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**Host-parasitoid avoidance behaviour in the context of  
contemporary evolution in insect classical biological control**

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A thesis  
submitted in partial fulfilment  
of the requirements for the Degree of  
Doctor of Philosophy

at  
Lincoln University  
by  
Morgan William Shields

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Lincoln University

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Abstract of a thesis submitted in partial fulfilment of the  
requirements for the Degree of Doctor of Philosophy in Ecology.

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in insect classical biological control

by

Morgan William Shields

Pests are increasingly invading novel environments due to global trade and travel and their management requires a greater emphasis on classical biological control than has previously been the case. This approach has been particularly successful in New Zealand pasture such as with the Argentine stem weevil, *Listronotus bonariensis* (Coleoptera: Curculionidae) (ASW). This pest was successfully managed by releasing the parthenogenetic parasitoid, *Microctonus hyperodae* (Hymenoptera: Braconidae), in the early 1990s with ASW parasitism rates quickly reaching over 75 %. However, these rates have substantially declined in the last decade. This decline was hypothesised to be due to contemporary evolution of enhanced avoidance behaviour by the sexual ASW and that these behaviours are influenced by the host plant.

The work in this thesis used microcosms in laboratory experiments to investigate whether there was any behavioural evidence supporting the above hypotheses, which if confirmed, may explain recent low parasitism rates. Experiments were conducted to examine the ASW behavioural responses to *M. hyperodae* and investigate whether these ASW responses differed between the ryegrass host plants; diploid hybrid (diploid *Lolium perenne* x diploid *Lolium multiflorum*), diploid *L. perenne* and tetraploid *L. multiflorum*. Furthermore, ASW behavioural responses to the presence of *M. hyperodae* were compared between ASW populations collected from areas with different parasitism rates, history and latitude. The sensitivity of ASW responses to different parasitoid species was also investigated.

The work in this thesis showed that the weevil's avoidance behaviour differed depending on the host plant, with ASWs on the diploid hybrid having the most consistent reduced feeding and plant abandonment responses to *M. hyperodae*. ASW on diploid *L. perenne* had similar responses to weevils on the diploid hybrid but these responses were delayed. ASW on tetraploid *L. multiflorum* showed a reduced feeding response but no plant abandonment. These results reflected recent ASW parasitism rates that were low on the diploid host plants compared to those in the 1990s and may explain the behavioural mechanisms behind the biological decline. Similarly, behavioural responses to *M. hyperodae* differed between ASW regional populations, which reflected current parasitism

rates and history. ASWs from the Waikato (Ruakura) region had the strongest behavioural responses to *M. hyperodae* where the greatest parasitism decline has occurred. There were delayed feeding reduction and plant abandonment responses to *M. hyperodae* from ASW populations (Canterbury: Lincoln; North of Auckland: Wellsford) with a medium decline in parasitism. There was minimal response from southern ASW populations (Otago: Invermay; Wiapiata) that have always had low parasitism rates with no decline detected. When comparing ASW responses to different parasitoid species, *M. hyperodae* showed the strongest responses with increased crouching and reduced feeding compared to other parasitoid species. There was some behavioural response to *Microctonus aethiopoides* (Hymenoptera: Braconidae), a parasitoid of the clover root weevil (*Sitona obsoletus*) (Coleoptera: Curculionidae), suggesting that ASW has pre-adaptation to this novel interaction.

The findings in this work strongly suggest that ASW behavioural responses are the mechanism behind the potential contemporary evolution causing the parasitism decline. Future work could investigate the behavioural effects of *M. hyperodae* from different populations, and what cues ASW uses to detect *M. hyperodae*. Furthermore, identifying the underlying genetic or symbiont mechanism for the increased sensitivity of ASW behaviour is essential to confirm contemporary evolution, which would be novel in the literature. This work also has implications for the persistence of other long-term successful insect biological control systems and points to what factors may lead to their future decline in efficacy.

**Keywords:** Asymmetric selection, Argentine stem weevil, behaviour, classical biological control, contemporary evolution, diffuse selection, feeding damage, *Listronotus bonariensis*, *Lolium*, microcosm, *Microctonus hyperodae*, natural enemies, non-consumptive effects, parasitoid, pasture, plant abandonment, ploidy, resistance, traits.

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# Chapter 1

## Introduction

### 1.1 Global agriculture

The global distribution of food must improve to sustainably feed the expanding human population (De Schutter 2010; Gustavsson et al. 2011; Alexander et al. 2017; Mc Carthy et al. 2018) that is predicted to grow from 7.6 billion in 2018 to 10 billion in 2050 (UN 2017). Over 40 % of harvested crops are lost prior to consumption (Alexander et al. 2017) and an estimated third of all food produced is wasted (Gustavsson et al. 2011). This post-harvest food waste puts pressure on conventional agriculture to meet the increasing food demand which relies on vast quantities of monocultural land that has high inputs of anthropogenic fertilisers and pesticides (De Schutter 2010; Godfray et al. 2010; Gurr et al. 2012; Pretty et al. 2018; Springmann et al. 2018; Shields et al. 2019).. This has caused an enormous loss of biodiversity (Sala et al. 2000) and the ecosystem functions that it provides, resulting in a rapid decline of ecosystem services such as biological control of pests (Evans 2016; Altieri & Nicholls 2018). The current global agricultural crisis may be mitigated if agricultural practices become more sustainable by restoring and enhancing ecosystem functions and the services to humans that they provide, by increasing functional biodiversity and reduce farm inputs (De Schutter 2010; Godfray et al. 2010; Struik & Kuyper 2017; Dumont et al. 2018; Gliessman 2018; Pretty et al. 2018). An essential ecosystem service to agriculture that can be restored and is an alternative to pesticides is biological control using insects (Naranjo et al. 2015; Gurr et al. 2017; Ingerslew & Finke 2018; LaCanne & Lundgren 2018; Shields et al. 2019). Insect classical biological control in particular is underutilised due to the economic investment at government and producer levels (Begum et al. 2017) and the potential risks. However, classical biological control it can manage pests at a national scale and may eliminate the need for insecticides and herbicides (Gurr & Wratten 2000; Naranjo et al. 2015; Gurr et al. 2016; Tomasetto et al. 2017b; Wyckhuys et al. 2019).

### 1.2 Classical biological control

Classical biological control using insects is a very valuable component of pest management (Naranjo et al. 2015; Hajek & Eilenberg 2018; Ferguson et al. 2019) and is becoming more relevant with pesticide withdrawals (Borel 2017; Gurr et al. 2017; Butler 2018) and increasing pest invasions through global trade (Bebber et al. 2014; Wyckhuys et al. 2018) and climate change (Thurman et al. 2017). Classical biological control (also known as importation biological control) is the management of invasive species by introducing exotic natural enemies, primarily from the pest's native range that establish self-perpetuating populations (Holt & Hochberg 1997; Goldson et al. 2014; Naranjo et al.



2015). These insect introductions first began in the late 1800s such as the release of a beetle (*Rodolia cardinalis* (Mulsant)) and a parasitic fly (*Chryptochaetum iceryae* (Williston)) to manage the cottony cushion scale, *Icerya purchasi* Maskall in California in 1888 (Caltagirone & Doult 1989). Since then insect biological control agents have successfully been used to manage at least 226 invasive insect pests leading to long term suppression (Heimpel & Cock 2018), although only around 10 % of attempted insect classical biological control is successful (Cock et al. 2016).

Prior to the 1980s, the risk of non-target effects were rarely considered, with research focused on the conditions and traits that make biological control successful (Beddington et al. 1978; Hall & Ehler 1979; Evans 2016). This resulted in some introductions having negative ecological impacts such as trophic cascades and extinctions (Howarth 1991; Simberloff & Stiling 1996; Henneman & Memmott 2001; Messing & Wright 2006; Bockerhoff et al. 2010; Barratt et al. 2016). For instance, the extinction of the Fijian coconut moth (*Levuana iridescens* Bethune-Baker) which was an endemic pest (Totthill et al. 1930). Another example is the local extinction of the United Kingdom population of the large blue butterfly (*Phengaris arion* L.) due to the biological control of rabbits in conjunction with changes in grazing practices (Simberloff & Stiling 1996; Thomas et al. 2009). This led to a paradigm shift in classical biological research to a risk-orientated paradigm that was strongly influenced by Howarth (1991) and Simberloff and Stiling (1996), with a major emphasis on ecological implications such as direct impact on non-target endemic species and indirect effects on ecological communities and food-webs (Heimpel & Cock 2018). This led to a rapid decline in classical biological control introductions worldwide (Cock et al. 2016) with 1.2 successful introductions per year between 1998 and 2012 compared to 4 in prior decades (Heimpel & Cock 2018). An exception to this global trend is New Zealand, which adopted a benefit-risk approach that is internationally recognised as a model for regulating classical biological control introductions (Hill et al. 2011; Heimpel & Cock 2018). New Zealand's classical biological success is in part due to its island biogeography and simplified agroecosystems that have resulted in severe pest problems such as in pasture (Ferguson et al. 2019).

### **1.3 New Zealand pasture**

New Zealand is an island nation dominated by pastoral agriculture (Ferguson et al. 2019) and involves around half the nation's land area comprised entirely of introduced plant species (Anon. 2018). A very small group of ryegrass species consisting of *Lolium perenne* L., *L. multiflorum* Lam., *L. perenne* x *L. multiflorum* hybrids and the clover species *Trifolium repens* L. and *T. pratensis* L. dominate these pastures (Goldson et al. 2015; Tomasetto et al. 2017b; Ferguson et al. 2019) with several other exotic plant species having minor contributions (Ferguson et al. 2019). The thesis focused on the following grasses with nil-endophyte; diploid *L. perenne* (cv. Samson); diploid hybrid (*L. perenne* x *L. multiflorum*) (cv. Manawa); Tetraploid *L. multiflorum* (cv. Tama). These grasses and

ploidies were used because they represented the major pasture types of New Zealand and were in previous but related experiments so comparisons could be made (Goldson 1982; Barker 1989; Goldson et al. 2015; Goldson & Tomasetto 2016). Additionally, nil-endophyte seed was used to remove the variable of endophyte as this would severely complicate experiments and was beyond the scope of this work. The above aspects are further discussed in Section 2.4.1.2. In conversation with Professor Stephen Goldson (pers. comm.) it was discussed how the assemblage of introduced plant species has created a very simplified agroecosystem that has low natural enemy diversity with a predominantly exotic invertebrate community that has little overlap with native New Zealand grasslands that mostly contain endemic species. Furthermore, the native and exotic arthropod natural enemies that do occur in New Zealand pastures generally have limited penetration into paddocks and tend to stay close to or within the field boundaries. The resulting low biotic resistance and available niches makes New Zealand pasture prone to pest invasions (Stephen Goldson pers. comm.), an example being the Argentine stem weevil (*Listronotus bonariensis* Kuschel) (Ferguson et al. 2019).



Figure 1.1 Adult Argentine stem weevil, *Listronotus bonariensis* Kuschel on a leaf blade and is 3-4 mm long. Photo: Jacquelyn Bennett with permission

#### **1.4 Argentine stem weevil classical biological control with *Microctonus hyperodae***

The Argentine stem weevil (ASW) is a major pest of New Zealand pastures, costing agriculturists up to \$NZ 200 million each year (Ferguson et al. 2019). ASW was first recorded in New Zealand in 1927 (Marshall 1937) and was probably introduced via stock feed on trading ships from South America (Williams et al. 1994) where the weevil had very low densities in its native ‘vega’ or ‘mallines’ habitats, feeding on a range of mainly South American endemic grasses (Lloyd 1966). In New Zealand ASW can reach densities of up to 723 adults/m<sup>2</sup> in the North Island (Barker & Addison 1993) causing severe damage to novel hosts in improved pastures of European origin (Goldson 1982; Barker 1989),

particularly from January to March when pasture may also water stress (Ferguson et al. 2019). Pasture damage is predominantly caused by the stem boring immature stages that kill 3-8 tillers per individual larva (Pottinger 1961; Barker et al. 1989). Adult ASW (3 - 4 mm long) also cause damage to a lesser extent by creating feeding scars or 'windows' in leaf blades (Goldson & Penman 1979; Barratt et al. 1996; Phillips 2002) and can devastate early grass seedlings (Barratt et al. 2016; Ruppert et al. 2017).



Figure 1.2 Adult weevil feeding scars or 'windows' in a leaf blade. Photo: Jacquelyn Bennett with permission.

ASW activity at all stages occurs above a 10.5 °C temperature threshold (Goldson & Proffitt 1990) and is considered nocturnal with peak activity in the hours after dusk in summer; in winter ASW feeds at any time above the temperature threshold (Barker & Pottinger 1986; Barratt et al. 1995). There are three generations per year in northern New Zealand (Barker et al. 1989) and 1-2 generations in the South Island (Goldson 1981a; Goldson et al. 2011). Adults generally undergo reproductive diapause during early March that is determined by photoperiod and which ends in late winter or spring (Goldson & Emberson 1980; Goldson 1981a).

ASW has few natural enemies (Vink & Kean 2013; Goldson et al. 2014; Tomasetto et al. 2017b) and substantial dispersal ability (Goldson 1981b). ASW was considered the biggest pasture pest prior to discovery and development of endophytes in the genus *Epichloë* ((Fr.) Tul. & C. Tul.) (Ascomycotina: Clavicipitaceae), in the 1980s and 1990s (Ferguson et al. 2019) as there are no effective or economical chemical management options available (Goldson et al. 1995; McNeill et al. 1996). Endophyte-based pasture resistance continues to reduce ASW damage. However, ASW populations can still reach over 200 adults/m<sup>2</sup> causing major damage (Barker & Addison 2006) and the extent of endophyte protection varies greatly between *Lolium* pasture types, endophyte strains and pasture management (Popay et al. 2017; Ferguson et al. 2019). Furthermore, early pasture seedlings are not protected by endophyte (Ruppert et al. 2017).

Around 1991, the same time as widespread endophyte adoption (Ferguson et al. 2019), the classical biological control agent *Microctonus hyperodae* Loan (1.5 mm long) from South America was released throughout New Zealand (Goldson et al. 1993). This solitary koinobiont endoparasitoid wasp (Loan & Lloyd 1974; Goldson et al. 1995) has restricted oligophagy in New Zealand, preferring ASW but can also successfully develop in endemic *Irenimus aequalis* Broun (Goldson et al. 1992) and has a high searching efficiency (Barlow et al. 1993). *M. hyperodae* reproduces by thelytokous parthenogenesis with c. 48 eggs per female and has three generations per year (Loan & Lloyd 1974; Goldson et al. 1995). Parasitism of ASW makes the host almost immediately sterile (Goldson et al. 1990; Goldson et al. 1995) and is part of the process from antennation followed by injecting an egg and venom between the sternites or in the anus of the weevil. This is followed by additional antennation and ASW hyperactivity (Phillips 2002). During the early 1990s there were eight *M. hyperodae* 'ecotypes' from different South American populations released simultaneously with equal numbers at 28 sites involving c. 270,000 parasitised ASWs with 90 % site establishment of *M. hyperodae* (Goldson et al. 1995). It is unknown how the released 'ecotypes' differed genetically and what 'ecotypes' have persisted but those from east of the Andes became dominant in New Zealand (Phillips & Baird 1996; Phillips et al. 1997; Phillips & Baird 2001). Once established *M. hyperodae* quickly achieved high parasitism rates of 75 % or higher in the mid-late 1990s (Goldson et al. 1998; Barker & Addison 2006). However, these rates have seriously declined in recent decades (Popay et al. 2011; Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b). Despite substantial investigation (Goldson et al. 2015; White et al. 2015; Goldson & Tomasetto 2016; Ferguson et al. 2019) the mechanisms behind the loss of biological control effectiveness are unknown (Tomasetto et al. 2017b).



Figure 1.3 Adult parasitoid *Microctonus hyperodae* Loan (1.5 mm long), the classical biological control agent of the weevil. Photo: Jacquelyn Bennett with permission.

## 1.5 Contemporary evolution

Insect pest resistance is where control measures become less effective due to changes in the insect pest's genetics, referred to as contemporary evolution (also known as resistance, rapid evolution or microevolution) (Hufbauer & Roderick 2005). Contemporary evolution in pest management is where relatively quick adaptation has occurred that overcame control measures by the proliferation and maintenance of heritable resistance-inducing genetic material throughout pest populations, such as new mutations or rare alleles that have become more beneficial (Hufbauer & Roderick 2005; Goldson et al. 2014; Mills 2017; Tomasetto et al. 2017b). This may occur when regular strong selection favours resistant traits and persistently removes most non-resistant pest individuals from the gene pool (Hufbauer & Roderick 2005; Thompson 2013; Liu 2015). Contemporary evolution has contributed to insect pest control failure for over 70 years (Hufbauer & Roderick 2005; Siegwart et al. 2015; Sparks & Nauen 2015). This has primarily occurred through repeated overuse of insecticides and bio-insecticides (Liu 2015; Siegwart et al. 2015; Sparks & Nauen 2015). Bio-insecticides are microbial biological control agents (often dead) and their products (Siegwart et al. 2015). Contemporary evolution has caused 586 insect pest species to become resistant to 325 insecticides since 1914 (Sparks & Nauen 2015). Ten of the most resistant insect pests are resistant to 38-91 insecticides with the diamondback moth *Plutella xylostella* (L.) being the most insecticide resistant insect pest in the world (Sparks & Nauen 2015). Conversely, at least 27 insect pest species are resistant to bio-insecticides that use *Bacillus thuringiensis* (Berliner) (Bt) toxins (Siegwart et al. 2015).

How contemporary evolution has caused this widespread insecticide and bio-insecticide control failure is well understood and can be traced back to the source evolutionary events. This is usually in the form of heritable random point mutations that alter existing traits (Liu 2015; Siegwart et al. 2015). Almost all mutations are detrimental, neutral or are lost from a population (Thompson 2013). However, when regularly exposed to insecticides or bio-insecticides, resistance inducing mutations proliferate in insect pest populations because typically, only individuals possessing these mutations survive (Siegwart et al. 2015). In addition to mutations, pre-existing resistance traits can occur if the insect pest has been previously exposed to the selection pressure such as toxins (natural or anthropogenic) that have a similar mode of action. This phenomena is known as cross-resistance (Siegwart et al. 2015). For example resistance to spinosad bacteria and neonicotinoid insecticides (Puinean et al. 2013; Bao et al. 2014). Cross-resistance of pre-existing defensive traits can also occur in insect classical biological control such as encapsulation of parasitoid larvae (Kapranas & Tena 2015).

Factors that affect contemporary evolution of insect pests occurring are likely to be similar between biological control, insecticides and bio-insecticides. Pre-existing mutations that confer insect pest

resistance that are spread and maintained within a population allows fixation in a population (Hufbauer & Roderick 2005; Siegwart et al. 2015). This is affected by the number of mutations required for resistance and the dominance of the resistant allele(s) (Curtis et al. 1978; Taylor & Georgiou 1979). Furthermore, transmission of resistance is affected by the chromosome in which the resistant genes occurs such as X or Y chromosome (Asser-Kaiser et al. 2007; Berling et al. 2008). Development of contemporary evolution and maintenance of resistance is strongly influenced by its impact on fitness in the absence of the selection pressure (Siegwart et al. 2015). Resistance can be lost if there is a high cost to maintain it such as reduced competitive ability, fecundity and dispersal (Georgiou 1983; Hufbauer & Roderick 2005; Siegwart et al. 2015). Gassmann et al. (2009) found that of 77 studies, 60 % showed a cost to maintaining Bt resistance. The cost associated with maintaining resistance is a major factor hypothesised to prevent resistance maintenance (Holt & Hochberg 1997; Hufbauer & Roderick 2005; Goldson et al. 2014). The number of generations per year determines the speed of contemporary evolution of resistant genotypes (Tabashnik & Croft 1985). Therefore, insect pests with short life cycles in insect classical biological control are more likely to develop contemporary evolution. Additionally, high dispersal ability of an insect pest species allows resistant genotypes to enter the gene pools of other populations, thus facilitating the spread and maintenance contemporary evolution (Taylor et al. 1983; Siegwart et al. 2015).

There is limited literature on contemporary evolution leading to insect biological control decline (Holt & Hochberg 1997; Hufbauer & Roderick 2005; Goldson et al. 2014; Mills 2017). The lack of evidence is hypothesised to be due to several factors can reduce the development of insect pest contemporary evolution (Holt & Hochberg 1997; Goldson et al. 2014; Tomasetto et al. 2017b). For instance, refuges in heterogenic landscapes for susceptible genotypes that can then immigrate (Curtis et al. 1978; Goldson et al. 2014) and dilute resistant genotypes in the population, preventing resistance genotype fixation and may make them susceptible to other deleterious processes such as genetic drift (Hufbauer & Roderick 2005). Additionally, pest defensive trait effectiveness in insect classical biological control varies depending on the individual involved (Thompson 2013) and cross-resistance to multiple natural enemies is argued to reduce the probability of biological control failure from contemporary evolution (Kraaijeveld et al. 2012). This is because even closely related natural enemies can use considerably different physiological mechanisms and have different antagonistic interactions with the pest (Hufbauer & Roderick 2005). Furthermore, creating different selection pressures by using a range of insecticides, bio-insecticides and biological control agents can also reduce the development of contemporary evolution (Dahlsten & Mills 1999; Siegwart et al. 2015; Slippers et al. 2015). Despite the above inhibiting factors, contemporary evolution has been recently hypothesised to be implicated in the failure of previously successful insect classical biological control

programs (Goldson et al. 2014; Slippers et al. 2015; Mills 2017; Tomasetto et al. 2017b) and is further discussed in subsequent chapters.

## 1.6 Thesis rationale and hypotheses

This thesis investigated if there was any laboratory-based behavioural evidence for the hypothesis that the ASW parasitism rate decline is because of ASW contemporary evolution in the form of enhanced avoidance behaviour as suggested by Goldson et al. (2015). This is currently the most parsimonious explanation given the temporal differences in ASW parasitism rates decades apart in the field (Goldson et al. 2015; Tomasetto et al. 2017b; Tomasetto et al. 2018b) and in the laboratory (Goldson & Tomasetto 2016). There are also several factors that support the notion that contemporary evolution is occurring, such as New Zealand's simplified pasture ecosystems with novel host plant associations and asymmetric selection in the favour of ASW (Holt & Hochberg 1997; Hufbauer & Roderick 2005; Goldson et al. 2014; Tomasetto et al. 2017b; Casanovas et al. 2018). Furthermore, there are predator avoidance behaviours of ASW (Gerard 2000; Phillips 2002) that intense *M. hyperodae* derived selection could act upon (Goldson et al. 2014; Tomasetto et al. 2017b). Although contemporary evolution of ASW behaviour was hypothesised to be the mechanism of the ASW parasitism rate decline, prior to the work in this thesis, there was little supporting evidence. ASW behavioural responses to *M. hyperodae* had been measured over 15 years ago (Gerard 2000; Phillips 2002) near the estimated start of the parasitism decline (Tomasetto et al. 2017b; Tomasetto et al. 2018b). Furthermore, ASW behaviour was measured only with *L. perenne* (Gerard 2000; Phillips 2002) and temporal non-independence within replicates was not accounted for (Gerard 2000). In addition, these investigations either used a ASW population (Otago, Invermay) (Gerard 2000) that has always had a low parasitism rate (Ferguson 1997) or an extremely artificial environment with Petri dishes (Phillips 2002). Therefore, if the overarching hypothesis of contemporary evolution is correct then current ASW behaviour will probably be different to the findings in past investigations. Undisputed contemporary evolution of pest resistance in an insect host-parasitoid classical biological control association has never been reported in the literature (Holt & Hochberg 1997; Goldson et al. 2014; Mills 2017; Tomasetto et al. 2017b). It is therefore imperative that the mechanisms behind such contemporary evolution are determined to provide insight into how to counter the mode of parasitism avoidance or prevent the driver of the mode occurring in other classical biological control programmes such as with the clover root weevil (*Sitona obsoletus* Gmelin) (formerly *S. lepidus* Gyllenhal) in New Zealand.

### 1.6.1 Chapter 2 evaluated hypotheses

Hypothesis 1: Differences in ASW behavioural responses to *M. hyperodae* can be detected using an artificial environment and living plants in microcosms in controlled conditions and are strongest at night.

Hypothesis 2: The ASW exhibits avoidance responses to *M. hyperodae* on diploid *L. perenne* and the hybrid but less so on the tetraploid *L. multiflorum*, this may explain why the recent changes in parasitism rates have occurred.

Hypothesis 3: The presence of *M. hyperodae* causes the non-consumptive effect of ASWs changing their within-plant distribution resulting in reduced feeding due to ASWs feeding on poorer quality food on the lowest section of the plant which may increase ASW mortality.

The context of the above three hypotheses is that recent ASW parasitism rates have been shown to substantially differ between *Lolium* host plant types (Goldson et al. 2015; Goldson & Tomasetto 2016). This pattern did not occur in the 1990s during peak parasitism levels when using similar methodology, cultivars and insect populations (Goldson & Tomasetto 2016). The above investigations revealed that bottom-up trophic effects are facilitating reduced ASW parasitism rates on dominant diploid pasture types whereas there has been less change in uncommon tetraploid *L. multiflorum* pastures (Goldson et al. 2015; Goldson & Tomasetto 2016). This supports the overarching contemporary evolution hypothesis as ASW has had more interactions with *M. hyperodae* on the common pastures where contemporary evolution is most likely to occur resulting in ASW having more sensitive responses than on tetraploid *L. multiflorum*. It was suggested that the enhancement of already occurring ASW avoidance behaviour towards *M. hyperodae* may be responsible (Goldson & Tomasetto 2016) such as reduced feeding and plant abandonment responses. Gerard (2000) reported these behaviours on diploid *L. perenne* after dusk and Barratt et al. (1996) to a lesser extent on diploid hybrid but it was unknown to what degree they reduced parasitism and within-plant ASW distributions were not measured. ASW is also known to have preferred feeding sections of host plants (Barker 1989). Therefore, exposure to *M. hyperodae* may cause a non-consumptive effect (NCE) that reduces the ASW fitness even when it has not been attacked. Chapter 2 aims to investigate whether *M. hyperodae* avoidance behaviour by ASW is plant-mediated and whether this may explain recent parasitism rates in the context of contemporary evolution.

### 1.6.2 Chapter 3 evaluated hypothesis

Hypothesis 4: ASWs from regions within New Zealand where the ASW parasitism decline had been greatest should exhibit the strongest avoidance behavioural responses. In contrast, ASWs from



regions where there had been no parasitism decline should have little or no detectable avoidance behavioural responses when exposed to *M. hyperodae*.

The context of the above hypothesis is that recent ASW parasitism rates vary greatly between ASW populations in different regions (Goldson et al. unpublished data) despite *M. hyperodae* been released around the same time in 1991-2 in all major pasture areas (Goldson et al. 1993). This geographic variation allows the behaviour of ASWs from populations with different parasitism history to be compared to determine whether that behaviour reflects this history and recent parasitism rates. The behavioural findings from Chapter 3 are currently the best evidence supporting the contemporary evolution hypothesis and are the phenotypes of an underlying unknown genetic mechanism, which other organisations are investigating.

### **1.6.3 Chapter 4 evaluated hypothesis**

Hypothesis 5: The ASW would have stronger avoidance responses to the parasitoid, *M. hyperodae*, where asymmetric selection has occurred in the weevil's favour compared to the novel interaction with *Microctonus aethiopoides* Loan and there would be no response to an aphid-specific parasitoid where no interaction has occurred.

