruption have been successfully employed. Mori and Evenden (2015), investigated the efficacy of Hecron Disrupt Micro-flakes on reducing red clover casebearer larval incidence and yield loss. They found that when these flakes

were evenly applied across a crop the mating of the red clover casebearer was disrupted, which in turn decreased the incidence of seed damage (Mori and Evenden 2015).

The relationship between male flight abundance and in-crop larvae abundance was also investigated. There was a strong correlation between the number of male moths caught in pheromone traps and the number of larvae within the crop, along with a higher abundance of adult moths and larvae correlating with a higher rate of seed damage (Mori and Evenden 2015).

1.3.15 Biocontrol

Because of the many challenges associated with managing the red clover casebearer, biocontrol is necessary for its sustainable control. The ideal agent would be one that is able to quickly respond to outbreaks (rapid population growth), along with being able to seek out and regulate the moth at low population densities to prevent future outbreaks.

1.3.16 Biocontrol in white clover crops in New Zealand

The pest pressures of the two clover casebearer species *C. alcyonipennella* and *C. mayrella* were not abated in white clover seed crops until the success of a 1960s DSIR biocontrol agent establishment programme. This programme aimed to establish many species of parasitoid wasps, but it is likely only two established; (*Bracon variegator* (Spinola 1808) Braconidae: Hymenoptera). and an unidentified *Neochrysocharis* sp. (Eulopholidae: Hymenoptera). These parasitoids appeared to quickly reduce the white clover casebearer populations below economically damaging levels, although there was no further investigation into the decline of *C. alcyonipennella* and *C. mayrella* that was observed following these parasitoid establishments (Thomas 1969; Pearson 1991).

B. variegator is a generalist wasp that lays a single egg in its hosts, which become immobilised. The adult wasps emerge 1-3 weeks later. Its native range is all of Europe and parts of the Middle East. Within this native range, *B. variegator* has been recorded as parasitising many insect species from over 10 families, most of which are leaf roller moths (Zikic et al 2012; Ghahari et al 2022).

Due to the wide host range of *B. variegator* and the similarities between clover feeding casebearer species in biology and habitat, it is hoped that *B. variegator* may become an effective agent in red clover casebearer biocontrol.

1.4 Conclusions and future investigation

It is unclear exactly what factors led to the sudden, widespread and intense outbreaks of red clover casebearer in New Zealand, but there are elements that can be explored now and into the future with more research that may hold useful information for biocontrol science in New Zealand (Chynoweth et al 2018).

Of further interest is the benefit of resident biocontrol agents, sometimes from previous biocontrol programmes, being able to adjust to, and effectively control, new pests. This is the biocontrol potential of an agro-ecosystem and is very important to building future biocontrol systems in agricultural landscapes.

The biocontrol potential within agro-ecosystems is advanced by increasing the diversity and abundance of species on farms through biocontrol agent releases and habitat enhancement. This thesis and numerous other works provide useful information on how these biocontrol agents were supported prior to the arrival of a new pest and how they were able to quickly adapt to and eventually suppress the invasive species (Gonzalez-Chang et al 2015; LaCanne and Lundgren 2018). It is important that teams of people with a variety of skills conduct this type of work because it is easy to be distracted by high abundances of parasitoids and sudden declines in pest populations instead of less obvious underlying processes.

By investigating all elements of a biocontrol system, including the less obvious and apparently less important ones, a foundational understanding of what effective biocontrol systems look like and how they are implemented can be developed. The following aims were developed to fill knowledge gaps in the understanding of the red clover casebearer and the ecology of New Zealand red clover seed crops.

1.5 Aims and hypotheses

- I. Aim 1: Improve understanding of the population dynamics of arthropod populations in red clover seed crops in New Zealand.
 - Hypothesis: the species composition of the arthropod community present in red clover seed crops is similar to that of white clover seed crops with some adjustment for host plant species-specific arthropods.
- II. Aim 2: Explore how these dynamics change over the growing season.
 - Hypothesis: The arthropod community will change through the growing season, with peak flowering being an important time.
- III. Aim 3: Develop understanding of the biocontrol provision within red clover seed crops by resident arthropods.
 - Hypothesis: The constant ground cover and provision of floral resources in summer will support a diversity of biocontrol agent species.
- IV. Aim 4: Investigate red clover casebearer moth outbreaks and general populations dynamics in New Zealand red clover seed crops.
 - Hypothesis: The red clove casebearer will continue to decline in population in New Zealand.
- V. Aim 5: Identify potential biocontrol agents of the red clover casebearer moth.
 - Hypothesis: It is unlikely that any of the resident predator insect species will be able to consume red clover casebearer larvae in appreciable numbers
- VI. Aim 6: Assess the potential of any biocontrol agents of the red clover casebearer identified.
 - Hypothesis: If a biocontrol agent exists it will likely be associated with a biocontrol programme for the white clover casebearer species in New Zealand.

1.6 Thesis structure

Chapter 1 Introduction and Literature review

An overview of the science underpinning the thesis approach relating to the need to develop more sustainable forms of food production.

Chapter 2 Aims, outline and preliminary experiments

Aims addressed: 1,2,3,4 and 5.

Explanation of the aims of this PhD and the approach used to inform the project as results were discovered. Five preliminary studies were used as the basis for the direction of the subsequent research. These experiments were simple forays into the ecology of red clover seed crops that aimed to highlight and confirm points of interest for exploration.

Chapter 3 Dynamics of arthropod communities in red clover crops

Aims addressed: 1, 2 and 3.

Exploration of the dynamics of the arthropod community present in red clover seed crops. This chapter is based around an in-depth sampling study undertaken in numerous red clover seed crops. This study consisted of sweep net sampling in the red clover seed crops and cataloguing those species collected, from multiple regions, dates and over two seasons.

Chapter 4 Red clover casebearer in red clover seed crops

Aims addressed: 4 and 5.

Investigation into the prevalence of the red clover casebearer in red clover seed crops and efforts to identify potential biocontrol agent(s).

Chapter 5 Biocontrol of the red clover casebearer and exploration of its agents

Aim addressed: 6.

Exploration into the identified biocontrol agent(s) of the red clover casebearer. This includes several experiments attempting to identify reasons for the different outcomes in biocontrol of casebearer species between New Zealand red and white clover seed crops.

Chapter 6 Summary and conclusions

Concluding discussions of the chapters and how this research is relevant to national/global efforts to develop sustainable agriculture. Potential areas of future work will also be discussed in this chapter.

Chapter 2

Preliminary exploration of the red clover seed crop ecosystem

2.1 Introduction

Cataloguing the insect community present in a crop is critical to understanding the ecosystem itself and any services or disservices it may be imparting to the crop (Ward and Stanley 2004; Dainese et al 2008; Kremen 2008). To use this approach to the pest issues facing red clover seed crops, it will be necessary to first catalogue what insect species are present in the crops, and then to investigate the functions of these insects within the ecosystem. Given that no biocontrol agents of the red clover casebearer have yet been identified, this required general experiments to find potential biocontrol agents and to better understand the agroecosystem overall.

The arthropod groups of interest in this work were biocontrol agents (predators and parasitoids) and the pests of red clover. In general, the pest species of agricultural crops are well known through their recorded impacts and spray efficacy studies. However, there has been less motivation to identify those biocontrol agents that inhabit crops and provide background biocontrol services. Further, even when studies are completed, they often are funded in terms of a biocontrol agent's impact on one important pest, commonly ignoring how the agent is supported in the crop beyond the one 'important pest' resource.

These initial studies reported in this chapter investigated the distinct aspects and seasonal changes affecting the red clover seed crop, along with the activity of the concomitant biocontrol agents. Due to the difficulty associated with providing a species level identification for every arthropod recovered, recognisable taxonomic units (RTUs) have been used to classify organisms and investigate diversity in the absence of a specific species identification (Ward and Stanley 2004).

2.2 The preliminary experiments:

- i. Post winter emergence: An investigation of the emergence of field-collected arthropods under greenhouse conditions during spring. Aims covered: 1 and 2.
- ii. Egg bait predation: A simple experiment evaluating predator activity in the crop by proxy of placed lepidopteran egg baits in the field. Aims covered: 2 and 3.
- iii. Video surveillance: Attempting to use a video surveillance camera to record predation on, and the behaviour of, the red clover casebearer moth. Aims covered: 1, 3 and 4.
- iv. Generalist predator bioassays: To determine if any of the generalist predators present in red clover seed crops predated on the red clover casebearer moth. Aim covered: 3.
- v. Laboratory parasitism experiment: The use of laboratory cultures of invertebrates collected from red clover seed crops to detect parasitism of key species. Aim covered: 5.

2.3 Location and study sites

The majority of the work on these preliminary experiments was conducted in red clover seed crops near to Lincoln University, Canterbury, New Zealand (-43°N , 172°E).

The three main fields used for this work were known as 'Kowhai', 'Tancreds' and 'Robinsons'. These three fields were all within 2km of Lincoln University.

Kowhai (Figure 2.1): This was a small crop of red clover within a study site managed by the Foundation for Arable Research. This crop received very little intervention (aside from mechanical topping in late November/early December and irrigation during summer) as it was a specific study site for work on the red clover casebearer. It was sown in 2017 and was 30m by 50m. The plant species composition was 50-60% red clover with a high level of weedy species, many of which flowered. At the time of the study, the crop was three years old.



Figure 2.1: Kowhai field outlined in red.

Tancreds (Figure 2.2): This was a commercial seed crop managed by PGG Wrightson Seed Ltd. This crop was under conventional management and received multiple interventions. It was grazed by sheep during spring to regulate growth and irrigated as needed over summer. This crop was in its first year during 2020 and was around 400m by 550m.



Figure 2.2: Tancreds field outlined in red.

Robinsons (Figure 2.3): This was a commercial seed crop managed by PGG Wrightson Seed Ltd. This crop was under conventional management and received multiple interventions. It was grazed by sheep during spring to regulate growth and irrigated as needed over summer. This crop was in its second year in 2020 and was around 100m by 500m.



Figure 2.3: Robinsons field outlined in red.

Further experiments, such as the laboratory parasitism experiments with samples from fields from other districts in Canterbury will be outlined where relevant.

2.4 Preliminary experiment 1: Post winter emergence

2.4.1 Background

The development of arthropod communities within agricultural fields is under constant pressure due to many factors, and the final community of arthropods that is present in a field at the end of a growing season is heavily influenced by the forms of farming practice and landscape management employed (Lundgren and Fergen 2010; Yang et al 2020).

Disturbances such as cultivation, spraying and burning off of crop residue negatively affect insect communities within agricultural fields (Tooker et al 2020; Yang et al 2020). Sometimes sensitive species are entirely removed from fields by these practices, pending re-colonisation. Regardless of how severe the initial impact of a disturbance is, most populations of insect species are slow to recover from these events. This recovery is also often further hindered by the low quality of habitat provided by agricultural landscapes (Tooker et al 2020).

The quality of the habitat that persists in fields over winter is important for supporting the insect species that remain within fields year-round (Lowenstien et al 2019). Some species maintain active generations or have periods of activity during winter, and the availability of key resources such as shelter, floral resources and other food sources is critical to maintaining their populations over these harsh months (Lundgren and Fergen 2010).

Red clover seed crops only flower during summer, and after harvesting and clean up treatment(s) the plants are largely left to grow until the next spring (Hardwick and Barton 2019). During this time, they provide habitat and shelter for insect species along with allowing some other small 'weedy plant' species to grow and provide further resources to arthropods, which allows many insect species to overwinter within them (Lundin et al 2016). Following winter, the most impactful common disturbances on the developing arthropod community in red clover seed crops are mechanical topping (to around 10 cm) and spring aphid insecticide sprays (Hardwick and Barton 2019).

Although these disturbances affect the developing insect community present within the crop, they do not eliminate any key species. The recovery from these disturbances is slow, but after 2-3 weeks, populations of insect species build to higher levels than before the disturbance as the growing season progresses (Hardwick and Barton 2019).

The species that emerge during the early weeks of the growing season can impact the pest pressure outcomes experienced in the final weeks of a crop's harvest cycle (Tenhumberg and Poehling 1995; Lundgren and Fergen 2010; Panel et al 2018). These processes are complex and difficult to understand due to factors such as those outlined above (Arrington et al 2007).

Of further concern is how climate change will affect insect communities in agricultural landscapes during critical times of the year, particularly post-winter emergence. Many modelling studies focusing on this issue find that in general, warmer winters will benefit less cold hardy species and negatively impact more cold hardy species (Bale and Hayward 2010). However, predicting exact outcomes for growing systems is hard due to other climatic changes that can be more localised and impact species differently, such as increased rain in winter favouring species of entomopathogenic fungi (Bale and Hayward 2010).

A study conducted by Zaller et al (2009) in August sown winter canola (*Brassica napus* L.) crops in North-Eastern Austria, found that overwintering predatory ground beetles are important regulators of some key pest species during spring and into summer. These experiments involved the containment, removal and exclusion of predatory ground beetles (typically *Anchomenus dorsalis* (Pontop 1763) and *Poecilus cupreus* (L.) (Coleoptera: Caribidae)) around canola plants in the field using pitfall traps and cages. The abundance of pollen beetles (*Meligethes* spp. (F.) Coleoptera: Nitidulidae) and stem weevils (*Ceutorhynchus pallidactylus* (Marsham 1802) Coleoptera: Curculionidae) was measured over the growing season and compared among the various treatments. It was found that that ground dwelling predatory beetles significantly reduced the emergence of populations of stem weevils but not pollen beetles. This result highlights the importance of maintaining populations of biocontrol agents within fields throughout the year, as well as showing that the abundance of some species can also be due to many factors that single experiments can struggle to capture (Zaller et al 2009).

2.4.2 Method

This initial experiment was designed to understand when key arthropod species emerged in red clover seed crops after winter, and to determine the arthropod species present in the crop. Because red clover seed crops are multiyear, the permanent habitat allows many species to overwinter within the crop in their respective life stages.

Clover plants grow biomass quickly as the temperature and sunlight hours increase during spring, which would make infield containment and sampling of plants very difficult. Because of this and the interest in all arthropod species that might emerge, this experiment was conducted within a greenhouse using clover plants and soil removed from local red clover seed crops.

Fifteen red clover plants were dug up from each of the Kowhai and Robinsons red clover seed crops during October and September 2020. The plant and its surrounding soil (about a 20cm diameter circle to 30cm deep, this soil included any weed that were amongst the red clover plant and effort was made not to disturb the soil) were then sealed in a large plastic rubbish bag for transportation

back to the University. The plants and accompanying soil were then transferred into 4L pots and placed in five bug dorms per field collection treatment (3 plants per dorm). The 'bug dorms' used in the current work were a small (50cm by 50cm) version of an interior of a dome tent (Figure 2.4). These bug dorms were then left in a greenhouse under a 16h daylight cycle to promote arthropod activity and emergence. Every three days the bug dorms were thoroughly searched for newly emerged arthropods, which were then removed for identification. After about three weeks (when new arthropods stopped emerging) the plants were removed and broken up to be searched (including the soil) for any remaining arthropods. After this a new set of plants were collected and the same steps repeated.

The searching method consisted of first shaking the cage while it was sealed to disturb and note any flying insects before opening it just enough to fit two arms through and catch the visible insects in containers to confirm their identity. Then the plant was closely examined for any flying insects before fully opening the cage and deeply searching the plant, cage, soil surface and under the pot for any more emerged arthropods, which were then removed and recorded. The arthropods were recorded as, recognisable taxonomic units (RTUs), which were determined by both prior knowledge of the species, the use of keys (Goulet and Huber; 1993; Landcare Research Fauna of New Zealand Series 2023 (Mound and Walker 1986; Klimaszewski and Watt 1997; Lariviere and Laroche 2004; Donovan 2007; Hoare 2017; Hoare 2019; Lariviere et al 2023)). As well as professional advice from Professor John Marris (Lincoln University), Dr Morgan Shields (Environment Canterbury) and Professor Cor Vink (Lincoln University)).

To reduce the risk of arthropods escaping, watering was undertaken directly after each search had been completed. Each run of this experiment consisted of fifteen red clover plants and the surrounding soil being removed from each of two red clover seed crops (total 30 plants). This was conducted twice during the spring of 2020 (on 15/9/20 and 20/10/20).



Figure 2.4: The bug dorms used for experiments.

2.4.3 Results and Discussion

A diverse range of species overwintered in red clover seed crops, and there was an even spread of pests and biocontrol agents (Tables 2.1-2.5). A higher average abundance of both pests and biocontrol agents was observed in plants collected from the three-year-old Kowhai field than the one-year-old Robinsons field (Table 2.3, 2.4). There was also a higher total diversity and abundance of RTUs observed in plants collected from the Kowhai field than the Robinsons field (Table 2.3).

The species of biocontrol agent included Tasman brown lacewing, five parasitoid wasps belonging to Ichneumonidae (including *Aphidius* spp.), and four other parasitoid wasps belonging to Braconidae, three species of ladybird (2, 7 and eleven-spot ladybird), the pacific damsel bug, two *Orius* spp., European harvestman and jumping spiders (Table 2.5).

Table 2.1: Average number of biocontrol agent and pest recognisable taxonomic units (RTUs) recorded from 30 red clover plants from the Kowhai field.

Kowhai	Number of Biocontrol RTUs (std.dv 2.3)	Number of pest RTUs (std.dv 0.8)
15/9/20	6	6
20/10/20	7	5

Table 2.2: Average number of biocontrol agent and pest RTUs recorded from 30 red clover plants from the Robinsons field.

Robinsons	Number of biocontrol agent RTUs (std.dv 1.0)	Number of pest RTUs (std.dv 0.7)
15/9/20	3	3
20/10/20	4	4

Table 2.3: Total number of RTUs belonging to different orders of arthropods recorded during the experiment from Robinsons and Kowhai fields collected from 30 red clover plants over two sampling dates.

Kowhai			Robinsons	
	15/9/20	20/10/20	15/9/20	20/10/20
Hymenoptera	4	5	2	1
Coleoptera	4	4	3	3
Aracena	4	4	2	1
Hemiptera	7	8	4	4
Thysanoptera	2	2	2	2
Neuroptera	1	1	0	1
Lepidoptera	3	3	0	0
Other	1	2	1	1
Total RTU	26	29	14	13

Table 2.4: Total number of individual arthropods observed from 30 red clover plants at each site during the experiment.

Date of collection	Kowhai	Robinsons
15/9/20	58	32
20/10/20	62	37

Table 2.5: Overview of the species recorded.

Biocontrol agent(s) known to consume this pest type	Pest species	References
<i>Aphidius</i> spp., European harvestman, Tasman brown lacewing, Pacific damsel bug, Hoverfly larvae and ladybird (all species)	Aphids	(Siddique 1985; Wratten et al 1995; Grasswitz and Resse 1998; Fagan et al 2009; Islam and Chapman 2001)
Ladybirds, Hoverfly larvae and European harvestman	<i>Thrips</i>	(Wratten et al 1995; Sandhu 2005)
Pacific damsel bug, European harvestman	Australian crop mirid and potato mirid	(Siddique 1985; Siddique and Chapman 1987)
European harvestman, <i>Orius</i> spp. and pacific damsel bug	Lepidopterans	(Siddique 1985; Siddique and Chapman 1987)

2.4.4 Conclusions

These results established that, at least in Lincoln, there was a diverse and even arthropod community present in the two red clover seed crops even in the early stages of the growing season. These species benefit from the habitat provision provided by the second and third year red clover seed crops, but this experiment did not attempt to explore how this occurs. For all of the pest species

recovered at least one biocontrol insect that was known to parasitise or prey on it was also recovered.

The higher number of species of all groups that emerged in the plants collected from the experimental Kowhai field than the plants collected from the Robinsons field (commercial seed crop) was expected. The Kowhai field was a third-year crop where presumably more species had had time to establish, but there was also a far lower rate of insecticide application at this site compared to the commercially managed crop. The importance of the age of the crop in the development of the insect community present has been noted from studying specific pests in clover seed systems including the red clover casebearer (Landry 1991; Schroeder and Clifford 1995; Hardwick and Barton 2019). The species recorded during this experiment are very similar to those sampled in white clover seed crops in New Zealand (Schroeder and Clifford 1995), with the exception of the red clover casebearer.

2.5 Preliminary experiments 2 and 3: Proxy measurements of egg bait predation and video camera surveillance

2.5.1 Background

It is difficult to estimate the biocontrol services of any biocontrol arthropod species, and it is even more difficult to understand the overall background biocontrol services provided by generalist predator species (Symondson et al 2002). This is due to factors such as the range in their diet and changes in their diet composition based on prey availability, along with competition and other inter-species interactions (Tscharntke et al 2005b; Michalko and Dvoryankina 2019; Krey et al 2021; Veronesi et al 2022). The biocontrol services of specialist species such as parasitoid wasps are much easier to quantify with parasitism rate experiments (Luna et al 2007; Abram et al 2020).

Proxy measurements of biocontrol services are useful tools for developing understanding of the activity of generalist biocontrol agents in the field (Hossain et al 2001). These proxy measurements can be things such as egg prey baiting, frass sampling, population sampling, artificial refuge, video surveillance and next generation sequencing (NGS) analysis of gut DNA (Shields et al 2021). These types of methods have been successfully employed in numerous studies and are often used when trying to determine the impact of a disturbance or habitat enhancement on a biocontrol system (Hossain et al 2001; Shields et al 2021). There are various costs and benefits associated with each method. For example, DNA techniques are costly, and results can be slow to return, but the results obtained are of a very high accuracy (Shields et al 2021).

Suckling et al (2006) investigated the efficacy of artificial shelter, lepidopteran (leaf roller) egg baits, artificial diet and frass sampling for monitoring earwig abundance and activity in relation to ground cover within apple orchards at Lincoln University, Canterbury, New Zealand. The artificial shelters and diets were useful in detecting earwigs in the study plots but there were issues with earwigs being less interested in shelters in the treatments with better ground cover. Frass sampling was also noted to be of limited use without DNA confirmation of the sample's origin. The leaf roller egg baits used were considered to be the most effective proxy measurement of earwig activity and a six-fold rate of egg bait predation was recorded in plots containing ground cover compared to the herbicide sprayed plots during late summer (Suckling et al 2006).

Hossain et al (2001) investigated insect biocontrol agent abundance and activity via *Helicoverpa* spp. egg bait predation and parasitism in strip harvested lucerne (12km west of Canowindra, New South Wales, Australia -33°N, 148°E). Both of these methods were considered effective, and the study found that harvesting reduced the abundance of all measured insect species (Hossain et al 2001).

Pennington et al (2018) investigated whether reduced fungicide application improved the biocontrol of *Lobesia botrana* (Denis and Schiffermuller 1775) (Lepidoptera: Tortricidae) on grapevines in Siebeldingen, Germany (49°N, 80°E). In this study, egg baits of *L. botrana* were used to assess biocontrol agent activity along with artificial inoculation with extra reared *L. botrana* eggs to assess damage rates on grapes after three weeks. They found that *L. botrana* egg bait predation increased with decreasing fungicide use, and that fruit damage in artificially inoculated treatments decreased with decreasing fungicide application. During these field experiments in the field, video surveillance was used to determine which arthropod biocontrol agents were preying on the egg baits. The cameras took a photograph of the egg bait every 10 seconds for 24h. Species belonging to Formicidae, Dermaptera and Chrysopidae were observed feeding on the baits. However, the images were not of high enough quality to discern the more specific groups they belonged to (Pennington et al 2018).

Shields et al (2021), used pitfall traps and *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) egg baits to assess arthropod biocontrol agent activity in three different field margin habitats 'Miscanthus plot' (*Miscanthus X giganteus* (Renvoize 2001)), uncultivated vegetation largely comprising agriculture grass species and 'weedy' flowering species and mown control boundaries) on a commercial dairy farm in Canterbury, New Zealand. In addition, video surveillance and Sanger sequencing were used to further explore the biocontrol system in these field margin habitats. Across the three sampling periods (April, August, September), many potential biocontrol agents were recorded using these methods, with the highest diversity and abundance of species being found within 'Miscanthus plots'. There were no significant differences in biocontrol agent species

abundances and diversity across the growing season between plots of the same treatment (Shields et al 2021). The highest rate of egg bait consumption occurred in the 'Miscanthus plot' (67%) compared to 10% in the pasture field margin. Slugs and harvestman were the only arthropods recorded consuming the egg baits around the pitfall traps. These events were recorded 23 times in the 'Miscanthus plot', but no predation was recorded in the pasture field boundary (Shields et al 2021).

Of interest, interactions between harvestmen and slugs were recorded on multiple occasions. The following behavioural account is from Shields et al (2021):

'P. opilio individuals were observed deterring slugs from feeding on the egg baits by prodding the slug with their legs, pedipalps and chelicerae until the slug moved on. These prolonged 'attacks' lasted 1 min, 8s and 2 min, 42s, after which P. opilio fed on the remaining egg baits. The opposite behaviour was also observed on one occasion where a slug prevented a P. opilio individual consuming egg baits by moving underneath the harvestman, covering the eggs and maintaining this position for 27 min and 50s. In response, P. opilio moved away. No predation was observed in video recordings from the field margin plots.'

Although only a small number of predation events were recorded, the recording of interactions between arthropods in the field is valuable to developing understanding of the biocontrol system. These methods can be applied to any species in a given crop and be used to observe its behaviour over the season.

Sanger DNA analysis involves removing the gut of biocontrol agents and then performing PCR analysis of the contents that is then checked for DNA of the egg bait species. This DNA technique was chosen because it provided a way to determine which agents had consumed the egg baits with certainty (Shields et al 2021). Sixty-six individual biocontrol agents were sampled using this technique and four were found to contain the target egg bait DNA. This low hit rate highlights the difficulty of using DNA techniques on field collected arthropod samples.

Shields et al (2021) found pros and cons with each of the biocontrol agent surveillance methods but overall, the combination of information gathered through the array of methods was considered beneficial to developing knowledge of the study system. This work emphasised the importance of using multiple methods in attempts to understand and explain biocontrol systems (Shields et al 2021).

2.5.2 Methods

Egg baits were chosen for use in this work because the early stages of this project were focussed on the suite of generalist predators present within red clover seed crops that were considered potential biocontrol agents of the red clover casebearer.

Video surveillance was also used as the equipment from the previous studies was available. This camera system consisted of three security cameras with HD video and fine focus ability along with accompanying battery packs that allowed for over 24h of video recording. The entire set up was weatherproof and able to be left in the field for extended periods.

The general aim of this experiment was to investigate if there was a lack of generalist predator activity in the crop spatially or temporally, because this was considered as a possible reason for the lack of biocontrol of the red clover casebearer in recent growing seasons.

Hossaine et al (2001) and Shields et al (2021) provided clear methods for the use of moth egg baits in the field for the proxy measurement of generalist biocontrol agent activity. Shields et al (2021) also provided methods for video surveillance in the field.

They are as follows:

Hossaine et al (2001): 'Helicoverpa egg baits were used to quantify predatory activity in the unharvested and harvested herbage within the strip harvest experiment. Baits were prepared by allowing caged Helicoverpa adults to oviposit onto filter paper. The paper was then cut into segments, each bearing 20 eggs. These baits were stapled to lucerne foliage 75mm from the ground in three positions in each strip of lucerne'.

Again, in Shields et al (2021):

'Each egg batch contained c. 200 eggs which had been laid on wax paper and were outlined with a pencil to improve detection in video recordings and to delineate the egg batches, including where they had been prior to predation, when examined under a microscope. Portions of the paper were cut to contain one egg batch and were pinned to the soil surface.'

'Sanyo HD4600 CCTV cameras with external infrared illuminators were used to investigate natural enemy activity and identify those RTUs that consumed egg batches. Two of these cameras were used per plot during the first trap night. Each camera continuously recorded invertebrate activity around one randomly selected pitfall trap, for 13h from 1800–0700. The videos were later analysed, and potential natural enemies' egg consumption behaviour and the duration of activity were recorded.'

In this experiment light brown apple moth egg baits were obtained from Plant and Food Research Ltd, Auckland. They were laid on wax paper which was then cut up into pieces (around 5 by 10cm)

containing four decent sized egg rafts. Each of these rafts were circled with pencil to assess overall 'size reduction' (Figure 2.5). Baits were placed from November to January, from the edge of the field to the centre. This experiment was conducted in the Kowhai and Tancreds red clover seed crops.

The layout of the egg baits in the field was by four transects that ran across the entire field, placed at a logarithmic scale from 0m-to the centre of the crop (Kowhai: 0m, 2m, 8m, 16m and centre and Tancreds: 0m, 2m, 4m, 8m, 16m, 32m, 64m and centre)

These baits were secured in the field by using toothpicks for 'ground baits' or spray on adhesive glue for 'canopy baits'. After 24h the egg baits were collected and assessed for damage/predation in the field. Damage/predation was classed as clear puncture holes and deflating of the raft, 'size' reduction, bite marks and missing pieces. This was recorded as the percentage damaged or missing and the type of damage that had occurred. The baits attached to the canopy frequently blew away resulting in very inaccurate data which have not been reported.



Figure 2.5: Light brown apple moth egg bait secured with toothpicks to the ground in a red clover seed crop. The 'egg masses' (light green) have been outlined in pencil.

2.5.3 Video surveillance



Figure 2.6: The video surveillance set up used.

Due to the frequent high winds experienced in Canterbury during this summer, efforts to video arthropod activity and egg bait predation (Figure 2.6) were hampered by the plants being blown in

and out of focus in the field. To combat this, three flowerheads in the centre of the shot were chosen and firmly staked, which improved the footage, but strong gusts sometimes still dislodged the stakes.

Overall, there were many issues with attempting to recorded arthropod activity within the crops. The impact of wind alone made much of the footage, even of staked plants, unusable. In addition, the complexity of the crop structure and varying sizes of arthropods in the field presented issues for the focus of shots.

Attempts were also made to film the predation of egg baits, live prey baits in the form of light brown apple moth larvae (instars three-five), and adults that were ether glued or pinned to a plant surface in the shot focus. Both of these methods of fixing live prey baits posed many issues that would have impacted the conclusions of any findings and no predation of these bait types was recorded.

2.5.4 Results

Overall, there was an active assemblage of predatory arthropods in both of the crops. The rate of egg bait parasitism did not differ with increasing depth into either of the fields (Table 2.6 and 2.7), but there was more egg predation in the late (January-February) season than in the early season (October-December). There tended to be more egg bait predation in the Kowhai field than the Tancred field (Tables 2.6 and 2.7).

Table 2.6: Percentage of egg bait predation in the Tancreds field.

Distance from edge of crop in meters	Average egg bait consumption (%) October-December std.dev 25.4%	Average egg bait consumption (%) January-February std.dev 20.1%
0m	29%	51%
2m	31%	50%
4m	31%	58%
8m	35%	58%
16m	37%	55%
32m	32%	55%
Centre of crop	30%	52%

Table 2.7: Egg bait predation rates in the Kowhai field.

Distance from edge of crop	Average egg bait consumption (%) October-December std.dev 27.7%	Average egg bait consumption (%) January-February std.dev 24.1%
0m	25%	57%
2m	41%	59%

4m	60%	59%
8m	44%	71%
Centre of crop	42%	68%

2.5.5 Conclusions

There was consistent generalist predator activity across both crops and both the early and later periods of the growing season. The early exploration of these aspects of the insect community dynamics helped establish that red clover casebearer outbreaks were probably due to issues with a specialist rather than a generalist biocontrol agent. Spatial factors, such as field depth, were not impacting biocontrol outcomes in these two red clover seed crops.

The use of video surveillance for monitoring the predation of the red clover casebearer in the field proved to be difficult. Although no red clover casebearer predation was recorded, a lot of footage of red clover casebearers in red clover flowerheads was recorded, and this technique may prove useful for answering a different question in the future.

Of further interest was the recording of the behaviour of the red clover casebearer in the field. The following is an account from observer video surveillance footage during this work:

'On one occasion as the sun was setting the following behaviour of the red clover casebearer cased larvae was recorded: for around 1hr the cased larvae in the shot remained lodged in the red clover flowerhead but wriggled their exposed cases intermittently with no real pattern. During this time some of the casebearers moved around the flowerhead and onto other flowerheads before re-lodging between florets and repeating the wriggling behaviour.'

2.6 Preliminary experiment 4: Bioassays

2.6.1 Introduction

Laboratory bioassays are important tools for assessing the biocontrol potential of arthropod agents against specific pests (Islam and Chapman 2001; Madsen et al 2004; Birkhofer et al 2017). These methods have been employed in many studies and have often provided clear results (Birkhofer et al 2017). Some aspects of parasitoid activity such as host-range and fecundity can be affected by laboratory conditions. Providing this is taken into account, the type of result obtained can be useful for understanding the biocontrol of a specific pest species (Birkhofer et al 2017; Bulgarini et al 2020).

Laboratory bioassays can also be designed to learn specific things about biocontrol agent habits, such as prey preference, foraging times and longevity under varying supplies of essential resources (Madsen et al 2004).

Pugh et al (2015) investigated the potential of the southern ladybird (*Cleobora mellyi* (Mulsant 1850) Coleoptera: Coccinellidae) for control of the invasive tomato potato psyllid on potato crops in New Zealand. Prey preference tests were performed between the tomato potato psyllid and *Myzus persicae*, *Trialeurodes vaporariorum* (Westwood 1856) (Hemiptera: Aleyrodidae) or *Macrosiphum euphorbiae* (Thomas 1878) (Hemiptera: Aphidae), three other common pests found in the study system on which the southern ladybird is known to predate. It was found that the southern ladybird preferred *M. persicae* and *M. euphorbiae* but not *T. vaporariorum* over the tomato potato psyllid (Pugh et al 2015).

The longevity of the southern ladybird was also tested under different floral (buckwheat and no prey or buckwheat + prey or control) and prey resource provisions (*B. cockerelli*, *T. vaporariorum*, *M. persicae* and *M. euphorbiae*). There were no statistical differences between prey resources but the presence of buckwheat significantly increased the longevity of the southern ladybird. Further greenhouse experiments were conducted to explore the provision of biocontrol by the southern ladybird against the tomato potato psyllid on potato plants. The southern ladybird reduced densities

of the tomato potato psyllid after three weeks and this reduction was maintained for seven weeks, which led to healthier potato tubers (Pugh et al 2015).

To explore the potential of the deployment of multiple biocontrol insects to enhance biocontrol of the tomato potato psyllid on greenhouse tomato crops, Veronesi et al (2020) conducted bioassays with key biocontrol agents (*Cleobora mellyi*, *Amblydromalus limonicus* (Garman and McGregor) (Mesostigmata: Phytoseiidae), *Engytatus nicotianae* (Kirkaldy 1908) (Hemiptera: Miridae), and *Tamarixia triozae*) against this pest. Each of these agents readily consumed the tomato potato psyllid during individual bioassays, however, no statistical differences were found in the tomato potato psyllid predation rate between different combinations of these biocontrol agents. Due to the promising laboratory results all these biocontrol agents show against the tomato potato psyllid when applied individually, further exploration into this system is required. This type of result is crucial to informing and improving the biocontrol system that will be implemented on these crops (Veronesi et al 2020).

Veronesi et al (2022) investigated the potential of *E. nicotianae* adults as biocontrol agents against the tomato potato psyllid on greenhouse tomato plants in New Zealand. This experiment employed similar laboratory predation bioassays as the previous experiments, as well as a larger experiment in the greenhouse, to assess the biocontrol potential of *E. nicotianae* against the tomato potato psyllid on tomato plants over the plant's growing cycle. The laboratory bioassays showed that *E. nicotianae* preferred to feed on the younger nymph stages of the tomato potato psyllid and predation on older life stages only occurred when other life stages were not available. The greenhouse experiment found that the tomato plants inoculated with *E. nicotianae* and the tomato potato psyllid produced a similar amount of fruit as the control plants, whereas, the plants only inoculated with the tomato potato psyllid produced the least fruit. These results helped provide a basis for field testing *E. nicotianae* as a biocontrol agent of the tomato potato psyllid in commercial tomato crops in New Zealand (Veronesi et al 2022).

2.6.2 Methods

The methods used by Veronesi et al (2020) and (2022) were:

'The assay was set up as a randomised complete block design with twelve treatments, each replicated in eight 24h blocks in a growing room set to 26 ± 1°C, 65–75% RH, and a L:D 16:8 photoperiod. Treatments comprised either eggs, first and second instars (five of each instar), third instar, or fourth instar B. cockerelli nymphs in feeding arenas to which a female, male or no adult E. nicotianae was added. Fifth instar nymphs were not included in this study due to the high chance of their becoming adults during the experiment and because preliminary assays showed that, the fifth instar is rarely preyed on by E. nicotianae. Treatment structure was therefore a four

(B. cockerelli life stages) by three (female, male or no adult E. nicotianae) factorial design. Arenas were 90 mm vented Petri dishes, with a piece of 10 cm diameter filter paper cut to fit into each dish. A small, fully expanded tomato leaflet was excised from the growing tip of a tomato plant, and its petiole was wrapped in a piece of moistened cotton wool inserted into a 0.5 ml Eppendorf tube to keep the leaflet fresh. Ten individuals each, of the B. cockerelli nymphal groups (see above) were transferred to each tomato leaflet using a fine painter's brush. For eggs, tomato leaflets on which B. cockerelli eggs had been laid over a 48h period were excised from whole plants.'

The predators selected for the bioassays in this study were ones that were of similar size to or larger than the fourth instar red clover casebearer larvae. This was because this is the only instar they would likely encounter in the field, as the earlier instars of this pest are concealed within the floret.

Table 2.8: Predator arthropods selected for laboratory bioassays against the red clover casebearer (RCCB).

Species	Diet	Relative size compared to fourth instar RCCB
Eleven-spot lady bird adult and larvae	Generalist but usually aphids	similar
Nine-spot lady bird adult and larvae	Generalist but usually aphids	smaller
European harvestman adult	Generalist	larger
Pacific damsel bug adult	Generalist	larger
Tasman brown lacewing larvae	Generalist but usually aphids	smaller
Large hoverfly larvae	Generalist	larger

The method used in these bioassays was adapted from Veronesi et al (2020) as follows: The petri dishes of the bioassay were laid out in a randomised complete block design with six predator treatments (Table 2.8), each tested in eight 24h, 48h or 72h periods in an incubator set to $21 \pm 1^{\circ}\text{C}$, 65–75% RH, and a L:D 16:8h photoperiod. Each treatment consisted of one petri dish with two individuals of the biocontrol agent species, six red clover casebearer fourth instar larvae and a sugar solution-soaked cotton bud.

The initial experiment ran for 24h and included a red clover flower in the petri dish. As no predation was recorded in this run, the following runs had the red clover flower removed and ran for 48h. Following the failure to detect predation in any of these runs of the experiment, a final bioassay was conducted with all of the potential predators and run for 72h.

2.6.3 Results and discussion

None of these candidate predator biocontrol agents consumed red clover casebearer fourth instar larvae in any of the experiments that ran for 24h, 48h and 72h. Therefore, all future work focused on possible parasitoids and non-arthropod causes of mortality of the red clover casebearer.

2.7 Important conclusions from this preliminary work

It would seem that growers are fortunate in that within the limited suite of arthropod and other biocontrol agents that make up the red clover seed crop arthropod community there appear to have been some that have been able to contribute to control of the red clover casebearer after the initial intense outbreaks in 2016-2017. However, these are yet to be determined.

It is hard to determine how much of the control of pest species is carried out by either biocontrol or human intervention, but it is clear that the current balance of resident biocontrol and crop management practices are effective in suppressing most pests of red clover seed crops.

These preliminary experiments helped direct the research towards further work to more thoroughly explore the arthropod communities in both red and white clover seed crops with a specific focus on the biocontrol of the red clover casebearer by now identified biocontrol agents.

It was still considered necessary to explore the arthropod communities present in clover seed crops generally, because the differences in biocontrol outcomes between red and white clover seed crops from 2016-2019 are poorly understood. At this stage, there were still many ecosystem and arthropod community driven possibilities for the sudden intense outbreak of the red clover casebearer, and an in-depth exploration of these respective seed crops arthropod communities would help to understand this process.

Chapter 3

The arthropod community in New Zealand clover crops

3.1 Introduction

As there are many gaps in the understanding of agro-ecosystems and thus the provision of biocontrol to agriculture itself (Crowder and Jabbour 2014; Krey et al 2021; Yang et al 2021; Sentis et al 2022), it is important to expand scientific research to entire agro-ecosystems when investigating the biocontrol of a key pest species (Macfadyen et al 2014; He et al 2019; Smith et al 2019; Hasan et al 2020). While much valuable information has been drawn from experiments focusing on singular pest-prey/biocontrol agent relationships in crops, it is essential to build a solid understanding of the agro-ecosystem that the study species exist within, and how this environment affects them (Olfert et al 2002; Krey et al 2021; Yang et al 2021).

For example, the species composition of a crop's arthropod community changes over time and this can lead to different pest pressures in the crop as the growing season progresses (especially in multiyear crops such as clover seed crops) (Togni et al 2019; Mhlanga et al 2020). Often how predators and other beneficial arthropods influence crop pest populations is poorly understood and many essentially free ecosystem services are not capitalised on (Crowder and Jabbour 2014; Bajwa et al 2020). Furthermore, many of the species present in a crop's arthropod community naturally compete with each other for a range of resources (Liu and Stiling 2006; Deutsch et al 2018). This competition can be naturally limiting to species and so pest species populations tend to rapidly build up in the absence of these natural pressures (Tscharntke et al 2005b; Brevault and Clouvel 2019; LaCanne and Lundgren 2019; Fortuna et al 2022).

The application of pesticides within conventional agriculture is often prophylactic, meaning they are usually applied in the anticipation of unknown pest populations building or known pest presences worsening (de Freitas Buenno et al 2011; Simon-Delso et al 2014; Vojvodic and Bazok 2021). While this limits the damage caused by developed pest populations within a conventional agriculture system, it does have undesirable ecological consequences and creates a reliance on the continued use of pesticides (Krauss et al 2011; Sluijs et al 2014; Sargent et al 2022).

In efforts to address these issues, a major goal of agro-ecological science is to build understanding of the ecology of agricultural systems that can be used to inform the development of practices that work to reduce the impact and cost of conventional agriculture, but still maintain the high levels of yield required to support society (Pingali 2012; Pretty 2018). Because modern agriculture techniques

were developed quickly, and their success immediately became integral to society, the impacts were difficult to anticipate, and environmental damage has occurred as a side effect (Lewontin and Berlan 1986; Pingali 2012; Lykogiannni et al 2021).

The role of agro-ecological research in this setting is to develop understanding of crop ecosystems to a point where management practices can be confidently informed by this knowledge (Krey et al 2021; Dorman et al 2022). With better-developed understanding of these elements, researchers in other fields can better inform their own approaches to the improvement of productive technologies and management practices (Ortiz et al 2021).

The effectiveness of agri-applications during the development of modern agriculture has meant that a lack of knowledge around agro-ecological elements has always been able to be worked around through the application of a range of powerful interventions (Pingali 2012; Pretty 2018; Sargent et al 2023). However, with increased pesticide resistance and undesirable environmental impacts this lack of understanding is becoming an increasing challenge to the improvement of these interventions and mitigation of their impacts (Krauss et al 2007; Lykogiannni et al 2021).

In natural ecosystems, monocultures are rare (Altieri 2009). Usually there is a diversity of plant species growing together that support a range of other species (Sanchez-Bayo 2021; Sanchez-Bayo et al 2021). In these natural ecosystems, a species' range is where it can live and includes a range of host plants and areas that are all overlapping but often limited due to environmental conditions, competition and predation/parasitism (Gurr et al 2003; Ortiz et al 2021). However, New Zealand agricultural landscapes include segregated blocks of monoculture, which represent low diversity ecosystems stitched together with a wider but still reduced diversity network of mainly exotic grasses and conifer shelter belts (Gurr et al 2003; Blackwell et al 2008; Smith et al 2012; Goldson et al 2020). This leads to lower diversity landscapes that are highly susceptible to pest invasion of all kinds; this includes the establishment of new alien species and the fast flow of new genetics and species within these landscapes through recolonisation (Norris 2008; Kramer et al 2011; Goldson et al 2020; Wilson and Fox 2020).

General surveys of arthropod communities are useful for building an understanding of crop ecosystems (Doxon et al 2011). They can be designed to explore aspects of how a crop influences its arthropod community and how the arthropod community itself changes over time. There are many methods used for these general surveys and those employed always depend on the goals and constraints of a given study (Cardoso et al 2009). With the above points in mind, the cataloguing of the arthropods present in a crop and their dynamics through a crop's growth cycle is always a worthwhile pursuit. This accumulation of information on the arthropod community present within

the crop can help explain some of the patterns and outcomes observed by growers and be key to developing more specific and effective management strategies (Caballero-Lopez et al 2010).

In this chapter, a general survey of the arthropod community is used both to explore those arthropod species present in clover seed crops, how this community is influenced by the season and other crop factors, and to provide a strong base to explore and evaluate hypotheses surrounding the biocontrol of the red clover casebearer in New Zealand.

The evaluation of hypotheses will carry on to further chapters as the potential roles of different species in the regulation of the red clover casebearer in New Zealand are more specifically explored. Special focus will be given to the presence of parasitoids that use the red clover casebearer as a host because of the important role that Hymenopteran parasitoids are suspected of having in the biocontrol of the white clover casebearer species (Pearson 1989).