The context of the above hypothesis is that given that changes in ASW behaviour is predicted to be responsible for the decline in parasitism rates (Goldson et al. 2015), it is unknown how ASW will respond to novel parasitoid species. This poses interesting evolutionary questions of how asymmetric selection has contributed to ASW's avoidance behaviour and how sensitive these responses are to novel parasitoid species that have different extents of interaction with the ASW. These questions can be investigated because as well as *M. hyperodae*, a related species (*M. aethiopoides*) also attacks ASW in New Zealand pasture but to a lesser extent (McNeill et al. 2002; Gerard et al. 2011; Gerard et al. 2012). This novel association began after the Moroccan strain of *M. aethiopoides* was introduced in the 1982 (Stufkens et al. 1987) and more so with the introduction of the Irish strain in 2006 (Gerard et al. 2011; Gerard et al. 2012).

## **1.7 Experimental chapter outlines**

### **1.7.1 Outline of Chapter 2: Plant-mediated avoidance behaviour of the Argentine stem weevil towards its biological control agent**

Chapter 2 developed methodology involving microcosms in five experiments to investigate differences in ASW behavioural responses to *M. hyperodae* and determined how different *Lolium* plant types influenced those responses. The frequency of weevil behaviour on entire plants and sections of plants as well as parasitism rate and post-experiment accumulated feeding data were measured. The protocols and experimental set-up changed based on the new insight gained from the

experience and results of the previous experiments (Section 2.4), hence a discussion section in the middle of Chapter 2 (Section 2.4.3). This was followed by further experiments (Section 2.5) and a chapter discussion (Section 2.6) (refer to Fig. 1.4).

Hypothesis 1: Differences in ASW behavioural responses to *M. hyperodae* can be detected using an artificial environment and living plants in microcosms in controlled conditions and are strongest at night.

Hypothesis 2: The ASW exhibits avoidance responses to *M. hyperodae* on diploid *L. perenne* and the hybrid but less so on the tetraploid *L. multiflorum*, which may explain why the recent parasitism rates have occurred.

Hypothesis 3: The presence of *M. hyperodae* causes the non-consumptive effect of ASWs changing their within-plant distribution resulting in reduced feeding due to ASWs' feeding on poorer quality food on the lowest section of the plant which may increase ASW mortality.

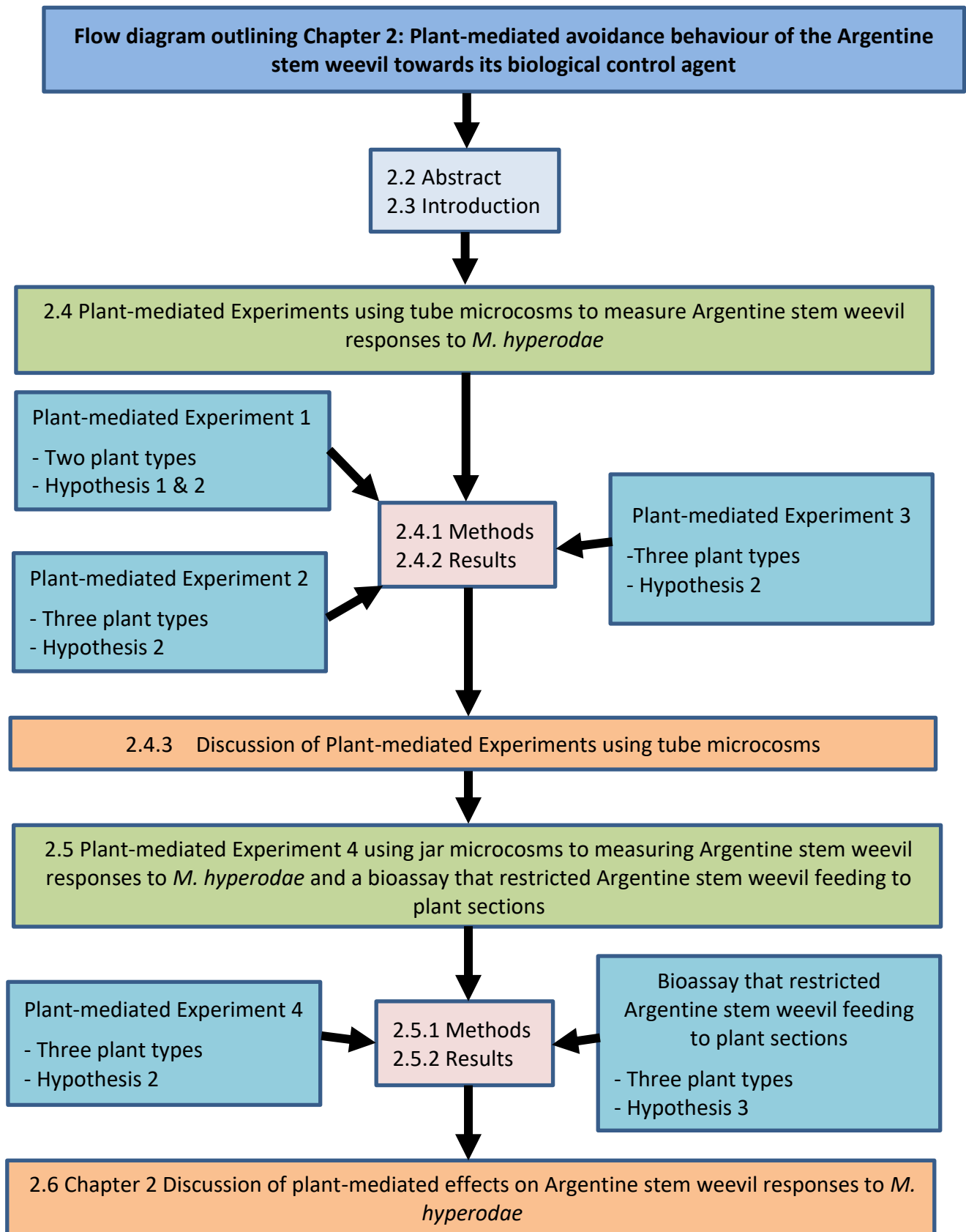


Figure 1.4 Flow diagram outlining Chapter 2: Plant-mediated avoidance behaviour of the Argentine stem weevil towards its biological control agent.

### 1.7.2 Outline of Chapter 3: Potential behavioural mechanisms for contemporary evolution driving a failing insect-parasitoid classical biological control system

Chapter 3 used microcosms in two experiments to investigate differences in weevil behavioural responses to *M. hyperodae* from four ASW regional populations (five localities) that varied in current parasitism rates, history and latitude (refer to fig. 1.5). The frequency of weevil feeding and plant abandonment behaviour on entire plants and sections of plants were measured. This work provided the first evidence that ASW behaviour reflects recent parasitism rates and supports the hypothesis that contemporary evolution has enhanced weevil behaviour leading to the decline in ASW classical biological control.

Hypothesis 4: ASWs from regions within New Zealand where the ASW parasitism decline had been greatest should exhibit the strongest avoidance behavioural responses. In contrast, ASWs from regions where there had been no parasitism decline should have little or no detectable avoidance behavioural responses when exposed to *M. hyperodae*.

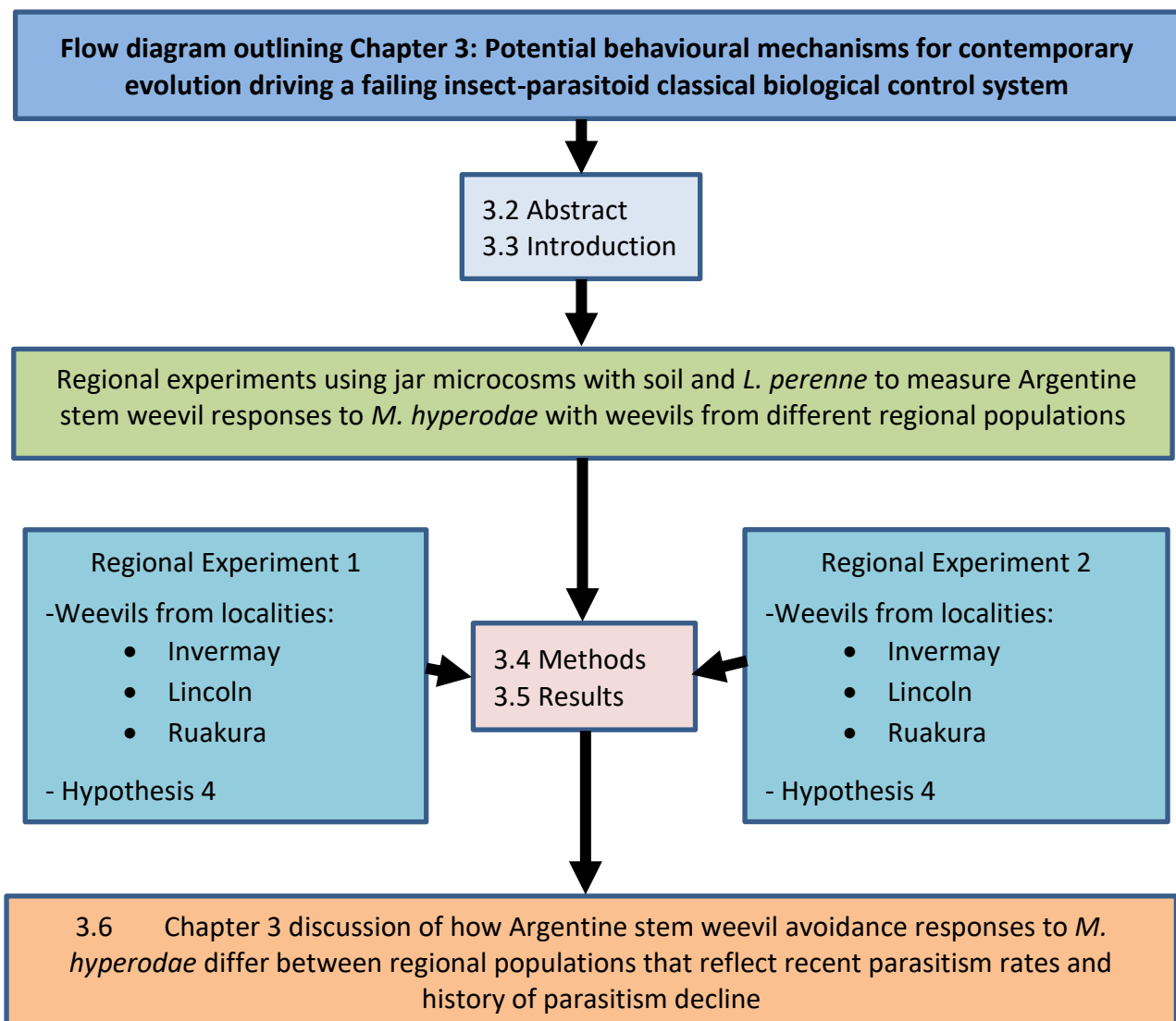


Figure 1.5 Flow diagram outlining Chapter 3: Potential behavioural mechanisms for contemporary evolution driving a failing insect-parasitoid classical biological control system

### 1.7.3 Outline of Chapter 4: Sensitivity of Argentine stem weevil behavioural responses to different parasitoid species

Chapter 4 used microcosms in an experiment with *L. perenne* to investigate differences in weevil behavioural responses to three parasitoid species that vary in the extent of interaction (refer to fig. 1.6). The frequency of weevil behaviour on entire plants and sections of plants were measured. This work showed that ASW may have specific responses to coevolved parasitoids and pre-adapted to responses novel parasitoids in the *Microctonus* genus.

Hypothesis 5: The ASW would have stronger avoidance responses to the parasitoid, *M. hyperodae*, where asymmetric selection has occurred in the weevil's favour compared to the novel interaction with *M. aethiopoides* and there would be no response to an aphid-specific parasitoid where no interaction has occurred.

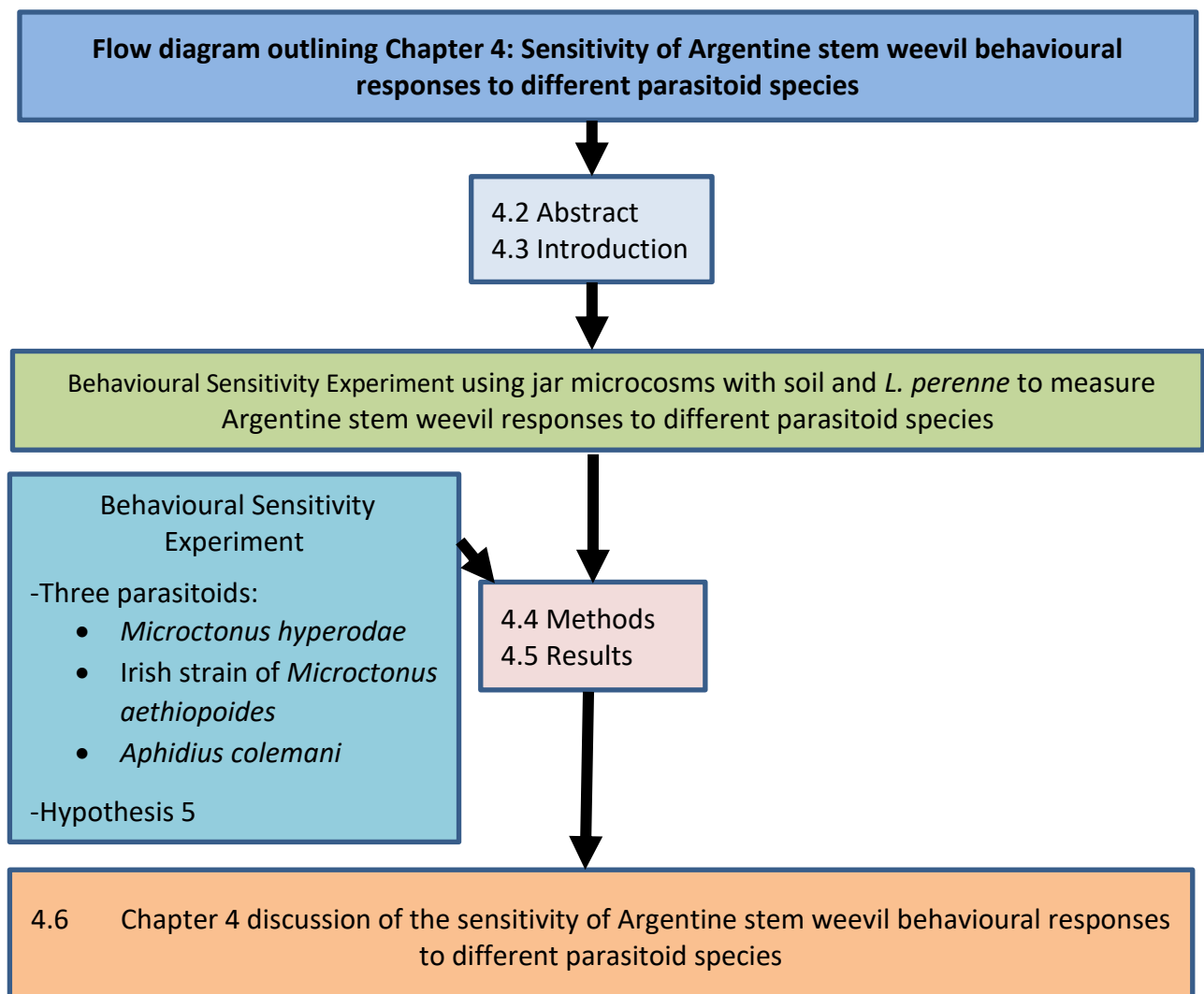


Figure 1.6 Flow diagram outlining Chapter 4: Sensitivity of Argentine stem weevil behavioural responses to different parasitoid species

## Chapter 2

# Plant-mediated avoidance behaviour of the Argentine stem weevil towards its biological control agent

### 2.1 Chapter 2 outline

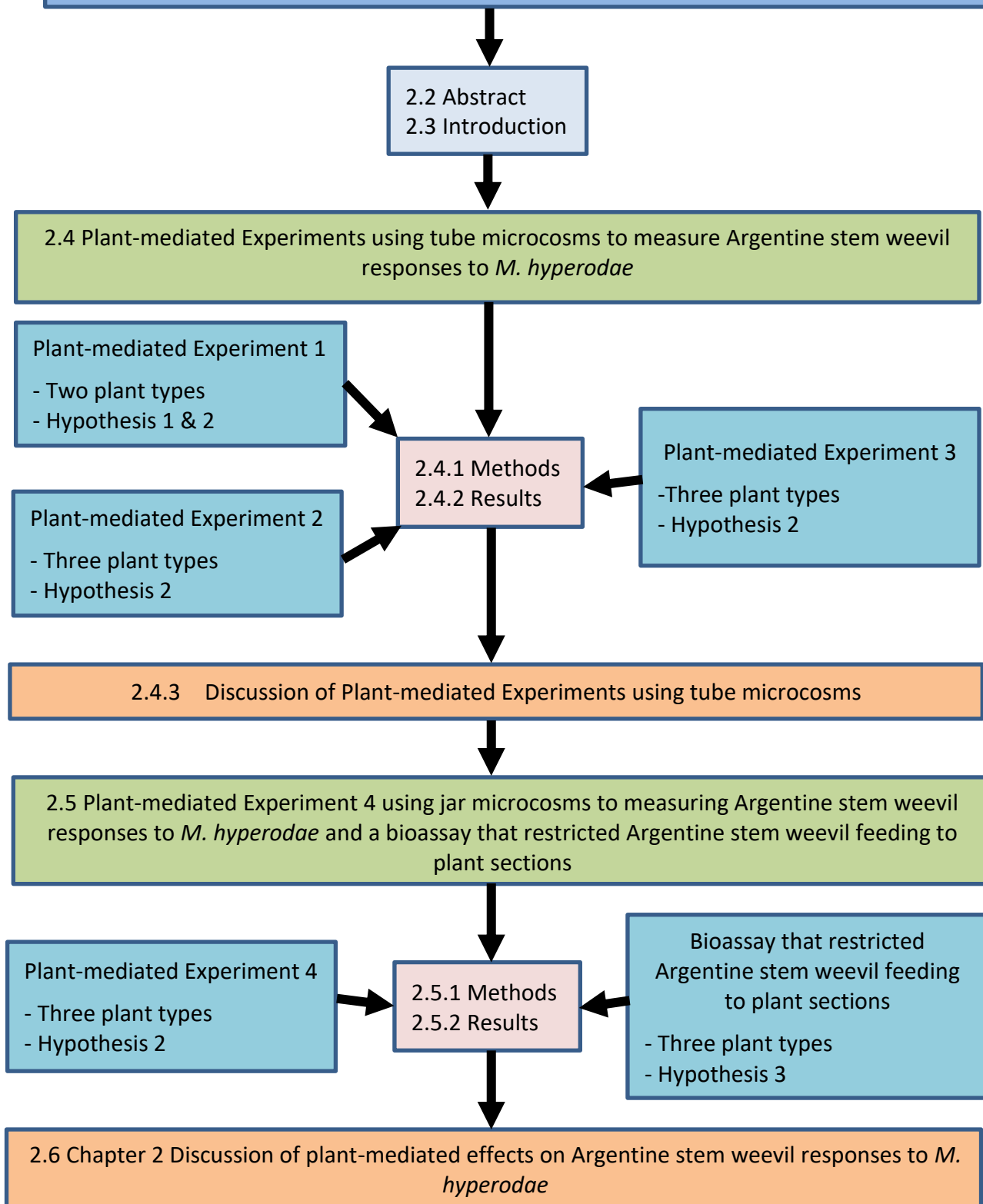
Chapter 2 developed methodology involving microcosms in five experiments to investigate differences in Argentine stem weevil (ASW) behavioural responses to *Microctonus hyperodae* and determined how different *Lolium* plant types influenced those responses. The frequency of weevil behaviour on entire plants and sections of plants as well as parasitism rate and post-experiment accumulated feeding data were measured. The protocols and experimental set-up changed based on the new insight gained from the experience and results of the previous experiments (Section 2.4), hence a discussion section in the middle of Chapter 2 (Section 2.4.3). This was followed by further experiments (Section 2.5) and a chapter discussion (Section 2.6).

Hypothesis 1: Differences in ASW behavioural responses to *M. hyperodae* can be detected using an artificial environment and living plants in microcosms in controlled conditions and are strongest at night.

Hypothesis 2: The ASW exhibits avoidance responses to *M. hyperodae* on diploid *Lolium perenne* and the hybrid (*L. perenne* x *L. multiflorum*) but less so on the tetraploid *L. multiflorum*, which may explain why the recent parasitism rates have occurred.

Hypothesis 3: The presence of *M. hyperodae* causes the non-consumptive effect of ASWs changing their within-plant distribution resulting in reduced feeding due to ASWs' feeding on poorer quality food on the lowest section of the plant which may increase ASW mortality.

**Flow diagram outlining Chapter 2: Plant-mediated avoidance behaviour of the Argentine stem weevil towards its biological control agent**



## 2.2 Abstract

Insect herbivore behavioural responses to predators are important factors in biological control success. However, host plant effects on behavioural non-consumptive effects are poorly understood. Here, Argentine stem weevil (ASW) avoidance behavioural responses to its classical biological control agent were investigated on *Lolium perenne*, *L. multiflorum* and hybrid pasture plants in no-choice microcosm laboratory experiments. The aim was to identify avoidance behaviour that may explain current parasitism rates of ASW in New Zealand pasture and the decline in biological control efficacy since the 1990s. ASW exhibited avoidance behavioural responses in the form of plant abandonment and reduced feeding. Furthermore, these responses differed between plant types, with weevils on the diploid hybrid having the strongest behavioural responses followed by diploid *L. perenne*. Weevils on tetraploid *Lolium multiflorum* had the most inconsistent reduced feeding response with no detection of plant abandonment. These behavioural findings reflect parasitism rates from field and laboratory experiments suggesting that the behavioural mechanisms identified are responsible. Potential reasons for the observed results in the context of New Zealand pasture and ASW parasitism decline are discussed. Potential implications of the results are discussed in Chapter 5.

## 2.3 Introduction

Host plants affect tri-trophic interactions through effects that influence insect herbivore behavioural responses to natural enemies (Price et al. 1980) which may impact the efficacy of biological control programmes (Pappas et al. 2016; Peterson et al. 2016). There is growing interest in this area of research with the increasing concern over pesticide use and a greater emphasis on biological control (Gurr & You 2016; Mills 2017; Hajek & Eilenberg 2018; Shields et al. 2019). However, there are still large knowledge gaps around the effects of host plants on insect behavioural interactions and how these impact biological control (Pappas et al. 2016; Peterson et al. 2016; Farrokhi et al. 2017; Hermann & Landis 2017). This invokes the consideration of the role of host plants in non-consumptive effects (NCEs) (Kessler & Baldwin 2002; Costamagna et al. 2013; Kersch-Becker & Thaler 2015). Also known as trait-mediated effects, NCEs are indirect effects that natural enemies have on potential prey without the prey being attacked (Preisser et al. 2005; Buchanan et al. 2017; Hermann & Landis 2017). These NCEs can be behavioural (Roitberg et al. 1979; Nelson et al. 2004), morphological (Wasserberg et al. 2013) or physiological (Lima 1998; Duong & McCauley 2016). There is often a trade-off between reduced predation risk and associated costs such as reduced fitness of the prey (Preisser et al. 2005; Buchanan et al. 2017; Hermann & Landis 2017). Natural enemy avoidance is a common NCE (Costamagna et al. 2013) that could be a contributing factor for the failure of biological control programmes in which insect behaviour can be overlooked (Mills & Kean 2010; Hermann & Landis 2017). Types of natural enemy avoidance include evasive behavioural responses (Wratten 1976; Hoefler et al. 2012; Lee et al. 2014), shifts in within plant distributions



(Costamagna et al. 2013), changes in host plant preference (Wilson & Leather 2012; Sidhu & Wilson Rankin 2016), reduced oviposition (Sendoya et al. 2009; Wasserberg et al. 2013) and reduced feeding (Rypstra & Buddle 2013; Kaplan et al. 2014; Thaler et al. 2014).

Herbivores assess predation risk predominantly by using chemical (odour) cues (Gonthier 2012; Hoefler et al. 2012; González & Rodríguez-Gironés 2013; Hermann & Thaler 2014). For instance, the Colorado beetle (*Leptinotarsa decemlineata* Say) has reduced feeding when a predatory stink bug (*Podisus maculiventris* Say) is nearby (Hermann & Thaler 2014). Another example is that some aphids can detect where ladybird larvae have foraged and as a result there is reduced colonisation on the affected parts of plants (Ninkovic et al. 2013). Visual cues may also contribute to NCEs such as natural enemy avoidance (Freitas & Oliveira 1996). This has been illustrated by there being reduced butterfly oviposition when dried ants have been pinned to plants (Sendoya et al. 2009). Furthermore, pollinator visitation has been reduced when models of crab spiders have been placed on flowers (Antiqueira & Romero 2016). However, NCEs do not occur in isolation but can be host-plant mediated through direct effects on natural enemies and herbivorous insects (Kersch-Becker & Thaler 2015). For instance, the aphid, *Aphis glycines* Matsumura prefers feeding on the higher nodes (fast growing) of soybean (*Glycine max* (L.) Merr.); however ladybird beetles also prefer to hunt aphids high on the plant. In response to beetles being on the soybean, the aphids feed on the less preferred lower nodes (slow growing) with lower nutritional value resulting in a population consequence of the aphids having a reduced rate of increase (Costamagna et al. 2013). Here, the NCE of reduced population increase due to having less nutritional food is mediated by the plant's nutrient distribution and aphids' within-plant feeding distribution.

There is a large body of literature on plant interactions with insect herbivores and natural enemies (Price et al. 1980; Mitchell et al. 2016; Peterson et al. 2016). Host plants have physical traits that have direct effects on herbivores and natural enemies such as waxy leaves (Hariprasad & van Emden 2010), external structures such as trichomes (Voigt et al. 2007; Karley et al. 2016) and tissues high in fibre (Barker 1989) and silica (Massey & Hartley 2009). However, many of these have additional functions such as minimising water loss (Edwards & Wratten 1980). Biochemical traits are a large component of the defence arsenal of plants against herbivores and are involved in attracting natural enemies (Mitchell et al. 2016; Peterson et al. 2016; Santolamazza-Carbone et al. 2016). Secondary metabolites can reduce herbivore pressure by making plant tissue toxic or indigestible to many taxonomic groups of herbivores as well as attract natural enemies (Schmelz et al. 2011; Huffaker et al. 2013; Santolamazza-Carbone et al. 2016). However, secondary metabolites are costly to plants and can be constitutive which influence host-plant choice, or herbivore-induced which help plants cope with herbivory pressure while conserving valuable biochemicals (Kessler 2015; Bixenmann et al. 2016; Mitchell et al. 2016; Peterson et al. 2016). Production of herbivore-induced secondary

metabolites will either be in the form of increased production of already-present secondary metabolites (Fuchs et al. 2017) or the production of new metabolites (Bixenmann et al. 2016). Secondary metabolites can also be volatile plant compounds that can attract natural enemies (Lucas-Barbosa et al. 2014; Tamiru et al. 2015), repel (Bruce et al. 2015) or attract (Bruce & Pickett 2011) herbivores and prime the defences of other plants (Erb et al. 2015). The discussed plant traits directly affect natural enemy and herbivore interactions through impacting their behaviour. Key behaviours affected by plants include searching efficiency, feeding behaviour (Barker 1989; Massey & Hartley 2009; Peterson et al. 2016; Santolamazza-Carbone et al. 2016; Farrokhi et al. 2017), on-plant mobility, time spent on plants (VanLaerhoven et al. 2006), distribution within plants (Costamagna et al. 2013) and utilisation of resource such as shelter, nectar and pollen (VanLaerhoven et al. 2006; Gillespie et al. 2016; Peterson et al. 2016). Furthermore, plant nutritional quality directly affects herbivore development time, size, and fecundity. (Price et al. 1980; Massey & Hartley 2009; Barkhordar et al. 2013; Costamagna et al. 2013; Kaplan et al. 2014; Mitchell et al. 2016). These traits in turn determine the herbivore quality and suitability as prey which impact the efficiency and fitness of natural enemies (Peterson et al. 2016). The role of these host-plant effects on natural enemy-herbivore behavioural interactions and their NCEs (Costamagna et al. 2013; Kersch-Becker & Thaler 2015) is becoming increasingly recognised in biological control research (Peterson et al. 2016). An example is the Argentine stem weevil (*Listronotus bonariensis* Kuschel) in New Zealand where plant-mediated behavioural NCEs may be contributing to a decline in the efficiency of its biological control agent (Goldson & Tomasetto 2016).