Surveys of white clover seed crops are also beneficial to these investigations as they provide a parallel system that faces similar pest pressures. Furthermore, the successful control of white clover casebearer species in New Zealand white clover seed crops provides an interesting comparison to red clover seed crops as the former represent a dense supply of developing clover seed for red clover casebearer larvae but are not reported to suffer the same level of red clover casebearer outbreaks. With these comparable systems it will be possible to explore how different aspects of clover seed growing systems influence red clover casebearer populations and the possible causes of outbreak and regulation of this pest species.

Some of the most common techniques used to sample arthropod communities present in crops are sweep netting, vacuum/suction sampling, pitfall trapping, sticky traps and light trap capture (Grootaert et al 2010). All these methods have unique applications and are the preferred technique for the capture of certain groups of arthropods or types of data (Hillhouse and Pitre 1974; Grootaert et al 2010)

Light traps involve using a powerful specially made light bulb, which draws adult moths from over a large distance at night (Morris 1960). Pitfall traps are useful for sampling crawling arthropods such as beetles and are most frequently used in systems with a high prevalence of predatory ground beetles (Luff 1975; Woodcock 2005). Other methods such as sticky traps are poor for arthropod diversity sampling but are frequently used to assess numbers of specific flying pests around crops (Kuno 1991; Preti et al 2021).

Overall, the most common methods of sampling used to assess the composition of crop arthropod communities are sweep netting and suction/vacuum sampling (Doxon et al 2011). Sweep net sampling has been a staple method of entomological research for nearly 200 years and is still considered effective for sampling arthropods (Buffington and Redak 1988; Doxon et al 2011). Sweep nets are able to catch arthropods that are flying in the air or stationary on surfaces. The strong wire-loop the net is fixed to allows the user to apply some force and pass the net through herbaceous shrubbery and crops, effectively knocking arthropods off plants and into the net.

Suction sampling is a much newer invention. Various forms were trialled from the 1930s through to the 1960s in America which led to the development of the D-vac which was widely used but not particularly user friendly. In the early 1990s, the 'Vortis' suction sampler, a handheld and more user-friendly version was invented (Arnold et al 1974; Arnold 1994). In more recent years modified leaf blowers have become the most popular version of this technology. Modified leaf blowers are light and very easy to use, with the user simply filling a sock on the end of the blower with the sample and emptying the collected sample into a container (Grootaert et al 2010).

How a method of sampling captures arthropods can limit its ability to detect the influence of factors that affect arthropods. Bakker et al (2022) found that sweep net sampling, but not sticky trap sampling, detected a decrease in arthropod diversity after insecticide was sprayed on to a range of crops in the Netherlands. While there are multiple reasons for this, a major one is that the species most commonly found on sticky traps are also those that are typically least affected by pesticide sprays at a population level such as Diptera species (Bakker et al 2022). Sticky traps also have a low rate of attraction and collection of most species so often they do not provide a strong enough sample to detect the population changes occurring in the majority of arthropod species (Bakker et al 2022).

3.2 Aims addressed in this chapter

- Aim 1: Improve understanding of the population dynamics of arthropod populations in red clover seed crops in New Zealand. Sampling will be conducted across the growing season with the aim of developing a sampling regime that best captures the diversity of arthropods present in the field. The data from this sampling will be used to explore the other aims for this chapter.

Hypothesis: That the species composition of the arthropod community present in red clover seed crops is similar to that of white clover seed crops with some adjustment for host plant species-specific arthropods.

- Aim 2: Explore how these dynamics change over the growing season. This sampling will also include white clover seed crops as a comparative growing system. The close genetic relation of red and white clover along with the studied impacts of clover casebearers on these crops will be useful for ruling out hypotheses relating to the biocontrol of these pests in these crops. Special attention will also be given to important arthropod species and how their populations are possibly impacted by shifts in resource availability or arthropod community composition.

Hypothesis: There will be changes in the composition of the arthropod community present in red clover seed crops as the environmental conditions change over the growing season.

- Aim 3: Develop an understanding of the biocontrol provision within red clover seed crops by resident arthropods. During this sampling factors relating to crop development will be explored. In particular, areas of interest are the mechanical topping that occurs in most fields and the impact of insecticide sprays. Through this sampling potential, biocontrol agents of the red clover casebearer that are resident within red clover seed crops will be identified. Much of this focus will be on exploring the hymenopteran parasitoid assemblage in red clover seed crops in preparation for further investigation of parasitoids of the red clover casebearer. For the further ecological aims of this exploratory work the biocontrol assemblage of the red clover seed crops will be analysed with the aim of understanding how this part of the arthropod community is impacted by the crop it resides in.

Hypothesis: the red clover casebearer's life history and protective casing will make it a difficult prey item for most arthropod predators.

3.3 Preliminary sampling experiment

3.3.1 Introduction

Initially this work began as an exploration of the arthropod community present in red clover seed crops using suction sampling and some sweep netting. As knowledge of the crop and the issues it faced was built, this initial work became the precursor for the larger field comparison experiment developed in this chapter. Suction sampling and sweep net sampling were conducted in two red clover seed crops to assess these collection methods, and to develop an understanding of the crop's arthropod diversity over the growing season. These data were used to explore the efficiency of the two collection methods, especially when it came to processing samples in the laboratory. The results were used to develop the methods for the final field comparison experiment.

3.3.2 Methods

The Kowhai and Tancreds fields (Chapter 2) at Lincoln were used for the initial sampling. They were sampled on three occasions from early spring until late summer in 2021. The suction sampling regime was four five-second suctions along four transects that intersected the field with sampling points set at a log scale (1, 2, 4, 8, 16m...centre). This regime aimed to gather data on which arthropod species were present in the crop and how each species changed in abundance through time and space. During January, sweep net samples were also taken to compare with the suction samples.

For each set of four five-second suction samples, the contents were collected in a 'mesh sock' fitted into the nozzle of the suction sampler, then emptied into a plastic container and frozen for sorting in the laboratory (Section 3.4.2). This process was time consuming for both sampling and sample sorting, taking around twice as long to complete compared to the sweep net sampling regime.

Species were identified and sorted into RTUs based on prior knowledge, the use of keys and professional advice as outlined in section (2.4.2).

3.3.3 Results and discussion

In the Kowhai field, arthropod numbers increased during November, but after the crop was mowed in early December, the number of beneficial arthropods substantially decreased (Table 3.1). At Tancreds, where the crop was grazed, total arthropod number was greater in early December than in November (Table 3.1).

Table 3.1: Total number of arthropods from suction sampling within two red clover seed crops.

Sampling Date	Beneficial arthropods		Pest arthropods	
	Kowhai	Tancreds	Kowhai	Tancreds
4/11/2020	182	113	38	6
28/11/2020	254	-	61	-
4/12/2020	-	189	-	64
18/12/2020	123	-	54	-

Suction sampling found a range of species including lacewing, ladybirds, damsel bugs and species of spiders across the season; these same species were also captured in sweep netting. As the efficiency of the methods was explored, it became apparent that long sweep net transects were the simplest method for collecting a good number of arthropods. This sweep net sampling regime quickly developed into 100m transects, as it was the most efficient way to collect this kind of sample.

While suction sampling can be effective at sampling clover seed crops, overall, this method is more time consuming than sweep netting as well, as the equipment is much heavier to transport in the field.

3.4 Field comparison study

3.4.1 Introduction

Expanding on the preliminary experiment, this field study was designed to further explore the arthropod community present in New Zealand clover crops. Could the arthropods present in the crops be related to the sudden decline of red clover casebearer from 2018 onwards? How widespread were the Hymenopteran parasitoids (*B. variegator* etc.) in red clover seed crops, and were they providing biocontrol of the red clover casebearer?

Some white clover seed crops were sampled in the second year, to explore whether there were fundamental differences (such as the absence of key biocontrol agents) in the arthropod community between the crops that could explain why there were outbreaks of the red clover casebearer but not white clover casebearer. However, the continued decline of the red clover casebearer during this time across New Zealand suggested that this was not the case.

3.4.2 Methods

A total of 19 red clover and 5 white clover seed crops (Figure 3.1, Table 3.2) were sampled once in a season. The sampling locations were chosen because they represent areas where red clover seed crops are common and in turn, where red clover casebearer outbreaks were previously recorded (Chynoweth et al 2018).

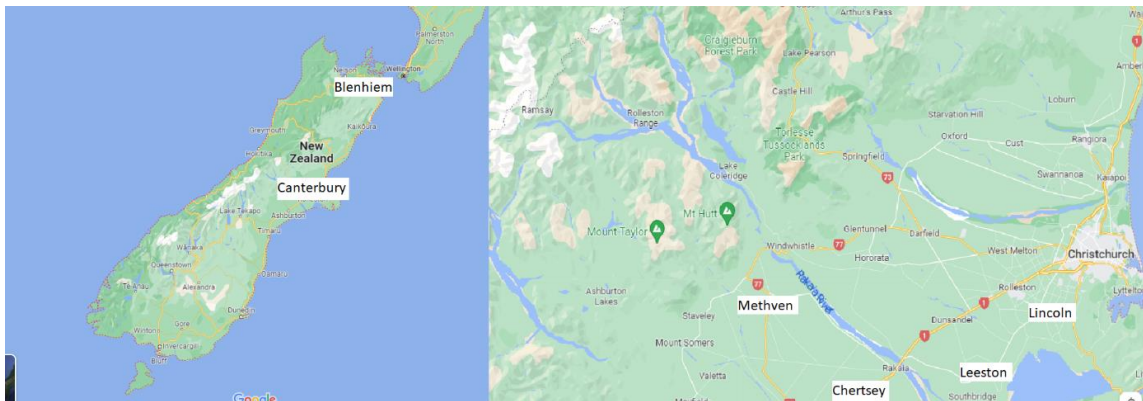


Figure 3.1: Map of the South Island showing the broad sampling regions of Blenheim and Canterbury (left) and more specifically those sampling areas in Canterbury; Lincoln, Leeston, Chertsey and Methven (right).

Table 3.2: The fields sampled along with their respective location, seed crop management type, crop species and crop age.

Site names	Location	Management	Clover species	Age (years) in first year of sampling	Years sampled
Kowhai	Lincoln	FAR ¹ (research)	Red	3	2021 and 2022
Tancreds	Lincoln	PGG ² (seed multiplication)	Red	2	2021
Robinsons	Lincoln	PGG (seed multiplication)	Red	1	2021
Chertsey	Chertsey	FAR (research)	Red	3	2021
Smith 1	Leeston	Farmer	Red	2	2021
Smith 2	Leeston	Farmer	Red	2	2021
Marr 1	Methven	Farmer	Red	1	2021 and 2022
Marr 2	Methven	Farmer	Red	1	2021
Marr 3	Methven	Farmer	Red	2	2021
Marr 4	Methven	Farmer	Red	2	2021
Ross Richards 1	Methven	Farmer	Red	3	2021 and 2022
Ross Richards 2	Methven	Farmer	Red	1	2021 and 2022
Grasslanz red	Lincoln	GrassLanz ³ (seed multiplication)	Red	1	2022
Grasslanz white	Lincoln	GrassLanz ³ (seed multiplication)	White	1	2022
Kowhai Pasture 1	Lincoln	FAR pasture (grazing)	White	5	2022
Kowhai Pasture 2	Lincoln	FAR pasture (grazing)	White	5	2022
Maw 1	Methven	Farmer	White	2	2022
Maw 2	Methven	Farmer	White	2	2022
Maw 3	Methven	Farmer	White	2	2022
B 1	Blenheim	Farmer	Red	2	2022
B 2	Blenheim	Farmer	Red	2	2022
B 3	Blenheim	Farmer	Red	2	2022
B 4	Blenheim	Farmer	Red	1	2022
B 5	Blenheim	Farmer	Red	1	2022

¹ Foundation for Arable Research, ² PGGWrightson Seed Ltd, ³ Grasslanz Technologies Ltd.

Each region has different climatic conditions, but they are all favoured seed production areas in New Zealand. Lincoln, Leeston and Chertsey are dry and windy in summer, and many of the roadsides and unirrigated pastures become brown (NIWA 2022). Methven is situated near the base of substantial mountains which leads to increased rainfall. Blenheim, at the top of the South Island, is a former seed producing area that has predominately moved into vineyards for wine production. The climate is similar to Lincoln, but all of the seed crops sampled were growing under centre pivot irrigation.

At peak flowering for the crops in each region a sweep net sample was collected. To determine peak flowering, farmers and other relevant experts were consulted. Visually this was the week or so where the crop is mostly covered in flower and little foliage is visible (this can occur from mid-January to early February across clover growing regions in New Zealand). Over this time, arthropod activity is at its highest, and it thus represents the best period to sample the highest diversity of arthropod species in the crop.

Aside from peak flowering, two other conditions were accounted for when sampling. Sampling was not conducted on rainy days or when dew was present. Temperature and wind were also considered important, as there was greater arthropod activity on sunny still days. Sampling was only conducted when it was above 20°C but below 25°C and was not conducted when there was more than a light breeze. Any of these adverse conditions reduced the diversity in the captured sample, and sometimes, also led to lower quality samples that were undesirable for laboratory processing.

A 100m sweep net sample was collected from each field. This was done by walking 100m sweeping the net in front of oneself from above the canopy and down through the middle height of the crop and up above the canopy again on the other side. This trajectory covered most of the red clover habitat and provided samples abundant with a range of arthropod groups. In fields under 100m in length, the longest path possible was swept. This was usually a diagonal across the field. The contents of this sweep were then transferred into a round 1L container that fitted flush with the sweep net. Afterwards the contained sample was placed on ice in a chilly bin before transportation to Lincoln University where the samples were stored in a freezer (at -30°C) for at least three days to kill all arthropods in the sample.

After this, the samples were processed in the laboratory. This consisted of spreading out the contents of a container across a large white tray under bright lights and using entomological forceps to first remove any plant material and then sort the arthropods on petri dishes by order. Finally, the arthropods sorted from the sample were grouped by RTUs (Recognisable taxonomic units) and the data were recorded.

The RTUs were developed first through the identity of known and easy to identify species such as the brown Tasman lacewing or red clover casebearer larvae. Other less easily identified species were assigned a name based on a unique and recognisable feature such as 'three white-striped fly' or '*Bracon* white-banded'. Close attention was paid to species of similar morphology, but some species that were very morphologically similar would have been grouped together.

The data were then analysed in R Studio using a principal coordinates analysis (PCoA). A PCoA is used to help visualise spatial and temporal data sets by reducing the dimensionality when mapping many

data (Mohammadi and Prasanna 2003). Data are run through various algorithms to fit linear connections of least difference. A PCoA graph aims to display the data in a way that highlights the strongest relationships between data points (R development team 2021).

3.4.3 Results

A wide range of arthropod species were sampled across the duration of this experiment. The most commonly found species were typically members of Diptera (Table 3.3). The most abundant biocontrol agents were money spiders, the eleven-spot ladybird, Tasman lacewing, small hoverfly, *Bracon variegator* and the pacific damsel bug (Table 3.3). The most sampled pest species were the Australian crop mirid, *Thrips* species, aphid species, the clover casebearer and the potato mirid (Table 3.3).

Invertebrate RTU diversity increased in red clover as the seed crops aged (Figure 3.2). However, there was only a significant difference between first- and third-year crops with second-year crops being intermediate (data averaged across both years of sampling).

In 2021, the parasitoid *B. variegator* was sampled from six of the fields where the red clover casebearer was present. However, there were still seven fields where the latter was present, but the former was not (Table 3.4). This wasp is therefore frequently present alongside the red clover casebearer, but these species also occur in the absence of each other, which partially confirms that *B. variegator* is not a *Coleophora* spp. specialist in New Zealand.

Figures 3.3 and 3.4 are principal component analysis (PCoA) graphs of the sampled arthropod diversity from each of the clover crops. On these graphs the ordinance of the fields is plotted by arthropod diversity X Abundance of each RTU in that field X the age of the crop. The result is a scatter plot showing the relationship between each sampled crop based on these factors.

Figure (3.3) produced a PCO1 value of 27% and PCO2 value of 14%. These statistical outputs indicate that the red clover crop age as a significant impact on arthropod RTU richness that is present. Highlighted in green and orange on Figure (3.3), is a wide grouping of the 1st year Methven crops, and on the right-hand side of the graph highlighted in red and blue is a much tighter grouping of points from Lincoln, Leeston and Chertsey (this grouping does include one 1st year crop Tancred's). Highlighted in orange and red, in between the two groups is the third year Ross Richards 1 crops located in Methven.

In Figure (3.4), all of the sample points are added to include 28 samples across two years (2021-2022). The relationships between the data points produce PCO1 and PCO2 values of 19.4% and 12.9% respectively (Figure 3.4). This represents a marginally weaker relationship between data points

in Figure (3.3). Highlighted in blue and red on Figure (3.4), is a tight grouping of the second- and third-year crops located in Lincoln, Leeston and Chertsey. At the top of the graph highlighted in brown are the Blenheim crops. Highlighted in yellow across the middle portion and to the right-hand side of the graph are the white clover seed crops. Highlighted in green on the right-hand side of the graph are the two pasture samples located in Lincoln.

As indicated by the outlined groupings in Figure (3.3) and (3.4), the region crops were located in also influenced the composition of the arthropod community present in the clover seed crops.

Table 3.3: Identified species sampled, these species are presented with the most specific identify able to be obtained by the author. Total abundance represents the total numbers of individuals sampled across all fields and both years (2021 and 2022), except in the case of aphid and *Thrips* species that were not counted in samples beyond presence and absence.

Species name	Higher classification	Function in relation to clover	Total Abundance
<i>Acalyptate</i> spp.	Diptera: Schizophora	Unknown/benign	643
money spiders (Linyphiidae spp.)	Araneae: Linyphiidae	Lie and wait predator	600
White stripped litter fly (<i>Poecilohetaerella bilineata</i>) (Hutton)	Diptera: lauxaniidae	Benign	600
Shore flies (Ephydriidae spp.)	Diptera: Ephydriidae	Benign	550
Eleven-spot ladybird (<i>Coccinella undecimpunctata</i>)	Coleoptera: Coccinellidae	Active searching predator	547
Spear winged fly species (<i>Lonchoptera bifurcate</i>) (Fallen)	Diptera: Lonchopteridae	Benign	400
Lacewing parasitoid (<i>Anacharis zelandica</i>)	Hymenoptera	Parasitoid of the lacewing	390
Vinegar flies (<i>Scaptomyza</i> spp.)	Diptera: Drosophilidae	Benign	350
Australian crop mirid (<i>Sidnia kinbergi</i>)	Hemiptera: Miridae	Pest	341
Tasman lacewing (<i>Micromus tasmaniae</i>)	Neuroptera: Hemerobiidae	Predator	340
Red clover casebearer (<i>Coleophora deauratella</i>)	Lepidoptera: Coleophoridae	Pest	250
Small hoverfly (<i>Melanostoma fasciatum</i>)	Diptera: Syrphidae	Pollinator and Predator	238
<i>Bracon variegator</i>	Hymenoptera: Braconidae	Parasitoid of red clover casebearer	198
Pacific damsel bug (<i>Nabis kinbergii</i>)	Hemiptera: Nabidae	Ambush predator	188
House flies (<i>Musca</i> spp.)	Diptera: Muscidae	Benign/pollinator	160
Aphid parasitoids (<i>Aphidius</i> spp.)	Hymenoptera: Braconidae	Aphid parasitoid	89

Nine-spot ladybird (<i>Coccinella novemnotata</i>)	Coleoptera: Coccinellidae	Active searching predator	79
Potato mirid (<i>Closterotomus norvegicus</i>)	Hemiptera: Miridae	Pest	78
Long tongue bumble (<i>Bombus hortorum</i>)	Hymenoptera: Apidae	Pollinator	75
Large hoverfly (<i>Melangyna novaezelandica</i>)	Diptera: Syrphidae	Pollinator and Predator	72
Dronefly (<i>Eristalis tenax</i>) (L.)	Diptera: Syrphidae	Pollinator	70
Seven-spot ladybird (<i>Coccinella septempunctata</i>)	Coleoptera: Coccinellidae	Active searching predator	64
European harvestman (<i>Phalangium opilio</i>)	Opiliones: Phalangidae	Active searching predator	60
<i>Pteromalus puparum</i>	Hymenoptera: Pteromalidae	Parasitoid	59
<i>Bracon</i> spp.	Hymenoptera: Braconidae	Parasitoid	58
Orb-weavers (Araneidae spp.)	Aranae: Araneidae	Lie and wait predator	47
Hoverfly parasitoid (<i>Diplazon laetorius</i>) (F.)	Hymenoptera: Ichneumonidae	Hoverfly parasitoid	43
Rove beetles (Aleocharinae spp. among others)	Coleoptera: Staphylinidae	Active searching predator incl. below ground	41
Wheat bug (<i>Nysius huttoni</i>) (White)	Hemipteran: Lygaeidae	Pest	40
Porina moth (<i>Wiseana</i> spp.)	Lepidoptera: Noctuidae	Pest	36
Two-spot ladybird (<i>Adalia bipunctata</i>)	Coleoptera: Coccinellidae	Active searching predator	27
<i>Leioproctus</i> spp.	Hymenoptera: Colletidae	Pollinator	25
Pirate bugs (<i>Orius</i> spp.)	Hemiptera: Anthracoridae	Possibly predator and herbivore	22
Cabbage white butterfly (<i>Pieris rapae</i>)	Lepidoptera: Pieridae	Pest	20
Wolf spiders (Lycosidae spp.)	Aranae: Lycosidae	Active searching predator	20
Jumping spiders (Salticidae spp.)	Aranae: Salticidae	Active searching predator	16
Caledonia seed bug (<i>Nysius caldoniae</i>) (Distant)	Hemipteran	Pest	15
Clover root weevil (<i>Sitona Lepidus</i>)	Coleoptera: Curculionidae	Below ground pest	13
European earwig (<i>Forficula auricularia</i>)	Dermaptera: Forficulidae	Omnivore	12
<i>Eupelmus messene</i>	Hymenoptera: Eupelmidae	Parasitoid	11
Weevil parasitoid (<i>Microtonus</i> spp.)	Hymenoptera: Braconidae	Weevil parasitoid	11
Blowflies (<i>Calliphora stygia</i>) (F.)	Diptera: Calliphoridae	Benign/pollinator	10

Meadow spittle bug (<i>Philaenus spumarius</i>) (L.)	Hemiptera: Aphrophoridae	Pest	10
Robber fly (<i>Neoitamus melanopogon</i>) (Schiner)	Diptera: Asilidae	Flying ambush predator	8
Harlequin ladybird (<i>Harmonia axyridis</i>) (Pallas)	Coleoptera: Coccinellidae	Biocontrol antagonist/predator	7
<i>Alloxysta virtrix</i>	Hymenoptera: Figitidae	Hyper-parasitoid of <i>Aphidius</i> spp.	5
Pea aphid (<i>Acyrtosiphon pisum</i>)	Hemiptera: Aphidoidea	Pest	very common
Black bean aphid (<i>Aphis fabae</i>)	Hemiptera: Aphidoidea	Pest	N/A very common
Honeybee (<i>Apis mellifera</i>)	Hymenoptera: Apidae	Pollinator	N/A common
Onion thrips (<i>Thrips tabaci</i>)	Thysanoptera: Thripidae	Pest	N/A common
Red clover thrips (<i>Halothrips leucanthemi</i>)	Thysanoptera: Phaeothripidae	Pest	N/A common
Short tongue bumble (<i>Bombus terrestris</i>)	Hymenoptera: Apidae	pollinator	n/a common
<i>Brachophagus gibbus</i>	Hymenoptera: Eurytomidae	Pest of seeds	5 or less
Broom seed beetle (<i>Bruchidius villosus</i>) (F.)	Coleoptera: Chrysomelidae	Seed pest	5 or less
Corticariinae spp.	Coleoptera: Latridiidae	Unknown/obligate fungi feeder	5 or less
European paper wasp (<i>Polistes dominula</i> Christ)	Hymenoptera: Vespidae	Predator	5 or less
Lemon borer parasitoid (<i>Glabridorsum stokesii</i>) (Cameron)	Hymenoptera: Ichneumonidae	Parasitoid	5 or less
<i>Helophilus seelandicus</i>	Diptera: Syrphidae	Pollinator	5 or less
Stone centipede (<i>Lithobiomorpha</i> spp.)	Lithobiomorpha: Lithobiidae	Active searching predator	5 or less
magpie moth (<i>Nyctemera annulata</i>) (Boisduval)	Lepidoptera: Noctuidae	Weed biocontrol agent	5 or less
<i>Orius insidiosus</i>	Hemiptera: Anthracoridae	Predator	5 or less
Common grass moth (<i>Orocrambus</i> spp.)	Lepidoptera: Crambidae	Pest	5 or less
southern blue butterfly (<i>Zizina</i> spp.)	Lepidoptera: Lycaenidae	Pollinator	5 or less
Yellow admiral (<i>Vanessa itea</i>) (F.)	Lepidoptera: Nymphalidae	Pollinator	5 or less
Blue green lucerne aphid (<i>Acyrtosiphon kondoi</i>)	Hemiptera: Aphidoidea	Pest	very common

Table 3.4: Abundance of red clover casebearer (RCCB) and *B. variegator* found in a 100m sweep net sample collected in 2021 (data is presented for crops where these species were found only).