Argentine stem weevil (ASW) is a major pasture pest in New Zealand where its once highly successful classical biological control programme is now failing (Ferguson et al. 2019). Contemporary evolution of enhanced ASW behavioural avoidance responses has been hypothesised to explain the decline in ASW parasitism by its parasitoid (*Microctonus hyperodae* Loan) which varies between different plant types and ploidies (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017a; Tomasetto et al. 2017b). Goldson and Tomasetto (2016) investigated whether there were plant-mediated effects on parasitism rates in controlled laboratory conditions. They found that ASW parasitism rates in the presence of tetraploid *Lolium multiflorum* Lam. were higher (75 %) than in the presence of both the diploid hybrid (diploid *L. perenne* × diploid *L. multiflorum*) (52 %) and diploid *L. perenne* L. (46 %) plants. These results were compared to experiments conducted in the 1990s using similar methods and the same plant cultivars where the parasitism rates for all plant types were c. 75 % (Goldson & Tomasetto 2016). The substantial reduction in laboratory derived parasitism rates reflect those in the field (Goldson et al. 2015) and ASW parasitism decline over the last 25 years detected by ASW dissections (Tomasetto et al. 2017b). Furthermore there is currently no evidence of other contributing variables to the parasitism rates on different plant types (Goldson & Tomasetto

2016) such as endophytes (Goldson et al. 2015), endosymbionts (White et al. 2015) and abiotic factors (Tomasetto et al. 2017b; Tomasetto et al. 2018b).

The findings are hypothesised to be the result of evolved resistance or enhanced behavioural responses which has occurred on common diploid *Lolium* pastures but not on the less-used tetraploid *L. multiflorum* (Goldson et al. 2015; Goldson & Tomasetto 2016). The contributing factors thought to create a suitable situation for such contemporary evolution to occur are described below, see (Hufbauer & Roderick 2005; Goldson et al. 2014). There was asymmetric selection as the sexual ASW has greater adaptive potential compared to the asexual *M. hyperodae*. There was no diffuse selection pressure as *M. hyperodae* provided the only predator pressure that impacts ASW at a population level and the simplified New Zealand pastoral ecosystem provides little refuge from *M. hyperodae* attack (Hufbauer & Roderick 2005; Goldson et al. 2014; Mills 2017; Tomasetto et al. 2017b; Casanovas et al. 2018). This combination of the factors described are thought to have created intense selection pressure which acted on already present adult ASW avoidance behaviours to *M. hyperodae* (Barratt et al. 1996; Gerard 2000; Phillips 2002). Avoidance behaviour in response to *M. hyperodae* included plant abandonment on diploid *L. perenne* plants (Gerard 2000) and Barratt et al. (1996) showed that ASW had reduced feeding and oviposition after *M. hyperodae* removal on the diploid hybrid. Therefore, a subtle shift in the strength or pattern of avoidance behaviours could explain the differences in parasitism rates recently observed on *L. multiflorum* (75 %), the hybrid (52 %) and *L. perenne* (46 %) (Goldson & Tomasetto 2016). These recent parasitism rates do not reflect feeding preference as adult ASWs prefer the diploid hybrid as much as tetraploid *L. multiflorum*, with diploid *L. perenne* been less preferred (Barker 1989). This feeding preference has been linked to fibre content (cellulose and lignin) in the leaf tissue (Goldson 1982; Barker 1989).

This chapter investigated adult ASW behavioural responses to *M. hyperodae* on the plant types mentioned above in no-choice laboratory experiments. The aim was to determine if adult ASW behavioural responses could be detected and if so then how such responses to *M. hyperodae* differ between plant types which may explain the observed parasitism rates in Goldson and Tomasetto (2016) and the potential feeding consequences. The hypothesis was that ASW would have *M. hyperodae* avoidance responses in the presence of diploid *L. perenne* and the hybrid but less so on the tetraploid *L. multiflorum* because the diploid plant types are dominant in New Zealand pasture. Therefore, if contemporary evolution had occurred it would have probably been detected on the diploid plants as the consequence of reduced parasitism rates shown by Goldson and Tomasetto (2016). Furthermore, the potential feeding consequences of the adult ASW responding to *M. hyperodae* by feeding on particular plant sections was investigated. The hypothesis was that the presence of *M. hyperodae* causes the non-consumptive effect of ASWs changing their within-plant distribution to decrease the risk of parasitism, resulting in reduced feeding due to ASWs feeding on

poorer quality food on the lowest section of the plant or abandoning the plant, such response may reduce ASW fitness. Plant-Mediated Experiments 1, 2 and 3 in Section 2.4 were primarily part of method development. The implications of this work are discussed in Chapter 5.

## 2.4 Developing methodology and investigating plant-mediated Argentine stem weevil avoidance responses to *M. hyperodae* using tube microcosms

### 2.4.1 Methods for Plant-mediated Experiment 1, 2 and 3 using tube microcosms

#### 2.4.1.1. Insect sampling and maintenance

Adult ASWs (c. 5000 individuals) were collected using a suction sampler (inverted leaf blower) with a mesh sock (Goldson et al. 2000) during mid-late January 2017. The sampling technique consisted of holding the suction sample on pasture with the sock end against the ground for 5 seconds then randomly taking a few steps and repeating the process for 15 minute intervals. Samples were taken from three farms in the Lincoln area on the Canterbury plains, New Zealand; AgResearch farm, -43.630334, 172.471321; Green Park Huts, -43.750747, 172.551187; Strathlahlan farm, -43.756444, 172.333902. This was during peak adult emergence (late December to late February) and moderate to low rates of *M. hyperodae* activity (Table 2.1) (Goldson et al. 1998; Phillips et al. 1998). This was to maximise ASW sampling success rates while increasing the likelihood of sampling 'naive' ASW adults which had little to no *M. hyperodae* exposure that were to be used in experiments. Further ASW sampling also took place during February and March 2017 when parasitism was relatively high (Table 2.1) (Goldson et al. 1998; Phillips et al. 1998; Goldson et al. 2011) from which *M. hyperodae* would later emerge to be used in experiments.

Table 2.1 Phenology of adult Argentine stem weevil and its parasitoid, *Microctonus hyperodae*, in Canterbury (data from; Goldson et al. 1998; Phillips et al. 1998; Goldson et al. 2011). Phenology is subject to climate variation between years and can differ from other regions.

Month	Jan.	Feb.	March	April	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	
Active adult <i>Micoctonus hyperodae</i>			summer generation Overlapping generations		second generation						overwintered generation		
Active adult Argentine stem weevil	summer generation high ----- low						diapausing summer & second generation					low - high	
				second generation high----- low									
Adult weevil parasitism %	low - moderate		highly variable				low ----- low - high					low	

Field collected adult 'naive' and 'exposed' ASWs were maintained separately were caged at around 23 °C and 60 % humidity in controlled temperature (CT) rooms with 16:8 light:dark photoperiod with simulated dusk and dawn in an attempt to make the artificial conditions more natural. Separate labelled plastic boxes were used for ASW purging to remove *M. hyperodae* and maintenance of ASWs in captivity, as used in Goldson and Tomasetto (2016). These primary translucent boxes had dimensions of 305 mm x 205 mm x 130 mm with 0.1 mm mesh lids and 1 mm gauze bases. Secondary translucent plastic boxes of the same dimensions (with no lids) were positioned tightly under the primary ones and had tissue paper added as a substrate for *M. hyperodae* larvae to pupate on as they fell through the gauze after emergence from adult ASWs (Goldson et al. 1993; Barratt et al. 1996; Phillips et al. 1996). The boxes were checked three times a week for *M. hyperodae* pupae. Once pupation had occurred, *M. hyperodae* pupa and the surrounding tissue paper were removed from the plastic container and put in a labelled Petri dish with a maximum of five pupae and 1-2 water dampened dental wicks to maintain humidity until eclosion (Barratt et al. 1996; Phillips et al. 1996). These Petri dishes were placed in a separate room from the ASWs but with the same conditions with the exception of being at 16 °C. Once eclosion had occurred adult *M. hyperodae* were maintained on 10 % honey-water soaked into a fresh dental wick (Phillips et al. 1996; Phillips & Baird 2001). This method allowed captive ASWs to be purged for 4 - 6 weeks of any *M. hyperodae* prior to experiments.

ASWs in captivity were fed a bouquet of nil-endophyte tetraploid *L. multiflorum* (cv. Grasslands Tama) with roots in a tied plastic bag with the tillers exposed. Two water-soaked wicks were added to plastic boxes to maintain humidity (Goldson & Tomasetto 2016). The *L. multiflorum* 'bouquet' and the water-soaked wicks were replaced every three days. *L. multiflorum* was solely used to maximise the survival of captive ASWs as it is the preferred host plant (Goldson 1982; Barker 1989). All ryegrass used was grown under greenhouse conditions in a Lincoln University Aluminex House using 150 mm x 100 mm x 50 mm seedling pots at 15 - 25 °C with standard 500 L potting mix composition; 400 L composted bark, 100 L pumice (1 - 7 mm), 1500 g Osmocote 3 - 4 month release, 500 g horticultural lime, 500 g Hydraflo (soil conditioner to help rehydrate mix if it gets dry), with no control of humidity.

#### **2.4.1.2. Plant selection**

The plants types tetraploid *L. multiflorum* (cv. Grasslands Tama), the diploid hybrid, (*Lolium boucheanum*, syn. *L. hybridum* (*L. perenne* × *L. multiflorum* (cv. Grasslands Manawa)) and diploid *Lolium perenne* (cv. Grasslands Samson) were selected to be used in plant-mediated behavioural experiments. These were chosen because they represent the main pastoral types in New Zealand and have been used in previous experiments investigating the ASW - *M. hyperodae* interactions (Goldson 1982; Barker 1989; Goldson et al. 2015; Goldson & Tomasetto 2016). Similarly, pasture cultivars with

no endophytes were used in this work because endophytes have been shown to have no effect on ASW parasitism rate (Goldson et al. 2015) and were absent in Goldson and Tomasetto (2016).

#### **2.4.1.3. Potential of video analysis of Argentine stem weevil and *M. hyperodae* behaviour**

To investigate adult ASW behavioural responses to *M. hyperodae* on different plant types, cameras in laboratory conditions and video analysis were originally planned to avoid prolonged observation in near-darkness and enabling continuous data collection compared to small segments of behaviour (Wratten 1994). This involved considerable time testing equipment and developing the experimental set-up. The planned experiment was a randomised block design (with blocks separated by time) with 10 replicates of 10 purged non-starved adult ASWs on plant treatments (see Section 2.4.1.1), with or without (the control) *M. hyperodae*. The same environmental conditions were used that the insects had experienced while maintained in captivity (see Section 2.4.1.1). Replicates consisted of individual vertical 150 mm plants of one type in potting mix covered with 2 cm of sand, in 140 mm diameter arenas with Insecta-a-slip Insect Barrier- Fluon® around the inside edges in cubic mesh enclosures. Each arena was to be filmed using four Axis P1428E video cameras with an infrared sensor, allowing all parts of the above ground plant to be filmed regardless of time of day (Fig. 2.1). ASWs were added 30 minutes prior to recording and an individual *M. hyperodae* was added at recording time if required. Recording took place over 6 h encompassing artificial dusk and night. The enclosures and arenas were washed with Decon 90 laboratory cleaning agent and all organisms were replaced between replicates. Behavioural video recordings were to be analysed using Behavioural Observation Research Interactive Software (BORIS) which allows event-based data collection to measure frequency of behaviours along with insect position on the plant. The experiment started in early February 2017. However, it was soon realised that this method was heavily restrained by time as only one replicate could be recorded each day and five blocks in one month. This was highly risky and not feasible when the logistics of setting up a replicate daily, technical issues and organism availability over several months were considered. Therefore, video data collection was stopped and an experimental setup using plastic tube containers and visual observation was developed (see Section 2.4.1.4).



Figure 2.1 Experimental set-up for planned video experiments using an arena within a mesh enclosure with four cameras and an infrared sensor. Photo: Morgan Shields.

#### **2.4.1.4. Plant-mediated Experiment 1 measuring Argentine stem weevil avoidance responses to *M. hyperodae* and if these mainly occurred at night**

Plant-mediated Experiment 1 investigated if ASW behavioural responses to *M. hyperodae* differed in the presence of different plant types and to determine what time of day ASW is most responsive under the experimental conditions (hypothesis 1). This no-choice experiment was conducted under controlled conditions and was a spatially randomised block design, with four blocks on February 10 and five blocks on February 15 2017. There were nine replicates each of *L. perenne* and *L. multiflorum* (see Section 2.4.1.2), with and without (the control) *M. hyperodae*. These plant species were used because they were the extremes in terms of plant preference by ASW used in Goldson and Tomasetto (2016). The same environmental conditions were used that the insects had experienced while they had been maintained in captivity (see Section 2.4.1.1). Replicates consisted of one plant type with several untrimmed 150 mm vertical individual plants with a total of five tillers/pseudostems that were 4 - 6 weeks old, in the centre of 50 mm diameter pots surrounded by potting mix. The mix was covered with 20 mm of sand to increase ASW visibility; this left 10 mm of the top inside edge of the pots exposed which were coated with a Fluon barrier to discourage ASWs from climbing the 240 mm x 70 mm transparent tubes that were placed tightly over the pots and plants. The plastic tubes had 0.1 mm mesh lids to provide ventilation and prevent the insects escaping (Fig. 2.2). All equipment was rinsed with hot water, washed with laboratory-grade detergent then rinsed again to reduce any potential effect of residual volatiles. All organisms were replaced after each replicate. There were 10 adult non-starved purged ASWs from the Lincoln area that were added to each randomised replicate 40 minutes prior to *M. hyperodae* introduction. Non-starved ASWs were used as this was thought to be more realistic as ASWs are surrounded by food in

the field. *M. hyperodae* individuals were fed 10 % honey water since emergence and were five days old or less when used in the experiment.

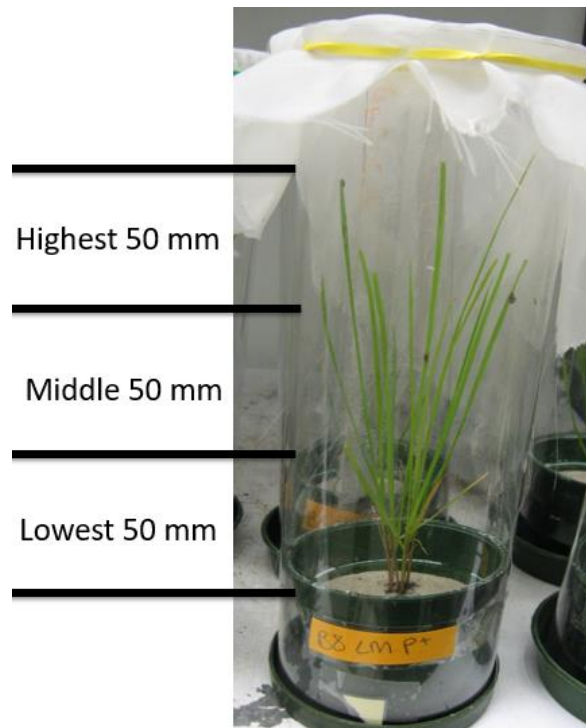


Figure 2.2 Tube microcosm set up with labelled 50 mm plant sections used in Plant-mediated Experiment 1 where Argentine stem weevil behavioural and temporal responses to its parasitoid, *M. hyperodae* were investigated in a laboratory no choice randomised block design experiment. Photo: Morgan Shields.

Data recording began around 30 minutes after *M. hyperodae* introduction and 2 h before dusk. The frequency of different ASW behaviours and the plant section each individual was on (lowest 50 mm, middle 50 mm and highest 50 mm) were recorded for each replicate over time creating a ‘snap shot’ of ASW behavioural responses. ASWs off the plant were considered to be in the process of hiding or escape. Observations were recorded at 1 h intervals for each replicate in the same order for 7 h. The data recording duration included two hours of daylight, two hours of dusk and three hours of night (near darkness). This incorporated the peak period of ASW activity when it is most vulnerable to *M. hyperodae* attack according to previous work (Barratt et al. 1995; Phillips 2002); however those studies were conducted under different conditions. A head torch with red light was used to observe ASW behaviour in dim light as ASW and *M. hyperodae* did not appear to respond to red light. Based on Phillips (2002) and Gerard (2000), ASW behaviour that made individuals vulnerable to attack or avoid *M. hyperodae* were recorded see, these behaviours included:

- On-plant (a measure of plant abandonment): All ASW individuals and their behaviour on host plants were recorded. ASW individuals on host plants were considered to be at risk of *M.*



*hyperodae* attack whereas individuals off-plant were considered to be hiding or escaping from *M. hyperodae*.

- Feeding behaviour: the ASW positioned head down with the rostrum in the plant surface, usually with the abdomen held high exposing the anus and sternites. A feeding initiation or scar is usually visible (highly vulnerable to *M. hyperodae* attack) (Phillips 2002) (Fig. 2.3).



Figure 2.3 Adult Argentine stem weevil in a feeding position with exposed sternites. This is when the weevil is most vulnerable to *M. hyperodae* attack (Phillips 2002). Photo: Jacquelyn Bennett with permission.

- Crouching behaviour: the ASW positioned head down with the abdomen low and not moving (very low vulnerability to *M. hyperodae* attack) (Gerard 2000; Phillips 2002).
- Stationary behaviour: the ASW not moving its legs with its head up, often with the antennae waving (moderately vulnerable to *M. hyperodae* attack). Stationary and crouching behaviour were recorded together as it was realised they had not been separated halfway through Plant-mediated Experiment 1.
- Walking behaviour: the ASW moving from one place to another (low vulnerability to *M. hyperodae* attack but may attract the parasitoid's attention) (Phillips 2002).
- Grooming behaviour: ASW mouthparts moving over antennae or leg segments (moderate vulnerability to *M. hyperodae* attack) (Phillips 2002).
- Mating behaviour: two ASWs in a mating position with male genitalia visible (moderate vulnerability to *M. hyperodae* attack) (Phillips 2002).

- ASW oviposition was not recorded as it does not occur after 10 days in captivity due to egg reabsorption (Goldson 1983).

After data recording finished the insects were recovered where possible, labelled and stored in a -20 °C freezer. The ASW sex ratio was later determined as 1:1.27 female:male from a sub-sample of 25 individuals using the Goldson and Emberson (1981) protocol. The sub-sample was small because the samples had been in the deep freeze for too long before dissection (several months). The ASW sex ratio in the current work was similar to field populations and previous experiments where ASW sex ratio was around 1:1 and there is no evidence that gender affects parasitism rate (Goldson et al. 1993; McNeill et al. 1996; Barker 2013). The number of ASW feeding scars per replicate were also recorded post-experiment as an accumulated indication of feeding behaviour as was done in previous studies (Barker 1989; Barratt et al. 1995; Barratt et al. 1996). To measure the accumulated feeding damage, the leaf blades were put between two microscope slides in order to facilitate accurate observation of feeding damage and measured using a microscope at 7-15 X magnification.

#### **2.4.1.5. Plant-mediated Experiment 2 investigating Argentine stem weevil avoidance responses to *M. hyperodae* on three plant types**

Based on the findings of the previous experiment, Plant-mediated Experiment 2 investigated how ASW behavioural responses to *M. hyperodae* differed between *L. multiflorum*, the hybrid (diploid *L. perenne* x diploid *L. multiflorum*) and *L. perenne* over four time steps with around 1 h between each observation. Plant-mediated Experiment 2 followed the protocol used in Plant-mediated Experiment 1 (see Section 2.4.1.4) with some modifications. The experiment was conducted on March 6 (5 blocks) and March 17 (5 blocks) 2017. Purged adult Lincoln ASWs were starved for 2 days prior to the experiment. This was to standardise ASW hunger to reduce variation (Gerard 2000). As discussed above, three plant types were used (see Section 2.3.2) meaning there were six treatment combinations. Individual plants were trimmed to a height of 150 mm with no leaf blades touching the inside of the plastic tubes. This was to reduce ASWs spending time on the microcosm surface and escaping. Data recording occurred over 4 time steps (4 h), two during dusk and two during night. Stationary and/or crouching behaviour was recorded as separate behaviours. The number of feeding scars per replicate and plant section was recorded post-experiment as an accumulated indication of feeding behaviour. The ASW sex ratio was 1.25:1 female:male from a sub-sample of 261 individuals.

#### **2.4.1.6. Plant-mediated Experiment 3 measuring Argentine stem weevil parasitism rates after 24 h exposure to *M. hyperodae* with three plant types**

The Plant-mediated Experiment 3 investigated whether ASW parasitism rates in the tube experimental set-up were similar to Goldson and Tomasetto (2016). The protocol was similar to Plant-mediated Experiment 2 (see Section 2.4.1.5), however 10 blocks of *M. hyperodae*-only

treatments with one parasitoid per replicate were used and the ASWs were exposed for 24 h. This exposure period was used because there were 10 ASWs per replicate in a small space whereas Goldson and Tomasetto (2016) used 23 ASWs with 48 h exposure in a larger space. The experiment was conducted on April 21-22 2017; no behavioural observation data was recorded. ASWs were maintained for 9 days (22 April - 1 May 2017) post-experiment for *M. hyperodae* larval development. During this time the ASWs were only fed *L. multiflorum* (see Section 2.4.1.1). ASWs were then frozen and later dissected to determine parasitism rate (see Section 2.4.1.4). The ASW sex ratio was 1.21:1 female:male from a sample of 181 individuals.

#### **2.4.1.7. Statistical analysis of Plant-mediated Experiments 1, 2 and 3**

All analysis was conducted in the statistical programme Rstudio 1.1.447 (RCoreTeam 2018) using base R code unless cited statistical packages were used. Untransformed data was used as they met the distribution requirements for the intended analyses. P-values  $\leq 0.05$  were used to show significant differences.

Plant-mediated Experiment 1 behavioural data were analysed with a generalised linear mixed effects model with penalised quasi-likelihood (GLMMPQL) and a binomial distribution using the packages lme4 (Bates et al. 2015), MASS (Venables & Ripley 2002) and nlme (Pinheiro et al. 2018). This was used to overcome the temporal non-independence between time steps allowing daylight (2 time steps combined), dusk (2 time steps combined) and night (3 time steps combined) to be compared and each plant section (including the entire plant) was analysed separately.

Plant-mediated Experiment 2 behavioural data for comparing between plant type and *M. hyperodae* treatments for each time step and plant section (including the entire plant) was analysed separately using a generalised linear model (GLM) with a binomial distribution (RCoreTeam 2018). Plant-mediated Experiment 2 behavioural data for comparing within plant type and *M. hyperodae* treatment for each time step, plant type and *M. hyperodae* treatment were analysed separately using a Log-linear analysis with a Poisson distribution (RCoreTeam 2018). The GLM and Log-linear analysis were used to investigate how patterns of ASW behaviour may change over the four time steps between and within different plant types and plant sections. Significant differences that occurred in more than one experiment or in two or more consecutive time steps were considered strong and consistent responses. The following ASW behaviours were not analysed due to lack of data or were represented by the main on-plant and feeding behaviours; stationary; crouching; grooming; mating; walking. Accumulated number of feeding scars (entire plant and by plant section) was analysed as count data using a GLM with a Poisson distribution or a negative binomial distribution (RCoreTeam 2018) if the data were greater than 1.5 times over-dispersed. Parasitism rates in the Plant-mediated Experiment 3 were analysed as percentage data using analysis of

variance (ANOVA) (RCoreTeam 2018) with the TukeyHSD post-hoc test using the package agricolae (de Mendiburu 2018). The multi-variate behavioural results were often presented as tables which were used when there was sufficient complexity of results. The same superscript numbers beside values indicates significant differences. The results were presented this way because possible treatment comparisons that had no biological meaning were not analysed when ANOVA was not appropriate. An example of such a comparison was the treatment combination of the hybrid with *M. hyperodae* present compared to the *L. perenne* control. For consistency, this presentation of significant differences using the same superscript numbers was used in all tables.

## **2.4.2 Results of Plant-mediated Experiments 1, 2 and 3**

### **2.4.2.1. Results of Plant-mediated Experiment 1 measuring Argentine stem weevil avoidance responses to *M. hyperodae* and if these mainly occurred at night**

#### **2.4.2.1.1. The proportion of Argentine stem weevil 'on-plant' in response to the presence of *M. hyperodae* in Plant-mediated Experiment 1**

GLMMPQL analysis indicated that ASW on-plant responses to *M. hyperodae* were strongest at night on both the *L. multiflorum* and *L. perenne* plants but only detected when analysing individual plant sections (see Table 2.2). On *L. multiflorum* at night, there was significantly fewer ASWs with *M. hyperodae* on the middle plant section (8.2 %) compared to the control (18.9 %,  $p = 0.006$ ) and a significant increased proportion of ASWs on the lowest plant section (28.9 %) compared to *M. hyperodae* control (14.0 %,  $p = 0.004$ ). Similarly, at night on the highest *L. perenne* section with *M. hyperodae* present there were significantly fewer ASWs (12.6 %) compared to the *L. perenne* control (20.7 %,  $p = 0.044$ ) and a significant increase in the proportion of ASWs on the lowest plant section (23.7 %) compared to the control (9.6 %,  $p = 0.002$ ). There were no other significant on-plant differences in response to *M. hyperodae*.

Table 2.2 Position of weevils on plants in Plant-Mediated Experiment 1, read table horizontally: Observed mean percentage of Argentine stem weevils on different plant types according to plant section (50 mm height), *Microctonus hyperodae* treatment and time of day. On-plant behaviour in each plant section was analysed separately using GLMMPQL (binomial distribution). The same superscript number beside values in each row and column indicates significant differences ( $p \leq 0.05$ ,  $n = 9$ ) (refer to Section 2.4.1.7). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid *Lolium perenne* = red; parasitoid absent (control) = purple; parasitoid present = black.