Field	RCCB abundance	<i>B. variegator</i> abundance	Crop age
Richards 1	38	2	3
Richards 2	3	15	1
Kowhai	57	127	3
Tancreds	0	2	2
Robinsons	0	1	1
Chertsey	0	25	3
Smith 1	0	8	2
Smith 2	0	8	2
Marr 2	5	0	1

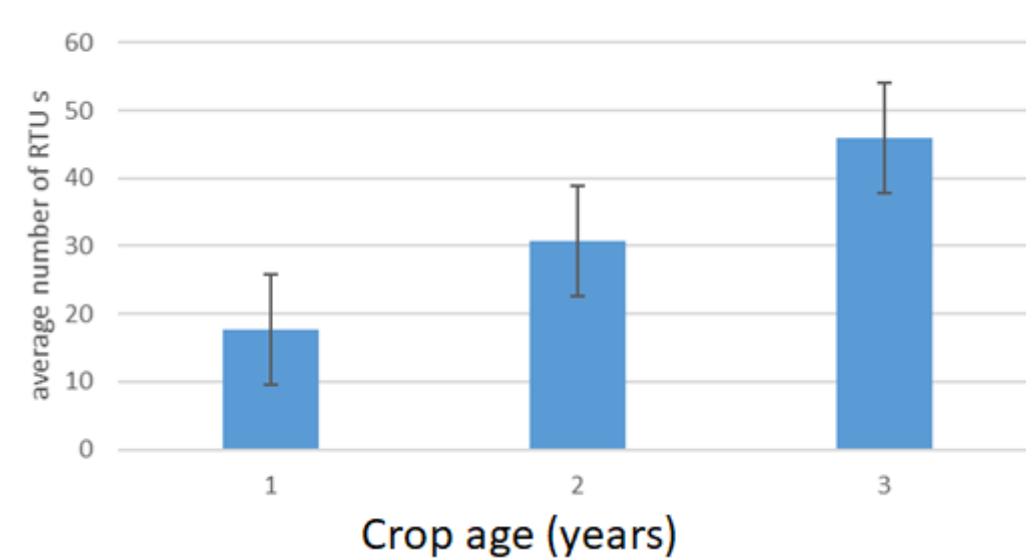


Figure 3.2: Average number of RTUs by red clover crop age (2021 and 2022).

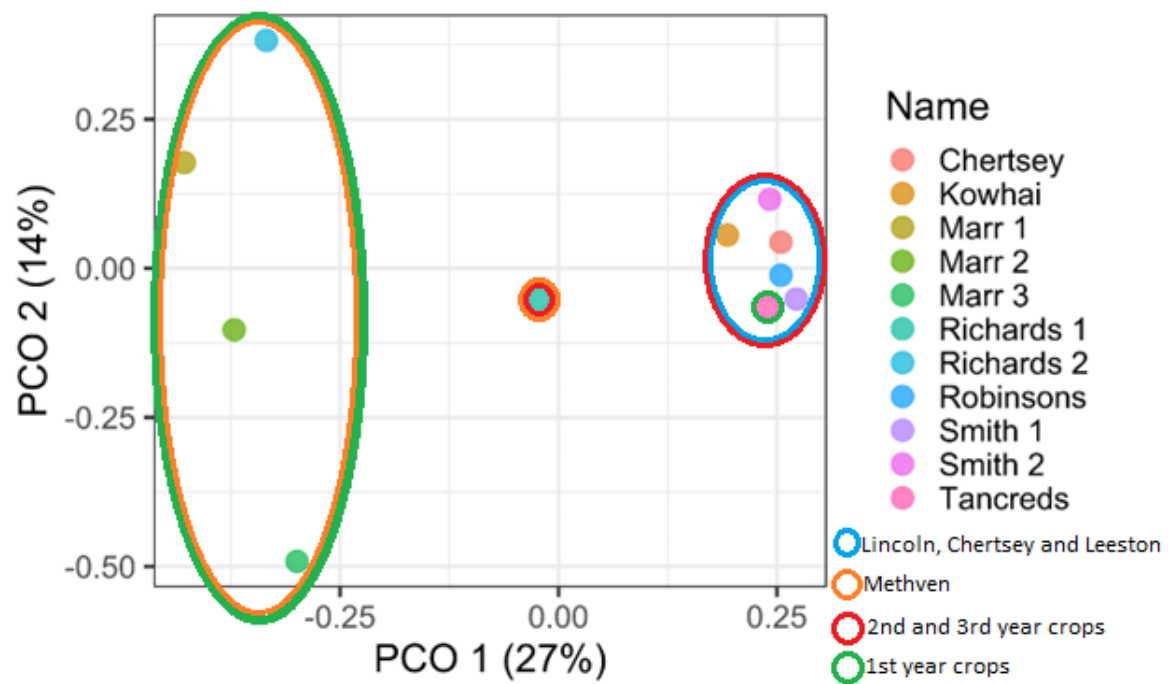


Figure 3.3: PCoA analysis of RTU diversity X abundance of RTUs X red clover crop age 2021.

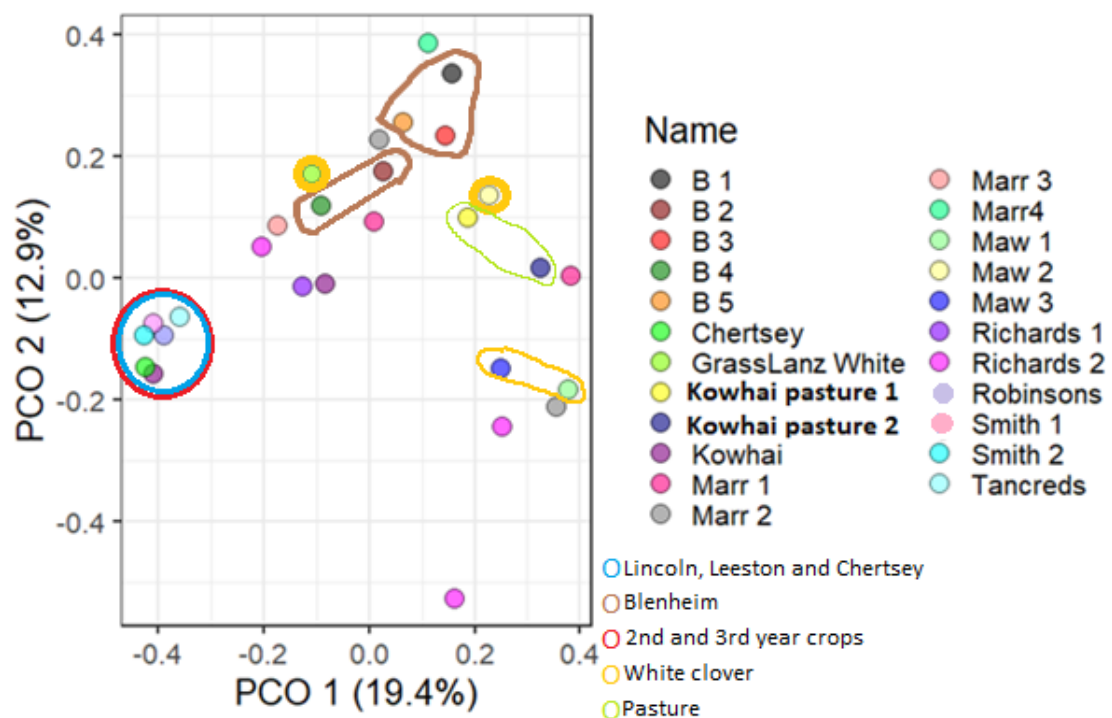


Figure 3.4: PCoA analysis of RTU diversity X abundance of RTUs X clover crop age 2021 and 2022 data.

3.4.4 Discussion

The age of the red clover seed crop influenced the insect community, and it was clear some important species such as the red clover casebearer were only ever abundant in older red clover seed crops. The pattern of a higher abundance of the red clover casebearer in older red clover seed crops has been shown overseas and was recorded during the initial outbreaks in New Zealand (Landry 1991; Hardwick and Barton 2019). However, the red clover casebearer is clearly now being suppressed effectively, and thus no peaks in abundance of this pest occurred in any of the first- or second-year red clover crops sampled.

The location of the crops had some influence of the composition of the arthropod community in the clover seed crops, however, the red clover casebearer and *B. variegator* were present in Methven, Lincoln, Chertsey and Leeston. Which indicates this factor was not particularly important to red clover casebearer pest issues.

Some changes must have occurred in the red clover seed crop ecosystem since 2018, as the red clover casebearer was not recorded at the infestation levels seen from 2016 to 2018 (Chynoweth et al 2018). Nonetheless, it remains unclear which biocontrol agents or process are directly responsible for this reduction of the red clover casebearer population. This point, although straightforward, is pivotal to understanding the state of the red clover casebearer as a pest in New Zealand.

The five Blenheim crops are under centre-pivot irrigation, a management form that was absent from the other sampled crops. This coupled with the regional affect may explain the similarities between the arthropod community of these crops.

The white clover fields sampled displayed some similarities to each other, however only a limited number of white clover crops were sampled. Some red clover seed crops such as Kowhai pasture 1 and 2 and Marr 1 and 2, were also fairly similar to the white clover crops sampled. This highlights that while white clover seed crops probably do support similar communities of arthropods, they are also not overly unique from the arthropod communities of red clover seed crops.

There are important physical differences between these two plants and the crop habitat they form, which may influence arthropod community composition. Red clover has an upright growth habit, that forms a dense herbaceous habitat through an overlapping network of plants, with abundant flowers from knee to waist height (Bowley et al 1984). Whereas white clover is stoloniferous, meaning it grows along the ground and produces less biomass leading to a lower and less dense canopy with flowers rarely occurring above knee height (Kendall and Stringner 1985). Because of the physiology of white clover, it flowers and is harvested earlier which means the yield of a white clover seed crop is more established before the peak of summer which creates a lower demand for water

(Caradus et al 1995; Barrett et al 2005). In contrast, red clover seed crops are harvested after summer and so these crops must receive sufficient water to produce profitable yields, leading to a lush crop habitat (Vleugels et al 2019; Riggi et al 2021).

Low growing white clover habitat is much more open to the elements than that of red clover, which can adversely affect populations of arthropods. This is due to the increased drying out of the area below the crop canopy, which means there is less suitable habitat for arthropods to shelter in during intense weather (MacLaren et al 2019). However, further research is required to determine the importance of these crop habitat differences.

The reduced impact of the red clover casebearer now allows growers to manage red clover seed without having to deal with the intense pressure of the red clover casebearer. In other terms, the apparent biological regulation of this pest means that human intervention is not required so intensely, and therefore, no major changes to the current growing system need to occur to avoid the damage caused by this pest.

Following outbreaks of the red clover casebearer a broad-spectrum insecticide (Chlorpyrifos) was used to reduce the rate of crop damage but still massive losses of up to 80% in seed yield occurred in New Zealand red clover seed crops between 2016 and 2018 (Chynoweth et al 2018). Although these insecticidal sprays do kill large numbers of the red clover casebearer, due to the concealed nature of its lifecycle many survive, while a high proportion of non-concealed pest and beneficial arthropod species die (Desneux et al 2007; Chynoweth et al 2018).

Insecticide use can lead to different balances of species and the release of pests from biocontrol pressure (Liu and Stiling 2006; LaCanne and Lundgren 2018; Gagic et al 2021). However, due to the red clover casebearer's niche bound life cycle, a lack of competition for key resources and few ecological enemies, it is unlikely this use of insecticide resulted in any kind of release of red clover casebearer populations from forces of control and competition (Tscharntke et al 2005b; Chynoweth et al 2018).

Earlier experiments investigated the impact of mechanical crop topping (mowing) on red clover casebearer outbreaks, but during the observed outbreak years the red clover casebearer was so abundant that no differences were found between topping and not topping the crop (Hardwick and Barton 2019). However, significant reductions in beneficial insect species that lasted for up to one month after topping were recorded (Hardwick and Barton 2019).

In addition, many of the arthropod species present in any agricultural crop are generalist species. These species will always colonise the crops they are able to survive in because they consistently

have abundant populations across farming landscapes (Alomar et al 2002; Tscharnkte et al 2005a; Michalko and Dvoryankina 2019). In contrast, specialised insect species are reliant on the abundance of their host(s), and so can be strongly affected by landscape composition (Kheirodin et al 2020). In areas with a high density of a pest herbivores host species (monocultures), damaging populations can quickly develop (O'Rourke et al 2011).

This is also true for the development of populations of specialised parasitoids of important pests providing they can thrive in a crop ecosystem (Plečáček et al 2014; Inclan et al 2015). Typically parasitoid wasp populations lag behind the growth of their host population (Mills and Getz 1996). This may be why outside of areas that have some abundant populations of the red clover casebearer, the incidence of *B. variegator* is sporadic and abundance is low.

The sampled crops, were all within regions that have been selected for seed production by farmers and where seed production continues to be profitable. While, there are some regional differences in climate. The majority of the crops were irrigated so presumably the within crop environment was similar. Further research could be conducted on the intensity of key pest species under different levels of red clover crop irrigation, to determine whether this crop management practice has an impact pest and/or beneficial insect species.

In general, there is a very limited diversity of arthropods in New Zealand farming landscapes due to the relatively recent modification of this land and the short time for the colonisation of new species to occur (Norton and Miller 2001; Tscharnkte et al 2007; Gillespie and Wratten 2012; Macdonald et al 2018). Thus, it is expected that there would be a lot of similarity among the sampled crops.

3.5 The incidence of red clover casebearer larvae

3.5.1 Introduction

Quantifying the incidence of a pest within an agricultural crop is broadly important, and in the current case provides useful context through which to understand other aspects of this work such as the field parasitism percentage. Due to their only being two known outbreaks of the red clover casebearer in red clover seed crops this sampling was limited in its scope.

3.5.2 Methods

Samples were taken from the Richards 1 and Kowhai field, and at varying distances from the field margin in the Richards 2 crop. The aim of this sampling regime was to investigate the incidence of fourth instar red clover casebearer in crops where it was abundant (Kowhai and Richards 1). As well as to investigate the colonisation of the red clover casebearer in a younger crop (Richards 2). The sampling regime used the same methods as described in section (3.4), with a specific sampling transect at 5, 15 and 40 meters into the crop. This sampling was conducted on two dates during late summer in 2022 and at each sampling 150 flowerheads were checked for the presence of red clover casebearer larvae.

A third sampling was attempted at the Richards 1 and 2 crops in mid-March, but recent rainfall events had left the remaining larvae either dead or in a degraded states, with no living fourth instar larvae being found in any flowerheads.

3.5.3 Results

In the Kowhai crop 38-40% of flowers contained red clover casebearer larvae in mid-January and early February. However, less than 10% did by mid-February (Table 3.5). In the Richards 1 crop around 70% of flowers contained red clover casebearer larvae in mid-February/ early-March (Table 3.6). In the Richards 2 crop only 1% or less of flowers contained red clover larvae at both sampling dates, and this did not differ with distance from the edge of the crop (Table 3.7).

Table 3.5: The percentage of flowers infested with red clover casebearer larvae in the Kowhai crop.

Sampling date	Percentage of flowers infested
18/1/2022	40%
11/2/2022	38%
25/2/2022	9%
28/2/2022	7%

Table 3.6: The percentage of flowers infested with red clover casebearer larvae in the Richards 1 crop.

Sampling date	Percentage of flowers infested
20/2/2022	68%
3/3/2022	70%

Table 3.7: The percentage of flowers infested with red clover casebearer larvae in the Richards 2 crop on 20/2/22 and 3/3/22.

Distance from edge of the crop (m)	Percentage of flowers infested 20/2/22	Percentage of flowers infested 3/3/22
5m	1%	0.3%
15m	0.5 %	0.6%
40m	0.45%	0.3%

3.5.4 Discussion

A high number of fourth instar red clover casebearer larvae were sample over summer form the Richards 1 and Kowhai crop. Sampling at the Richards 1 crop was limited due to a population crash occurring after heavy rainfall, in this sampling attempt no live fourth instar larvae were found but many cadavers were, making it impossible to infer a proper incidence percentage. In the Richards 2 crop, red clover casebearer were establishing well into the field, however, there was never a high abundance of it recorded in this crop. It is unclear what factors are limiting the expansion of the red clover casebearer population in this crop.

The large differences in the percentage of flowers infected between the two third year crops is of interest, however, it is unclear what factors of crop management or development might contribute to this pattern.

3.6 General chapter discussion

No new outbreaks of the red clover casebearer were detected in any of the fields sampled over the two seasons. Both of the outbreaks recorded (Kowhai and Richards 1) were already known by the crop managers and had existed from previous seasons when the red clover casebearer was abundant across New Zealand. In addition, there were no reports of new outbreaks of the red clover casebearer reported elsewhere in New Zealand between 2020 and 2023 (Chynoweth pers. coms. 2020-2023).

Therefore, the development of high red clover casebearer populations in these two crops is probably related to higher rates of colonisation and population growth associated during the overall higher prevalence of the red clover casebearer that occurred during 2016-2018.

This is supported by fact a new red clover seed crop sown in 2020 (Richards 2) which is adjacent to a crop (Richards 1) containing a high abundance of the red clover casebearer did not develop outbreaks themselves. In addition, the two Kowhai pasture (Kowhai pasture 1 and 2) sample locations also had only low levels of the red clover casebearer despite being in close proximity to the Kowhai crop infestation.

The low rate of red clover casebearer colonisation in new red clover seed crops is unlikely because of it being a poor flyer or that it does not readily leave its birth habitat. Various pheromone surveys have shown that the red clover casebearer commonly travels hundreds of meters during dispersal flights (Chynoweth et al 2018). Therefore, it is likely that there are no species physiological traits reducing the red clover casebearer's ability to build populations from low densities (Chynoweth et al 2018). Furthermore, it is unlikely that an arthropod or arthropods are a sole major factor in the regulation of the red clover casebearer at high densities. Therefore, it is probable that a combination of factors outside of the arthropod biocontrol assemblage is important in regulating the red clover casebearer below damage causing levels.

These unidentified factors may be related to the decline of the red clover casebearer larvae in the fields as shown in the latter two sampling dates undertaken at the Kowhai crop. The decline was more drastic in the Richards crop and appeared to be related to weather conditions, namely, heavy rainfall events.

The arthropod species recovered through sweep netting, represent an expected diversity of species for New Zealand flowering crops (Wratten et al 1995). Many of these species present are generalists that are successful across the New Zealand, as well as is in the Northern Hemisphere farming landscape such as parasitoid wasps, hoverflies and weevil and *Thrips* pests (Schroder and Clifford 1995; Lundin et al 2012; Shields et al 2021; Goldson et al 2022). The major differences between New

Zealand and North-Hemisphere red clover seed crops are much lower numbers of pollen beetles (Coleoptera: Nitidulidae) (sampled in this work *Bruchidius villosus* (5 individuals)), ground beetles (Coleoptera: Carabidae) and bumblebees (Dupont et al 2011; Lundin et al 2016).

Two parasitoids that negatively impact biocontrol provision were identified among the sampled Hymenopterans. *Alloxysta virtrix* which is a hyper-parasitoid of *Aphidius* spp. (Grasswitz and Reese 1998) and *Anacharis zealandica* which parasitises the brown Tasman lacewing (Robinson 2009). While neither of these parasitoids are considered to significantly impact the biocontrol provision of their respective host species (Grasswitz and Reese 1998; Robinson 2009), further monitoring of these species may provide more information.

One species of concern that was sampled is the harlequin ladybird. Whilst initially reared and dispersed as a biocontrol agent of aphid pests in Europe and the United States (Roy et al 2016), in New Zealand it is considered invasive and was first recorded in Auckland in 2016 (Martin 2016). The harlequin ladybird has become problematic more recently as it specialises in predating on the young of aphidophagous insects (in particular other ladybird species), and it has been associated with the decline of biocontrol and native ladybird species in the United States (Roy et al 2016).

3.7 Summary

3.7.1 Improve understanding of the population dynamics of arthropod populations in red clover seed crops in New Zealand

This work provided a red clover focussed study of the arthropods present in clover seed crops, and this contributes to the overall body of work on the arthropod community in New Zealand.

In recent years, work on arthropods in clover seed crops has been focussed on key weevil pests. The present results help to generally expand on this knowledge base by providing new information on the surface-dwelling arthropod species in these crops.

3.7.2 Explore how these dynamics change over the growing season and the crop's life cycle

Over a year there are multiple events that affect the arthropod community of red clover seed crops; winter is cold and combined with little available resource within the field, there is much less arthropod activity. However, as red clover seed is a multiple year crop, many arthropod species diapause in the crop after its first growing season, allowing for more rapid development of subsequent arthropod populations (Pfiffner and Luka 2000). In late spring/early summer, seed crops are mechanically topped or grazed by sheep, both of which affect the arthropod community (although, mechanical topping appears to be more impactful) (Hardwick and Barton 2019).