Treatment combinations			Time of day		
Plant sections	Plant type	<i>Microctonus hyperodae</i> treatment	Daylight	Dusk	Night
Entire plant	Tetraploid <i>Lolium multiflorum</i>	absent	53.3	55.0	48.5
		present	50.6	42.3	53.0
	Diploid <i>Lolium perenne</i>	absent	42.2	40.6	47.0
		present	53.9	46.1	55.6
Highest	Tetraploid <i>Lolium multiflorum</i>	absent	5.6	7.2	11.5 <sup>3</sup>
		present	6.7 <sup>1</sup>	3.9 <sup>2</sup>	14.1 <sup>1,2</sup>
	Diploid <i>Lolium perenne</i>	absent	8.9 <sup>4</sup>	10.0 <sup>5</sup>	20.7 <sup>3,4,5,6</sup>
		present	8.9	7.2	12.6 <sup>6</sup>
Middle	Tetraploid <i>Lolium multiflorum</i>	absent	8.3 <sup>1</sup>	7.8 <sup>3</sup>	18.9 <sup>1,3,4</sup>
		present	3.3 <sup>2</sup>	5.0	8.2 <sup>4,5</sup>
	Diploid <i>Lolium perenne</i>	absent	6.7 <sup>6</sup>	7.2 <sup>7</sup>	15.2 <sup>6,7</sup>
		present	10.6 <sup>2</sup>	6.7 <sup>8</sup>	15.9 <sup>5,8</sup>
Lowest	Tetraploid <i>Lolium multiflorum</i>	absent	39.4 <sup>1,2</sup>	37.8 <sup>3,4</sup>	14.0 <sup>2,4,5</sup>
		present	40.6	32.8	28.9 <sup>5</sup>
	Diploid <i>Lolium perenne</i>	absent	23.3 <sup>1,6</sup>	21.7 <sup>3,7</sup>	9.6 <sup>6,7,8</sup>
		present	32.2	32.2	23.7 <sup>8</sup>

#### 2.4.2.1.2. The proportion of Argentine stem weevil feeding in response to the presence of *M. hyperodae* in Plant-mediated Experiment 1

GLMMPQL analysis indicated that in response to *M. hyperodae* exposure, ASWs had a reduced feeding response which was more frequent on *L. multiflorum* compared to *L. perenne* and predominately occurred during dusk and night. There were significantly fewer ASWs feeding on the entire *L. multiflorum* during daylight (5.0 %) than the entire *L. perenne* with *M. hyperodae* present (18.9 %,  $p = 0.024$ ). This corresponds with a significantly higher proportion of ASWs feeding during daylight on the lowest plant section of *L. perenne* with *M. hyperodae* present (8.9 %) compared to the control (1.7 %,  $p = 0.000$ ). There were significantly fewer ASWs feeding on the entire *L. multiflorum* during dusk when *M. hyperodae* was present (2.3 %) compared to the control (11.7 %,  $p = 0.031$ ). On the middle *L. multiflorum* section at night there were significantly fewer ASWs feeding with *M. hyperodae* present (1.1 %) compared to the control (5.6 %,  $p = 0.026$ ). On the middle *L. perenne* section at dusk with *M. hyperodae* there was reduced ASW feeding (0.6 %) compared to at daylight (5.0 %,  $p = 0.007$ ) and at night (4.1 %,  $p = 0.044$ ). There were no other significant differences

in ASW feeding patterns in response to *M. hyperodae*. Accumulated total number of feeding scars did not have any significant differences between treatments.

#### **2.4.2.1.3. The proportion of Argentine stem weevil combined stationary and/or crouching responses to the presence of *M. hyperodae* in Plant-mediated Experiment 1**

GLMMPQL analysis revealed that ASWs responded to *M. hyperodae* with increased stationary and/or crouching behaviour predominantly at night and on *L. multiflorum* and on the lowest section of *L. perenne*. There was a significantly higher proportion of ASWs stationary or crouching at night on the entire *L. multiflorum* plant when *M. hyperodae* was present (33.7 %) compared to the control (16.7 %,  $p = 0.01$ ). This can be attributed to the higher proportion of ASWs stationary and/or crouching when *M. hyperodae* was present on *L. multiflorum* at night on the highest plant section (9.3 %) compared to the control (2.6 %,  $p < 0.015$ ) and on the lowest plant section (19.6 %) compared to the control (7.8 %,  $p < 0.001$ ). On *L. perenne* the only significant stationary and/or crouching responses to *M. hyperodae* occurred on the lowest plant section at night (14.4 %) compared the control (5.9 %,  $p = 0.024$ ). On both host plants the behavioural pattern at night with *M. hyperodae* present was in contrast to when *M. hyperodae* was absent where significantly fewer ASWs did stationary and/or crouching behaviour at night ( $< 8\%$ ) compared to earlier time periods ( $\geq 20\%$ ,  $p < 0.034$ ). There were no other significant differences in ASW combined stationary and/or crouching patterns in response to *M. hyperodae*.

#### **2.4.2.2. Plant-mediated Experiment 2 results investigating Argentine stem weevil avoidance responses to *M. hyperodae* on three plant types**

##### **2.4.2.2.1. The proportion of Argentine stem weevil 'on-plant' in response to the presence of *M. hyperodae* in Plant-mediated Experiment 2**

GLM analysis showed that ASWs had inconsistent on-plant responses to *M. hyperodae* on the hybrid and *L. perenne* with no response on *L. multiflorum* when compared between host plants, and there was no indication of plant abandonment for all host plants (Table 2.3; Table 2.4). There were significantly fewer ASWs on the lowest hybrid section when *M. hyperodae* was present (27 %) compared to the control (40 %,  $p = 0.05$ ) in time step 1 (dusk). In time step 3 (night) there were significantly fewer ASWs with *M. hyperodae* present on the highest plant section of the hybrid (10 %) compared to *L. multiflorum* (23 %,  $p < 0.016$ ) and *L. perenne* (22 %,  $p < 0.024$ ). In time step 4 (night) with *M. hyperodae* present, the *L. perenne* middle section had significantly fewer ASWs (10 %) compared to the control (22 %,  $p < 0.024$ ). Contrastingly, in response to *M. hyperodae*, the lowest *L. perenne* section had an increased proportion of ASWs (33 %) compared to the control (15 %,  $p < 0.004$ ). There were no other significant differences in ASW on-plant behaviour between host plants in response to *M. hyperodae* (Table 2.3).

Table 2.3 Position of weevils on plants in Plant-Mediated Experiment 2, read table horizontally: Observed mean percentage of Argentine stem weevils on plant sections (50 mm height) compared between plant types and *Microctonus hyperodae* treatments at each time step. On-plant behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 10$ ) (refer to Section 2.4.1.7). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid hybrid = orange; diploid *Lolium perenne* = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1 (dusk)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	46	42	48	36	49	47
	Highest	8	6	2	5	7	4
	Middle	6	4	6	4	4	4
	Lowest	32	32	40 <sup>1</sup>	27 <sup>1</sup>	38	39
Time step 2 (dusk)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	58	49	52	50	54	51
	Highest	18 <sup>1</sup>	15	6 <sup>1</sup>	13	12	13
	Middle	8	10	7	5	8	3
	Lowest	32	24	39	32	34	35
Time step 3 (night)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	66	53	55	47	62	59
	Highest	29	23 <sup>1</sup>	18	10 <sup>1,2</sup>	27	22 <sup>2</sup>
	Middle	11	12	15	11	15	9
	Lowest	26	18	22	26	20	28
Time step 4 (night)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	69	56	66	59	65	61
	Highest	29	21	22	17	28	21
	Middle	11 <sup>1</sup>	9	15	16	22 <sup>1,2</sup>	10 <sup>2</sup>
	Lowest	29 <sup>1</sup>	26	29 <sup>2</sup>	26	15 <sup>1,2,3</sup>	33 <sup>3</sup>

Plant-mediated Experiment 2 Log-linear analysis indicated that the proportion of ASWs on the hybrid with *M. hyperodae* present was significantly higher on the lowest plant section compared to the upper sections for time steps 1-3, this was similar to the hybrid control for time steps 1-2 and time step 4 (Table 2.4). On *L. perenne* with *M. hyperodae* present there were significantly fewer ASWs on at least one of the upper plant sections compared to the lowest plant section for all time steps. A similar pattern also occurred in the *L. perenne* control in time steps 1-2 and time step 4 (Table 2.4). ASWs on *L. multiflorum* had a similar within-plant distribution for most time steps regardless of the *M. hyperodae* treatment with a higher proportion of ASWs on the lowest plant section and the highest section to a lesser extent compared to the middle section, although this pattern was less consistent with *M. hyperodae* present (Table 2.4). There were no other significant differences in ASW on-plant behaviour within host plant and *M. hyperodae* treatments.



Table 2.4 Weevil within-plant distribution in Plant-Mediated Experiment 2, read table vertically: Total observed Argentine stem weevils on plant sections (50 mm height) within each plant type and *Microctonus hyperodae* treatment at each time step. *Microctonus hyperodae* treatments of each plant type within each time step were analysed separately using a Log-linear analysis. The same superscript number beside values in each column indicates significant differences ( $P \leq 0.05$ ,  $n = 10$ ) (refer to Section 2.4.1.7). Colours; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid hybrid = orange; diploid *Lolium perenne* = red; parasitoid absent (control) = purple; parasitoid present = black

Time step 1 (dusk)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	8 <sup>1</sup>	6 <sup>1</sup>	2 <sup>1</sup>	5 <sup>1</sup>	7 <sup>1</sup>	4 <sup>1</sup>
	Middle	6 <sup>2</sup>	4 <sup>2</sup>	6 <sup>2</sup>	4 <sup>2</sup>	4 <sup>2</sup>	4 <sup>2</sup>
	Lowest	32 <sup>1,2</sup>	32 <sup>1,2</sup>	40 <sup>1,2</sup>	27 <sup>1,2</sup>	38 <sup>1,2</sup>	39 <sup>1,2</sup>
Time step 2 (dusk)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	18 <sup>1</sup>	15	6 <sup>1</sup>	13 <sup>1</sup>	12 <sup>1</sup>	13 <sup>1,2</sup>
	Middle	8 <sup>2</sup>	10 <sup>1</sup>	7 <sup>2</sup>	5 <sup>2</sup>	8 <sup>2</sup>	3 <sup>2,3</sup>
	Lowest	32 <sup>1,2</sup>	24 <sup>1</sup>	39 <sup>1,2</sup>	32 <sup>1,2</sup>	34 <sup>1,2</sup>	35 <sup>1,3</sup>
Time step 3 (night)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	29 <sup>1</sup>	23	18	10 <sup>1</sup>	27	22 <sup>1</sup>
	Middle	11 <sup>1,2</sup>	12	15	11 <sup>2</sup>	15	9 <sup>1,2</sup>
	Lowest	26 <sup>2</sup>	18	22	26 <sup>1,2</sup>	20	28 <sup>2</sup>
Time step 4 (night)							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	29 <sup>1</sup>	21 <sup>1</sup>	22	17	28 <sup>1</sup>	18 <sup>1</sup>
	Middle	11 <sup>1,2</sup>	9 <sup>1,2</sup>	15 <sup>1</sup>	16	22	10 <sup>2</sup>
	Lowest	29 <sup>2</sup>	26 <sup>2</sup>	29 <sup>1</sup>	26	15 <sup>1</sup>	33 <sup>1,2</sup>

#### **2.4.2.2.2. The proportion of Argentine stem weevil feeding in the presence of *M. hyperodae* in Plant-mediated Experiment 2**

The GLM analysis indicated that there were few feeding responses to *M. hyperodae*. ASWs on *L. multiflorum* had a significantly reduced feeding response over the entire plant at night (time steps 3-4) ( $p < 0.009$ ) as did ASWs on *L. perenne* in time step 4 (night) ( $p < 0.006$ ). ASWs on the hybrid in the first time step had an anomalous increase in ASWs feeding (11 %) over the entire plant treatment with *M. hyperodae* present compared to the control (4 %,  $p = 0.018$ ). However, in the same time step there was significantly fewer ASWs feeding (8 %) on the lowest hybrid section compared to the hybrid control (21 %,  $p = 0.012$ ). There were no consistent differences within host plant ASW feeding distributions in response to *M. hyperodae* with the exception of ASWs on the hybrid with *M. hyperodae* present having significantly fewer individuals on the middle plant section ( $\leq 2$  %) compared to the lowest plant section ( $\geq 8$  %,  $p < 0.038$ ) for time steps 1-3.

#### **2.4.2.2.3. The number of accumulated feeding scars made by Argentine stem weevil in the presence of *M. hyperodae* in Plant-mediated Experiment 2**

The GLM analysis showed there was no significant difference in the number of ASW feeding scars between entire plant treatments in response to *M. hyperodae*. On the highest *L. multiflorum* section, there was a significantly reduced feeding response with *M. hyperodae* present (9.9) compared to the *L. multiflorum* control (16.4,  $p = 0.023$ ). In the absence of *M. hyperodae* the highest *L. multiflorum* section had significantly more feeding scars (16.4) than the hybrid (9.0,  $p = 0.006$ ). At the middle plant section in the absence of *M. hyperodae*, *L. multiflorum* had significantly fewer feeding scars (5.7) than the hybrid (10.5,  $p < 0.011$ ) and *L. perenne* (9.8,  $p < 0.024$ ). There were no other significant differences between plant types and *M. hyperodae* treatments.

Log-linear analysis indicated that with *M. hyperodae* present, an accumulated feeding scar distribution occurred on the hybrid with significantly more feeding scars on the lowest plant section (14.3) compared to the upper plant sections (7.7,  $p = 0.016$ ). Similarly with *M. hyperodae* present, on *L. perenne* there were significantly more feeding scars on the lowest plant section (12.4) compared to the middle plant section (6.5,  $p = 0.014$ ). There were no other significant within plant feeding scar distributions with the exception of *L. multiflorum* with *M. hyperodae* absent where there were significantly more feeding scars on the highest plant section (16.4) compared to the lower plant sections ( $< 8.8$ ,  $p < 0.012$ ).

### 2.4.2.3. Results of the Plant-mediated Experiment 3 measuring Argentine stem weevil parasitism rates after 24 h exposure to *M. hyperodae* with three plant types

#### 2.4.2.3.1. Argentine stem weevil parasitism rates for the Plant-mediated Experiment 3

ANOVA revealed ASW parasitism rate in the presence of *L. multiflorum* (54 %) was significantly higher than in the presence of *L. perenne* (32 %,  $p = 0.044$ ). There was no significant difference in ASW parasitism rate between the hybrid (36 %) and the other plant types (Fig. 2.4).

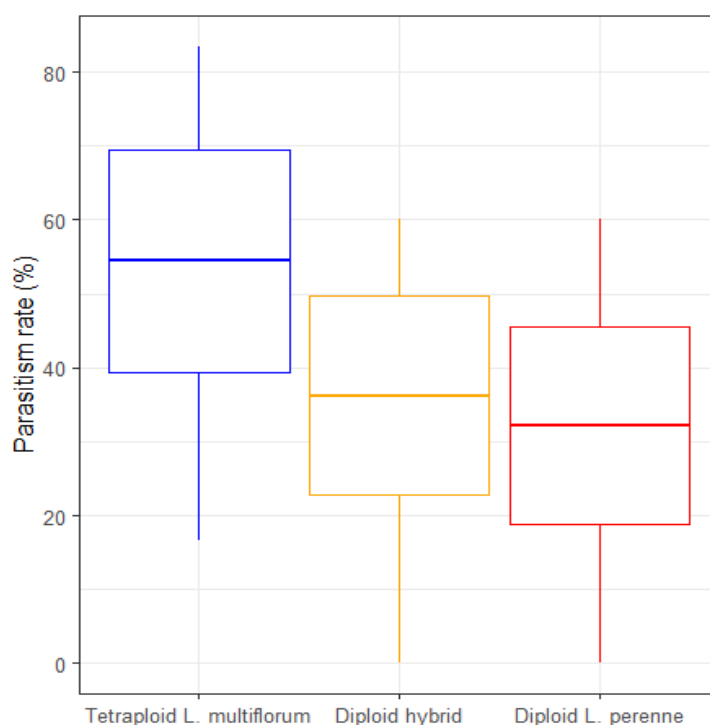


Figure 2.4 Parasitism rate (%) of Argentine stem weevil after 24 h exposure on different plant types in Plant-mediated Experiment 3. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a normal distribution,  $n = 10$ .

#### 2.4.2.3.2. Argentine stem weevil accumulated feeding scar results in response to *M. hyperodae* for the Plant-mediated Experiment 3

The GLM analysis showed there were no significant differences in the accumulated feeding scars between entire host plant treatments. *L. multiflorum* had a significantly higher number of feeding scars on the highest plant section (11.5 scars) compared to the hybrid (6.6 scars,  $p < 0.03$ ). Within plant types the hybrid had a significantly higher number of feeding scars (11.9 scars) on the lowest plant section compared to the highest section (6.6 scars,  $p = 0.015$ ). There were no other significant differences in the number of feeding scars between or within plant types.

## **2.4.3 Chapter 2 Discussion of plant-mediated effects on Argentine stem weevil responses to *M. hyperodae***

### **2.4.3.1. Main findings**

The results indicate that differences in Argentine stem weevil (ASW) behavioural responses to *M. hyperodae* could be detected, predominantly at night, using the artificial environment and living plants in microcosms. These findings also confirm that ASW behavioural responses to *M. hyperodae* are plant-mediated non-consumptive effects (NCE) in the form of changing ASW within-plant distributions and reduced feeding, although consistent patterns were difficult to detect due to highly variable data. It appears that ASWs on the diploid hybrid and *L. perenne* respond to *M. hyperodae* through a within-plant distribution change to the lowest plant section. This was only weakly detected on tetraploid *L. multiflorum* (Table 2.2; Tables 2.3-4). However, ASWs also responded to *M. hyperodae* by having reduced feeding, particularly in later time steps on tetraploid *L. multiflorum* and diploid *L. perenne* (Sections 2.4.2.2-3). Conversely, this was only weakly detected on the diploid hybrid (Section 2.4.2.2.3; Section 2.4.2.3.2). The ASW behavioural responses need further investigation to be more conclusive but the results suggest that the combination of within-plant distribution and feeding responses play an important role in the current ASW parasitism rates observed by (Goldson & Tomasetto 2016) as indicated by the results of the Plant-mediated Experiment 3 (Fig. 2.4). These complex interactions were further investigated in Sections 2.5-6 and their implications are discussed in Chapter 5. The individual avoidance responses exhibited by ASW are common NCE among herbivorous insects (Kessler & Baldwin 2002; Costamagna et al. 2013; Rypstra & Buddle 2013; Kaplan et al. 2014; Thaler et al. 2014). However, biological control decline being caused by the potential contemporary evolution of the relatively common insect avoidance behaviours mentioned above is unheard of in the literature.

It appears that the within-plant ASW distribution and reduced feeding NCEs only weakly support the hypothesis that ASW behavioural responses to *M. hyperodae* occur on the dominant diploid *L. perenne* and the hybrid but less so on the relatively uncommon tetraploid *L. multiflorum* (Goldson et al. 2014; Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b). A proportion of ASW individuals exhibit avoidance behaviour on *L. multiflorum* (Section 2.4.2) which is less effective at reducing parasitism rates than on the diploid plant types as shown by the Plant-mediated Experiment 3 (Fig. 2.4) and Goldson and Tomasetto (2016). This requires further investigation as ASW may have different behavioural patterns between plant types for the same behavioural response, which could affect parasitism rates (refer to Section 2.4.2.2). The findings also suggest that ASWs on the lowest plant section are less likely to be attacked by *M. hyperodae* and is particularly evident on the hybrid and *L. perenne* (refer to Section 2.4.2). This suggests there is preliminary evidence of a

trade-off between avoiding *M. hyperodae* attack and having low quality food, this is investigated in later sections (Section 2.5.2.2; Section 2.6.5).

#### **2.4.3.2. Time of day for Argentine stem weevil response activity**

Plant-mediated Experiment 1 indicated that within the experimental conditions used, ASW behavioural responses to *M. hyperodae* on both *L. multiflorum* and *L. perenne* were strongest at night and to a lesser extent during dusk (Section 2.4.2.1) and is supported by Plant-mediated Experiment 2 findings (Section 2.4.2.2). These results are similar to previous studies where ASWs were found to be most active after dusk for at least the first 4 h of night in summer conditions (Barratt et al. 1995). ASW night activity was best demonstrated by the on-plant distributions of ASWs on *L. multiflorum* and *L. perenne* in Plant-mediated Experiment 1. Here, ASW had fewer individuals on the upper plant sections (highest and middle) and an increased proportion of stationary and/or crouching individuals on the lowest plant section (Section 2.4.2.1.1; Section 2.4.2.1.3). These results confirmed that ASW responses to *M. hyperodae* predominantly occurred at night, could be detected using the experimental set-up and for the first time, indicated that ASWs respond to *M. hyperodae* on *L. multiflorum*. Furthermore, in Plant-mediated Experiment 1 with the controls, the lowest plant section at night had the lowest proportion of ASWs on it compared to during daylight and dusk regardless of plant type (Table 2.2). This indicates that the lowest plant section is the least preferred when the ASWs are most active and generally feeding which is supported by previous findings (Barratt et al. 1995; Gerard 2000; Phillips 2002).

#### **2.4.3.3. Comparing on-plant responses of Plant-mediated Experiments 1 and 2**

At night, ASWs on *L. perenne* had similar on-plant distribution responses to *M. hyperodae* in Plant-mediated Experiments 1 and 2 where the proportion of ASWs on the lowest plant section increased (Table 2.2; Table 2.3; Table 2.4). By this response occurring in different experiments it points to a very strong trend and is comparable to response behaviour observed in Gerard (2000). The similar behavioural findings on *L. perenne* of the current work and that of Gerard (2000), suggest that these ASW behavioural responses to *M. hyperodae* concur with the current parasitism rates (Goldson & Tomasetto 2016). Unlike in the Plant-mediated Experiment 1 (Table 2.2), weevils on *L. multiflorum* had no substantial on-plant distribution change with *M. hyperodae* present in Plant-mediated Experiment 2 (Table 2.4). This confirms a weak distribution response to the *M. hyperodae* on *L. multiflorum*. The lack of response to *M. hyperodae* on *L. multiflorum* is consistent with the high ASW parasitism rate of c. 75 % on same plant type in Goldson and Tomasetto (2016).

#### **2.4.3.4. Comparing Argentine stem weevil feeding responses of Plant-mediated Experiments 1 and 2**

In Plant-mediated Experiment 1, there was a general lack of reduced feeding behaviour when *M. hyperodae* was present, with the exception of *L. multiflorum* at dusk (Section 2.4.2.1.2). ASWs instead responded to *M. hyperodae* by being stationary or crouching, particularly those on *L. multiflorum* (Section 2.4.2.1.3). This was probably because the ASWs were not starved, therefore there was not a high proportion of them feeding at any one time. This made detection of feeding behavioural responses difficult. There was probably also no incentive to feed, due to the lack of hunger, if it meant substantially increasing the risk of *M. hyperodae* attack. Contrastingly, in Plant-mediated Experiment 2 on *L. multiflorum*, ASW had reduced feeding in response to *M. hyperodae*, particularly on the lowest section in the later time steps (Section 2.4.2.2.2). Furthermore there were more accumulated feeding scars on the highest section of *L. multiflorum* compared to the hybrid in the Plant-mediated Experiment 3 where ASWs were exposed to *M. hyperodae* for 24 h (Section 2.4.2.3.2). These findings suggests that the ASW is more vulnerable to *M. hyperodae* attack on the upper *L. multiflorum* sections, particularly at night.

In Plant-mediated Experiment 2, ASWs on *L. perenne* had a delayed reduced feeding response to *M. hyperodae* on the upper plant (highest and middle) sections (Section 2.4.2.2.2) and an increase in the accumulated feeding scars on the lowest *L. perenne* section compared to the middle section (Section 2.4.2.2.3). This suggests that ASWs on *L. perenne* do not quickly respond to *M. hyperodae* with detectable strength and that spending more time on the lowest plant section may reduce the risk of parasitism. The feeding response is weaker than expected given that *L. perenne* is the most common pasture type in New Zealand and a large parasitism rate reduction has occurred on it (Goldson & Tomasetto 2016) and is further discussed in Section 2.6. ASWs on the hybrid in Plant-mediated Experiment 2 only had a short reduced feeding response to *M. hyperodae* (Section 2.4.2.2.2). However, the accumulated feeding scars in Plant-mediated Experiment 2 (Section 2.4.2.2.3) and Plant-mediated Experiment 3 (Section 2.6) when *M. hyperodae* was present indicates more feeding on the lowest hybrid section compared to the upper sections. This feeding response was expected as the hybrid is a preferred host plant of ASW (Goldson 1982; Barker 1989) but has had a parasitism rate reduction similar to *L. perenne* (Goldson & Tomasetto 2016). Furthermore, Barratt et al. (1996) indicated some reduced feeding response in the presence of the hybrid once *M. hyperodae* had been removed using ASW from the Otago region. In the current work it is unclear why a stronger feeding response was not detected on the hybrid with the behavioural data but it could be due to the highly artificial experimental set-up where there was nowhere for ASWs to hide off the plants.

#### **2.4.3.5. Experimental set-up issues**

The tube experimental set-up used in the above plant-mediated experiments produced meaningful behavioural results which were confirmed to be relevant to the field by the parasitism rates in the plant-mediated Experiment 3 (Fig. 2.4) which have a similar pattern to those in Goldson and Tomasetto (2016). However, the tube experimental set-up was flawed and encountered several problems. The plant leaf blades were not trimmed in Plant-mediated Experiment 1 and in Plant-mediated Experiment 2 the trimmed leaf blades often collapsed on to the tube walls due to ASW activity, allowing the ASWs to spend a lot of time away from the plants making the data highly variable. There were some instances where ASWs escaped from the tubes due to design issues and most importantly, sand was used at the base of the plants to increase visibility and stop the ASWs becoming obscured following the Gerard (2000) protocol. However, using sand effectively prevented the ASWs from hiding from *M. hyperodae*. This could have severely affected the results such as the limited ASW responses on the hybrid (Section 2.4.2.2) and might be why the lowest plant section was popular when *M. hyperodae* was present (Section 2.4.2). The lack of places to hide may also explain why there were never any significant differences when comparing the proportion of ASWs on the entire plants (Table 2.2, Section 2.4.2.2.1). These issues might explain why it was difficult to detect consistent behavioural responses over multiple time steps. For the above reasons the tube experimental set-up could be considerably improved and a new design (see Section 2.8) was developed to further investigate how ASWs respond to *M. hyperodae* on different plant types and whether this may explain the parasitism rates observed in Goldson and Tomasetto (2016).

### **2.5 Plant-mediated Experiment 4 using jar microcosms and a bioassay that restricted Argentine stem weevil feeding to plant sections**

#### **2.5.1 Methods of Plant-mediated Experiment 4 and the bioassay that restricted Argentine stem weevil feeding to plant sections**

##### **2.5.1.1. Plant-mediated Experiment 4 investigated Argentine stem weevil avoidance responses to *M. hyperodae* on three plant types using jar microcosms**

ASW plant-mediated behavioural responses to *M. hyperodae* were further investigated with an improved design based on a jar microcosm experimental set-up to overcome issues encountered in previous experiments (Section 2.4.3.5). This was to quantify more accurately how ASW responds to *M. hyperodae* on different plant types.

### 2.5.1.2. Methods of the Plant-mediated Experiment 4 which investigated Argentine stem weevil avoidance responses to *M. hyperodae* on three plant types using jar microcosms

Adult ASWs were collected in 2018, purged of *M. hyperodae* and maintained using the same method as that described in Section 2.4.1.1. Plant-mediated Experiment 4 used the same plant types as Plant-mediated Experiment 2 (Section 2.4.1.2; Section 2.4.1.5). The experimental design comprised a spatially randomised block design with 15 replicates and eight treatments. Behavioural data were recorded over three dates with five blocks per date (March 30, April 7 and April 12 2018). Treatments included those that were used in Experiment 2 (Section 2.4.1.5) with three plant types in the absence (the control) or presence of *M. hyperodae* with an additional two non-plant treatments with *M. hyperodae* absent (control) or present. The two non-plant treatments each used one 15 mm high glass rod with a 3 mm diameter and artificial diet on the end (Pilkington 1988) per replicate instead of a plant (Fig. 2.5). The glass rods had a height of 150 mm above the soil. Non-plant treatments were included to test whether the ASW needed a plant to show avoidance behaviour.



Figure 2.5 Argentine stem weevils on artificial diet at the top of a 3 mm diameter glass rod (Pilkington 1988) in a non-plant treatment in Plant-mediated Experiment 4. Photo: Morgan Shields.

The experimental set-up consisted of jar microcosms where 160 mm labelled pots with 165 mm diameters were filled to a height of 110 mm with potting mix that was covered with a 150 mm diameter filter paper. Pasteurised immature pallic pasture soil from Lincoln University demonstration dairy farm (Landcare 2016) was placed on top of the filter paper, filling another 40 mm of the pot with a space in the centre for a plant or glass rod. The day before data recording a trimmed individual 150 mm high plant with 7-10 tillers of one of the plant types or a glass rod was placed in the centre of each pot and watered. Plants were 4-6 weeks old. Cleaned transparent plastic jars (230 mm x 12 mm x 8 mm) and Fluon suspension applied to a 20 mm height on the inside of the jar starting from the lower 10 mm. The jars had screw-top lids with 0.1 mm mesh for ventilation. The jars were placed over the plants and glass rods and pushed into the soil so the Fluon barrier was just above the soil



surface. Any leaf blades that were touching the plastic jars were trimmed. These precautions were taken to prevent any insects escaping so the ASWs were confined to the plants and soil (Fig. 2.6). Once made, the replicates were placed in a randomised order in controlled temperature rooms under the same conditions in Section 2.4.1.1.



Figure 2.6 Microcosm experimental set-up for Plant-mediated Experiment 4 using plastic jars with screw-top lids and a Fluon barrier. Jars were pushed into the soil to prevent insects escaping and Argentine stem weevils spending time on the plastic surfaces. Photo: Morgan Shields.

On the day of the experiment live adult Lincoln ASWs (10 per replicate) which had been starved for 3 days, were added to each jar 1 h before night, during automated dusk. Where appropriate, one *M. hyperodae* was added to each replicate 1.5 h after the ASWs and around 30 minutes before data recording began. Behavioural data recording started 1 h after night began where data from each replicate was recorded in the same order at 1 h intervals for four consecutive hours during the 'night' 2-5 pm (inclusive) in near darkness. The same ASW behaviours and plant sections that ASWs were on for plant treatments were recorded as in Plant-mediated Experiment 2 (2.4.1.5), using red light from a head torch. Only on-glass behaviour was recorded for the non-plant treatments as this was the only behaviour observed. Post experiment, plants were potted and labelled with all ASWs removed. A sub-sample of insects were frozen post experiment in case ASW dissections and *M. hyperodae* strain identification was needed later (see Section 2.4.1.4). Accumulated feeding damage was later recorded in the form of number of feeding scars, feeding area and feeding scar size for entire plants and plant sections for all plant treatments, using a microscope. The feeding scar sizes were; size 1, < 1000  $\mu\text{m}^2$ ; size 2, 1000 - 4999  $\mu\text{m}^2$ ; size 3, 5000 - 9999  $\mu\text{m}^2$ ; size 4, 10000 - 20000  $\mu\text{m}^2$ ; size 5, > 20000  $\mu\text{m}^2$ .

#### **2.5.1.3. Bioassay that restricted Argentine stem weevil feeding to plant sections of three plant types**

The previous plant-mediated experiments indicated that reduced feeding and being on the lowest section of the plant where there is less nutritional food were ASW avoidance behaviours in response to *M. hyperodae* (see Section 2.4.3). These non-consumptive effects (NCEs) could have consequences, such as reduced ASW fitness and may contribute to ASW mortality but because of density dependant effects this is likely to be inconsequential to pasture damage (Goldson et al. 2011). Here, the feeding consequences as a proxy of fitness was investigated using ASWs that were independently restricted to different plant sections and plant types.

The hypothesis was that ASWs restricted to the lowest plant section, which simulated *M. hyperodae* avoidance behaviour, would feed less and have lower percentage weight gain than those restricted to the upper plant sections due to the increased fibre content of the plant sheath (Barker 1989). This potential feeding pattern may differ between plant types and contribute to the high parasitism observed on tetraploid *L. multiflorum* compared to diploid *L. perenne* and the hybrid (Goldson et al. 2015; Goldson & Tomasetto 2016).

#### **2.5.1.4. Methods of the bioassay that restricted Argentine stem weevil feeding to plant sections**

ASWs were collected in early November 2017 using the same method and locations as in Section 2.4.1.1 and were immediately starved for 2-4 days prior to the experiment. The same three plant types with no endophyte were used as in previous experiments; *L. multiflorum*, the hybrid (*L. perenne* x *L. multiflorum*) and *L. perenne* (see Section 2.4.1.2). Plants were grown in a glasshouse at Lincoln University Nursery (refer to Section 2.4.1.1.) and were 38 days old at the start of the experiment. A randomised block design was used with 20 replicates of nine treatments consisting of the three plant types and three different plant sections, lowest, middle and highest (Fig. 2.7; Fig. 2.8). No *M. hyperodae* were used. The experiment ran from November 9-13 2017 in ambient laboratory conditions using live vertical plants in pots. In each replicate at the appropriate plant section there was a 50 mm height of living plant tissue encased in a 50 mm x 80 mm hinged plastic compartment with foam surrounding the plant tissue at either end to prevent ASW from escaping (Fig 2.7; Fig. 2.8). The plastic compartment was held in place using two stakes in the pot on either side of the living plant. One randomly selected ASW was placed in the plastic compartment of each replicate which was then sealed (Fig 2.7; Fig. 2.8). ASWs were weighed immediately before and after the four-day experiment. After the experiment ASWs that had been weighed were dissected (see Section 2.4.1.4)

to determine the female:male sex ratio. Accumulated feeding area and number of feeding scars were measured post-experiment (see Section 2.4.1.7).



Figure 2.7 Experimental set-up of the bioassay restricting Argentine stem weevil feeding to plant sections showing independent compartments at different plant heights. Photo: Morgan Shields



Figure 2.8 Lowest plant section in the bioassay restricting Argentine stem weevil feeding to plant sections showing an individual weevil and plant sheath. Photo: Morgan Shields

#### **2.5.1.5. Statistical analysis of the Plant-mediated Experiment 4 and the bioassay that restricted Argentine stem weevil feeding to plant sections**

Plant-mediated Experiment 4 measured the same behaviours and used the same statistical analysis as was used in Plant-mediated Experiment 2 involving GLMs and the Log-linear models using Rstudio 1.1.447 (RCoreTeam 2018) and the results were presented in the same manner (refer to Section 2.4.1.7). Some behaviours were not analysed due to lack of data or were represented by the on-plant distribution; stationary; crouching; grooming; mating; walking. Accumulated feeding scar number (entire plant and by plant section) was analysed as count data using a GLM with a Poisson distribution or a negative binomial distribution (RCoreTeam 2018) if the data were greater than 1.5 times over-dispersed. Accumulated feeding area (entire plant and by plant section) was analysed as continuous data using ANOVA (RCoreTeam 2018) and the TukeyHSD post-hoc test using the package agricolae (de Mendiburu 2018).

Statistical analysis for the bioassay that restricted ASW feeding to plant sections involved the analysis of the accumulated feeding area and feeding scar number (see above). Log10 transformation of feeding area data was also investigated but made no difference to the results. ASW weight change was analysed as percentage data using ANOVA (RCoreTeam 2018) and the TukeyHSD post-hoc test using the package agricolae (de Mendiburu 2018). Significant differences were often presented in tables using the same superscript numbers beside values as described in Section 2.4.1.7.

#### **2.5.2 Results of the Plant-mediated Experiment 4 and the bioassay that restricted Argentine stem weevil feeding to plant sections on three plant types**

##### **2.5.2.1. Results of Plant-mediated Experiment 4 that investigated Argentine stem weevil avoidance responses to *M. hyperodae* on three plant types using jar microcosms**

##### **2.5.2.1.1. The proportion of Argentine stem weevil ‘on-plant’ in response to the presence of *M. hyperodae* in Plant-mediated Experiment 4**

GLM analysis showed that ASWs were significantly more active and responsive to *M. hyperodae* in host plants treatments (< 60.2 %) compared to the non-plant treatments (< 15.5 %,  $p = 0.000$ ) where there were no non-plant effects. With reference to the plant treatments with the plant types used there was significant reoccurring plant abandonment on the diploid hybrid (*L. perenne* x *L. multiflorum*) in response to *M. hyperodae* of at least 21.5 % ( $p < 0.032$ ) in all but one time step ( $p = 0.059$ ) (Table 2.5). A similar but delayed plant abandonment response of 29.3 % occurred on *L. perenne* in the last time step ( $p < 0.006$ ). Furthermore, there was a significantly lower proportion of ASWs in on the entire *L. perenne* with *M. hyperodae* present (< 48 %) in the last two time steps and the entire hybrid in time step 4 (41 %) compared to on the entire *L. multiflorum* (> 54 %,  $p < 0.037$ ) (Table 2.5). There was no significant plant abandonment response detected when *M. hyperodae* was

present in the *L. multiflorum* treatment despite changes in the within plant distribution in the last two time steps (Table 2.5). Log-linear analysis of the within-plant ASW distributions revealed there were no significant differences when *M. hyperodae* was present. However, in the controls there were consistently significantly higher proportions of ASWs on the middle *L. multiflorum* section ( $> 40\%$ ) compared to the other *L. multiflorum* sections ( $< 26\%$ ,  $p < 0.013$ ). Additionally, in the last two time steps there were fewer ASWs on the highest hybrid section ( $\leq 17\%$ ) compared to the lower hybrid sections ( $\geq 30\%$   $p < 0.032$ ).

Table 2.5 Position of weevils on plants in Plant-Mediated Experiment 4, read table horizontally: Observed mean percentage of Argentine stem weevils on sections of plants (50 mm height) compared with between plant types and *Microctonus hyperodae* treatments at each time step in Plant-mediated Experiment 4. On-plant behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 15$ ) (refer to Section 2.4.1.7). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid hybrid = orange; diploid *Lolium perenne* = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	55	50	59 <sup>1</sup>	42 <sup>1,2</sup>	50	58 <sup>2</sup>
	Highest	15	17	17	16	23	24
	Middle	23 <sup>1</sup>	17	20	12	13 <sup>1</sup>	16
	Lowest	17	16	22	14	14	18
Time step 2							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	61	59	61	49	55	51
	Highest	17	17	16	18	17	21
	Middle	29 <sup>1</sup>	23	18 <sup>1</sup>	16	23	18
	Lowest	14 <sup>1</sup>	19	27 <sup>1,2,3</sup>	16 <sup>2</sup>	14 <sup>3</sup>	13
Time step 3							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	59	58 <sup>1</sup>	60 <sup>2</sup>	47 <sup>2</sup>	53	46 <sup>1</sup>
	Highest	14	17	12	19	17	14
	Middle	33 <sup>1,2</sup>	17 <sup>1</sup>	26 <sup>3</sup>	11 <sup>3</sup>	22 <sup>2</sup>	18
	Lowest	12 <sup>1,2</sup>	23 <sup>1,3</sup>	21 <sup>2</sup>	16	14	14 <sup>3</sup>
Time step 4							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	61	55 <sup>1,2</sup>	61 <sup>3</sup>	41 <sup>1,3</sup>	58 <sup>4</sup>	41 <sup>2,4</sup>
	Highest	16	11	11	11	15	13
	Middle	34 <sup>1,2</sup>	22 <sup>1</sup>	27 <sup>3</sup>	17 <sup>3</sup>	22 <sup>2</sup>	16
	Lowest	12 <sup>1,2</sup>	21 <sup>1</sup>	22 <sup>2</sup>	14	22	13

#### **2.5.2.1.1. The proportion of Argentine stem weevil feeding in the presence of *M. hyperodae* in Plant-mediated Experiment 4**

The GLM analysis showed that ASW had a reduced feeding response to *M. hyperodae* on all host plants; however, the pattern of this response varied between plant types (Table 2.6). The reduced feeding response was strongest on the hybrid with consistently fewer ASW feeding on the entire plant in all time steps than on the hybrid control and was strongest on the lowest and middle plant sections (Table 2.6). On *L. perenne* the reduced feeding response to *M. hyperodae* was delayed to the last two time steps (Table 2.6). On *L. multiflorum* the reduced feeding response to *M. hyperodae* was less consistent with significantly fewer ASW feeding on the entire plant in the first and last time steps (Table 2.6).

In response to *M. hyperodae* there were also significantly fewer ASW feeding in the middle *L. multiflorum* section in all time steps (Table 2.6). This is supported by the Log-linear analysis within-plant feeding distribution findings, where on the *L. multiflorum* control the middle plant section had significantly higher proportions of ASW feeding compared to the other plant sections in all time steps. Within the ASW feeding distribution on *L. multiflorum* when *M. hyperodae* was present, there were significantly fewer ASW feeding on the highest plant section compared to the lowest section in time steps 2-3. ASW on the hybrid control had a within plant feeding distribution with significantly more ASWs feeding on the lowest plant section compared to the highest section for most time steps. There were no other significant within plant feeding distributions detected. The above findings are supported by significant differences not reported here but these can be viewed in Table 2.6.



Table 2.6 Percentage of weevils feeding in Plant-Mediated Experiment 4, read table horizontally: Observed mean percentage of Argentine stem weevils feeding on each plant section (50 mm height) compared between plant types and *Microctonus hyperodae* treatments at each time step. Feeding behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 15$ ) (refer to Section 2.4.1.7). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid hybrid = orange; diploid *Lolium perenne* = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	40 <sup>1</sup>	29 <sup>1</sup>	38 <sup>2</sup>	23 <sup>2</sup>	35	32
	Highest	8	6	10	6	14	10
	Middle	20 <sup>1,2,3</sup>	11 <sup>1</sup>	10 <sup>2</sup>	6	9 <sup>3</sup>	11
	Lowest	12	11	18	11	11	11
Time step 2							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	37	30	41 <sup>1,2</sup>	21 <sup>1</sup>	27 <sup>2</sup>	21
	Highest	5	6	11	7	7	6
	Middle	21 <sup>1,2</sup>	11 <sup>1</sup>	12	6	11 <sup>2</sup>	9
	Lowest	11	14 <sup>1</sup>	18 <sup>2</sup>	8 <sup>2</sup>	10	6 <sup>1</sup>
Time step 3							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	31	23 <sup>1</sup>	34 <sup>2</sup>	18 <sup>2</sup>	29 <sup>3</sup>	11 <sup>1,3</sup>
	Highest	5	4	3	7	7	4
	Middle	19 <sup>1</sup>	8 <sup>1</sup>	16 <sup>2</sup>	4 <sup>2</sup>	11 <sup>3</sup>	3 <sup>3</sup>
	Lowest	7	11 <sup>1</sup>	16 <sup>2</sup>	7 <sup>2</sup>	10	4 <sup>1</sup>
Time step 4							
Plant type		Tetraploid <i>Lolium multiflorum</i>		Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )		Diploid <i>Lolium perenne</i>	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	37 <sup>1</sup>	19 <sup>1</sup>	33 <sup>2</sup>	15 <sup>2</sup>	38 <sup>3</sup>	13 <sup>3</sup>
	Highest	8	3	6	4	11 <sup>1</sup>	3 <sup>1</sup>
	Middle	20 <sup>1,2</sup>	8 <sup>1</sup>	13 <sup>3</sup>	4 <sup>3</sup>	11 <sup>2</sup>	6
	Lowest	9	8	14 <sup>1</sup>	6 <sup>1</sup>	16 <sup>2</sup>	5 <sup>2</sup>



#### 2.5.2.1.2. Argentine stem weevil accumulated feeding area results in response to the presence of *M. hyperodae* in Plant-mediated Experiment 4

The ANOVA analysis indicated the mean total accumulated feeding area of the ASW in the presence of *M. hyperodae* was significantly higher on *L. multiflorum* (467 mm<sup>2</sup>) than on *L. perenne* (246 mm<sup>2</sup>,  $p = 0.032$ ). There were no other significant differences. Although not significant, the pattern of total feeding appears to be different between all plant types (Fig. 2.7). There were no significant differences when comparing feeding area on plant sections between and within plant types and *Microctonus hyperodae* treatments.

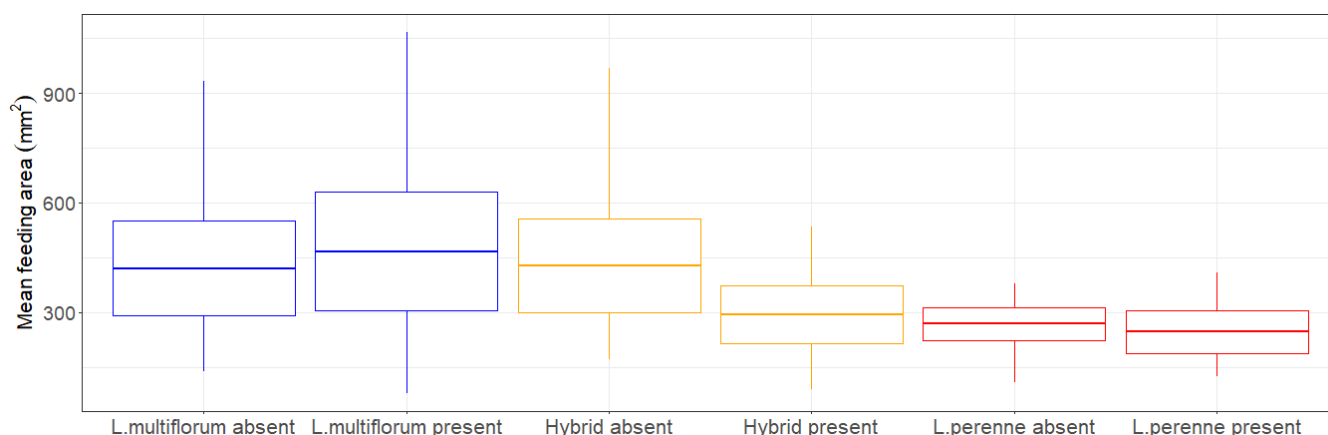


Figure 2.9 Mean accumulated feeding area (mm<sup>2</sup>) of Argentine stem weevil on different plant types in the absence or presence of *Microctonus hyperodae* in Plant-mediated Experiment 4. The boxplot was modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a normal distribution,  $n = 15$ .

#### 2.5.2.1.3. Number of accumulated feeding scars by Argentine stem weevil in the presence of *M. hyperodae* in Plant-mediated Experiment 4

The GLM analysis showed there were no significant differences between the total number of accumulated feeding scars between plant types and *M. hyperodae* treatments. There were significantly more scars on the highest section of *L. multiflorum* compared to *L. perenne*, regardless of the *M. hyperodae* treatment ( $p = 0.037$ ). In the controls there were significantly fewer feeding scars on the lowest *L. multiflorum* section (5.8 scars) compared to the hybrid (13.0 scars,  $p = 0.001$ ) and *L. perenne* (10.7,  $p = 0.01$ ). This is supported by the ASW distribution in the *L. multiflorum* control where there were significantly fewer feeding scars on the lowest plant section (5.8 scars) compared to the highest (13.2 scars,  $p = 0.001$ ) and middle (11.7 scars,  $p = 0.005$ ) plant sections. There were no other significant differences in the number of feeding scars between and within treatments.

#### **2.5.2.1.4. Distribution of accumulated feeding scar size by Argentine stem weevil in response to the presence of *M. hyperodae* in Plant-mediated Experiment 4**

The GLM analysis showed there were limited ASW feeding scar distribution responses to *M. hyperodae*. On *L. multiflorum* there was an increase in small scars (sizes 1-3) on the lowest and to a lesser extent middle plant sections, whereas there was no change in regard to large scar (sizes 4-5) distributions. The hybrid had sporadic feeding scar responses with fewer size 2 scars on the highest plant section (2.5 scars) compared to the hybrid control (4.6 scars,  $p = 0.048$ ). There were also fewer size 5 feeding scars (0.5 scars) on the lowest hybrid section when *M. hyperodae* was present compared to the hybrid control (1.9 scars,  $p = 0.024$ ). There were no significant scar size feeding responses to *M. hyperodae* on *L. perenne*.

ASWs on *L. multiflorum* in the presence of the *M. hyperodae* generally had fewer small feeding scars (sizes 1-3) than on the hybrid and *L. perenne* in the controls but these differences did not occur when *M. hyperodae* was present. On the highest *L. multiflorum* section the number of large size 4 feeding scars (3.3 scars) was significantly higher with *M. hyperodae* present compared to the hybrid (1.7 scars,  $p < 0.05$ ) and *L. perenne* (1.3 scars,  $p = 0.009$ ). There were significantly more large (size 5) scars when *M. hyperodae* was present on the entire *L. multiflorum* plant (7.4 scars) compared to the hybrid (3.9 scars,  $p = 0.015$ ) and *L. perenne* (3.0 scars,  $p < 0.002$ ). Both *L. multiflorum* and the hybrid had a higher number of size 5 feeding scars on the highest plant section compared to *L. perenne* when *M. hyperodae* was present. The Log-linear analysis showed that the distribution of ASW feeding scar sizes within hybrid treatments involved significantly more large feeding scars (size 4-5) on the middle plant section compared to the lowest section when *M. hyperodae* was present. There were no other significant feeding scar size distributions when *M. hyperodae* was present for the hybrid and *L. perenne*. On *L. multiflorum* when *M. hyperodae* was present there were significantly fewer size 4 scars on the lowest plant section compared to the upper plant sections, this feeding scar distribution was similar for all scar sizes in the *L. multiflorum* control.

#### **2.5.2.2. Results of the bioassay that restricted Argentine stem weevil feeding to plant sections on three plant types**

##### **2.5.2.2.1. Argentine stem weevil number of accumulated feeding scars in the bioassay that restricted Argentine stem weevil feeding to plant sections**

GLM analysis revealed there were significantly more ASW accumulated feeding scars on *L. perenne* (33 scars) than on *L. multiflorum* on the lowest plant section (20 scars,  $p = 0.004$ ). All plant types had a feeding scar distribution of significantly fewer feeding scars on the lowest plant section compared to the upper ones ( $p < 0.009$ ) (Table 2.7).

Table 2.7 Read table vertically: Mean number of accumulated feeding scars of Argentine stem weevils within plant types in the bioassay that restricts weevil feeding to plant sections. Plant types were analysed separately using a GLM (negative binomial),  $n = 20$ . Colours; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid hybrid = orange; diploid *Lolium perenne* = red.

Plant type		Tetraploid <i>Lolium multiflorum</i>	Diploid hybrid ( <i>Lolium multiflorum</i> $\times$ <i>Lolium perenne</i> )	Diploid <i>Lolium perenne</i>
Sections of plants	Highest	49 <sup>1</sup>	45 <sup>1</sup>	50 <sup>1</sup>
	Middle	44 <sup>2</sup>	47 <sup>2</sup>	50 <sup>2</sup>
	Lowest	20 <sup>1,2</sup>	26 <sup>1,2</sup>	33 <sup>1,2</sup>

#### 2.5.2.2.2. Accumulated feeding area by Argentine stem weevil in the bioassay that restricted Argentine stem weevil feeding to plant sections

Similar to the number of feeding scars, ANOVA showed accumulated feeding area was higher on the upper plant sections compared to the lowest section at the plant section treatment level ( $p < 0.001$ ). ASWs restricted to the highest *L. multiflorum* section had a greater feeding area ( $46.7 \text{ mm}^2$ ) compared to ASWs restricted to the lowest *L. multiflorum* section ( $16 \text{ mm}^2$ ,  $p < 0.006$ ). The ASWs restricted to the middle hybrid section had a higher feeding area ( $50.0 \text{ mm}^2$ ) compared to those restricted to the lowest hybrid section ( $18.9 \text{ mm}^2$ ,  $p < 0.001$ ) (Fig. 2.10). There were no other significant feeding area results.

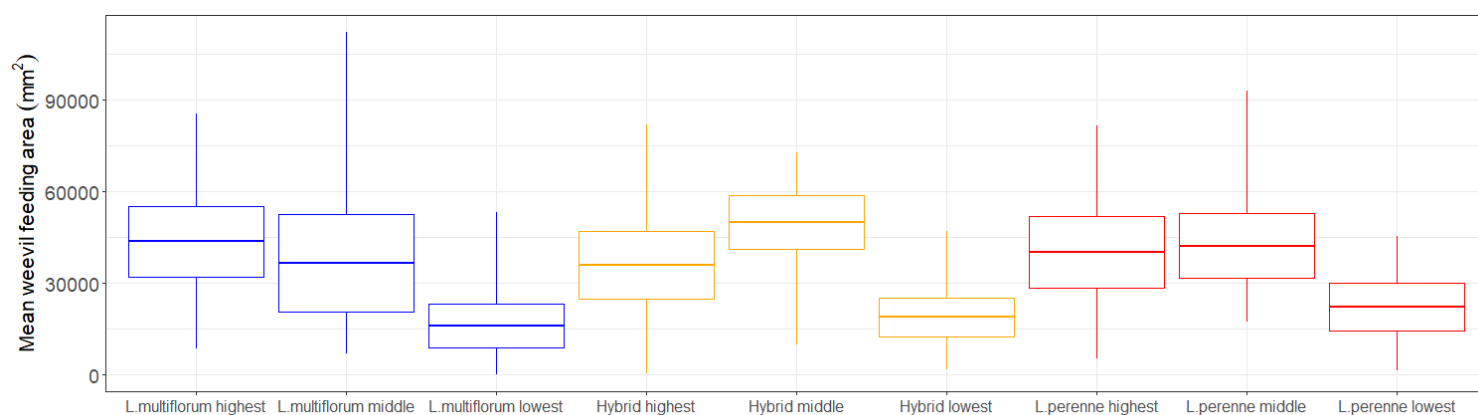


Figure 2.10 Mean accumulated feeding area ( $\text{mm}^2$ ) of Argentine stem weevil on different plant types and plant sections in the bioassay that restricts weevil feeding to plant sections. The boxplot was modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a normal distribution ( $n = 20$ ). Note that the figure uses raw data.

### 2.5.2.2.3. Argentine stem weevil percentage weight gain, sex ratio and parasitism rate in the bioassay that restricted Argentine stem weevil feeding to plant sections on three plant types

ANOVA analysis showed there were no significant differences for ASW percentage weight (Table 2.8). The ASW female:male sex ratio of 1.37:1 and parasitism rate (21.5 % from a sample of 132 individuals). These values were not considered large enough to impact the feeding results as there is no evidence that gender or parasitism impacts ASW feeding (Stephen Goldson, pers. comm.).

Table 2.8 Read table vertically: Mean percentage weight increase of Argentine stem weevils in the bioassay that restricts weevil feeding to plant sections. Plant type and sections were analysed using ANOVA (normal distribution). The same superscript number beside values in each column indicates significant differences ( $P \leq 0.05$ ,  $n = 20$ ). Colours; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; tetraploid *Lolium multiflorum* = blue; diploid hybrid = orange; diploid *Lolium perenne* = red.

Plant type		Tetraploid <i>Lolium multiflorum</i>	Diploid hybrid ( <i>Lolium multiflorum</i> x <i>Lolium perenne</i> )	Diploid <i>Lolium perenne</i>
Sections of plants	Highest	9.4	3.7	7.9
	Middle	14.4	9.3	6.7
	Lowest	9.5	6.4	10.2

## 2.6 Chapter 2 discussion of the plant-mediated effects on Argentine stem weevil responses to *M. hyperodae*

Laboratory experiments in Chapter 2 investigated whether Argentine stem weevil (ASW) behavioural non-consumptive effects (NCEs) triggered by the presence of the *M. hyperodae* were affected by plant type. These NCEs were expressed in the extent of reoccurring plant abandonment, reduced feeding, and within-plant distribution changes in response to *M. hyperodae* (see below) and were detected only on a population basis rather than individual level. Avoidance behaviour by ASW towards *M. hyperodae* is currently the most parsimonious explanation for the difference in parasitism rates between *Lolium* plant types observed in the field (Goldson et al. 2015) and the laboratory (Goldson & Tomasetto 2016) and has not been previously reported in the literature.