3.7.3 Develop understanding of the biocontrol provision within red clover seed crops by resident arthropods

This survey work provided population data on many biocontrol agents and other beneficial arthropods that inhabit red clover seed crops in New Zealand. Although limited work was carried on all but a few of these species their cataloguing and relative abundances are useful information to gather.

Although, the red clover crop system is typically stable and not especially prone to outbreaks of known pests, new pest arrivals can have a massive impact. This is clearly shown by the red clover casebearer's sudden and intense impact on red clover seed yields in New Zealand (Chynoweth et al 2018; Hardwick and Barton 2019). In this case, it appears that biotic agents already present within the system were able to adjust to this new pest and provide effective control.

Within red clover seed crops, only the red clover casebearer was posing a serious threat to seed yields. However, by the time this work was completed in 2022, the red clover casebearer was clearly being suppressed and apparently unable to initiate new outbreaks. However, other pest species continue to adapt to growing systems and management strategies as well as accumulate further

genetic diversity through new incursions. Therefore, it is important that pest pressures continue to be monitored in clover seed crops into the future.

There has clearly been some kind of adjustment of the agroecosystem to the red clover casebearer since the time of the initial outbreaks of this pest. Whatever this is, it has had a very strong impact on the ability of the red clover casebearer to develop crop yield-threatening populations. There have not been major changes to red clover seed crop management or the introduction of any new biocontrol agents. The further experiments pursued in this study will not be able to fully explore all the possibilities, because often these dynamics involve various interacting forces and organisms that require expertise from a range of fields to understand.

Chapter 4

Investigations into *Bracon variegator*

4.1 Introduction

The parasitoid *Bracon variegator* (Spinola) (Hymenoptera: Braconidae) was first encountered through the experiments reported in Chapter 3, and during this time this parasitoid's high abundance in relation to red clover casebearer outbreaks was observed in the two crops. Based on the previous successful biocontrol programme of the white clover casebearers in the 1960s, a parasitoid wasp was considered to be a probable biocontrol agent of the red clover casebearer in New Zealand (Thomas 1969; Pearson 1989; Hardwick and Barton 2019), and so the following research was developed to investigate this.

Knowledge of natural enemies of pest insects is crucial to understanding their functions, limitations and habitat preferences in the field (Le Hesran et al 2019; Leung et al 2020). This information can be used to develop more effective biocontrol systems on farms through means such as habitat enhancement, further biocontrol agent introductions and adjustment of agricultural management practices to better suite these biocontrol agents (Tscharntke et al 2005a; Prather et al 2012). For many reasons standard agricultural practice is not always developed with biocontrol agent enhancement in mind (Dainese et al 2019). Thus, a crucial function of this type of research is to contribute to allow the formation of the necessary knowledge base needed by farmers and other developers of agriculture practice, to form more sustainable practices (Eyre and Leifert 2012; Cock et al 2013). Learning more about the function of biocontrol agents also helps to avoid over-investing in agents that are not overly effective or compete excessively with other biocontrol agents already in the system (Tancoigne et al 2014; Wood et al 2015; Michalko and Dvoryankina 2019; McGrath et al 2020; Lemay et al 2022).

The work undertaken in this chapter was developed with these points in mind, and the overarching aim was to contribute some knowledge of *B. variegator's* ability as a biocontrol agent of the red clover casebearer, as well as to further address research aims 5 and 6.

In an agro-ecological context this is important because the conclusions reached following research are often used to inform agricultural policy and practice (Dupraz and Guyomard 2019; Clay et al 2020). For this reason, it is very important that there is strong communication between agricultural science researchers, advisors and farmers (Pe'er et al 2020). This ensures that there is feedback between groups which is important to developing useful and impactful research for industry (Bouma 2019).

4.2 Laboratory parasitism investigation

4.2.1 Background

It has been clearly demonstrated that parasitoid wasps will parasitise viable host species in captivity. There are also many papers that explain how to support parasitoid wasps in the laboratory conditions to maximise longevity and parasitism (Tylianakis et al 2004; Barreto-Barriga et al 2021).

Supporting parasitoid wasps in the laboratory requires the provision of sugar-soaked cotton buds and fresh flowers (Berndt and Wratten 2005). These non-prey resources provide them with pollen, nectar and sugar. Many parasitoid species are also multivoltine and their development and emergence can be promoted under certain conditions (Tylianakis et al 2004; Berndt and Wratten 2005). These conditions can be achieved under a variety of incubation settings and shock treatments, such as cold exposure, to simulate winter (Baeza and Ohno 2007; Petrice et al 2019; Shields et al 2022a).

The parasitoids of pests discovered through these types of laboratory experiments can help identify potential biocontrol agents that may not be commonly sampled in the field, as well as allowing the exploration of the behaviour and biology of the pest and parasitoids under controlled conditions (Chailleux et al 2012; Murillo et al 2019).

Tylianakis et al (2004) combined field and laboratory experiments to explore the effects of floral resources on the reproduction of aphid parasitoids in Lincoln, Canterbury, New Zealand. They found that sugar resources significantly increased the longevity and fecundity of *Aphidius rhopalosiphii* and *Diaeretiella rapae* (McIntosh 1855) (Hymenoptera: Aphidiidae). This same mechanism was observed during field experiments where floral patches in wheat crops increased parasitism rates for up to 14 meters from their boundary compared to control areas. The benefits of increased longevity and fecundity for parasitoid wasps in biocontrol systems, is that they reduce the chances of individuals becoming limited in their biocontrol provision by time, or egg availability during the experiment (Tylianakis et al 2004).

Berndt and Wratten (2005) conducted a laboratory experiment to assess the effects of the floral resources supplied by Alyssum (*Lobularia maritima* (L.)) on the longevity, fecundity, and sex ratio of *Dolichogenidea tasmanica* (Cameron) (Hymenoptera: Braconidae), a parasitoid of leafroller moths. The longevity of individual wasps was increased up to seven times for females and three times for males with the provision of alyssum flowers. The lifetime fecundity but not daily fecundity was also increased by the provision of these flowers. It was also found that the absence of floral resources led to the sex ratio of captive *D. tasmanica* offspring being male biased, but it was approximately equal when floral resource was provided (Berndt and Wratten 2005).

Rahat et al (2007) investigated longevity of the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenopter: Scelionidae) in laboratory experiments when provided with floral resources from a range of plant species. Ten nectar-bearing flowering plant species were tested: *Lobularia maritima* L., *Brassica napus* (L.), *Tagetes patula* (L.), *Ocimum basilicum* (L.), *Fagopyrum esculentum* (Moench.), *Cosmos bipennatus* (Cav.), *Calendula arvensis* (L.), *Coriandrum sativum* (L.), *Tropaeolum majus* (L.) and *Phacelia tanacetifolia* (Bentham). The longevity of *T. basalis* was greatest (33 days) on flowering *T. patula* shoots and significantly lower (8 days) in control treatments that lacked floral resources or a simulation (sugar water). Of the ten plant species, only *L. maritima* flowers did not enhance parasitoid longevity. These results show the importance of a range of floral resources to foraging parasitoids. However, these results also emphasise that information must be sought on the suitability of flowering plant species to specific parasitoids, especially when parasitoid species will only be provided with a limited number of floral sources (Rahat et al 2007).

4.2.2 Development of methods

This laboratory experiment was devised to detect parasitoids of the red clover casebearer found in red clover seed crops. For research aims 5 and 6, it was necessary to investigate parasitism of the red clover casebearer specifically, as it was clear none of the generalist predator species present in red clover seed crops consumed red clover casebearer larvae. The work on releasing parasitoids in white clover seed crops against two other *Coleophora* spp. during the 1960s in New Zealand provided evidence for there possibly being parasitoid species that would emerge from the fourth instar larval stage of the red clover casebearer (Thomas 1969; Pearson 1989).

The 'rearing method' outlined below was chosen due to unfamiliarity with the red clover casebearer's development and its associated parasitoids (along with the benefits of using a wide search of host parasitoid species interactions in the event no parasitoids of the red clover casebearer could be detected). Furthermore, the chance of observing rare parasitism events is possibly enhanced by these contained rearing methods, due to the prolonged periods of proximity between potential host and parasitoid species (Luna et al 2007; Ahissou et al 2021; Ghosh et al 2021). Even though these less common parasitoid-host interactions are very rare in the field, it is important to document the potential host ranges of parasitoids in agricultural systems, as these findings may allude to new groups of biocontrol agents to explore for insect pest species management (Giorgini et al 2018; Gomina et al 2020; Ahissou et al 2021).

4.2.3 Methods

Eight fields (Table 4.1, selected from those described in Chapter 3) were sweep netted extra vigorously for 100m across the crop, and the contents of this sweep were transferred into a rearing box for observation. The rearing boxes were large Tupperware containers with a metal mesh bottom and plastic netting section in the lid to allow airflow (Figure 4.1). This box was then fitted into another bottom of the same sized box with no modifications to seal the mesh bottom (Figure 4.1). This sampling occurred on three dates (20/1/2021, 10/2/2021 and 20/2/2021) and each set of samples was reared for 2-3 weeks in a rearing box in the laboratory. The actual termination of each rearing box depended on the activity of the arthropods inside. After two-three weeks, rearing boxes were considered finished when the active species in the box reduced to the few that could sustain populations under these conditions, such as aphids, wheat bugs and money spiders.

Due to the vigorous sweeping method many clover flowerheads were collected in the sweep net and, these plus a sugar-water-soaked cotton bud comprised the first few day's food. On every second day the food contents of the boxes were changed, and fresh clover flowerheads with their stems wrapped in moist tissue paper were added, along with a new sugar-soaked cotton bud to provide more easily accessed resources to foraging arthropods. Careful attention was paid to the material being removed and any attached arthropods were placed back into the rearing box. Each time the rearing box was emptied of clover material the contents were searched for pupating or otherwise possibly parasitised arthropods. These arthropods were then placed in a petri dish with a small piece of moist tissue paper that was transferred to an incubation unit. The incubation unit was set to the typical conditions ($26 \pm 1^{\circ}\text{C}$, 65–75% RH, and a L:D 16:8 photoperiod) experienced during the longest photoperiod in Canterbury to promote the emergence of adult insects. Adult insects were recorded as they emerged, and then a select sample was sent to Dr Darren Ward Landcare Research Ltd, Auckland to confirm the species identification of the parasitoids.

Over the course of this experiment and into the next year attempts were made to culture the red clover casebearer through rearing pupating larvae in the laboratory. Over one hundred larvae were exposed to a variety of methods that could potentially promote the emergence of adult moths that are difficult to work with in laboratory incubation conditions. The first was cold shock treatment in a fridge at 4.6°C degrees for two weeks, four weeks and eventually two months. After each of these cold shock periods, the larvae were returned to the standard summer incubation conditions for one month. The second was standard constant incubation under summer conditions. There was no emergence after one year but many still apparently healthy pupae. Fifty pupating larvae were also kept at room temperature for a year, but this also failed to yield adult moths. The final method

attempted was leaving pupating larvae in a rearing box outside for one month over winter and then returning them to summer incubation conditions, but again no adult moths emerged.

None of these steps were performed on those larvae that were part of the parasitism experiment. But during the summer of 2021 and 2022 extra fourth instar red clover casebearer larvae were collected for the purpose of exploring issues related to promoting emergence in captivity in the hope of resolving some of these issues for the parasitism rate experiment.

In every sample of pupating red clover casebearer larvae there were three outcomes for individuals: parasitoid wasp emergence, degradation of the case/pupae or no emergence or visible degradation. Eventually all cases in incubation did visibly desiccate or degrade and this had occurred after about 10 months, or early in the summer following their collection (Figure 4.2).

Table 4.1: Red clover seed crops sampled for this experiment (all of these fields are located within Canterbury, New Zealand).

Field Name	Location	Crop age (years)
Kowhai	Lincoln	3
Tancreds	Lincoln	1
Robinsons	Lincoln	2
Chertsey	Chertsey	2
Richards 1 and 2	Methven	1 and 3
Smith	Leeston	2
Marr 1 and 2	Methven	1



Figure 4.1: Rearing box used in this experiment.

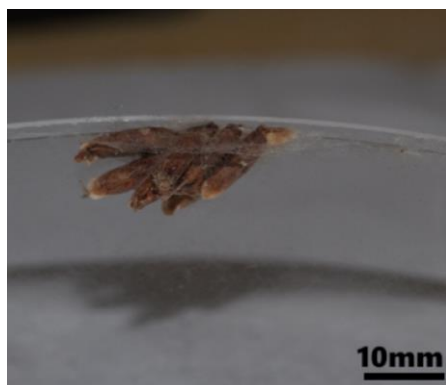


Figure 4.2: An example of fourth instar red clover casebearer larvae pupating.

4.2.4 Results

The red clover casebearer larvae were often observed amalgamating in the top corners of the rearing boxes in a mass of grey and white silk (Figure 4.2). These larvae, which had stopped moving, appeared to be in pupation or parasitised, and so were transferred to the incubator from which after about three weeks *B. variegator* often emerged.

In this experiment three parasitoids of the red clover casebearer fourth instar larvae were identified *Bracon variegator*, *Pteromalus puparum* (L. Hymenoptera: Pteromalidae) and *Eupelmus (Macroneura) messene* (Walker Hymenoptera: Eupelmidae) (Figures 4.3-4.6 and Table 4.3). *B. variegator* was by far the most prevalent parasitoid among those emerged (Table 4.2). All these methods did produce some adult *B. variegator* (Figure 4.7), however none yielded any adult red clover casebearer moths, and so determination of the total parasitism percentage must consider this.

Table 4.2: Total number of each parasitoid species recovered.

Parasitoid name	Total numbers recovered through incubation
<i>B. variegator</i>	140
<i>P. puparum</i>	12
<i>E. messene</i>	3

Table 4.3: Parasitoids of the red clover casebearer.

Species name	Introduction to New Zealand	Hosts globally	Other information and references
<i>Bracon variegator</i> (Figure 4.3 and 4.4)	For the control of <i>Coleophora</i> spp. in white clover seed crops	Lepidopteran generalist, prefers Tortricidae	(Aydogdu 2012; Piekarska-Boniecka et al 2019)
<i>Pteromalus puparum</i> (Figure 4.5)	For the control of the cabbage white butterfly in brassica crops	Lepidopteran generalist	Gregarious egg laying and the offspring compete with other parasitoids (Barron et al 2003)
<i>Eupelmus</i> (<i>Macroneura</i>) <i>messene</i> (Figure 4.6)	For the control of <i>Mayetiola destructor</i> (Diptera: Cecidomyiidae)	Broad generalist that is recorded attack a range of 'concealed hosts' such as gall wasps	The is a wingless 'strain' of the species (Figure 4.6) (Nikelshparg et al 2021)



Figure 4.3: *B. variegator* female.



Figure 4.4: *B. variegator* male.

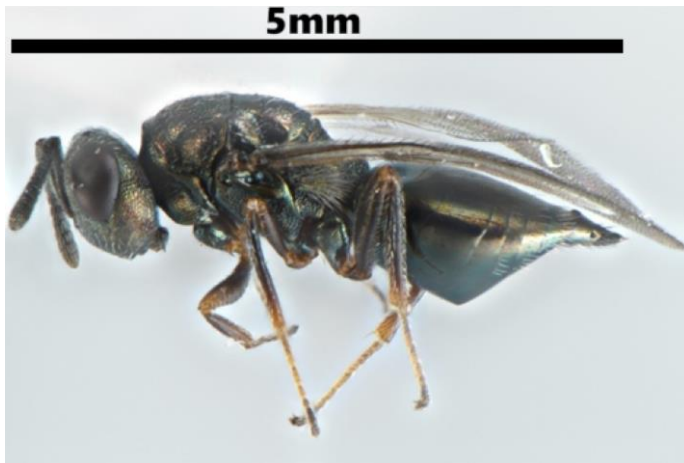


Figure 4.5: *P. puparum* female.



Figure 4.6: *Eupelmus (Macroneura) messene* female.



Figure 4.7: Anterior (left) and dorsal (right) view of a red clover casebearer case after a parasitoid has exited. The exit hole is visible (right).

4.2.5 Discussion

Through this laboratory experiment, it became apparent that the fourth instar larvae of the red clover casebearer could be readily parasitised. Prior to this there had not been any substantial parasitoid activity observed on the red clover casebearer in the field. However, following the observed decline of this pest over the previous growing season this finding was not unexpected. *P. puparum* and *E. (Macroneura) messene* both proved to be rare parasitoids of the red clover casebearer. However, it is possible certain strains of these species or other members of these genera could also be of use in the biocontrol of Coleophoridae pests in New Zealand.

From these observations, it was decided that only *B. variegator* was of importance for further investigation because of how strongly this species dominated in the emerging incubated samples. The following experiments in this chapter explored aspects of the biology of *B. variegator* and provided a hypothesis of possible limiting factors for this parasitoid species in the red clover seed crop ecosystem and as a parasitoid of the red clover casebearer generally.

4.3 Parasitism rate

4.3.1 Methods

To test the extent of red clover casebearer parasitism by *B. variegator* in the field, 30 red clover casebearer larvae within their cases were collected from either the Kowhai or Richards fields, from late-January to mid-February. This was done by identifying which clover flowers contained larger cased individuals and plucking the entire flowerhead. The flowerhead was then placed into a container to be taken back to Lincoln University campus. Five larvae were placed into a petri dish with two fresh red clover flowerheads to feed on (Figure 4.8). The petri dishes containing the red clover casebearer larvae were placed in an incubator set at $26 \pm 1^\circ\text{C}$, 65–75% RH, and a L:D 16:8 photoperiod. Once larvae started to pupate, they were transferred to a separate petri dish and monitored to see if an adult red clover casebearer moth or parasitoid wasp would emerge.

The red clover casebearer larvae selected for this experiment were the thirty largest individuals of the sampled fourth instar larvae, as these individuals were the closest to pupation and had been viable hosts for *B. variegator* for some time in the field. Collections were conducted when the crop was dry so that there was minimal chance of microbial contamination. Efforts were constantly made to ensure the interior of the Petri dishes was not so dry it would desiccate the pupating insects, but dry enough to hinder other growth. This was achieved with a small piece of moistened tissue paper and constant monitoring to dry any condensation forming on the underside of the petri dish lid.

The food source was supplied to larvae in the Petri dishes until such time that no larvae were observed to be using it for up to two weeks. Typically, the pupation of red clover casebearer larvae parasitised or otherwise occurred within one week of field collection, and these larger fourth instar individuals appeared to require minimal food to enter pupation by this stage. Pupation was determined by a lack of activity by the individual larvae and the closing of the proximal end of the case with silk.



Figure 4.8: Petri dishes containing fourth instar red clover casebearer individuals with two fresh red clover flowerheads to test parasitism rate (left in this Figure).

4.3.2 Results

Out of the 330 larvae collected for the experiment, 103 produced a parasitoid after 6 months of incubation. A further 72 were degraded (primarily by fungal contamination) leaving the remaining 125 from which no adult insects emerged. At the Kowhai field, parasitism was less than 20% in early and mid-January but increased thereafter reaching 75% in the third week of February (Table 4.4). In contrast at the Richards 1 field no parasitism was recorded in January, and less than 10% in February (Table 4.5).

Table 4.4: Kowhai field larval parasitism percentage.

Date	Larval parasitism (%)
11/1/2022	17%
19/1/2022	14%
31/1/2022	53%
8/2/2022	39%
16/2/2022	67%
24/2/2022	75%

Table 4.5: Richards 1 larval parasitism percentage.

Date	Larval parasitism (%)
19/1/2022	0%
31/1/2022	0%
2/2/2022	3%
16/2/2022	6%
28/2/2022	8%

4.3.3 Discussion

During the incubation, it was often difficult to determine the developmental state of the red clover casebearer larvae and/or parasitic *B. variegator* larvae (even though *B. variegator* is an ectoparasitoid it could not be readily observed due to the larvae's case, however, it was sometimes seen when looking through the opening at the proximal end of the red clover casebearer larvae's case). Although, care was taken to ensure the survival of any developing larval specimens in the incubator, a portion of specimens either did not produce any adult insects or became visibly degraded. This highlights some major caveats of laboratory parasitism experiments in general but especially when investigating species that do not emerge under laboratory conditions. The clover casebearers as a group have posed many issues for laboratory work and they have never been

successfully reared in captivity (Pearson 1975; Pearson 1989). The environmental trigger(s) that initiate clover casebearer adult emergence have not yet been identified or replicated under controlled conditions (Pearson 1975).

During 2021 and 2022, attempts were made, but failed to initiate the emergence of incubating cases that had not produced an adult parasitoid wasp or had visibly degraded during the first two months of incubation.

In particular the Richards 1 field had a high number of degraded cases and often these cases exhibited signs of fungal growth. In terms of determining the parasitism percentage it was difficult to confirm what proportion of degraded cases were parasitised, and if this was partially responsible for the lower recorded field parasitism. Between 10-40% of the incubating larvae collected from the Richards 1 field in each petri dish were visibly degraded during the course of the incubation period, which is much higher than the 5-10% that was observed for the Kowhai field. Because of irrigation the conditions in the Richards crops were wetter than those at the Kowhai crop, and this may have led to a higher prevalence of fungi and possibly other microbes.

A further sampling was undertaken one week after the final recorded parasitism for both of these fields, but it was found that there were very low numbers of red clover casebearer larvae in the crops by this stage, and those that were present were of poor health and either succumbed to fungal infection or did not successfully pupate. The red clover casebearer is a species with fairly synchronised development across individuals in all life stages. There is about a month in variation around the emergence time to account for the range in clover seed development but within a seed crop that is also managed very uniformly, the pupations/cessation of clover casebearer larval activity seems to be very synchronised (Chynoweth et al 2018; Walenta et al 2018). The maximum parasitism delivery of *B. variegator* in both crops aligned with the peak in abundance of the red clover casebearer in the crops during the growing season.

Interestingly, the red clover casebearer was still present at a very high density in the flowerheads despite a maximum parasitism incidence of 75%. This casts a lot of doubt on *B. variegator* being the most important biocontrol agent of the red clover casebearer. Even though this parasitism rate would substantially limit most species (Cameron et al 1993), it appears that despite this, the red clover casebearer can still cause a high level of crop damage and successfully develop a substantial population of new adults. This may be due to issues with synchronism between *B. variegator* and the red clover casebearer. It is possible that because *B. variegator* only parasitises the fourth instar of this pest (Thomas 1969), many of the larvae make it through to adulthood because the parasitoids in the field are saturated with an over abundant host. As this level of abundance was consistent across

New Zealand red clover seed crops until 2019 (Chynoweth et al 2018) it seems unlikely that this parasitoid alone could have so effectively regulated the pest across New Zealand.

This variation in parasitism rate between the two fields shows how active this parasitoid can be but also that it can be severely limited due to some crop, environmental and/or host conditions. The causes of why *B. variegator* can have a high variation in parasitism rate are outside of the scope of this research and yet to be determined.