### 2.6.1 Main findings

ASW plant-mediated behavioural responses to *M. hyperodae* differed between the plant types investigated and were more pronounced in Plant-mediated Experiment 4 with jar microcosms and soil compared to previous plant-mediated experiments which used tube microcosms and sand (refer to Section 2.4.1.4; Section 2.4.3.5). The diploid hybrid (diploid *L. perenne* x diploid *L. multiflorum*) induced the strongest *M. hyperodae* avoidance responses of the plant types investigated (see Section 2.5.2.1). This work also supports the hypothesis that avoidance behaviour by ASW occurs on the diploid *Lolium* plant types but to a lesser extent on tetraploid *L. multiflorum* (Goldson & Tomasetto

2016). Unlike ASW on the diploid plant types, ASWs on *L. multiflorum* did not respond to *M. hyperodae* by having a lower proportion of those on the entire plant (plant abandonment) (Table 2.5) which was similar to findings in Plant-mediated Experiment 1 (Table 2.2). The avoidance behaviour of plant abandonment was identified as an important behaviour which occurred on the diploid plant types only (Table 2.5). This suggests that ASWs on *L. multiflorum* are more vulnerable to *M. hyperodae* attack because the evolved abandonment response is less sensitive due to fewer interactions with *M. hyperodae* on tetraploid *L. multiflorum* compared to the diploid plants. This explains the parasitism rates observed in Goldson and Tomasetto (2016) and the Plant-mediated Experiment 3 (Fig. 2.4).

## 2.6.2 Behavioural responses on the hybrid

The data show greater avoidance behaviour on the diploid hybrid (diploid *L. perenne* x diploid *L. multiflorum*) compared to diploid *L. perenne* and tetraploid *L. multiflorum* (see Section 2.5.2.1). When ASW on the hybrid were exposed to *M. hyperodae*, there were fewer weevils on the entire plant (Table 2.5) as well as reduced feeding (Table 2.6). This is weakly supported by the ASW responses in Plant-mediated Experiment 2 (refer to Section 2.4.2.2) and previous work (Barratt et al. 1996). The ASW behavioural responses on the hybrid are also supported by the accumulated feeding scar results in response to *M. hyperodae* (Sections 2.5.2.1.2-4). On the hybrid there were fewer medium (size 2) ASW feeding scars on the highest plant section and fewer very large (size 5) feeding scars on the entire hybrid plant, particularly the lowest plant section in response to *M. hyperodae* (see Section 2.5.2.2.3). Furthermore, in the presence of *M. hyperodae*, on the hybrid there were generally fewer accumulated large (size 4-5) ASW feeding scars than on *L. multiflorum* (refer to Section 2.5.2.2.3). ASW feeding results on the hybrid control were similar to that on the *L. multiflorum* control but there were strong *M. hyperodae* avoidance responses on the hybrid when exposed to the parasitoid (Section 2.5.2.1). These findings clearly indicate how ASWs on the hybrid can feed as if on *L. multiflorum* (Fig. 2.9) (Barker 1989) but have similar parasitism rates to those weevils on diploid *L. perenne* (Fig. 2.4) (Goldson et al. 2015; Goldson & Tomasetto 2016). The underlying mechanisms driving this strong avoidance response are unknown but it is likely that it could involve the hybrid containing genes from both *L. multiflorum* and *L. perenne* (Langer 1973). One resulting trait from the hybridisation is less fibre (lignin and cellulose) than both parent plants making the hybrid more palatable to the ASW (Barker 1989). This could have made the hybrid a preferred host plant (Goldson 1982; Barker 1989) but unlike tetraploid *L. multiflorum*, it has been common in New Zealand pasture (Popay et al. 2017). The relatively widespread use of hybrid pasture may have allowed the contemporary evolution of enhanced avoidance behaviour to occur due to consistently high selection pressure by *M. hyperodae* in the 1990s (Tomasetto et al. 2017b). Furthermore, the avoidance behaviours would have to be strong to overcome the attractiveness of

the hybrid which is reflected by the current findings (see above) and the significantly reduced parasitism rate of c. 52 % compared to c. 75 % on the tetraploid *L. multiflorum* and in the 1990s (Goldson & Tomasetto 2016).

### 2.6.3 Behavioural responses on *L. perenne*

Argentine stem weevils (ASWs) on *L. perenne* had delayed extensive plant abandonment (Table 2.5) and reduced feeding (Table 2.6) responses in Plant-mediated Experiment 4 which were consistent with those responses detected in Plant-mediated Experiments 1 and 2 (Table 2.2-4). Furthermore, ASWs on *L. perenne* had less accumulated feeding than on *L. multiflorum* when *M. hyperodae* was present (Fig. 2.9) and fewer feeding scars, particularly very large (size 5) scars (Section 2.5.2.1.5). The plant abandonment and reduced feeding responses indicate why ASWs on diploid *L. perenne* have lower parasitism rates than on tetraploid *L. multiflorum* (Fig. 2.4) (Goldson et al. 2015; Goldson & Tomasetto 2016). These responses were similar to that observed by Gerard (2000) but substantially delayed to the last two time steps. It is unclear why the avoidance behaviours were not detected earlier on *L. perenne*, given that it is the dominant plant type used in New Zealand pasture. Potential explanations include methodology such as the combination of the snap-shot observations, highly variable behaviour between ASW individuals and the artificial environment. If the data recording ran later, more consistent avoidance behaviour may have been detected on *L. perenne*. Furthermore, ASWs were maintained on *L. multiflorum* in captivity to maximise survival prior to being starved before most of experiments which may have resulted in the ASW not responding to the same extent as if they had been maintained on the less preferred *L. perenne*. If the contemporary evolution hypothesis (Goldson et al. 2014; Tomasetto et al. 2017b) is correct, where a small genetic change in ASW populations has enhanced already occurring avoidance behaviours, then the behavioural responses should be more easily detected using ASWs from Ruakura where a stronger parasitism decline has occurred compared to Lincoln (Goldson et al. unpublished data). However, it is abundantly clear how important *M. hyperodae* avoidance responses are in the presence of *L. perenne* given that they have occurred across multiple experiments (Sections 2.4.2; Section 2.5.1.1). These findings support the hypothesis originally developed by Goldson et al. (2015), which states that contemporary evolution in the form of enhanced avoidance behaviour may have occurred on diploid *Lolium* plant types but less so on tetraploid *L. multiflorum* in part due to the former dominating the New Zealand pastoral landscape. This is because any form of resistance to *M. hyperodae* is most likely to occur on the plant types where on which the ASW has experienced the most interactions with *M. hyperodae* and therefore undergone the strongest selection pressure (Goldson et al. 2015; Goldson & Tomasetto 2016).

#### **2.6.4 Behavioural responses on *L. multiflorum***

ASWs on tetraploid *L. multiflorum* exhibited *M. hyperodae* avoidance behaviours but to a lesser extent than on the diploid plant types. Avoidance behaviours in the form of a within-plant distribution change towards the lowest plant section and reduced feeding were often not consistent between time steps and experiments (Section 2.4.2; Section 2.5.2.1). ASW scar size distributions on *L. multiflorum* indicate that weevils were spending less time feeding at the same scar on the lowest and middle plant sections when exposed to *M. hyperodae* (Section 2.5.2.1.4). However, ASWs on the highest *L. multiflorum* remained vulnerable to *M. hyperodae* attack. Avoidance behaviours on tetraploid *L. multiflorum* may have occurred because the weevils used in this work were collected from the dominant diploid *Lolium* pastures where ASW parasitism has declined (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2018b). Therefore, collected ASWs from this field population are likely to exhibit at least some aspects of avoidance behaviour when exposed to *M. hyperodae* on tetraploid *L. multiflorum* (Section 2.5.2.1.1).

#### **2.6.5 Discussion of the the bioassay that restricted Argentine stem weevil feeding to plant sections on three plant types**

The current work partially confirms the hypothesis that the presence of *M. hyperodae* causes the non-consumptive effect of ASWs changing their within-plant distribution and feeding on poorer quality food on the lowest section of the plant (Table 2.7; Fig. 2.10). Goldson (1982) suggested that this was the case as the plant sheath and stems on the lower plant have a higher fibre content than the upper plant tissues and that area is thought to be less palatable and nutritious (Barker 1989). This is supporting evidence of a trade-off between reducing the risk of *M. hyperodae* attack with being in a less desirable area. The current work showed that ASW is often driven to the lowest plant section or off the plant by *M. hyperodae* (see Sections 2.4.2; Section 2.5.2.1) which may result in reduced ASW fitness through increased exposure to pathogens (Abram et al. 2019) and epigeal predators (Losey & Denno 1998; Snyder et al. 2006). Such trade-offs can occur with other herbivorous insects which show within-plant distribution change and reduced feeding avoidance responses (Hermann & Landis 2017; Khudr et al. 2017), which can affect insect fitness such as reduced population growth (Costamagna et al. 2013). Although a trade-off may occur that reduces ASW fitness, this is unlikely to impact the weevil population and reduce pasture damage due to female weevils having density-dependant oviposition and a high egg-load (Goldson 1982; Barker 1989; Goldson et al. 2011).

There were some differences in feeding patterns across plant types as there was a higher number of feeding scars on the lowest plant section of *L. perenne* compared to *L. multiflorum* (see Section 2.5.2.2.1) but no significant difference in feeding area (Fig. 2.10). This suggests that the lowest section of *L. perenne* was less palatable than on *L. multiflorum* so the ASWs had to make more

feeding initiations to consume a similar volume of plant tissue. This is supported by the general finding that ASW prefers feeding on tetraploid *L. multiflorum* compared to diploid *L. perenne* (Goldson 1982; Barker 1989; Goldson et al. 2015; Goldson & Tomasetto 2016). There were no significant ASW percentage weight change differences despite *L. multiflorum* having the highest mean percentage values for the highest and middle plant sections (Table 2.8). Therefore, there is little evidence from this experiment suggesting an increase in ASW mortality due to the NCE of feeding on the lowest plant section.

#### **2.6.6 Potential bottom-up mechanisms driving behavioural responses**

It is currently unknown what underlying drivers account for the differences in avoidance responses on the different plant types investigated but it has shown that plant ploidy has an influence (Goldson et al. 2015; Popay et al. 2017; Tomasetto et al. 2017a). The notion that ploidy affects NCEs which has potentially contributed to ASW parasitism decline is supported by parasitism rates being higher on tetraploid plant types compared to diploid counterparts, regardless of *Lolium* species (Goldson et al. 2015; Tomasetto et al. 2017a). Goldson et al. (2015) found that autumn parasitism rates on tetraploid *L. perenne* and tetraploid *L. multiflorum* were significantly higher than diploid *L. perenne* in a field experiment. Similarly, Tomasetto et al. (2017a) found that tetraploid *L. multiflorum* had higher parasitism rates than diploid *L. multiflorum*, *L. perenne* and the hybrid (*L. perenne* x *L. multiflorum*) in a laboratory experiment. These findings illustrate that ploidy affects parasitism rates, probably due to the ASW being reluctant to leave the *Lolium* tetraploid varieties that have higher nutritional quality compared to diploids (Barker 1989; Sun et al. 2010). It can be speculated that the superior nutritional quality of tetraploid *L. multiflorum* may limit avoidance behaviour to the extent that it is not effective. This is because ASW gain a fitness benefit by staying on the plant for the high nutrition but have the risk of *M. hyperodae* attack.

#### **2.6.7 Plant-mediated Experiment 4 constraints**

The Plant-mediated Experiment 4 artificial set-up with jar microcosms and soil substantially improved the quality of data collected compared to earlier plant-mediated experiments. The main advantage was that ASWs were able to hide away from the host plants in soil that was more realistic and substantially clarified behavioural patterns. This allowed the behavioural avoidance response of having a lower portion of ASW on the entire plant (plant abandonment) when *M. hyperodae* was present to be identified as an important behaviour that may determine parasitism rates. However, improvements in the experimental design could be made if this work were to be continued, such as using Ruakura ASWs as these have had stronger selection pressure by *M. hyperodae*, associated with the highest parasitism rate declines (Goldson et al. unpublished data). Therefore, stronger behavioural responses should be detected (see Chapter 3). Although planned, parasitism rates in the



Plant-mediated Experiment 4 set-up were not investigated due to time constraints and insect availability. This should be done for every new laboratory experimental set-up to test how similar the tri-trophic level interactions are to those observed in the field.

#### **2.6.8 Chapter 2 conclusions, constraints and future research**

The current work has achieved the aim of providing an explanation for the current parasitism rates observed on the plant types investigated. Here, the avoidance behaviour by ASW in response to *M. hyperodae* was stronger on the diploid hybrid and *L. perenne* compared to tetraploid *L. multiflorum*. This involved the behavioural responses of the ASW hiding off the plant in conjunction with a feeding reduction when on the plant. These findings may be the result of potential contemporary evolution of enhanced avoidance behaviour. However, the underlying mechanisms driving the differing behavioural responses between plant types have not been determined. Future work could investigate using different ASW populations and 'climate' conditions as well as potentially expanding the plant types, ploidy of the same *Lolium* species and endophyte treatments used to make the work more relatable to field conditions and different regions. Furthermore, there is ongoing genetic work into the avoidance behaviour and how this may vary across New Zealand. The implications of pastoral plant type affecting biological control success and potential contemporary evolution of an insect pest to its classical biological control agent through the NCE of avoidance behaviour are novel in pest management and far reaching. These ideas are explored in more depth in Chapter 5 (thesis discussion).

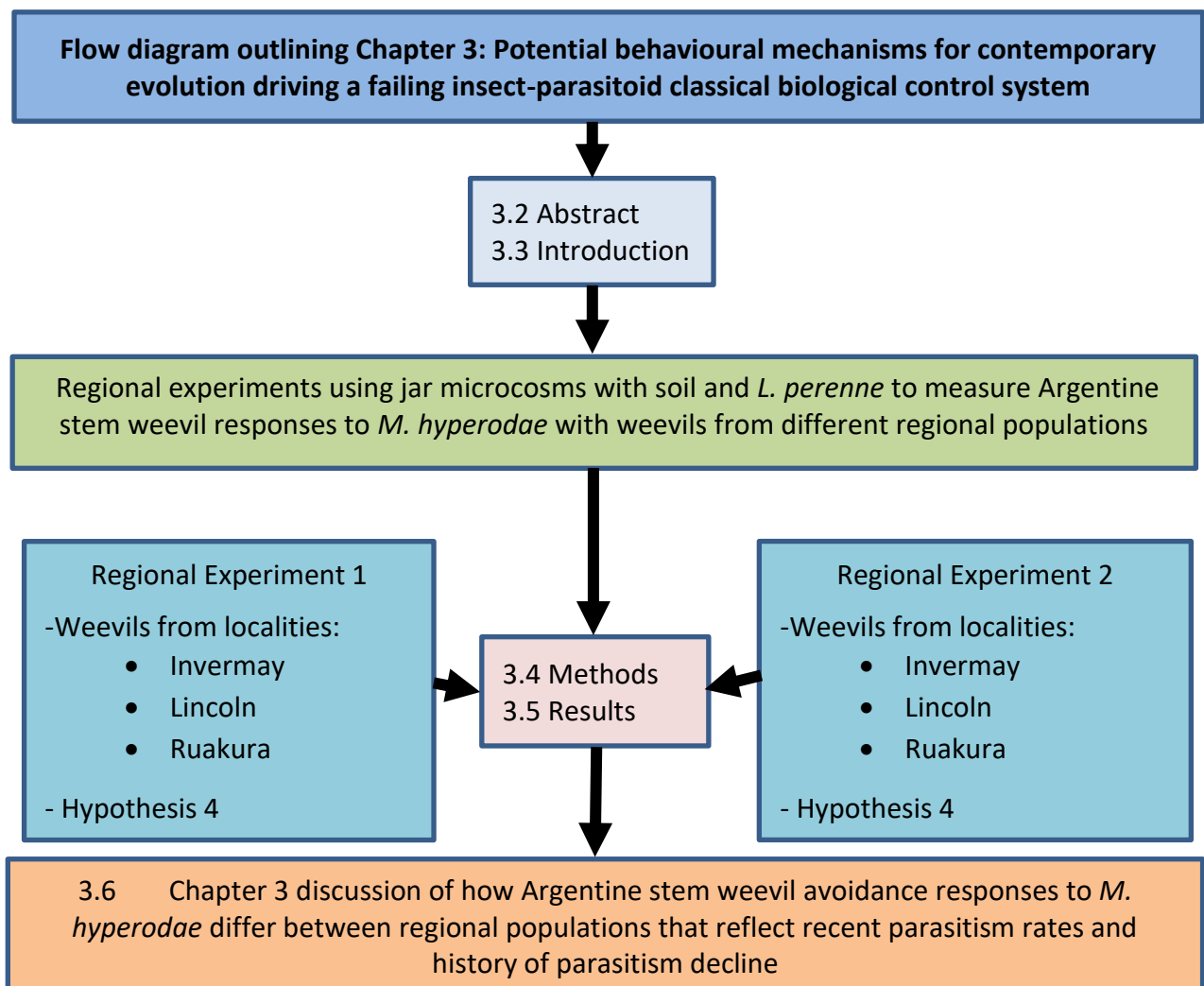
## Chapter 3

### Potential behavioural mechanisms for contemporary evolution driving a failing insect-parasitoid classical biological control system

#### 3.1 Chapter 3 outline

Chapter 3 used microcosms in two experiments to investigate differences in Argentine stem weevil (ASW) behavioural responses to *Micrcotonus hyperodae* from four ASW regional populations represented by five localities, that varied in current parasitism rates, history and latitude. The frequency of weevil feeding and plant abandonment behaviour on entire plants and sections of plants were measured. This work provided the first evidence that ASW behaviour reflects recent parasitism rates and supports the hypothesis that contemporary evolution has enhanced weevil behaviour leading to the decline in ASW classical biological control.

Hypothesis 4: ASWs from regions within New Zealand where the ASW parasitism decline had been greatest should exhibit the strongest avoidance behavioural responses. In contrast, ASWs from regions where there had been no parasitism decline should have little or no detectable avoidance behavioural responses when exposed to *M. hyperodae*.



### 3.2 Abstract

Insect host-parasitoid classical biological control can be extremely successful. However, even after working for close to a decade or more it could fail due to contemporary evolution of the pest. Here, the behavioural mechanisms behind the potential contemporary evolution of the changes in Argentine stem weevil (*Listronotus bonariensis*) (ASW) towards its parasitoid (*Microctonus hyperodae*) in New Zealand pasture were investigated. This was done in microcosm experiments with a spatial randomised block design under laboratory conditions. The behaviour of ASW from regions with either high, medium or no parasitism rate decline (Goldson et al. unpublished data) were compared in the presence and absence (the control) of *M. hyperodae*. ASWs from a population in the 'high' category exhibited the strongest avoidance behaviour to *M. hyperodae*, showing reduced feeding and to a lesser extent, a higher rate of plant abandonment, whereas ASWs from populations in the 'moderate' category had a delayed reduced feeding response to *M. hyperodae*. ASWs from populations with no history of parasitism decline and minimal exposure to *M. hyperodae* had either a very delayed reduced feeding response to *M. hyperodae* or what appeared to be a positive within-plant distribution response which may increase the ASWs vulnerability to *M. hyperodae* attack. The different strengths of avoidance behaviours by ASW reflect the parasitism rate decline that each population had previously experienced, indicating that these responses are probably responsible for the different decline rates. It is hypothesised that these already-present avoidance behaviours by ASW were enhanced by contemporary evolution after *M. hyperodae* was released, due to the unique combination of factors in this host-parasitoid biological control system, leading to the parasitism decline.

### 3.3 Introduction

Biological control using insect natural enemies to manage insect pests can be extremely beneficial when successful (Gurr & Wratten 2000; Ferguson et al. 2019; Shields et al. 2019) and is generally considered evolutionarily stable, unlike pesticide use (Holt & Hochberg 1997; Hufbauer & Roderick 2005). However, if contemporary evolution of pest resistance occurs in currently successful insect biological control programmes in the future, there could be devastating implications (Goldson et al. 2014). This is becoming a more important prospect with the growing reliance on biological control, due to pesticides having increased regulatory pressures imposed on them (Butler 2018), pesticide resistance (Sparks & Nauen 2015; Borel 2017) and concerns of human health and the environment (Gurr et al. 2012; Barzman et al. 2015; Czaja et al. 2015). Contemporary evolution (also known as microevolution and rapid evolution) is defined as a change in the genetic composition of a population (Hartl & Clark 1989) which needs only current genetic variation to occur, without the requirement of mutations or recombination (Hufbauer & Roderick 2005). Contemporary evolution of pesticide resistance is common in insects, plants and pathogens (Gould 1991; Sparks & Nauen 2015). This is

because a large proportion of pest populations is affected by a very strong selection pressure which favours beneficial mutations or standing genetic variation to become fixed within populations (Hufbauer & Roderick 2005). Furthermore, the pesticide active ingredients are unchanging and their modes of action are often interrupted by a single mutation that may provide resistance against multiple pesticides (Gould 1991; Sparks & Nauen 2015). Selection pressure from natural enemies is generally considered to be weaker than from pesticides due to spatial and temporal heterogeneity (Holt & Hochberg 1997). Furthermore, contemporary evolution towards predators often does not develop because among other explanations, resistant genotypes may not persist such as over winter or after crops have been removed. Thus, there can be large associated costs to resistance and there may be constant immigration of susceptible genotypes (Holt & Hochberg 1997; Hufbauer & Roderick 2005; Thompson 2013). However, although uncommon, contemporary evolution of resistance to biological control has occurred with pathogens and to a lesser extent, plants (Burdon et al. 1981; Fenner 1983; Chaboudez 1994; Holt & Hochberg 1997; Hufbauer & Roderick 2005; Marshall et al. 2017).

Unlike with other organism interactions, contemporary evolution in insect prey through resistance to insect predators or parasitoids is extremely rare in the literature (Henter & Via 1995; Holt & Hochberg 1997; Hufbauer & Roderick 2005; Goldson et al. 2014; Mills 2017). Currently, the most convincing example of insect-parasitoid contemporary evolution is male crickets (*Teleogryllus oceanicus* Le Guillou) developing a novel flatwing trait to avoid parasitism by a fly (*Ormia ochracea* Bigot) in Hawaii. Flatwing removes sound-producing structures on the crickets' wings and arose through convergent evolution of two independent mutations each of which occurred and proliferated in cricket populations on separate Hawaiian islands (Pascoal et al. 2014). There is also limited evidence that suggests parasitoids could benefit from contemporary evolution in host-parasitoid interactions (Waage et al. 1988; Zarrabi et al. 2003). For instance, parasitoids in classical biological control can become more effective after establishment than those remaining in their native range and in quarantine (Waage et al. 1988). A potential example of contemporary evolution is the parasitoid *Bathyplectes curculionis* (Thomson) overcoming the immune response of its host, the alfalfa weevil (*Hypera postica* Gyllenhal) in North America. However, the underlying mechanism has not been identified. This loss of defence is indicated by the alfalfa weevil's decreased parasitoid egg encapsulation efficiency over a 27 year period (Zarrabi et al. 2003).

Despite the lack of host-parasitoid examples of contemporary evolution in the literature, insects have sufficient genetic diversity for contemporary evolution to occur e.g. (Salt & van den Bosch 1967; Hufbauer 2001). There is evidence that the pea aphid (*Acyrtosiphon pisum* Harris) contains genetic variation of resistance to its parasitoid (*Aphidius ervi* Haliday) within (Henter & Via 1995) and between populations (Hufbauer & Via 1999). Parasitoid eggs failed to develop within resistant hosts

in laboratory experiments compared to susceptible clonal lines from a single pea aphid population. However in the field experiments there was no indication of contemporary evolution due to selection pressure exerted by the parasitoid over multiple aphid generations which could be due to the fitness costs associated with immune responses (Henter & Via 1995). Studies on the fruit fly (*Drosophila melanogaster* Meigen) and its parasitoids show they have genetic variation in resistance and counter-resistance traits (Kraaijeveld & Godfray 2009). Resistance to fruit fly parasitoids often takes the form of egg encapsulation in the host's haemolymph. However there is a significant trade-off between the host's ability to mount an immune response and cost to reproduction which may slow the development of contemporary evolution (Kraaijeveld & Godfray 2009). An alternative form of parasitoid resistance by a fruit fly is avoidance behaviour where adults prefer to oviposit in parasitoid-free areas compared to parasitoid-infested ones. This is likely to reduce parasitism, with a lower reproduction cost compared to an immune response (Lefèvre et al. 2012; Lynch et al. 2016). There is now substantial evidence that contemporary evolution in insect host-parasitoid interactions can occur if there is sufficient standing genetic variation in resistance traits acted upon by strong selection pressure (Hufbauer & Roderick 2005; Goldson et al. 2014; Pascoal et al. 2014; Mills 2017; Tomasetto et al. 2017b).

Contemporary evolution of the Argentine stem weevil (*Listronotus bonariensis* Kuschel) (ASW) is hypothesised to be responsible for the decline in parasitism rates by the parasitoid, *Microctonus hyperodae* Loan, leading to failure of this once highly successful biological control programme (Goldson et al. 2014; Tomasetto et al. 2017b; Ferguson et al. 2019). The South American parasitoid was introduced in 1991 (Goldson et al. 1993) and was extremely successful, with parasitism rates averaging 75 % over much of the country (Goldson et al. 1998; Barker & Addison 2006) with the exception of the ASW's range extremes such as the region of Otago in southern New Zealand where rates were around 10-15 % (Ferguson 1997) (Fig. 3.1). These rates were much higher than the c. 5 % in South America (Loan & Lloyd 1974; Goldson et al. 1990). However, in regions such as the Waikato and Canterbury where *M. hyperodae* was highly successful in the 1990s, there has been up to an 80 % decline in parasitism (Goldson et al. 2014; Tomasetto et al. 2017b).

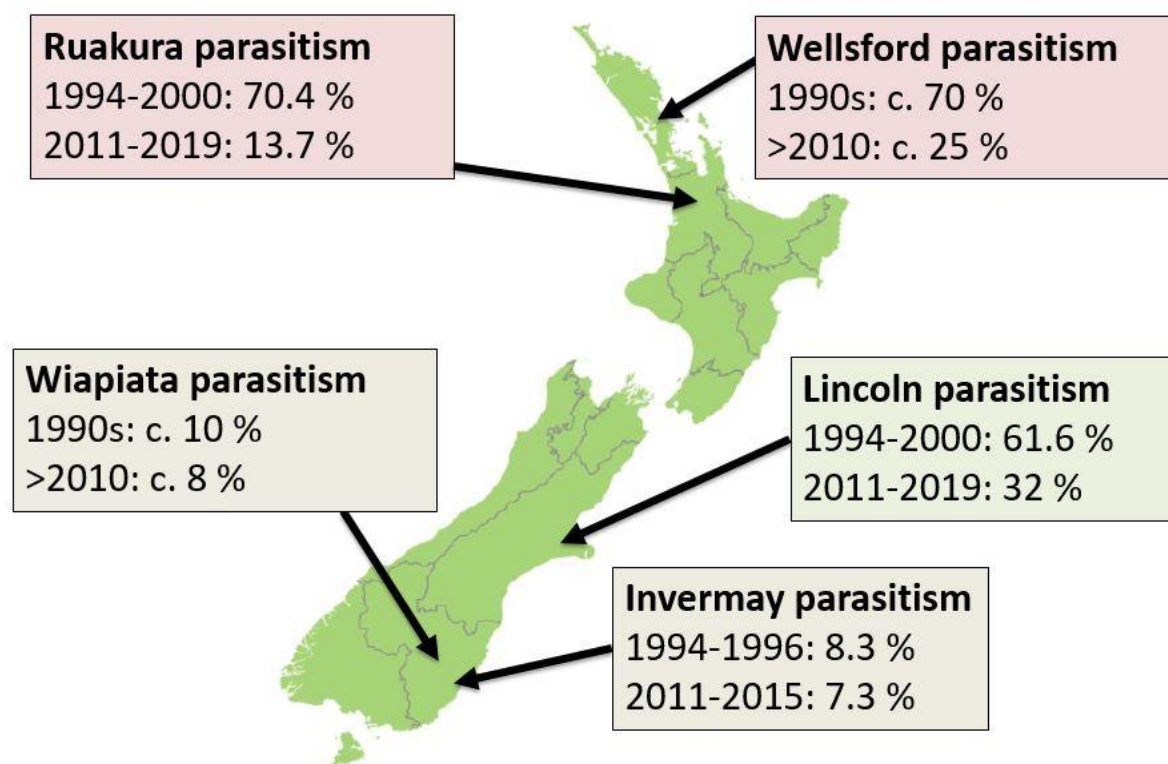


Figure 3.1 Historical and recent overwintering field parasitism rates of Argentine stem weevil by the parasitoid *Microctonus hyperodae* in various regions around New Zealand. Image of New Zealand: Ozhiker (edited by Greg O'Beirne) with permission.

Contemporary evolution of *M. hyperodae* resistance is thought to be responsible because of a unique combination of factors affecting this host-parasitoid system. New Zealand pasture is a highly simplified ecosystem with low plant and natural enemy diversity, which may have created unoccupied niches open to pest invasion (Goldson et al. 1997; Gerard et al. 2010; Goldson et al. 2014). This simplified ecosystem dominated by productive *Lolium* pastures was ideal for both ASW and *M. hyperodae* because it allowed the ASW to reach high densities but also provided few refuges from *M. hyperodae* (Tomasetto et al. 2017b) which has a high searching efficiency (Barlow et al. 1993; Tomasetto et al. 2018a). The combination of the above factors and virtually no other natural enemies of ASW is thought to have created an extremely strong selection pressure (Goldson et al. 2014). This pressure is thought to have then acted on standing genetic variation of resistance traits that ASW possessed in the form of behavioural avoidance (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b). ASW behavioural responses are thought to be the mechanism for parasitism decline because behavioural responses often have a lower reproductive cost than physiological immune responses (Lefèvre et al. 2012; Lynch et al. 2016). There is also no evidence of encapsulation by the ASW (Goldson et al. 2015) whereas there are known ASW avoidance responses on which selection could act, such as reduced feeding (Barratt et al. 1996; Gerard 2000; Phillips 2002) and plant abandonment when the *M. hyperodae* is present (Gerard 2000). In the biological control system studied here, there is asymmetric selection as the sexually reproducing ASW has far greater

adaptive capacity than the asexual *M. hyperodae* (Goldson et al. 2014; Casanovas et al. 2018) which reproduces by ameiotic thelytoky (Iline & Phillips 2004). This is supported by ASW having a large and highly complex genome that has recently been sequenced (Jeanne Jacobs, pers. comm.), indicating that ASW has high genetic variation on which selection can act. Furthermore, there is no evidence of other direct factors driving the parasitism decline despite investigation e.g. climate (Tomasetto et al. 2017b; Tomasetto et al. 2018b), pasture endophytes (Goldson et al. 2015) and endosymbionts (White et al. 2015). The novel combination of factors described above points towards contemporary evolution of enhanced avoidance behaviour as the most parsimonious explanation for the ASW parasitism decline in this insect host-parasitoid classical biological control system.

This chapter investigated whether *M. hyperodae* avoidance behaviour of ASW from different regions reflected regional differences in current parasitism rates (Goldson et al. unpublished data) as the result of the parasitism decline. Avoidance behaviours of ASW from localities representing regions that have differed in parasitism rate decline were compared in no-choice microcosm laboratory experiments. The hypothesis was that ASWs from regions where the parasitism decline had been greatest (c. 80 %) such as in the Waikato region (Goldson et al. unpublished data), should exhibit the strongest avoidance behavioural responses. ASW from regions where there had been a medium parasitism decline (c. 30 %) such in the Canterbury region (Goldson et al. unpublished data), should have medium level responses whereas ASW from regions where there had been a no parasitism decline and have always had very low parasitism rates such as Otago (Ferguson 1997) (Goldson et al. unpublished data), should have little or no avoidance behavioural responses when exposed to the *M. hyperodae*. Therefore, individuals from these ASW populations with no parasitism decline are the closest example of ASWs having little or no historical *M. hyperodae* exposure, allowing the potential contemporary evolution of avoidance behaviour to be investigated.

### **3.4 Methodology of Regional Experiment 1 and 2 where Argentine stem weevil avoidance responses to *M. hyperodae* were compared between weevils from different regions**

These methods were developed in close collaboration with AgResearch entomologists, particularly Professor Stephen Goldson (unpublished data), who have used historical and contemporary Argentine stem weevil (ASW) parasitism data to access ongoing changes in overwintering parasitism rates across New Zealand climate zones. Reference to this will be made as Goldson et al. (unpublished data).

### 3.4.1 Regional Experiment 1 methods

#### 3.4.1.1. Insect sampling and maintenance

Adult ASWs were collected with a suction sampler (inverted leaf blower) with a mesh sock (Goldson et al. 2000) using the same protocol as described in Chapter 2 (see Section 2.4.1.1). Samples were taken from three regions in New Zealand that have different current parasitism rates and history. They were; Waikato which has had a steep parasitism decline (Goldson et al. unpublished data), represented by Ruakura ASW (sampled January 8 2018, Ruakura Research Centre farm, -37.770933, 175.304805); Canterbury which has had a medium parasitism decline (Goldson et al. unpublished data) and is represented by Lincoln ASW (sampled January 8 2018, AgResearch farm, -43.630334, 172.471321; Greenpark Huts, -43.750747, 172.551187); Otago which has had no parasitism decline (Goldson et al. unpublished data), represented by Invermay (Dunedin) ASW (sampled February 13 2018, -45.858707, 170.387355). Sampled localities were at least 100 km away from each other. Sampling took place in January and early February 2018 to maximise ASW sampling success rates while increasing the likelihood of sampling 'naive' ASW adults that had little to no *M. hyperodae* exposure (refer to Table 2.1). Sampling ASWs with minimal *M. hyperodae* interaction allows the measurement of innate behavioural responses that may reflect genotypes rather than learned behaviours, however this is limited by practicality. Subsamples of 50 adult ASWs from each region were dissected to check parasitism which was under 25 % and therefore *M. hyperodae* purging was not considered necessary. ASWs from each region were maintained in separate labelled plastic boxes using the same protocol described in Chapter 2 (see Section 2.4.1.1). Further Lincoln ASW sampling took place during February and March 2018 when parasitism rates were relatively high (Goldson et al. 1998; Phillips et al. 1998; Goldson et al. 2011) from which Lincoln *M. hyperodae* were purged to be used in experiments. Once enough Lincoln *M. hyperodae* were available, Regional Experiment 1 was conducted.

#### 3.4.1.2. Regional Experiment 1 protocol

The no-choice experimental design was a spatial randomised block design with 11 replicates and 6 treatments. Behavioural data were recorded over two dates, with five blocks on March 9 and six blocks on March 23 2018. Treatments included ASWs from one region with or without (control) *M. hyperodae*. To standardise and simplify the experiment as much as possible, all ASWs used in the experiment were starved for three days prior, only Lincoln *M. hyperodae* that were  $\leq 5$  days old were used. Nil-endophyte diploid *Lolium perenne* (cv. Grasslands Samson) was the only host plant used (refer to Section 2.4.1.2). The experimental jar microcosm set-up was the same as that described in Chapter 2 (see Section 2.5.1.2). On the day of the experiment, 10 starved adult ASWs from one of the regions were added to each replicate 2 h before the onset of night. Where appropriate, one *M.*



*hyperodae* individual was added to each replicate 1.5 h after the ASWs were added and around 30 minutes before data recording began at the onset of night. Behavioural data recording from each replicate was done in the same order for four consecutive 40-60 minute intervals during the night, using red light from a head torch. ASW behaviours that could be either *M. hyperodae* avoidance or vulnerable behaviour on each 50 mm-high plant section (high, middle and low) was recorded (Gerard 2000; Phillips 2002). These behaviours have been defined in Section 2.4.1.4. Insect and plant individuals were only used once and a sub-sample of insects were frozen post-experiment in case ASW dissections and *M. hyperodae* strain identification were needed later (see Section 2.4.1.4).

### **3.4.2 Regional Experiment 2 methodology**

Using the protocol of Regional Experiment 1 (see Section 3.4.1), samples were taken from two localities not previously compared, with Waikato (Ruakura) ASWs as the control. All regions have different current parasitism rates and history of decline. These were; North of Auckland which has had a medium parasitism decline (Goldson et al. unpublished data), represented by Wellsford ASW (sampled on April 20 2018, Grace farm, -36.237237, 174.513526); Waikato which has had a steep parasitism decline (Goldson et al. unpublished data), represented by Ruakura ASWs (sampled on April 19 2018, Research Centre farm, -37.770933, 175.304805); Otago which has had no parasitism decline (Goldson et al. unpublished data), represented by Wiapiata (Central Otago) ASW (sampled on April 26 2018, -45.176350, 170.156429). Subsamples of up to 50 adult ASWs from each region were dissected to check parasitism which was under 25 % and therefore *M. hyperodae* purging was not considered necessary. ASWs from each region were maintained in separate labelled plastic boxes for up to 21 days using the same protocol described in Chapter 2 (see Section 2.4.1.1). Regional Experiment 2 used the same protocol as the Regional Experiment 1 (Section 3.4.1.2) with some modifications. The randomised block design had 18 replicates from which data were recorded over three dates, with six blocks on each date (April 30, May 4 and May 10 2018). Additionally, the behavioural data from each replicate were recorded in the same order for four consecutive 35-60 minute intervals instead of the 40-60 minute intervals in Regional Experiment 1 (see Section 3.4.1.2).

### **3.4.3 Statistical analysis of regional experiments**

All analysis was conducted using the statistical programme Rstudio 1.1.447 (RCoreTeam 2018) with base R code unless cited statistical packages were used. Untransformed data was used as transformations were not necessary for analyses and P-values were used to determine significant differences (refer to Section 2.4.1.7). Only on-plant and feeding ASW behavioural data were analysed because the other recorded behaviours had a lack of data or were represented by the main on-plant and feeding behaviours. Behavioural data were first analysed with a generalised linear mixed effects model with penalised quasi-likelihood (GLMMPQL) and a binomial distribution the packages lme4

(Bates et al. 2015), MASS (Venables & Ripley 2002) and nlme (Pinheiro et al. 2018). The GLMMPQL used all the data in all the time steps to indicate any very strong ASW behavioural responses to the *M. hyperodae*. Responses compared between localities and *M. hyperodae* treatments for each time step and plant section (including the entire plant) were analysed separately using a generalised linear model (GLM) with a binomial distribution (RCoreTeam 2018). Within-plant distributions and behavioural patterns for ASWs from each locality and *M. hyperodae* treatment for each time step were analysed separately using a Log-linear analysis with a Poisson distribution (RCoreTeam 2018). The GLM and Log-linear analysis was used to investigate how patterns of ASW behaviour may change over the four time steps between and within different localities and plant sections. Significant differences that occurred in two or more time steps or in more than one experiment were considered consistent strong responses. Significant differences in tables are indicated by the same superscript numbers beside values. This was done because ANOVA was not appropriate without transformation and the analysis of possible treatment comparisons that had no biological meaning. An example of such a comparison is the treatment combination of the Lincoln ASWs with *M. hyperodae* present compared to Ruakura control. For consistency, this presentation of significant differences using the same superscript numbers was used in all tables.

### 3.5 Results of Regional Experiment 1 and 2 where Argentine stem weevil avoidance responses to *M. hyperodae* were compared between weevils from different regions

#### 3.5.1 Results of Regional Experiment 1 which compared Argentine stem weevil avoidance responses to *M. hyperodae* between weevils from Otago (Invermay), Canterbury (Lincoln) and Waikato (Ruakura)

##### 3.5.1.1 Results of Regional Experiment 1 'on-plant' responses of Argentine stem weevil towards *M. hyperodae*

The GLMMPQL analysis revealed that Ruakura ASWs had a significant plant abandonment response with a lower proportion of ASWs on plants in the presence of *M. hyperodae* (31 %) compared to in the Ruakura control (45 %,  $p = 0.043$ ). There were significantly fewer Ruakura ASWs on plants in the presence of *M. hyperodae* (31 %) compared to ASWs from Lincoln and Invermay (52 %,  $p < 0.004$ ). There were no significant overall on-plant responses to *M. hyperodae* from Lincoln and Invermay ASWs (Fig. 3.2).

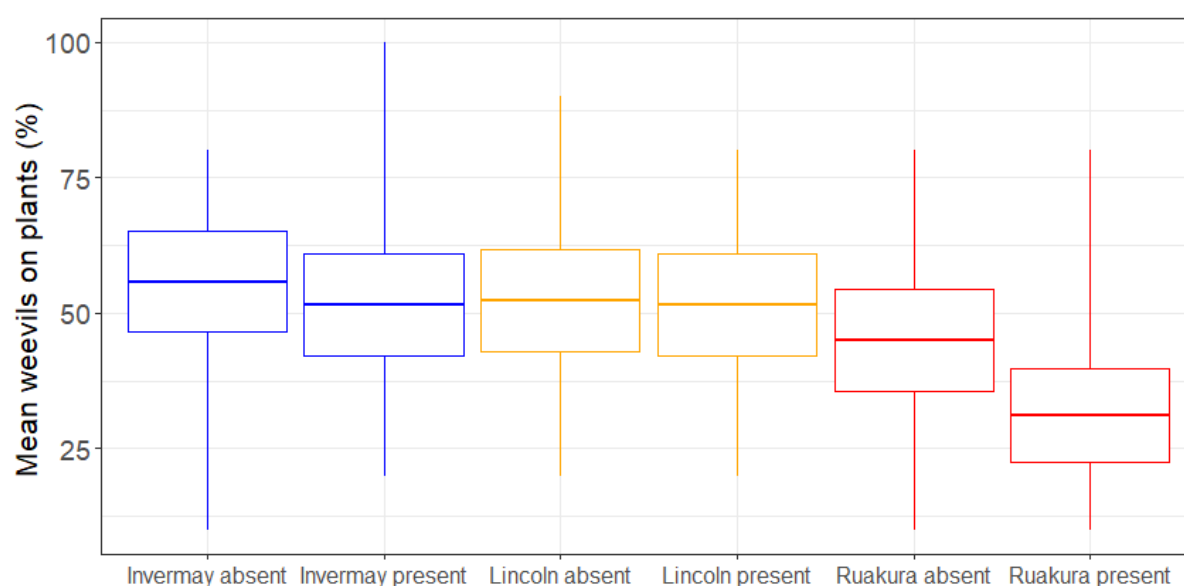


Figure 3.2 Mean percentage of Argentine stem weevils from Invermay, Lincoln and Ruakura on diploid *Lolium perenne* in the absence (control) or presence of *Microctonus hyperodae* in Regional Experiment 1. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a binomial distribution,  $n = 11$ .

The Regional Experiment 1 GLM analysis indicated that Ruakura ASWs responded to *M. hyperodae*, leading to fewer ASWs on the entire plant in most time steps (Table 3.1). For all time steps, Ruakura ASWs had fewer individuals on the entire plant when *M. hyperodae* was present compared to Lincoln and Invermay ASWs (Table 3.1). Lincoln ASWs had no significant on-plant responses to *M. hyperodae*. In time steps 2 and 4, with *M. hyperodae*, there were significantly fewer Invermay ASWs on the lowest plant section and an increase in the proportion of ASWs on the middle plant section was present (Table 3.1). Ruakura and Lincoln ASWs had no consistent significant differences in within-

plant distribution patterns for all time steps. In the Invermay control, there were more ASWs on the lowest plant section (> 22 individuals) compared to the upper sections (highest or middle) (< 15 individuals,  $p < 0.05$ ) in most time steps, whereas there was no significant within-plant distribution pattern when *M. hyperodae* was present.

Table 3.1 Position of weevils on plants in Regional Experiment 1, read table horizontally: Observed mean percentage of Argentine stem weevils on plant sections (50 mm height) compared between locality and *Microctonus hyperodae* treatments at each time step. On-plant behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 11$ ) (refer to Section 3.3.4). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; Invermay = blue; Lincoln = orange; Ruakura = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	56	51 <sup>1</sup>	51	53 <sup>2</sup>	43	33 <sup>1,2</sup>
	Highest	14	11	23	19	17	12
	Middle	10	16	8	15	16	9
	Lowest	31 <sup>1</sup>	24 <sup>2</sup>	20 <sup>3</sup>	18	9 <sup>1,3</sup>	12 <sup>2</sup>
Time step 2							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	61 <sup>1</sup>	57 <sup>2</sup>	51	46 <sup>3</sup>	46 <sup>1,4</sup>	30 <sup>2,3,4</sup>
	Highest	18	15	18	14	19	13
	Middle	13	24 <sup>1,2</sup>	15	10 <sup>1</sup>	17 <sup>3</sup>	8 <sup>2,3</sup>
	Lowest	30 <sup>1,2,3</sup>	18 <sup>1,4</sup>	17 <sup>2</sup>	23 <sup>5</sup>	10 <sup>3</sup>	9 <sup>4,5</sup>
Time step 3							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	54	49 <sup>1</sup>	54	55 <sup>2</sup>	48 <sup>3</sup>	33 <sup>1,2,3</sup>
	Highest	13	12 <sup>1</sup>	19	25 <sup>2</sup>	20	16
	Middle	14	20 <sup>1</sup>	15	18 <sup>2</sup>	18 <sup>3</sup>	7 <sup>1,2,3</sup>
	Lowest	27 <sup>1</sup>	17	19	12	10 <sup>1</sup>	11
Time step 4							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	53	49 <sup>1</sup>	54	53 <sup>2</sup>	43 <sup>3</sup>	29 <sup>1,2,3</sup>
	Highest	19	15	21	17	22 <sup>1</sup>	11 <sup>1</sup>
	Middle	11	21 <sup>1</sup>	19	15	14	10 <sup>1</sup>
	Lowest	23 <sup>1,2</sup>	13 <sup>1</sup>	14	20 <sup>3</sup>	7 <sup>2</sup>	8 <sup>3</sup>

### 3.5.1.2 Results of Regional Experiment 1 feeding responses of Argentine stem weevil towards *M. hyperodae*

GLMMPQL analysis of observed ASW feeding data found that Ruakura ASWs had a lower proportion of individuals feeding when *M. hyperodae* was present (14 %) compared to the Ruakura control (30 %,  $p < 0.002$ ). There were also fewer Ruakura ASWs observed feeding compared to Lincoln (24 %,  $p = 0.032$ ) and Invermay ASWs (30 %,  $p = 0.002$ ) when *M. hyperodae* was present (Fig. 3.3). There were no significant overall feeding responses to *M. hyperodae* from Lincoln and Invermay ASWs.

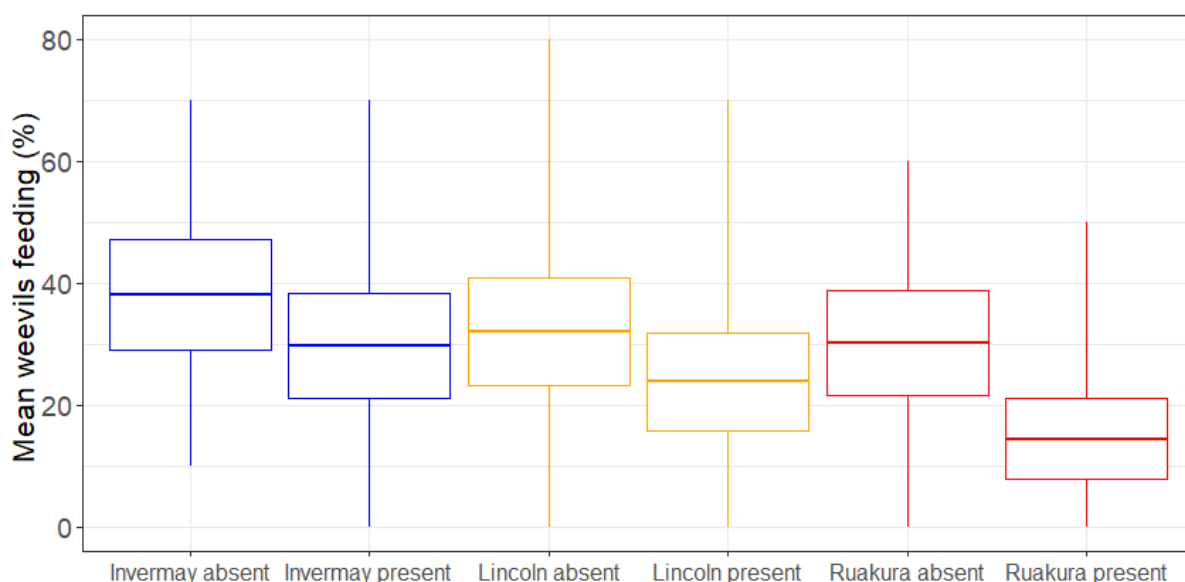


Figure 3.3 Mean percentage of Argentine stem weevils from Invermay, Lincoln and Ruakura feeding on diploid *Lolium perenne* in the absence (control) or presence of *Microctonus hyperodae* in Regional Experiment 1. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a binomial distribution,  $n = 11$ .

The GLM analysis showed that Ruakura ASWs consistently had a reduced feeding response in the presence of *M. hyperodae* in all time steps (Table 3.2). Those ASWs were less frequently feeding ( $< 20$  %) when *M. hyperodae* was present compared to those from Invermay in time steps 1-3 ( $> 28$  %,  $p < 0.017$ ) (Table 3.2). Lincoln ASWs showed a lower proportion of observed feeding in response to *M. hyperodae* in time step 3 ( $p < 0.011$ ), particularly on the lowest plant section ( $p < 0.007$ ). Invermay ASWs showed a lower proportion of observed feeding in response to *M. hyperodae* in time step 4 ( $p < 0.007$ ), particularly on the highest plant section ( $p < 0.006$ ) (Table 3.2). In the absence of *M. hyperodae* (controls), there were significantly fewer Ruakura ASWs on the lowest plant section ( $< 10$  %) compared to Invermay ASWs ( $> 20$  %,  $p < 0.019$ ) for time steps 1-3 and Lincoln ASWs (15 %,  $p = 0.036$ ) for time step 3 (Table 3.2). The Log-linear analysis showed that there were no consistent significant within-plant feeding distributions with the exception of the Invermay control. This had a significantly higher number of ASWs feeding on the lowest plant section ( $> 18$  individuals) compared to the upper sections (highest and middle) for time steps 1-3 ( $< 9$  individuals,  $p < 0.041$ ), whereas this pattern did not occur with *M. hyperodae* present.

Table 3.2 Read table horizontally: Observed mean percentage of Argentine stem weevils feeding on plant sections (50 mm height) compared between locality and *Microctonus hyperodae* treatments at each time step in Regional Experiment 1. Feeding behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 11$ ) (refer to Section 3.3.4). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; Invermay = blue; Lincoln = orange; Ruakura = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	40	34 <sup>1</sup>	35	25	31 <sup>2</sup>	19 <sup>1,2</sup>
	Highest	10	5	12	7	9	4
	Middle	9	11	6	5	13	6
	Lowest	21 <sup>1</sup>	18 <sup>2</sup>	16	12	9 <sup>1</sup>	9 <sup>2</sup>
Time step 2							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	43 <sup>1</sup>	38 <sup>2,3</sup>	27 <sup>1</sup>	25 <sup>2</sup>	32 <sup>4</sup>	11 <sup>2,3,4</sup>
	Highest	7	7	11	5	13	5
	Middle	9	15 <sup>1</sup>	6	5	14 <sup>2</sup>	1 <sup>1,2</sup>
	Lowest	28 <sup>1,2,3</sup>	15 <sup>1,4</sup>	10 <sup>2</sup>	15 <sup>5</sup>	5 <sup>3</sup>	5 <sup>4,5</sup>
Time step 3							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	35	29 <sup>1</sup>	40 <sup>2</sup>	24 <sup>2</sup>	31 <sup>3</sup>	15 <sup>1,3</sup>
	Highest	8	5	14	11	14	7
	Middle	6	12 <sup>1</sup>	11	9 <sup>2</sup>	11 <sup>3</sup>	2 <sup>1,2,3</sup>
	Lowest	21 <sup>1</sup>	12 <sup>2</sup>	15 <sup>3,4</sup>	4 <sup>3,2</sup>	6 <sup>1,4</sup>	6
Time step 4							
Locality		Invermay		Lincoln		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	35 <sup>1</sup>	18 <sup>1</sup>	26	22	27 <sup>2</sup>	12 <sup>2</sup>
	Highest	14 <sup>1</sup>	2 <sup>1</sup>	6 <sup>2</sup>	7	15 <sup>2,3</sup>	2 <sup>3</sup>
	Middle	8	10	8	6	6	5
	Lowest	13	6	12	8	6	5

### 3.5.2 Regional Experiment 2 which compared Argentine stem weevil avoidance responses to *M. hyperodae* between weevils from Otago (Wapiata), North of Auckland (Wellsford) and Waikato (Ruakura)

#### 3.5.2.1 Results of Regional Experiment 2 ‘on-plant’ responses of Argentine stem weevil towards *M. hyperodae*

In the GLMMPQL analysis, there were no on-plant responses to *M. hyperodae* detected in Regional Experiment 2 (Fig. 3.4). In the parasitoid’s absence (controls) there were significantly more Ruakura ASWs observed on the plants (61 %) compared to Wapiata ASWs (50 %,  $p < 0.05$ ).

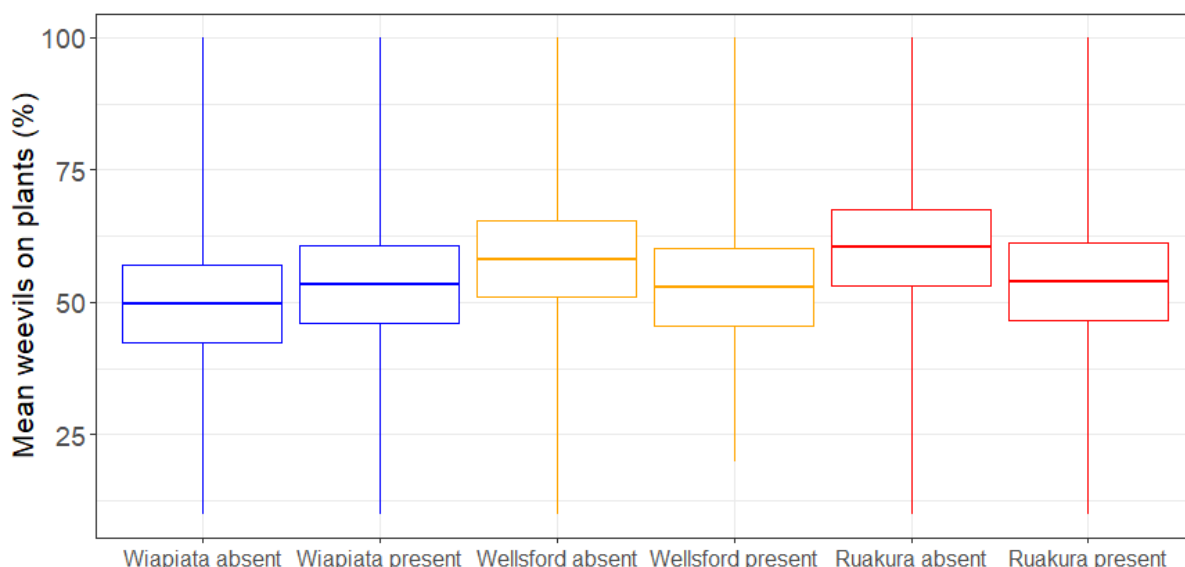


Figure 3.4 Mean percentage of Argentine stem weevils from Wapiata, Wellsford and Ruakura on diploid *Lolium perenne* in the absence (control) or presence of *Microctonus hyperodae* in Regional Experiment 2. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a binomial distribution,  $n = 18$ .