The distance sampling in the Richards 2 crop found only low numbers of red clover casebearer despite the crop's proximity to the infested Richards 1 crop. Although these data are insufficient to make claims about the dispersal and colonisation ability of the red clover casebearer, they do pair nicely with the very low numbers of red clover casebearer found in red clover pastures adjacent to the Kowhai crop as well (Chapter 3).

4.4 Wasp longevity

4.4.1 Introduction

Due to the high level of parasitism by *B. variegator* on the red clover casebearer from the Kowhai field observed in the laboratory, floral resource suitability was explored as a possible reason for differences in the biocontrol outcomes between red and white clover.

The hypothesis was that structural differences between the red and white clover flowers and florets meant that *B. variegator* was less able to access floral resources in red than white clover seed crops.

These structural differences between red and white clover floral parts do affect some pollinators species (such as the honeybee) which struggle to push through the long tight corolla of red clover flowers especially in cooler weather (Figure 4.9 and 4.10) (Dennis and Holm 1977; Jing et al 2021). Overall, the white clover floret is much more open with the top and bottom corollas splaying apart (Figure 4.11 and 4.12). The larger flower structure itself is also much more open which may ease navigation for small insects.

Buckwheat was added to the selection of flowers due to its high level of preference among foraging insects from a range of orders, in particular those foraging agricultural systems. Previous studies have established the effectiveness of buckwheat in prolonging the lifespan of parasitoid wasps in particular (Vattala et al 2006; Araj et al 2019).

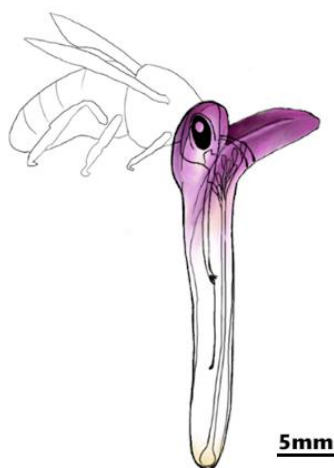


Figure 4.9: A honeybee pollinating the longer and tighter red clover floret (Drawing by Katie McIvor).



Figure 4.10: Front view of the entry into a red clover floret (Drawing by Katie Mclvor).

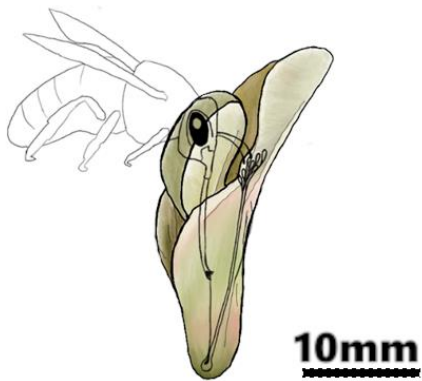


Figure 4.11: A honeybee pollinating a white clover floret (Drawing by Katie Mclvor).



Figure 4.12: Front view of the entry into a white clover floret (Drawing by Katie Mclvor).

4.4.2 Methods

This first experiment involved placing *B. variegator* wasps in containers with an insect netting lid to allow an oxygen supply, which were then left in an incubator simulating summer conditions ($26 \pm 1^\circ\text{C}$, 65–75% RH, and a L:D 16:8 photoperiod). There were three treatments; white clover, red clover and buckwheat each replicated twice (along with a control that consisted of *B. variegator* in the same container set up with no flowers, just the container and the moist paper the flowers were held in). Twelve to seventeen wasps were placed in each container and the flowers were refreshed every two days. For a second larger experiment, eighteen to twenty-two *B. variegator* wasps were placed in cages in a glasshouse. Each cage contained a red clover plant, supplemented with extra red clover or buckwheat flowers. Unfortunately, no data could be collected from this experiment, as a heat wave three days into the experiment led to very high temperatures in the glass house, and all of the wasps died as a result.

4.4.3 Results

In the absence of any floral resources, the average wasp lifespan was five days (Table 4.6). All three floral resources increase the lifespan to 15-23 days (Table 4.6).

Table 4.6: Effect of floral resource type on wasp life span.

Floral resource	Average wasp life (days \pm SE)
Red clover	19 ± 5
White clover	20 ± 4
Buck wheat	23 ± 11
Nil	5 ± 2

4.4.4 Discussion

Results from this simple experiment suggest that floral resource availability is not a cause of the differences in biocontrol outcomes for clover casebearers between white and red clover seed crops in New Zealand.

4.5 Strains of *Bracon variegator*

4.5.1 Introduction

Understanding the fine details of biocontrol agent genetics is very important to successful biocontrol and is employed in many advanced biocontrol programmes (Szucs et al 2019a; Jensen et al 2022). One useful application is that genetic diversity can be managed during the rearing and acclimatisation phases to prevent the insects being selected for laboratory conditions so that they are less effective in the field (Hoddle 2006). It also gives researchers the ability to diversify the genetics of biocontrol agents overall, as well as developing strains for local and dynamic conditions, such as dispersing versus more stationary fecundate strains to develop a more stable biocontrol system overall (Sentis et al 2022). *B. variegator* is a generalist parasitoid with strong preferences for certain groups of species such as *Coleophora* spp. and leaf roller moth (Thomas 1969; Zikic et al 2019).

It was considered possible that with the continued suppression of the white clover casebearer spp., populations of *B. variegator* in New Zealand could only persist at very low levels on the few white clover casebearers present in a given seed crop. However, it is more likely that *B. variegator* populations have sustained themselves on other Lepidopteran hosts in New Zealand as they do overseas.

There were two distinct collection areas for the *B. variegator* released into New Zealand, Avignon, France and Western Germany (Pearson 1989). It is unclear what impact this sourcing might have on the genetics and behaviour of individuals collected from each region and if any differences in genetics occurring via this route would have any implications for biocontrol. It is likely that any genetic differences detected within *B. variegator* populations will be due to a combination of these and other factors, but how these differences affected the population trajectory of the red clover casebearer, and its biocontrol outcomes remains unknown.

4.5.2 Methods

The genetic profile of some field sampled adult wasps (all of which were *B. variegator*, apart from one *Dolichogenida* sp.) was analysed to see if there were any substantially different strains of this parasitoid present in red clover seed crops. This analysis was conducted by Dr Nicky Richards at AgResearch Ltd and Plant and Food Research Ltd facilities in Lincoln, New Zealand.

Individual wasps were reared out of red clover casebearer larvae collected from some of the fields used for the field comparison experiment. This ensured that these individuals were using the red

clover casebearer as a host. The fields were located in Chertsey, Lincoln, Methven and Leeston, and the rearing techniques used were those outlined in the Chapter 3 rearing experiment.

The methods used by Dr Richards are as follows: The analysis was conducted on the CO1 gene and used a non-Braconid out-group (*Eupelmus vesicularis*) as well as including an *Apanteles* sp. due to it being the closest related Hymenopteran species to the *Dolichogenidea* sp. available in the BOLD database.

Individual wasps were reared out of red clover casebearer larvae collected from some of the fields used for the field comparison experiment. This ensured that these individuals were using the red clover casebearer as a host. The fields were located in Chertsey, Lincoln, Methven and Leeston, and the rearing techniques used were those outlined in the Chapter 3 rearing experiment.

DNA was extracted from whole adults using the Genomic DNA Mini Kit Tissue (Geneaid™, Taiwan) following manufacturer's specifications. Polymerase chain reactions (PCRs) contained i-StarTaq™ DNA polymerase (iNtRON Biotechnology, Korea) at approximately 1 unit/20µL PCR, 2mM MgCl₂, 0.2mM dNTPs, 0.2µM primers (Integrated DNA Technologies, Singapore) and 1µL template DNA at ~1ng per 20µL of PCR reaction. Cycling conditions were 95°C for 2min, followed by 40 cycles of 95°C for 30s, 45°C for 45s and 72°C for 45s; the final step was 5min at 72°C. All PCR runs included a template-free reaction as a negative control. Existing primers (LCO1490, HCO2198) were used to amplify the 5' mitochondrial cytochrome oxidase subunit I (mtCOI) gene or barcode region (Folmer et al 1994). PCR products and 100bp ladder (DNA Marker 1, A and A Biotechnology, Poland) were run on 1% agarose gels containing RedSafe™ (iNtRON Biotechnology, Korea), in 0.5 × TBE buffer. Sanger (BigDye® v 3.1, Applied Biosystems) sequencing was conducted at Macrogen (Korea) and the sequences were tidied and analysed using Geneious version Prime (<http://www.geneious.com>, Kearse et al 2012). Multiple alignments of the sequences from the unknown wasp adult specimens and their closest online matches were generated using the program MUSCLE (Edgar 2004). Distance analyses were performed using the Neighbour-Joining tree building method with the Tamura-Nei genetic distance model (Saitou and Nei 1987). This approach provides a simple measure of the level of variation between the barcode sequences. The robustness of the nodes was assessed with 1000 bootstrap replicates (Felsenstein 1985). Closely related sequences were downloaded from the Barcode of Life Data System (BOLD; <http://www.boldsystems.org/>) (Ratnasingham and Hebert 2007) and GenBank (Benson et al 2013).

4.5.3 Results

Sequence analysis of the barcode region (5' end of the mitochondrial cytochrome oxidase subunit I (COI) gene) revealed two haplotypes of *B. variegator* (as identified morphologically by Dr Darren

Ward of Landcare Research Ltd) as well as confirmation of the presence of a *Dolichogenidea* sp. from the Grasslanz red clover crop located in Lincoln. Figure (4.13) is a distance tree showing the sequences compared to the closest matches on the BOLD and Genebank databases, and included the samples morphologically identified by Dr Darren Ward as references for *B. variegator*. Larvae collected from the Kowhai and Chertsey fields contained wasps from both haplotypes of *B. variegator*, but those from 'Lincoln' (Grasslanz red clover crop), only contained the first haplotype, while those from Methven only had the second haplotype (Figure 4.13).

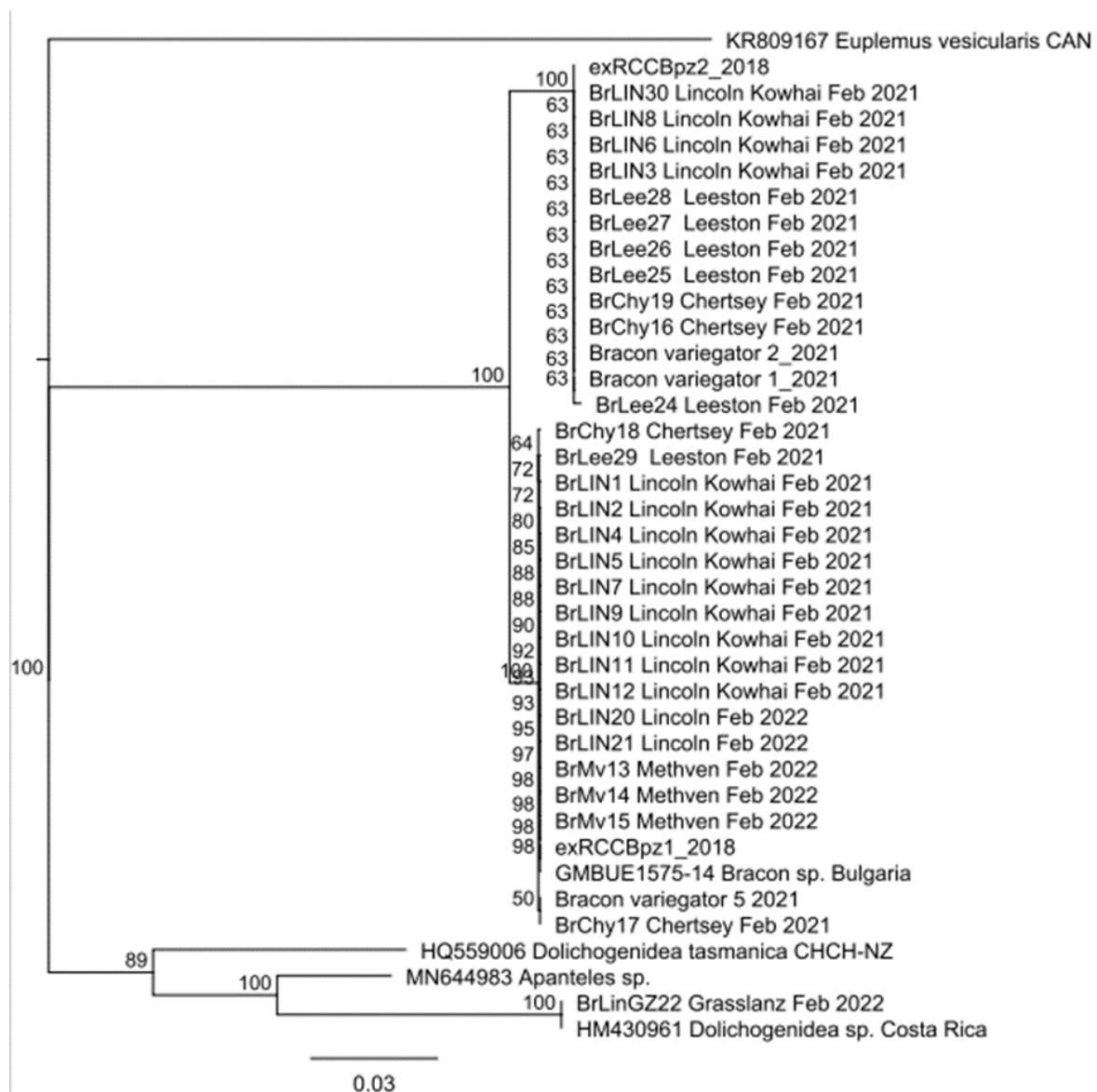


Figure 4.13: Neighbour joining tree of Mt-COI sequences from parasitoids of red clover casebearer with their closest related Hymenopteran species found on BOLD and GenBank®. A *Eupholidae* spp., *Eupelmus vesicularis*, is used as the out-group. Bootstrap values over 50% are shown in the node. The three samples labelled 'Bracon variegator 1_2021, 2_2021 and 5_2021' were morphologically identified by Darren Ward and are from the Kowhai crop.

4.5.4 Discussion

The sequences for the two haplotypes of *B. variegator* show approximately 2% variation. This level of variation is seen between haplotypes of other parasitoids (de Leon and Setamou 2010), e.g. the Irish and Moroccan strains of the clover root weevil (*Sitona obsoletus*) parasitoid *Microctonus aethiopoides* exhibit 2.3% variation within the mitochondrial COI barcode region (Vink et al 2012). However, it is worth noting that the mitochondrial COI barcode region can also be highly similar between species e.g. *Wiseana mimica* and *W. jocosa* are 99.5% identical (Richards et al 2017) and, hence it should not be ruled out that the two groups of Braconid parasitoids may represent two closely related species and not just haplotypes of *B. variegator*.

In summary, whilst the two clades have some segregation in that Methven and Leeston each appear to support one clade of *B. variegator* and, Lincoln supports both clades, the latter suggests that *B. variegator* individuals from each clade are able to persist in the same red clover seed crops on the red clover casebearer. Whether these small genetic differences are due to simply the two original locations of *B. variegator* in Europe or something more substantial such as host preference, it is interesting that the differences were still found fifty years after, *B. variegator* was introduced into New Zealand. No investigations were undertaken to explore the implications of these differences for *B. variegator* and its provision of biocontrol. The wider implications of genetics for biocontrol will be explored in the final discussion. The presence of the *Dolichogenida* sp. is of interest because an unidentified *Dolichogenida* sp. was imported for biocontrol of the white clover casebearer species in the 1960s DSIR programme (Thomas 1969). However, it was not possible to determine a species level identification of this specimen to confirm if it was a parasitoid related to the earlier programme.

4.6 Alternative hosts

4.6.1 Introduction

In their native ranges *Coleophora* spp. are usually attacked by a diversity of specialist and generalist parasitoids. Typically, these parasitoids target the second through to fourth instar of the larvae (Bousfield and Lood 1971; Ellis and Bjørnson 1996). In addition, the red clover casebearer is only present as a suitable host to *B. variegator* during the late summer in New Zealand (Kelsey 1958; Ellis and Bjørnson 1996; Chynoweth et al 2018). Therefore, the red clover casebearer cannot be *B. variegator*'s only host over 10-11 months, as this would require an extremely long diapause period for a parasitoid wasp. Research overseas has found many non-Coleophorid hosts of *B. variegator*. These include members of Depressariidae, Gelechiidae, Gracillariidae, Oecophoridae, Pyralidae, Tortricidae and Yponomeutidae (Aydogdu 2012; Gadallah et al 2021; Žikić et al 2021). There are many species belonging to these families that are present within *B. variegator*'s range in New Zealand (Suckling et al 1998; Aydogdu 2012; Ghahari and Fischer 2012; Ghahari and Beyarslan 2017; Gadallah et al 2021; Žikić et al 2021). Thus, there should be other insect host species in agricultural landscapes that support *B. variegator* outside of fourth instar red clover casebearers in New Zealand. This work aimed to identify possible alternative host species of *B. variegator* that might be present in New Zealand red clover seed crops. This information was seen as useful to further developing and possibly enhancing this parasitoid's delivery of red clover casebearer in the field. In their native ranges *Coleophora* spp. are usually attacked by a diversity of specialist and generalist parasitoids. Typically, these parasitoids target the second through to fourth instar of the larvae (Bousfield and Lood 1971; Ellis and Bjørnson 1996).

4.6.2 Methods

The methodology and equipment used for this experiment were derived from Hardwick et al (2016) and Hardwick and Phillips (2021). They were originally developed to improve the monitoring capability for the parasitoid *Microtonus aethiopoides*, following its controlled release as part of a biocontrol programme carried out against the weevil pest *Sitona obsoletus* in New Zealand.

There was a need to develop more specific and less labour-intensive monitoring strategies for this biocontrol system. This was due to the limited available techniques for data collection, relying on good weather, and monitoring only being able to be undertaken during winter, when host larvae diapause at high densities. These methods were further constrained by the long travel time between sites and heavy time requirement for laboratory processing. In addition, the limited time window that this sampling could occur did not cover the period of the year that *M. aethiopoides* was dispersing in the field (Hardwick and Phillips 2021).

This led to the investigation of devices that could be left in the field for monitoring parasitism rates over time. Hardwick et al (2018) investigated various factors of the modified plastic Sistema box system that was decided on. Their experiments included assessing if adult parasitoids would enter the box and parasitise, if parasitoid oviposition within boxes would produce adult offspring and if adult offspring would exit the devices. It was found that *M. aethioides* could easily pass through the mesh and parasitise host weevils inside which successfully produced adult wasp offspring.

This description of the developed boxes is from Hardwick et al (2018):

'Each device comprised two chambers, both constructed from plastic rectangular Klip-it boxes (Sistema style 1850; 265 x 235 x 120 mm). For the upper chamber, the floor and most of the lid (190 x 160 mm) were substituted with 900 µm plastic mesh. For the lower chamber, the same mesh was used to create four ventilation slots in the side-walls (two of 35 x 200 mm, and two of 35 x 170 mm). The upper chamber served as a lid for the lower chamber. Mesh edges were secured to chambers with continuous beads of Bostik HM Glue Stick clear glue.'

For the work undertaken by Hardwick and Phillips (2018) the box from the previous research was modified slightly and is described as follows: (from Hardwick and Phillips 2021)

*'The monitoring device was based on the emergence device described in Hardwick et al. (2016) and comprised a single chamber. It was constructed from a plastic 5 l rectangular Klip-it® box (Sistema style 1850) with dimensions 265 × 235 × 120 mm. Most (190 × 160 mm) of the lid of the Klip-it® box was removed and replaced with 900 µm plastic mesh. The 900 µm mesh size was used because it allows transit of *M. aethioides* adults, but not *S. obsoletus* adults (Hardwick et al. 2016). In addition, one hole (35 × 200 mm) was made in each side end of the box, and another (35 × 170 mm) in each end, and these were also covered with 900 µm plastic mesh. Mesh was fixed at its edges with a continuous bead of Bostik HM Glue Stick Clear glue. Upon placing monitoring devices in the field, they were covered with a 400 mm length of half-round 285 mm diameter plastic culvert to prevent flooding by rain or irrigation water.'*

This final design developed by Hardwick and Phillips (2021) was used for the current experiment and the units were borrowed from AgResearch Ltd, Lincoln.

Table 4.7: Species of Lepidopteran larvae used in the host range experiment.

Name	Family	Descriptor
<i>Planotortrix octo</i> (Dugdale 1990)	Tortricidae	Introduced leaf roller pest
<i>Planotortrix excessana</i> (Walker 1863)	Tortricidae	Native leaf roller
<i>Ctenopseustis obliquana</i> (Walker 1863)	Tortricidae	Native leaf roller (pest of plantation pine)
<i>Ctenopseutis herana</i> (Felder and Rogenhofer 1875)	Tortricidae	Native leaf roller
<i>Cnephasia jactatana</i> (Walker 1863)	Tortricidae	Native leaf roller
<i>Epiphyas postvittana</i>	Tortricidae	Introduced pest of many crops
<i>Spodoptera litura</i> (F. 1775)	Noctuidae	Introduced pest of vegetables
<i>Helicoverpa armigera</i>	Noctuidae	Introduced pest of vegetables

Eight species of moth caterpillar (Table 4.7) were tested as alternative hosts for *B. variegator*. This took place from December-February in Canterbury and once in Blenheim during February. Red clover seed crops were predominantly used but some white clover seed crops were also included.

These caterpillars were raised by Anne Barrington at Plant and Food Research Ltd, Auckland, and were couriered to Lincoln University in rearing tubes with a food supply during their third instar. They were then reared further in these tubes in the laboratory until they reached the fourth instar before being placed in the field. These caterpillars initially were selected for being members of Tortricidae, which is the family most frequently parasitised by *B. variegator* in Europe. Initially preference was given to species from a genus already known to be parasitised by *B. variegator*.

Caterpillar individuals were placed into Petri dishes containing a layer of caterpillar food prepared to the specifications of Teakle and Jensen (1985) (the brown paste visible inside the petri dishes in Figure (4.14)). This 'Caterpillar food' consisted of; pre-boiled water (47%), navy beans (8.6%), plain agar (12.5g agar and 303ml water) (31%), wheat germ (6%), brewer's yeast (5%), honeybee pollen (1%), ascorbic acid (approx. 0.3%), hydroxyl benzoate (approx. 0.3%) sorbic acid (approx. 0.1%), 40% concentration Formaldehyde (approx. 0.1%) (In addition to the caterpillar food, clover stems containing leaves and flowers were also placed within the container with the stems poked into wet floral foam, to provide supplementary food and shade for the caterpillars. The clover material was plucked from the same field as the box was being placed in, to remove any possible interference or disruption to parasitoid wasp hunting patterns, which may have occurred with clover material foreign to the site.

Four boxes containing eight caterpillars were left in the Kowhai, Grasslanz red clover, Grasslanz white clover, Maw 1, Marr 1, Richards 1 and Richards 2 fields for a period of 5 hours before being removed and taken back to Lincoln University campus. Once back on campus, the surviving caterpillars were transferred onto new petri dishes containing fresh caterpillar food (Figure 4.14). The caterpillars were then moved to an incubator ($26 \pm 1^\circ\text{C}$, 65–75% RH, with a L:D 16:8 photoperiod). Any of the caterpillars which had started to pupate, were transferred to a petri dish (1 caterpillar per petri dish) with a wad of damp paper towel to ensure that moisture was retained within the petri dish. This petri dish was then put into an incubator (with a lid on) with the same environmental conditions as listed above. These caterpillars were then monitored to see if a parasitoid wasp or an adult of the specified caterpillar emerged.



Figure 4.14: *Planotortrix excessana*, *Ctenopseutis herana* and *Cnephasia jactatana* individuals resting on caterpillar food after being left in clover fields to be exposed to parasitoid wasps for a period of 5 hours.

4.6.3 Direct exposure of leafroller caterpillars to *Bracon variegator*

B. variegator is a generalist parasitoid whose hosts include multiple insect families (Aydogdu 2012; Piekarska-Boniecke 2019). The host range experiment was therefore, expanded to include the native leafroller caterpillars: *Planotortrix octo*, *Planotortrix excessana*, *Ctenopseutis obliquana*, *Ctenopseutis herana* and *Cnephasia jactatana*, also supplied by Anne Barrington. They were directly exposed to *B. variegator* individuals to test if they would be attacked when kept in close proximity to the wasps. The caterpillars were kept in the test tubes in which they were supplied. There was one individual caterpillar per test tube sitting on a layer of caterpillar food with a cotton bud at the entrance to the test tube to prevent its escape (Figure 4.15). *B. variegator* individuals from either the Kowhai or Richards 2 field were then inserted into the test tubes with the caterpillars. A male and female *B. variegator* were placed into each test tube to ensure that a pregnant female would be present to oviposit on a caterpillar if it decided to use the caterpillar as an alternative host. An

example of this experiment is shown in (Figure 4.15). These test tubes were placed in an incubator ($26 \pm 1^{\circ}\text{C}$, 65–75% RH, with a L:D 16:8 photoperiod).



Figure 4.15: *Cnephasia jactatana* individuals being exposed to male and female *B. variegator* wasps within test tubes to test if the *C. jactatana* individuals can be used as alternative hosts by *B. variegator*.

4.6.4 Results and discussion

At the conclusion of this experiment, no adult wasps had emerged from any of the incubated caterpillars (including from those caterpillars used in the direct exposure trial). However, 80% of the caterpillars that pupated produced an adult moth. This result does not add any information about the host range of *B. variegator*. It is unclear if this was due to a failure to select viable and/or desirable lepidopteran hosts of *B. variegator* or was related to some feature of the boxes used in the field for this work. Although the ability of *B. variegator* to enter and exit through the mesh lid of the boxes was confirmed in the laboratory, it is possible that this box design caused *B. variegator* some kind of aversion in the field. In addition, it may also be that the tested host species of caterpillar are not particularly attractive to foraging *B. variegator* adults in the field. How *B. variegator* is supported outside of red clover casebearer abundance remains unclear. Whilst this is not immediately an issue for clover seed crop management, it may prove important if the biocontrol of *Coleophora* spp. ever begins to fail.

Chapter 5

Entomopathogenic Fungi

5.1 Entomopathogenic fungi

Entomopathogenic fungi are found in more or less every arthropod population worldwide (Shah and Pell 2003; Korosi et al 2019). Some of these fungi are host specific but others are known to have very broad interkingdom host ranges (Vega et al 2008; Litwin et al 2020; Gielen et al 2022).

There is a lack of information on the impact of these fungi in the field and their wider role in ecosystems because it is usually difficult to study them in action (Vega et al 2009; Moreno-Gavira et al 2020). In addition, the evolution of these fungi and the selection pressures they apply to arthropod populations are poorly understood (Korosi et al 2019; da Silva Santos et al 2020).

A range of studies have found that fungal isolates belonging to species of *Fusarium* and *Penicillium* among others, that were previously only regarded as plant pathogens can also infect and kill species of insects (Laith et al 2018; da Silva Santos et al 2020). It is suggested that such occurrences went unnoticed initially due to the design of research on lepidopteran mortality which mainly focused on the pupal and adult stages of pest species, and known causes of mortality, such as that caused by parasitoid wasps (da Silva Santos et al 2020). There is however, increasing evidence for the role of plants in the relationship between arthropods and entomopathogenic fungi (McKinnon et al 2017). It is suggested that these antagonist fungi can be entophytic in the host plant of the insect pest; this has been confirmed in some cases, but it is far from a complete model (Amatuzzi et al 2017).

Amatuzzi et al (2017) isolated fungi colonies from strawberry leaves, and eight potentially entomopathogenic isolates (*Fusarium solani*, *Aspergillus terreus*, *A. sydowii*, *Diaporthe endophytica*, two isolates of *Paecilomyces lilacinus*, *Cladosporium sphaerospermum* and *C. cladospirdaes*) were chosen for pathogenicity testing against third-instar larvae of *Duponchelia fovealis* (Zeller) (Lepidoptera: Crambidae). They found that, the two isolates of *P. lilacinus* killed 80% of *D. fovealis* larvae. This was the first report of this genus as an entomopathogen.

Akello and Sikora (2012) found that faba bean plants grown from seed treated with isolates of some endophytic fungi were protected against two species of aphid (*Acyrtosiphon pisum* and *Aphis faba*). Isolates of *Trichoderma asperellum*, *Gibberella moniliformis* and *Beauveria bassiana* had the largest impacts on aphid populations, whereas those of *Metarhizium anisopilae* and *Hypocrea lixi* had the least impact on aphid populations (Akello and Sikora 2012).

This role of fungi in plant protection is an extension of the 'bodyguard hypothesis' that suggests plants attract and support insects among other natural enemies to protect themselves from herbivory and microbial infection, may help explain the prevalence of entomopathogenic fungi in arthropod populations (Bentley 1977; Elliot et al 2000). Rivas-Franco et al (2020) demonstrated that the presence of beetle pests of maize roots but not beetle pests of roots of other plant species increased the presence of entomopathogenic fungi in the maize rhizosphere. These endophytic fungi have also been shown to have multiple functions and benefits they confer to plants, including the suppression of plant pathogens and enhancement of nutrient and water uptake (Tewari et al 2019; Sharma et al 2020; Diagne et al 2020).

Developing insect nymphs, larvae and pupae can be more susceptible to pathogen attacks than their adult counterparts (Erasmus et al 2021; Srivasta and Shukla 2021 Eski and Gezgini 2022). This is because the cuticle of developing insects offers less protection from attack, and their humoral and cellular defences are less developed than those of adults (Dubovskiy et al 2013; Araujo and Hughes 2016).

Aand and Tiwary (2009) infected the eggs and second instar larvae of the rice cut worm (*Spodoptera litura* (F) Lepidoptera: Noctuidae) with different concentrations of conidial suspensions of six isolates of fungi belonging to five species, *Metarhizium anisopliae* var. *anisopliae*, *Metschnikov sorokin*, *Lecanicillium muscarium*, *Cordyceps cardinalis*, *Fusarium lateritium*, and *Aspergillus* sp. Both *S. litura* eggs and second instar larvae were susceptible to all tested fungal isolates with higher concentrations producing higher mortality rates. The authors recommended further investigation of *M. anisopliae*, *F. lateritium* and *L. muscarium* as potential biocontrol agents because they produced the highest larval mortality rates.

There are some microbe-based pesticide applications commercially available. *Trichoderma* spp., *Metarrhizium* spp. and *Beauveria bassiana* are widely used (Gul et al 2014; Samada and Tambunan 2020). However, it is generally accepted that the use of entomopathogenic fungi as bio-insecticides remains underutilised (Ayilara et al 2023). There are many positive and negative elements of these fungi that provide unique solutions as well as challenges to researchers and farmers (Sharma et al 2019). The benefits are generally that they are host specific, can be applied alongside synthetic chemicals in many cases, they can often self-perpetuate, they are generally safe to ecosystems and they are unique in their modes of actions which essentially eliminates issues associated with resistance to synthetic pesticides (Sharma et al 2019; Bamisile et al 2021). However, there are also limitations to their use, which include the need for favourable conditions (warm and humid) and careful management of synthetic fungicides to be effective, some of their secondary metabolites such as alkaloids, polyketides and phenols can be harmful (Li et al 2020; Xu et al 2021), and they can

have a short shelf life (Zeilinger et al 2016; Essiedu et al 2020; Bamisile et al 2021). Their use also requires good crop scouting and accurate pest information to ensure success. In short, they require a higher level of expertise to use them (Teetor-Barsch and Roberts 1983; Islam et al 2021).

5.2 Background and Methods

Laboratory experiments tend to offer much more favourable conditions for developing entomopathogenic fungi as they are not unimpeded by ecological constraints such as UV light and competition (Panyasiri et al 2007; da Silva Santos et al 2016).

During laboratory experiments in 2021, many red clover casebearer larvae were found to have various fibrous growths on the exterior surface of their cases (Figures 5.1 and 5.2). These growths appeared to be different from the clean case exterior and bright white silk plug in the opening of the case that was typical of parasitised red clover casebearer larvae and pupating non-parasitised larvae.

In addition, many of the larvae showed positive gravi-taxis when kept in laboratory conditions. Large numbers of the larvae were fixing themselves as high as possible in the corners of the cages after about a week in captivity. Once in this position a large greyish fibrous mass formed on the exterior of the cases (Figures 5.1 and 5.2).

To further investigate this, cases that had developed the fibrous growth were kept in an incubator ($26 \pm 1^{\circ}\text{C}$, 65–75% RH, and L:D 16:8 photoperiod), for a further six-months (during 2021) to observe developments. Usually after about three months in incubation, the case was desiccated and produced no living insect from inside.

Dissection of these cases showed that the contents were also desiccated with no obvious reasons to why this had occurred. It also appeared that these dead cases contained both parasitised and non-parasitised larvae, as many partially formed –desiccated parasitoid cadavers were found in them. It did not appear that this phenomenon was caused by competition between two parasitoid species, as there was no clear evidence of multiple species of parasitoid cocoons in any of the dissected dried out cases.

In the following year (2022), cases exhibiting this fibrous growth were gathered and immediately dissected to see if this could provide more information. It appeared from observation, that a fungal species may have attacked the larvae/cases. The fungus was then isolated from the larvae (Figure 5.3), and identified by sequencing courtesy of Dr Jo Narciso and Professor Travis Glare of Lincoln University.



Figure 5.1: Various cases that produced *B. variegator* parasitoids notice the white silk which is produced by the developing *B. variegator*.



Figure 5.2: Two angles of the same formation of fourth instar larvae that formed in the top corners of the rearing containers.

The methods used for the culturing and identification of the fungi were based on reported by (Alizadeh et al et al 2017):

- i. A sterilised glass slide was laid on the surface of a plain agar plate in a Petri dish. An infected larvae including its case was then placed on top of the clean glass slide and the dish covered with its lid.
- ii. The inoculated plate was placed in a tight lid container with wet paper towel to ensure high humidity needed for fungal sporulation. The container was incubated at 23°C for 6 days.
- iii. At 6 days of incubation, the infected larval material was then transferred onto a fresh Potato Dextrose Agar (PDA) plate to allow maximum growth and incubated at 23°C.
- iv. Three days later, genomic DNA was extracted using 5% Chelex (chelating material produced by Bio-Rad Laboratories Inc.) from mycelia of the growing fungal colony (Figure 5.3).
- v. PCR was done on the extracted DNA using elongation factor primers 983F and 2218R. The amplified PCR product was sequenced with both forward and reverse primers.
- vi. Following this the forward and reverse sequences were compared to BLAST fungal databases for identification.

5.2.1 Results

The sequence produced in this work (figure 5.4) matched BLAST records for *Fusarium pseudograminearum*, a possibly entomopathogenic fungus that is commonly known as a wheat root rot causing plant pathogen (Kazan and Gardiner 2017).

During 2022, an attempt was made to investigate the incidence of *F. pseudograminearum* in overwintering red clover casebearer larvae in the field. However, due to the small size of these larvae and the dark brown colour of the case, it was impossible to locate a sufficient number of these cases to provide reliable data.

Material was removed from the surface and soil of the Kowhai red clover crop in April and June of 2022, but only three larvae were found during the April sampling and none in May. These three larvae were cultured using the same methods as outlined above and *F. pseudograminearum* was isolated from one of these larvae.

Due to the highly labour-intensive nature of finding overwintering red clover casebearer larvae, this experiment was terminated at this point. Larvae have been reported to migrate to fence posts of the fields used (Pearson 1975), but no fence posts at the Kowhai field were found to harbour any accessible larvae.



Figure 5.3: Fungal colony isolated from the red clover casebearer larvae growing on PDA.

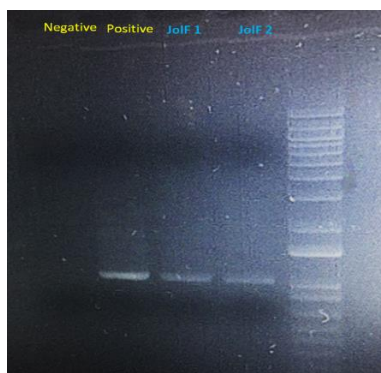


Figure 5.4: Gel electrophoresis of the PCR products of the fungus isolated from the red clover casebearers using 983F and 2218R primers. JoIF 1 full strength DNA (left) and JoIF 2 1:1 diluted gDNA (right).

5.3 Discussion

Fusarium spp. are a group of fungi that can be found in almost any environment or organism; they have been recorded as both pathogens and mutualists of plants and animals as well as saprophytes (Torbati et al 2018; Sharma et al 2020). Currently there up to 30 recognised *Fusarium* spp. complexes that are divided into around 2000 genetic isolates with widely different functions, although, there are undoubtedly many more undiscovered members of this genus (Summerall 2019; da Silva Santos et al 2020; Saad et al 2023)

The most thoroughly researched *Fusarium* species are plant pathogens responsible for yield reductions in many crops (O'Donnel et al 2013). They cause diseases such as wheat root rot, corn ear rot, sudden soybean death disease and certain vascular wilting conditions (Kazan and Gardiner 2017; Summerall 2019). Two species (*F. oxysporum* and *F. pseudograminearum*) are among the world's five most prevalent plant pathogenic species (Dean et al 2012).

The plant antagonist aspect of *Fusarium* biology makes some species at least of dubious suitability for biocontrol programmes (Dean et al 2012). However, there are an increasing number of documented cases of *Fusarium* spp. that are known to produce insecticidal metabolites, such as *F. oxysporum* (da Silva Santos et al 2020; Gielen et al 2022). Nevertheless, their adoption as insecticidal treatments must be dependent on the specificity of genetic isolates used and would require trust to be built up in farmers who are familiar with these fungi as the cause of serious plant diseases (Samada and Tambunan 2020; da Silva Santos et al 2020).

Substantial work on cataloguing *Fusarium* species was carried out by Gordan (1959) who studied 9000 *Fusarium* isolates over 30 years from a wide range of hosts. Of these hosts, however, only 1% were insects (Gordon 1959). Since then, other researchers have reported various isolates of *Fusarium* on a range of insect species (Teetor-Barsch and Roberts 1983; Batta 2012; Laith et al 2018). Studies that are more recent have found many more isolates of *Fusarium* spp. that are entomopathogenic; these include isolates of *F. oxysporum*, *F. avenaceum*, *F. proliferatum* and *F. pseudograminearum* (Laith et al 2018; da Silva Santos et al 2020).

Because of their cosmopolitan and hardy nature, *Fusarium* spp. are a potentially useful avenue to explore for the control of pesticide-resistant pests. Mantzoukas et al (2022) isolated seven *Fusarium* species from the cotton bollworm (*H. armigera*) crop habitat and tested them against *H. armigera* larvae in the laboratory. A range of larval death rates were recorded, with the most effective fungi being *F. solani*. This work provides further evidence for the potential of entomopathogenic fungi as biocontrol agents. Further research must be conducted on the possible impacts these fungi could have on important pests.

5.3.1 Entomopathogenic fungi and clover casebearers

There have been various entomopathogenic fungi cultured from and identified on *Coleophora* spp. material in New Zealand and overseas. In New Zealand, fungi from the genera *Beauveria*, *Fusarium*, *Hymenostillbe* and *Paecilomyces* were found on the white clover casebearers (*Coleophora mayrella* and *C. alcyonipennella*) by Pearson (1975 and 1989). In addition, two separate *Neoaplectana* spp. were recorded by Dumbleton (1952). Whilst there are no data on the field infection rates of these fungi on *Coleophora* species, Pearson (1975) reported an unusual factor he described as 'shrivelling', and suggested various entomopathogenic fungi were one of the major causes of mortality in fourth instar white clover casebearer larvae (despite the presence of at least two successful parasitoid wasps) (Pearson 1975). Whilst the work carried out by these researchers does not go into depth about the biology of these fungi, they do provide a good record of this interaction in New Zealand (Glare et al 1993).

Because this shrivelling factor remains undefined, it is possible that it is a result of larvae that have died from one of, or a combination of, entomopathogenic fungi or parasitoid wasp(s) that could persist under the experimental conditions.

5.3.2 Hypothesis

F. pseudograminearum alone or in combination with other fungi is responsible for the sudden and widespread red clover casebearer population reductions in New Zealand. The fungus may have taken time to adapt to the new red clover casebearer host in red clover crops. However, it is thoroughly established in white clover crops and readily attacks white clover casebearers.

It is also important to note that currently the fungus has only been cultured from the case and larvae together, so it is unclear if it attacks and kills red clover casebearer directly. There are two possibilities for its action as a biocontrol agent of the red clover casebearer.

- i. It is an entomopathogenic fungus that attacks and kills the larvae itself.
- ii. It does not directly kill the larvae, but its presence within the case creates an environment inhospitable to red clover casebearer larvae; this may be due to a combination of fungal produced toxins, physical degradation of the case, or the kind of conditions (raised moisture levels etc.) that the fungi need to thrive.

5.4 Conclusions

F. pseudograminearum has been widely established in New Zealand for a long time (Monds et al 2005). However, it is possible that this entomopathogenic isolate of *F. pseudograminearum* may have been accidentally released during the 1960s biocontrol program, as a large quantity of red clover casebearer material was imported from Europe over a decade, and researchers noted that there were often fungal outbreaks that killed the larvae during incubation before a wasp or a moth could emerge (Thomas 1969; Pearson 1989). In addition, a *Pyeotes* spp. (Acari: Trombidiformes) that is a parasite of the clover casebearers was accidentally established during this programme (Pearson 1975).

There was no investigation over the time that white clover casebearer species were in decline to determine whether an entomopathogenic fungus may have been responsible. Instead, it was assumed that the two parasitoid wasp species that were easily established and readily dispersed were responsible (Thomas 1969). A biocontrol system that is reliant on multiple species from different kingdoms is likely to be more stable and effective over time than one that relies on a singular specialist species (Rannback et al 2015). Therefore, it would be beneficial to the system if there were in fact multiple biocontrol agents responsible for the control of *Coleophora* spp. in New Zealand.

This was only a preliminary look into a possible alternate biocontrol agent for *Coleophora* spp. in New Zealand. Further investigation is required to determine whether the fungus is directly responsible for larval death, and if so, is it only active against fourth instar larvae? Information on its presence in red clover seed crops, conditions which favour it as an entomopathogen and its effectiveness as a biocontrol agent are also required.

Chapter 6

Discussion

6.1 Contemporary biocontrol programmes and directions of development

There are many cases of systems featuring singular biocontrol agents being very successful. For example, the AgResearch programmes that successfully controlled three serious weevil pests of pastures as discussed in Goldson et al (2020). However, this is not always the case for most consistent long-term background biocontrol (Lewis 1975; Hawkins et al 1999; Dainese et al 2019). Within any productive landscape a range of potential pests may be kept below damaging levels through naturalised biocontrol agents and other competitive species (Fielder et al 2008; Dunn et al 2020). The heightened potential risks growers face regarding new pest arrivals, and the emergence of new domestic pest issues, suggest that more complete biocontrol systems should be developed for crops (Pingali 2012; Barratt et al 2018; Han et al 2019). Nonetheless, the complex nature of more diverse approaches to pest regulation means that progress is slow, even if potential benefits to agriculture production and other humanitarian goals are substantial (Barratt et al 2010; Abram et al 2020; Andow et al 2021).

6.1.1 The general approach to biocontrol used in this thesis and exploring some options for improvement/cohesion

Science can both support and work against a transition towards sustainable agriculture, and there are many counterproductive, although, equally scientific pursuits being undertaken in regard to this end (Lewontin and Berlan 1986; Sarewtiz 2004). This is not so much a flaw within science but the obvious limitation of it. Science on its own cannot be relied on to produce solutions to climate change independently of environmental movements and the re-contextualisation of findings within other fields (Lewontin and Berlan 1986; Kallis et al 2013; Fisher 2012; Diaz et al 2015; Mahnkopft 2019)

Traditionally modern science has been largely hypothesis driven (Verma 2002). However, in recent decades more varied, often observational, techniques have also been employed to great success (Kell and Oliver 2003; Havens and Auman 2000; Read and Sharma 2021). Whilst there are various strong adherents to either end of the spectrum, most contemporary scientists employ and support a mixed use of inductive and deductive reasoning amongst whatever else they might find useful (Kell and Oliver 2004). The approach used in this thesis (Table 6.1), represents a mixed approach of hypothesis

driven objectives that are based on more general observational experiments aimed at collecting data and exploring the study system (Gaffney et al 2019; Lykogianni et al 2021).

Table 6.1: Outline of research approach.

General exploration of the agroecosystem	Preliminary observations; basic sampling (key species, times of year) and understanding how major farm processes impact arthropod life
Targeted study of species and processes thought to be important	Bioassays, pest species rearing to discover parasitoids and pathogens and exploration of crop characteristics and regions
What is the impact of key species and events? Are they universal to the question?	Parasitism rate, biocontrol limitations, genetics of agents and pests
What can be done to enhance or stabilise the system?	Future research

Due to the confusion surrounding the spread and decline of the red clover casebearer in New Zealand, this work was able to take a broader approach that benefited from being able to work backwards from a typical biocontrol programme's start point. This situation of red clover seed production in New Zealand presented unique angles in that there was an historic analogous biocontrol programme (that of the white clover casebearers) and the red clover casebearer was clearly undergoing rapid population decline by the time this research began.

Therefore, this research was able to be centred on an exploration of a biocontrol system that was now functioning and why this might be the case, rather than aiming to expand on a current 'failing' biocontrol system with the introduction of new agents. It attempted to assess methods for understanding agroecosystem dynamics in regard to biocontrol provisions, and employed a range of simple and scalable methods for assessing biocontrol systems and potentially enhancing them.

In the early stages, this involved a range of observations based on the use of post winter emergence cages, egg bait predation and the exploration of suction and sweep net sampling efficacy within a general red clover seed crop context (Table 6.1). This was crucial in developing broad understandings of the ecosystem present in the crop and the general state of its biocontrol provision. This quickly revealed that there was a high level of generalist predator activity within red clover seed crops supported by a well-rounded suite of arthropods. In addition, this also aligned with the industry suspicion that the red clover casebearer, *Thrips* and the two mirid species (in order of decreasing concern) were the most important pest species in this system (Rolston pers. com. 2020). Additionally, the exploration of sweep net and suction sampling provided solid data on which to base further sampling for this research.

With this information available, it became easier to begin planning further specific experiments around the red clover casebearer itself and its potential biocontrol system (Table 6.1). The next step of this process was to explore the various aspects of red clover casebearer pressure and biocontrol by monitoring its abundance in the field and conducting laboratory experiments and observations.

These approaches first aimed to identify potential parasitoid and predator biocontrol agents of this pest. Laboratory bioassays were used to determine if predation of red clover casebearer larvae was occurring, and the parasitism rearing box experiment was conducted to detect potential parasitoids. The results revealed that the potential for predation of the red clover casebearer was very low; however, multiple parasitoids were detected in the rearing box experiment. This led to further experiments focusing on the most common parasitoid (*Bracon variegator*) as well as the red clover casebearer's biology.

It is important that the types of laboratory studies and the conditions they impose on test subjects are understood (Casas et al 2004), as many laboratory experiments increase the likelihood of parasitoids and pathogens attacking potential host species (Stiling and Cornelissen 2005). This can present an issue for drawing conclusions, but it does also provide researchers with a chance to observe unknown pathogens, especially in the case of rearing field-collected arthropods (Morin et al 2009). An important aspect of conducting this general research was the way that it increased the chance of observing unknown/unexpected elements within the study system. There are many reasons that the entirety of an agroecosystem or even a single species life history cannot be realistically observed (Casas et al 2004). Because of this, general assumptions of the biology of a species are often drawn from similar species. However, things are not always as they seem and so a mixture of laboratory experiments and fieldwork appears to lead to more consistent biocontrol outcomes (Morin et al 2009)

6.1.2 Using historical work: many patterns that have previously occurred can and will repeat within similar crops.

For this research historical work was very useful as a parallel study system for comparison, along with providing many substantial points from which to start investigations. There is not always such a direct comparison available to every new biocontrol programme, but there will often be similar historical work to draw from. However, it is also important to prevent the programme being defined by historical work.

Conversations with Dr Dan Pearson led to many new perspectives on researching this difficult genus. These conversations were especially useful regarding the intricacies of working with *Coleophora* spp. in the laboratory, which helped avoid many time-consuming errors. Pearson's 1975 thesis was also

crucial in developing an understanding of Coleophorid pests of clover seed crops in New Zealand over the last 100 years and highlighted the usefulness of this information in assisting future biocontrol efforts.

6.1.3 Within the context of New Zealand agricultural production

New Zealand features a very simple agricultural landscape that is largely composed of exotic species; these exotic species represent fragmented parts of largely European farmland ecosystems (Moller et al 2008; Gillespie and Wratten 2012; Goldson et al 2020; Norton et al 2020; Pearson 2020; Tran et al 2022). Because of this reduced diversity of species, there are unique outcomes for biocontrol, or at least, the observation of agroecosystem intricacies becomes much easier in this type of simplified ecosystem (Goldson et al 2020; Shields et al 2022a and 2022b). With reduced avenues of competition, predation and other evolutionary pressures, species can rapidly adapt to specific pressures they face and become unusually challenging pests (Jonsson et al 2015; Goldson et al 2020; Gagic et al 2021). It also appears that biocontrol agents can rapidly adapt new pests as they invade the crop ecosystem resulting in stark population patterns (Liebhold and Tobin 2008). There is possibly some kind of enhanced potential for genetic change in species (and their biocontrol agents) invading these simple agroecosystems (Szucs et al 2019a). However, the details of this are yet to be established.

If it is possible, increasing the suite of predator biocontrol agents that are present in agricultural landscapes can increase biocontrol efficacy. However, there can be negative consequences such as increased predation of biocontrol agents themselves (Letourneau 2009; Moreno et al 2010). Partly this can be due to a lack of understanding regarding ecosystem function and species traits as well as a lack of understanding regarding species interactions, farm management impacts and impacts on non-target species (Barratt et al 2018; Gardarin et al 2018). These negative effects can be avoided through developing an improved understanding of species functional traits (Symondson et al 2002; Stiling and Cornelissen 2005; Trajkovic and Zikic 2023).

6.1.4 Future experiments, caveats and conclusions

Concealed and internally feeding insect pests present a unique challenge to agriculture. Because these insect pests spend periods of their lifecycle concealed in one way or another, making them generally inaccessible to non-systemic chemicals and a range of biocontrol agents (especially generalist predators), this presents a unique challenge to pest management (Jackson et al 2000; Wilby and Thomas 2002; Xiaoyi and Zhongqi 2008). In some cases, the application of a systemic pesticide (such as a neonicotinoid) is effective in ‘plugging’ this weakness in the pest management system (Bakker et al 2020). However, there are many situations where the use of systemic formulations is not suitable due to impacts on the environment and non-target species such as in highly bee pollinator-dependent crops where loss of pollinators leads to yield loss (Cresswell 2011; Singla 2019; Obregon et al 2022). Therefore, non-pesticidal forms of pest control, that can be used for concealed and internally feeding pests are of great benefit (Obregon et al 2022); in the case of the red clover casebearer this appears to be *B. variegator* and possibly an entomopathogenic fungus.

If *F. pseudograminearum* is a biocontrol agent, it is possible that it will be able to attack the red clover casebearer at any time during its contact with the red clover plant, although, this is yet to be determined. *B. variegator* can attack the red clover casebearers fourth instar, that is usually protected by its case. Parasitoid wasps can be more effective than other insect taxa at controlling these concealed and cased life stages of pests, due to their ability to search for hosts through solid surfaces using a range of sensory techniques (Tumlinson et al 1993; Xiaoyi and Zhongqi 2008)

Particular consideration should be given to concealed and internally feeding pests both in New Zealand and globally (Leroy et al 2020; Steyn et al 2020). For example, *Thrips* are of particular concern for many crops and the occurrences of their parasitoids globally are largely unknown. In New Zealand, there is only one case of an introduction of a parasitoid (*Thripobius javae* (Girault 1917) Hymenoptera: Eulophidae) for the biocontrol of a *Thrips* species (*Heliothrips haemorrhoidalis* (Bouche 1883) Thysanoptera: Thripidae) (Morales-Rodriguez et al 2022). Because *Thrips* and other small, often internally feeding insect species inhabit such a large range of plant features, including deep inside cuticles and growth tips, they often avoid many conventional insect controls including systemic pesticides to which they can evolve resistance (Stuart et al 2011; Nilon et al 2021). The increased unpredictability of pest pressure, due to climate change and to many concealed pest’s unique avoidance of lethal pesticide thresholds, the possibility for further resistance developing is high (Nilon et al 2021).

The widespread decline of the red clover casebearer after such a short and intensive period of outbreaks is not sufficiently explained by the discovery of the fairly active parasitoid *B. variegator*. As shown in previous chapters, it even appears that red clover casebearer populations can persist at

high levels despite heavy parasitism pressure from *B. variegator*. Due to this lack of convincing parasitoid regulation pattern, it is suspected that there are other non-arthropod pressures (Rannback et al 2015; Miranda-Fuentes et al 2019), that are important in explaining the red clover casebearer population dynamics. In addition, genetic process could also be impacting red clover casebearer populations. In invading species that have reached the limit of their expansion a reduction in population growth rate is often observed (Burton et al 2010; Zaviezo et al 2017).

It is possible that this population regulation of the red clover casebearer is driven by *F. pseudograminearum*, which may be highly effective at high red clover casebearer densities (especially during over-wintering) (Kovac et al 2021). Low-density regulation may possibly be supplemented by *B. variegator* populations that are largely supported by other host species in the landscape.

Within New Zealand Coleophorid clover seed pests, *Coleophora* spp. were prevalent in white clover seed crops until the long-term biocontrol importation programme for the control of these pests in the 1960s through to the 1980s (Pearson 1975). The white clover specialised *Coleophora* species (*Coleophora alcyonipennella* and *C. mayrella*) have not made a recorded resurgence since this long-term programme (Pearson 1989; Chynoweth et al 2018). The patterns in red clover casebearer population dynamics may be best explained by this; there was an initial outbreak period where the red clover casebearer was relatively free from typically associated control pressures from within its native range, and therefore, able to increase to very high abundances across New Zealand. While the pathogen (*F. pseudograminearum*) and parasitoids (*B. variegator* etc.) existed in red clover seed crops, their populations may have been sustained on non-Coleophorid host species prior to the red clover casebearer's arrival. These biocontrol agents then needed time to adjust to this new prevalent host species (the red clover casebearer). These periods of adjustment might be explained by the red clover casebearer's very specific univoltine lifecycle (the entire population is very synchronised with fourth instar larvae only being active in crops for a few weeks in late summer). It is therefore possible that pathogen and parasitoids could have taken a number of seasons to build sufficient population levels at key times of the year to deliver significant biocontrol of the red clover casebearer in red clover seed crops.

Within the simple agricultural landscapes of New Zealand these periods of adjustment are very hard to predict, as any new species can cause major shifts within a crop ecosystem relatively quickly; e.g. the arrival of a new sap feeding species can massively increase the level of sugars on plant surfaces allowing more fungi to grow on stems (Liere et al 2015). Because of this it is likely that the disease profile a pest faces is dynamic and responds quickly to system level changes (Lacey et al 2015). For

the red clover casebearer this could mean that after reaching high abundances nationally, this pest resource itself was colonised by sufficient pathogen and parasitoid elements to regulate it at the population level (Gouli et al 2013).

It is possible that *Coleophora* species are often regulated largely by a complex of fungal isolates that may or may not be endophytes of respective Coleophorid species host plants in their native environments. These fungal pathogens may be encountered during case construction or the internal plant living habits of larvae in the more host specialised *Coleophora* species. It is suggested that under normal native conditions these pests are kept in check by a combination of these fungi which largely impact the species at a higher population density, and synchronised or unsynchronised parasitoids that impact Coleophorid larvae at all population densities (Brown and Kulhavy 1978). While the current research has provided some information on the biocontrol of the red clover casebearer, an exploration of the adaption to New Zealand by red clover casebearer, *B. variegator* and *F. pseudograminearum* would also be beneficial to understanding the wider picture.

So far, it has been established that the red clover casebearer can be a highly aggressive invading pest; however, it appears to have succumbed to some ecological or genetic pressure, within 5 years, which has greatly lessened its impact as a pest. It is also possible more typical genetic processes may be in play, such as inbreeding depression which can quickly establish following the colonisation limits of a pest being reached (Burton et al 2010; Zaviezo et al 2017). Although, genetic bottle necks do not always have negative impacts on a species their impacts have been observed within five generations of a Hymenoptera species (Burton et al 2010; Zaviezo et al 2017).

In addition, it is important to understand how aspects of a cropping system and Individual crop/field characteristics affect pest dynamics. In the case of the red clover casebearer much of the pest issue can be avoided by harvesting red clover seed crops in the first and second years only, as this pest usually only builds to serious numbers in the third and fourth year a clover crop is harvested for seed (Pearson 1989; Chynoweth 2018).

6.1.5 Contemporary evolutionary processes and biocontrol

Populations of alien species in new landscapes that are undergoing active range and population expansion experience many complex evolutionary dynamics that can occur more rapidly than 'traditional evolution' (speciation) (Berlocher and Feder 2002; Pelisse et al 2018; Simon and Peccoud 2018; Szucs et al 2019a). However, most modes of genetic change such as the development of ecotypes, do not always lead to speciation due to adequate genetic flow from the wider species population among other factors (Dermauw et al 2018; O'Neal et al 2018). An example of these rapid genetic changes is the development of resistance to insecticidal *Bt* cotton by Western corn root

worm (*Diabrotica virgifera virgifera* (LeConte 1868) Coleoptera: Chrysomelidae) populations in the United States (Grassman et al 2014).

These forms of genetic change are described and substantiated to various degrees through many papers and studies, but it is still not a fully articulated or predictable force of ecology (Schooner 2011; Szucs et al 2019a). There are many factors driving these genetic developments in range expanding species, such as totalising environmental pressure. This pressure can come from conventional agriculture practice occurring on the bulk of any farmland which determines a pest species inhabitable range in this landscape, genetic diversity of the founding population and fitness trade-offs between dispersal and remaining in areas of high species abundance (Schooner 2011; O'Neal et al 2018).

The small genetic aspect of this research provided some interesting information on the diversity of *B. variegator* in New Zealand. While no conclusions could be drawn, there are at least two clades present in this species in Canterbury within individuals collected from the same field. Often, within imported and released biocontrol agents, strains will naturally hybridise and maintain purity in the field, but over time selection pressures will lead to a more favourable purity or combination of strains being dominant (Szucs et al 2019b). However, this appears to not be the case within *B. variegator* established in New Zealand, suggesting that these genetic differences are not relevant to the fitness of *B. variegator* as a parasitoid of the red clover casebearer. Whilst heterosis and inbreeding depression do occur in introduced parasitoid populations (Vayssade et al 2014; Zaviezo et al 2017), a lot of information needs to be gathered on the relative ecotypes of the species to be able to draw conclusions (Benevenuto et al 2012).

It is evident that the conditions of parasitoid capture/sourcing, transportation and rearing all play a role in the final array of variation and traits in the population of a species that is released for a biocontrol programme (Hufbauer 2002). Furthermore, these conditions and intricate genetic processes can result in these small recently introduced populations being prone to extinction (Fauvergue et al 2015).

Shields et al (2021; 2022) proved parasitoid-mediated avoidance evolution in a host species for an introduced biocontrol system. This case represented the first time this process of evolution had been observed in an introduced pest and biocontrol system. This development occurred very quickly and points to some rapid form of evolution being possible within simple modified landscapes such as those of New Zealand farming (Shields et al 2021; Shields et al 2022b; Sentis et al 2022).

The case: A weevil pest (Argentine stem weevil) that reproduces sexually in its introduced New Zealand population developed an avoidance behaviour to its asexual parasitoid wasp (*Microtonus*

hyperboda) over a period of 30 years. This development of behaviour was also proven to be occurring faster in the warmer Northern latitudes of New Zealand. This has led to an observable decrease in the effectiveness of the biocontrol provisions over time (Shields et al 2021; Shields et al 2022b).

There may well be more such cases in development or already completed in parts of the world; currently this has only been observed in a system featuring a singular asexual parasitoid in a very simple modified landscape. There may be other mechanisms that lead to biocontrol breakdowns through pest evolution. The revisiting of previous biocontrol programmes with a contemporary lens may well avoid more cases of biocontrol breakdown (Cock et al 2016).

One immediately problematic genetic process to biocontrol programmes is inbreeding, which can occur relatively quickly within limited captive populations. Inbred parasitoid populations often have lower biocontrol efficacy rates (Vayssade et al 2014; Zaviezo et al 2017). Typically, attempts to avoid inbreeding have been based on widening collection of the parasitoid species (Szucs et al 2019a). However, due to the variation in local ecotype adaptations, recombining genes of species blindly from around its range can be detrimental to biocontrol programmes as well (Szucs et al 2019a). Contemporary biocontrol programmes gather genetic information on ecotypes and perform a range of laboratory experiments to avoid these issues (Vayssade et al 2014; Zaviezo et al 2017).

From 2019 to 2022, the red clover casebearer was never observed outside of dense red clover seed crops, even when relatively abundant supplies of clover seed occurred in pastures nearby. Potentially, this pattern shows that due to the red clover casebearer population collapsing, it is no longer dispersing in the same way it was in the earlier phases of its invasion of New Zealand.

This could be due to adaptations during the dispersal and colonisation phase of this pest leading to the colonisation of less dense host plant resources (Burton et al 2010). This would likely be due to events of adaptation/mutation followed by high immediate levels of the proliferation of these adaptations (Pelisse et al 2018). Multiple factors could explain the decline of the red clover casebearer's presence on non-red clover seed crop red clover plants. This habitat is more disturbed and marginal (mowers, random grazing and other crushing) which would kill off a high proportion of developing offspring. Strong pheromone cues from other moths/dense crops stabilise the distribution of the red clover casebearer over time (the bulk of the red clover casebearer population is constantly being drawn to the strongest local cues).

Red clover crops are managed via topping and closing dates so that flowering occurs within one month. This generally means that non-crop red clover plants operate on a different phenological timeline to seed crop red clover plants, generally flowering much earlier and for a longer period of time. Adaptation

to the earlier flowing host plant resources may be difficult for such a highly specialised insect pest in an agricultural landscape (Reznick and Ghalambor 2001; Estoup and Guillemaud 2010; Sculz et al 2019a).

Thomas et al (2001) demonstrated how four insect species in Britain displayed changes of traits in response to increasing habitat breadth. Two butterfly species *Hesperia comma* (L. Lepidoptera: Hesperidae) and *Aricia agestis* (Debis and Schiffermuller Lepidoptera: Lycaenidae) both used a wider range of host plants in populations colonising a new habitat compared to historic populations that typically used a single host plant species and were thus restricted to that plant's distribution. Low density populations of two cricket species *Concephalus discolour* (Thunberg Orthoptera: Tettigoniidae) and *Metroptera roselii* (Hagenbach Orthoptera: Tettigoniidae) in the marginal areas of their respective ranges were found to have a higher proportion of winged and longer winged adults better suited for dispersal. This finding runs contrary to the belief that the more dispersive forms are developed due to denser populations of a species (Thomas et al 2001).

Whilst this is speculative theorising of pest population patterns, it is important to consider the real randomness and intensity of pest invasion dynamics. These processes largely cannot be predicted because a new genetic assemblage in a new landscape is essentially an unstudied species. It will be able to adapt and progress in new ways due to new pressures and fuels (Sentis et al 2022).

6.2 Future research

- Explore the mode of mortality and pathway of infection of *F. pseudograminearum* as an entomopathogen of the red clover casebearer. This research could investigate whether *F. pseudograminearum* is specifically active on red clover casebearer larvae (or just on the case), as well as determining the path of infection and cause of the eventual death of larvae exposed to the pathogen. In addition, the possibility of this entomopathogenic strain of *F. pseudograminearum* being an endophyte of red clover plants should be explored. Due to the difficulties of locating substantial overwintering clover casebearer larvae, a field mortality rate may be unobtainable. However, generally important aspects of this ecological interaction can be established in the laboratory, which may reveal a way to measure mortality in the field.
- Survey of the fungal pathogens of crop pest *Coleophora* spp. in their native and pest ranges. Largely this research would be undertaken to build the body of information regarding these lepidopterans and possible avenues for their control in agricultural landscapes. Currently the tri-trophic systems of host plants, fungi (that interact with both host plant and pest insect) and insect pests are poorly understood and in many cases entomopathogenic interactions of typically plant pathogenic fungi are often undocumented.
- Of particular interest in this area will be whether there are differences in the composition of fungal pathogens of *Coleophora* spp. between their native European ranges and introduced ranges.
- How *B. variegator* is sustained in the landscape outside of the clover casebearer species in New Zealand. How this parasitoid sustains populations in the landscape outside of the majority of the year remains to be established. Whilst it may not have serious implications for the biocontrol of *Coleophora* pests currently, it is of interest to understand *B. variegator*'s function and lifecycle within New Zealand habitats.

6.3 Conclusions

How the aims of this work were addressed: Aim one, *Improve understanding of the population dynamics of arthropod populations in red clover seed crops in New Zealand*. This research showed how the richness of the arthropod community present in red clover increased with age, and whilst a higher range of pests may be present in older crops, there were also more biocontrol agents to help regulate them.

Aim two: *Explore how these dynamics change over the growing season*. The topping of the red clover seed crop in late spring and early summer was found to disturb the arthropod community however, the populations of arthropod species quickly recovered from this intervention. Furthermore, the arthropod community peaked in abundance and diversity during crop flowering due to the increased provision of floral resource in the crop at this time being able to support the full suite of species.

Aim three: *Develop understanding of the biocontrol provision within red clover seed crops by resident arthropods*. A high number of parasitoid wasp species were found, whilst most were only identified as RTU's due to the high level of expertise and time needed for species identification. It can be stated that this diversity of parasitoid wasps is essential to pest regulation in the red clover seed crop system. Of importance was the discovery that three parasitoid wasp species *B. variegator*, *P. puparum* and *E. messene* could all parasitise the red clover casebearer. Furthermore, the preliminary egg baiting experiment and field comparison experiment showed that there was a high level of generalist predator activity in the crop that increased during the growing season.

Aim four: *Investigate red clover casebearer moth outbreaks and general populations dynamics in New Zealand red clover seed crops*. It was found through the field comparison experiment and all other sampling the red clover casebearer had become rare in New Zealand red clover seed crops, and that outbreaks were only occurring in two red clover seed crops that were planted in 2018. This aim was difficult to investigate given the remission of the red clover casebearer as a pest of red clover, however, abundances of this pest in instances of outbreak were shown as well as its rapid decline when faced with unsuitable conditions such as high rainfall.

Aim five: *Identify potential biocontrol agents of the red clover casebearer moth*. Resident predator arthropods in red clover seed crops were unable to predate of red clover casebearer larvae, however, three parasitoid wasp species, as well as, a potentially entomopathogenic fungal pathogen *F. pseudograminearum* were found to cause mortality of red clover casebearer larvae.

Aim six: *Assess the potential of any biocontrol agents of the red clover casebearer identified*. Of the three parasitoid species recorded parasitising red clover casebearer larvae, only *B. variegator* was found to be active at a meaningful rate and was shown to be an important parasitoid of the red clover casebearer that is essential to its regulation in New Zealand. *F. pseudograminearum* and other fungal pathogens also appear to be especially deadly to developing red clover casebearer larvae based on field and laboratory observations.

This thesis represented a unique opportunity to explore issues relating to biocontrol of an insect pest in New Zealand. The sudden explosion of the red clover casebearer populations over all red clover-growing regions in New Zealand and then its rapid decline made it a valuable case study from a biocontrol perspective. Having a similar pest issue in white clover crops previously was very helpful in understanding this pest.

The arrival of the red clover casebearer provides another example of how vulnerable New Zealand is to sudden and severe pest outbreaks, and prior detection cannot always be relied on. To improve the resilience of growing systems, pest regulation endeavours must be multi-disciplinary and not rely on singular methods or species for effective control.

While many questions still remain regarding this pest issue, although this work has shown that a parasitoid (*Bracon variegator*) and maybe even more so a fungal pathogen (*Fusarium pseudograminearum*) are likely to be important to the biocontrol of the red clover casebearer.

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