The Regional Experiment 2 GLM analysis revealed that there were limited of on-plants responses to *M. hyperodae* (Table 3.3) where Ruakura ASWs only had significantly fewer individuals on the entire plant in the last time step when *M. hyperodae* was present (55 %) compared to the Ruakura control (65 %,  $p < 0.05$ ), particularly on the lowest plant section ( $p = 0.028$ ). Wellsford ASWs had no significant on-plant responses to *M. hyperodae*. In time steps 1-2, Wapiata ASWs had a positive response to *M. hyperodae* with an increase in on the highest plant section (28 %) compared to the Wapiata control (*M. hyperodae* absent) (19 %,  $p < 0.038$ ). The Log-linear analysis showed that Ruakura and Wellsford ASW within-plant distributions did not change in response to *M. hyperodae* in Regional Experiment 2 with significantly more ASWs on the upper plant sections (highest and middle) (Table 3.3). In the presence of *M. hyperodae*, Wapiata ASWs had significantly more individuals on the highest plant section ( $< 52$  individuals) compared to the middle section ( $< 32$  individuals,  $p < 0.029$ ) for time steps 1-3 and the lowest plant section for all time steps ( $< 25$  individuals,  $p < 0.036$ ) (Table 3.3). Contrastingly, in the Wapiata control there were significantly fewer individuals on the lowest plant section ( $< 18$  individuals) compared to the upper plant sections (highest and middle) ( $>$

31 individuals,  $p < 0.043$ ) for time steps 1-2 with no pattern in time steps 3-4 (Table 3.3). There were no other on-plant ASW responses to *M. hyperodae* to report.

Table 3.3 Distribution of weevils within-plants in Regional Experiment 2, read table vertically: Total observed Argentine stem weevils on plant sections (50 mm height) within each locality and *Microctonus hyperodae* treatment at each time step. *M. hyperodae* treatments of each plant type within each time step were analysed separately using a Log-linear analysis. The same superscript number beside values in each column indicates significant differences ( $P \leq 0.05$ ,  $n = 18$ ) (refer to Section 3.3.4). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; Wiapiata = blue; Wellsford = orange; Ruakura = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	32 <sup>1</sup>	51 <sup>1,2</sup>	40 <sup>1</sup>	42 <sup>1</sup>	36	51 <sup>1,2</sup>
	Middle	32 <sup>2</sup>	29 <sup>1</sup>	36 <sup>2</sup>	28	40	20 <sup>1</sup>
	Lowest	17 <sup>1,2</sup>	24 <sup>2</sup>	16 <sup>1,2</sup>	23 <sup>1</sup>	25	15 <sup>2</sup>
Time step 2							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	34 <sup>1</sup>	51 <sup>1,2</sup>	41 <sup>1</sup>	40 <sup>1</sup>	46 <sup>1</sup>	48 <sup>1</sup>
	Middle	38 <sup>2</sup>	31 <sup>1</sup>	42 <sup>2</sup>	40 <sup>2</sup>	41 <sup>2</sup>	30 <sup>2</sup>
	Lowest	19 <sup>1,2</sup>	21 <sup>2</sup>	17 <sup>1,2</sup>	16 <sup>1,2</sup>	24 <sup>1,2</sup>	16 <sup>1,2</sup>
Time step 3							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	31	44 <sup>1,2</sup>	42 <sup>1</sup>	41 <sup>1</sup>	35	40 <sup>1</sup>
	Middle	32	25 <sup>1</sup>	34	44 <sup>2</sup>	46 <sup>1</sup>	33
	Lowest	24	21 <sup>2</sup>	24 <sup>1</sup>	14 <sup>1,2</sup>	25 <sup>1</sup>	20 <sup>1</sup>
Time step 4							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Highest	30	38 <sup>1</sup>	40 <sup>1</sup>	41 <sup>1</sup>	42	44 <sup>1</sup>
	Middle	33	29	43 <sup>2</sup>	39 <sup>2</sup>	42	32 <sup>2</sup>
	Lowest	31	20 <sup>1</sup>	21 <sup>1,2</sup>	13 <sup>1,3</sup>	33	17 <sup>1,2</sup>



### 3.5.2.2 Results of Regional Experiment 2 feeding responses of Argentine stem weevil towards *M. hyperodae*

GLMMPQL analysis of observed ASW feeding for Regional Experiment 2 showed that Ruakura ASWs had a highly significant reduced feeding response ( $p < 0.001$ ) (Figure 3.5). There were fewer Ruakura ASWs feeding (16 %) compared to Wellsford ASWs when *Microctonus hyperodae* was present (24 %,  $p < 0.02$ ) whereas in the absence (controls) of *M. hyperodae* there were fewer Wiapiata ASWs feeding (18 %) compared to Ruakura and Wellsford ASWs (32 %,  $p < 0.002$ ) (Fig. 3.5).

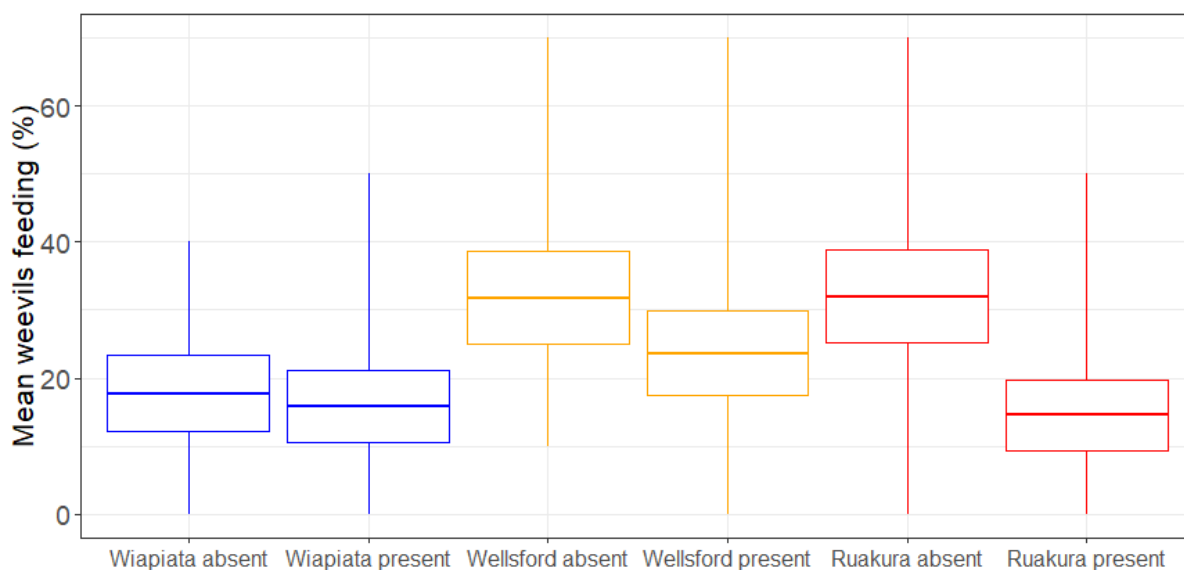


Figure 3.5 Mean percentage of Argentine stem weevils feeding from Wiapiata, Wellsford and Ruakura on diploid *Lolium perenne* in the absence (control) or presence of *Microctonus hyperodae* in Regional Experiment 2. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a binomial distribution,  $n = 18$ .

In the Regional Experiment 2, GLM analysis between localities and *M. hyperodae* treatments Ruakura ASWs had significantly reduced feeding in response to *M. hyperodae* in all time steps ( $p < 0.001$ ) with the exception of time step 3 ( $p = 0.068$ ), particularly in the middle and lowest plant sections (Table 3.4). Wellsford ASWs had significantly reduced feeding in response to *M. hyperodae* in the last two time steps ( $p < 0.01$ ) with reduced feeding detected in different plant sections depending on time step involved (Table 3.4). There was no significant feeding response by Wiapiata ASWs detected and generally fewer Wiapiata individuals observed feeding in all time steps compared to Ruakura and Wellsford ASWs (Table 3.4). In the Log-linear analysis there were no significant within-plant feeding distributions detected for Ruakura and Wiapiata treatments. In the presence of *M. hyperodae* for time step 2-4, Wellsford ASWs had significantly more ASWs observed feeding on the middle plant section ( $> 15$  individuals) compared to the lowest section ( $< 10$  individuals,  $p < 0.024$ ) whereas there was no consistent within-plant feeding distribution in the Wellsford control.

Table 3.4 Read table horizontally: Observed mean percentage of Argentine stem weevils feeding on plant sections (50 mm height) compared between locality and *Microctonus hyperodae* treatments at each time step in Regional Experiment 2. Feeding behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 18$ ) (refer to Section 3.3.4). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; Wiapiata = blue; Wellsford = orange; Ruakura = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	19	17 <sup>1</sup>	28	25 <sup>1,2</sup>	28 <sup>3</sup>	14 <sup>2,3</sup>
	Highest	4	7	9	9	7	5
	Middle	8	4	13	8	14 <sup>1</sup>	8 <sup>1</sup>
	Lowest	7	5	6	8	7	4
Time-step 2							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	17 <sup>1,2</sup>	17 <sup>3</sup>	32 <sup>1</sup>	29 <sup>3,4</sup>	35 <sup>2,5</sup>	14 <sup>4,5</sup>
	Highest	7	7	12	9	9	6
	Middle	6 <sup>1,2</sup>	6 <sup>3</sup>	12 <sup>1</sup>	15 <sup>3,4</sup>	16 <sup>2,5</sup>	4 <sup>4,5</sup>
	Lowest	5	3	8	5	10 <sup>1</sup>	4 <sup>1</sup>
Time step 3							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	16 <sup>1,2</sup>	17	34 <sup>1,3</sup>	22 <sup>3</sup>	27 <sup>2</sup>	19
	Highest	4 <sup>1</sup>	8	15 <sup>1,2,3</sup>	7 <sup>3</sup>	5 <sup>2</sup>	9
	Middle	6 <sup>1</sup>	5 <sup>2</sup>	12	13 <sup>2,3</sup>	13 <sup>1,4</sup>	6 <sup>3,4</sup>
	Lowest	6	3	7 <sup>1</sup>	2 <sup>1</sup>	9	4
Time step 4							
Locality		Wiapiata		Wellsford		Ruakura	
<i>Microctonus hyperodae</i> treatment		Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present	Parasitoid absent	Parasitoid present
Plant section	Entire plant	18 <sup>1,2</sup>	13	32 <sup>1,3</sup>	18 <sup>3</sup>	38 <sup>2,4</sup>	12 <sup>4</sup>
	Highest	6 <sup>1</sup>	6	12	7	12 <sup>1,2</sup>	5 <sup>2</sup>
	Middle	6 <sup>1,2</sup>	2 <sup>3</sup>	13 <sup>1</sup>	9 <sup>3</sup>	12 <sup>2,4</sup>	5 <sup>4</sup>
	Lowest	6 <sup>1</sup>	4	8 <sup>2</sup>	3 <sup>2</sup>	13 <sup>1,3</sup>	2 <sup>3</sup>

### **3.6 Chapter 3 discussion of how Argentine stem weevil avoidance responses to *M. hyperodae* differ between weevil regional populations**

This chapter investigated whether Argentine stem weevil (ASW) behavioural responses to *M. hyperodae* differed between ASW populations from different regions of New Zealand and whether these behavioural responses reflected current and historical parasitism rates of each region. These experiments provide the first evidence (see Section 3.5) of a behavioural mechanism for the ASW parasitism decline at a national scale that is consistent with the hypothesis that the decline is due to contemporary evolution in the ASW. The implications of this are discussed in Chapter 5.

#### **3.6.1 Main findings**

It is clear that ASWs from different populations differ in behavioural responses to *M. hyperodae* (see Section 3.5) and that these differences reflect current parasitism rates and the extent of ASW parasitism decline (Goldson et al. unpublished data). The Ruakura ASW population had the strongest and most consistent avoidance behaviour response to *M. hyperodae* with reduced feeding in both regional experiments (Fig. 3.3; Table 3.2; Fig. 3.5; Table 3.4). A plant abandonment response to *M. hyperodae* was also exhibited by Ruakura ASWs, where there were fewer individuals on the entire plant in the presence of *M. hyperodae* compared to the control. This response by Ruakura ASWs was very strong (Fig. 3.2) and consistent (Table 3.1) in Regional Experiment 1 but was very delayed in Regional Experiment 2 (refer to Section 3.5.2). The apparent inconsistency is discussed further in Section 3.6.2. The *M. hyperodae* avoidance responses of the Ruakura locality reflect the parasitism history of the greater Waikato region where stable overwintering parasitism rates were c. 85 % in 1990s (Barker & Addison 2006) but have since declined to c. 15 % (Goldson et al. unpublished data). This large decline could be due to the contemporary evolution of enhanced avoidance behaviours hypothesised by Goldson et al. (2015) and Tomasetto et al. (2017b) due to the unique combination of factors described in Section 3.3. These *M. hyperodae* avoidance responses by the ASWs are known from previous studies (Gerard 2000; Phillips 2002) and were consistently detected in the regional experiments (see Section 3.5).

The hypothesis that contemporary evolution of enhanced avoidance behaviour has led to the decline in ASW parasitism rates (Goldson et al. 2014; Goldson et al. 2015; Goldson & Tomasetto 2016) is further supported by the delayed behavioural responses by ASWs from regions where there has been a moderate decline in parasitism rates. ASW populations in Lincoln and the greater Canterbury region as well as Wellsford and the surrounding region north of Auckland have experienced a moderate parasitism decline from c. 75 % in the 1990s (Goldson et al. 1998; Barker & Addison 2006) to c. 25-45 % (Goldson et al. unpublished data). This less intense decline is reflected by the delayed and weaker reduced feeding behavioural responses of Lincoln and Wellsford ASWs compared to the

strong responses of those from Ruakura (see Section 3.5.1.2; Section 3.5.2.2). The delayed reduced feeding response of Lincoln ASWs was detected only in one time step (Table 3.2) and the Wellsford ASW delayed reduced feeding response to *M. hyperodae* occurred in the last two time steps (Table 3.4). Unlike Ruakura ASWs, there was no plant abandonment response detected for those from Lincoln (Table 3.1) and Wellsford (refer to Section 3.5.2.1) which indicates that this response is harder to detect than the reduced feeding response.

ASW populations that have experienced no parasitism decline (Goldson et al. unpublished data) and have always had low overwintering parasitism rates such as in the Otago region (15 %) (Ferguson 1997) were hypothesised to have little or no avoidance behaviour in response to *M. hyperodae*. This is crucial to the over-arching contemporary evolution hypothesis. Similar to the regions with a moderate parasitism decline (e.g. Canterbury and north of Auckland), there was no plant abandonment avoidance behaviour in Otago ASWs (Invermay and Wiapiata localities) (Fig. 3.2; Table 3.1; Fig. 3.4; Section 3.5.2.1). Furthermore, Invermay ASWs had a minimal reduced feeding response to *M. hyperodae* (Table 3.2) and no response from Wiapiata weevils (Table 3.4), indicating that the feeding response is rarely expressed in these weevil populations. The feeding response to *M. hyperodae* by Invermay ASWs has been more prominent in Gerard (2000) where a more artificial experimental set-up and questionable statistical analysis was used compared to the current work. Contrastingly, Wiapiata ASWs appear to have a positive within-plant distribution response to the presence of *M. hyperodae*, with an increased proportion of individuals on the highest plant section (Section 3.5.2.1; Table 3.3). This indicates a lack of ASW resistance to *M. hyperodae*, which could be due to fewer interactions in Central Otago, leading to insufficient selection pressure for contemporary evolution of enhanced avoidance responses to occur. These findings support Thomson's hypothesis of coevolution mosaics (Thompson et al. 2002; Thompson 2005; Thompson 2013), where in this case there are 'hot spots' of ASW-*M. hyperodae* interactions (e.g. Waikato (Ruakura)) where *M. hyperodae* resistance is high, and 'cold spots' of ASW-*M. hyperodae* interactions (e.g. Otago (Invermay and Wiapiata)) where *M. hyperodae* resistance is low. This idea is supported by the behavioural findings, which reflect the different historical parasitism pressure which each ASW population experienced in the 1990s (Ferguson 1997; Goldson et al. 1998; Barker & Addison 2006).

### 3.6.2 Contrasting results between regional experiments

It was remarkable that significant signals and trends were detected in the regional experiments given the artificial experimental conditions and that insect behaviour is generally considered highly variable (Godfray 1994; Szendrei & Rodriguez-Saona 2010; Inghilesi et al. 2015) but there were some notable differences detected in results between the Regional Experiment 1 and Regional Experiment 2. This was primarily the plant abandonment response to *M. hyperodae* by Ruakura ASWs indicated by the proportion of ASWs on the plant which is highly variable and difficult to detect because it involves all the ASWs in treatments not just those individuals doing a specific behaviour such as feeding. In Regional Experiment 1 Ruakura ASWs had a very strong and consistent plant abandonment response (Fig. 3.2; Table 3.1) but this response was very delayed in Regional Experiment 2 (see Section 3.5.2.1). Ruakura ASWs used in the Regional 1 Experiment were sampled on January 8 2018 before diapause (Goldson 1981a) and maintained in captivity for up to 75 days before the experiment. Contrastingly, the Ruakura ASWs used in Regional Experiment 2 were sampled from the same farm on April 19 2018 after the onset of ASW diapause (Goldson 1981a) and kept in captivity for up to 22 days. Thus, ASW behaviour could have been influenced by environmental conditions that the ASWs experienced prior to sampling, which would have varied greatly between mid-summer and the end of autumn. Furthermore, diapause could also impact ASW behaviour through indirect effects due to physiological changes, although it is not known to affect ASW feeding (Goldson 1981a; Ferguson 1997; Goldson et al. 2011) which is supported by the current work (Fig. 3.3; Table 3.2; Fig. 3.5; Table 3.3). There is also potential of a caging effect that influenced behaviour, as ASWs used in Regional Experiment 1 were kept in captivity substantially longer than those used in Regional Experiment 2 due to logistical constraints and *M. hyperodae* availability. Such a caging effect is known to alter ASW physiology where females reabsorb their eggs after around 10 days in captivity (Goldson 1983) and could potentially also affect ASW behaviour. Despite the discrepancy in the Ruakura plant abandonment findings, the reduced feeding response to *M. hyperodae* of Ruakura ASWs was very strong and consistent in both experiments (Fig. 3.3; Table 3.2; Fig. 3.5; Table 3.3). This suggests that only the more variable plant abandonment response was affected by the potentially influential factors of sampling season, diapause, and caging effects discussed above.

### 3.6.3 Contrasting results between regional experiments and Plant-mediated Experiment 4

There were differences in avoidance behaviour by Lincoln ASWs on *L. perenne* between Regional Experiment 1 (see Section 3.5.1) and Plant-mediated Experiment 4 (see Section 2.5.2.1), both of which used very similar methodology (see Section 2.5.1.2; Section 3.4). Lincoln weevils used in both experiments were sampled at the same location and date (January 8 2018) and the experiments were conducted within 35 days of each other, Regional Experiment 1 occurred on March 9 and March 23 2018 and the Plant-mediated Experiment 4 occurred on March 30 – April 12 2018 (see Section 2.5.1.2; Section 3.4.1.1). Regardless of these similarities, Lincoln ASWs in Regional Experiment 1 had no detectable plant abandonment response (Table 3.1) and showed a reduced feeding response (Table 3.2). Conversely, Lincoln ASWs in Plant-mediated Experiment 4 had a stronger response to *M. hyperodae* on *L. perenne* than in Regional Experiment 1 with a delayed plant abandonment (Table 2.5) and reduced feeding response (Table 2.6). The differences in the strength of detectable avoidance behaviours between individuals from the same ASW population suggest that the expression of behaviours is highly variable even within weevil populations. This behavioural variation may reflect the large and diverse ASW genome (Jacobs, unpublished data) and the potential impact of an individual's nutritional needs and physiological state (Simpson & Abisgold 1985; Camus et al. 2018; Akami et al. 2019; Cook et al. 2019). These confounding variables are difficult to remove or test but substantial effort was devoted to standardising all possible variables that may affect ASW behaviour (see Section 2.5.1.2; Section 3.4). It is also important to note that while the reduced feeding response was consistently the dominant avoidance response in the regional experiments (Fig. 3.3; Table 3.2; Fig. 3.5; Table 3.4), the plant abandonment behaviour was the dominant response in the plant-mediated experiments, followed closely by the reduced feeding response (see Sections 2.5.2.1; Section 2.6). This is probably because it is easier to detect behavioural responses when comparing different plant types where plant-mediated effects can impact ASW response sensitivity (refer to Chapter 2) compared to detecting behaviours with ASWs of different origins using the same plant type as no such plant-mediated effects occur. When considering all the natural behavioural variation and potential variables affecting behaviour that make it difficult to detect significant differences, any reoccurring behavioural trends or signals that do occur are probably very strong and important, such as the reduced feeding response from the data which is particularly prevalent with Ruakura ASWs.

### 3.6.4 Limitations of the regional experiments

There were considerable logistical challenges and restrictions when planning and conducting the regional experiments. ASWs needed to be sampled around the same time in different parts of the country, which was weather dependent and AgResearch had a major role in achieving this. *M. hyperodae* availability was a major restriction as this determined when and how many replicates could be measured, resulting in data recording occurring on dates sometimes weeks apart. Furthermore, due to the nature of these ambitious experiments, six blocks of replicates could not be included in the Regional Experiment 1 analysis due to delayed *M. hyperodae* exposure and therefore delayed recording, which was different to the other dates of data recording. This resulted in 11 replicates used in the Regional Experiment 1 analysis instead of the intended 17. A statistical investigation showed that this outcome did not affect the GLMMPQL analysis but it may have affected the significance of the GLM results. Once it was apparent that significant differences were detected in Regional Experiment 1, because of logistical issues there was deliberation of whether another regional experiment was possible, including the planning of localities with different known parasitism histories and when ASWs could be sampled. This resulted in a four-month gap between ASWs sampled for the regional experiments. As discussed in Section 3.6.2, this temporal difference in sampling may have contributed to variation in the Ruakura results between the two regional experiments and emphasises the need for ASW sampling around similar times to standardise future experiments as much as logistically possible.

### 3.6.5 Future research

Future research could test for possible behavioural plasticity by comparing behaviour of ASWs under different climate conditions, most notably temperature. This may provide supporting evidence for the hypothesis that the differences in behavioural responses between ASW populations observed in the current work were derived from contemporary evolution due to parasitism pressure rather than from ASWs with different genetic variation responding to the same environmental conditions used in the experiments. The impact of using *M. hyperodae* from different populations on ASW behaviour would be interesting to investigate as it is currently unknown what *M. hyperodae* 'ecotypes' and clonal lines from the original eight South American 'populations' have persisted in New Zealand (Goldson et al. 1997; Phillips et al. 1997). It is also unknown to what extent these populations differed genetically and therefore to what degree *M. hyperodae* from different collections differ in behaviour. This in turn may result in different ASW behavioural responses, as there are at least two *M. hyperodae* haplotypes, 'east' and 'west' of the Andes which differ in phenology (Phillips et al. 1994) and fecundity (Phillips et al. 1997). The 'east' *M. hyperodae* haplotype is dominant in New Zealand (Phillips et al. 1994) and was identified as the haplotype used in the current work (Craig Phillips, pers. comm.). Given the variation in traits identified by previous studies (Goldson et al. 1997;

Phillips et al. 1997; Iline & Phillips 2004) advancement in molecular technology in the last decade means that there is now an intense whole genome study into the genetics of *M. hyperodae* (Peter Dearden, pers. comm.)

### **3.6.6 Conclusions**

The current work emphasises that in classical biological control there is a need to have a detailed understanding of host-parasitoid interactions at a regional and national scale and that behaviour must be compared between different localities as it is unlikely to be universal. This also applies to establishing new classical biological control if there is no prior information on the intra-specific genetic variation in the introduced agents. Behaviour can be a crucial component in pest management that can be easily overlooked due to its variability and difficulty to measure but it can contribute or cause attempted biological control to be unsuccessful. Furthermore, this work has highlighted the need for long-term monitoring of biological control systems. The implications of this work are further discussed in Chapter 5.



## Chapter 4

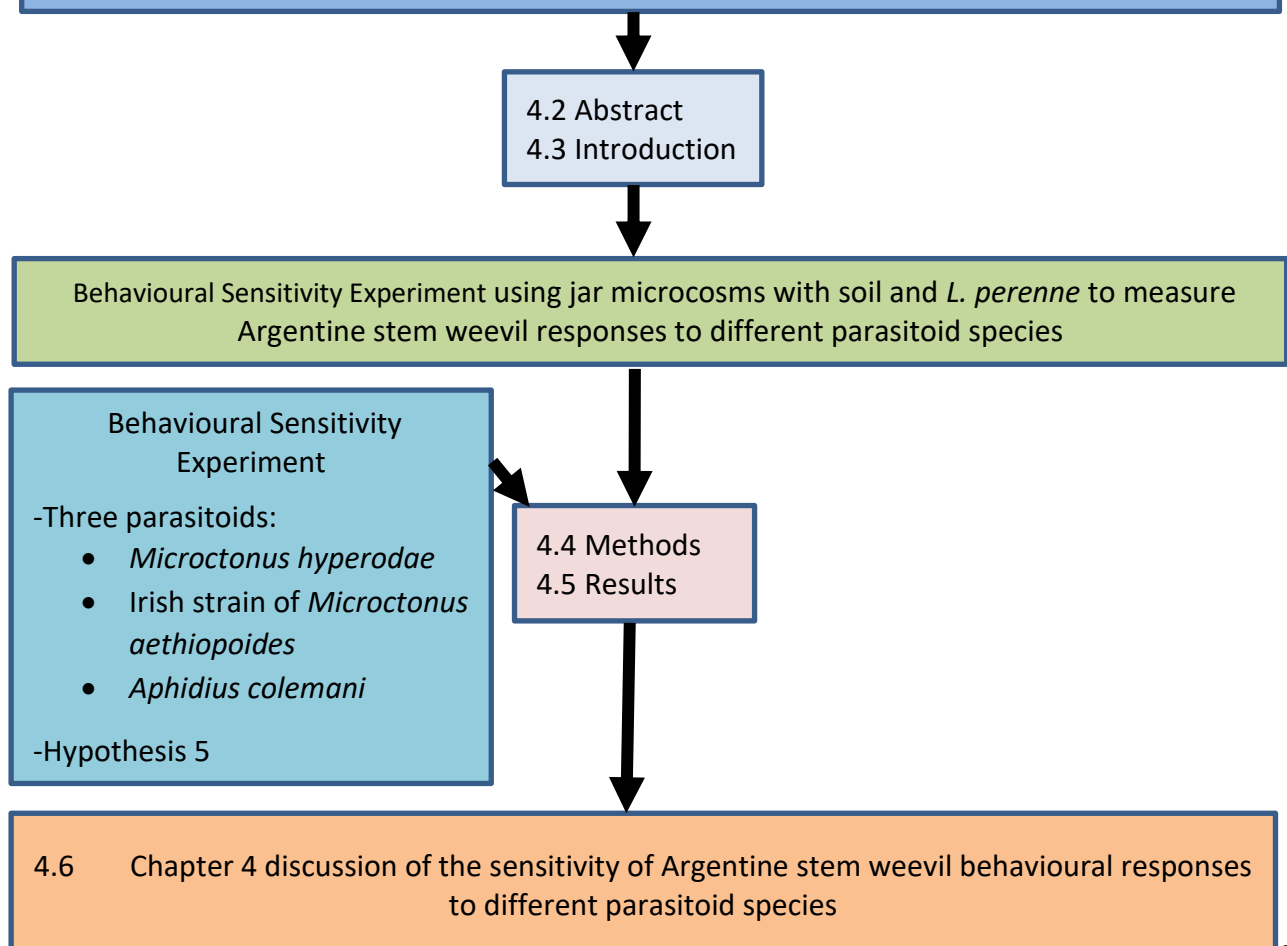
### Sensitivity of Argentine stem weevil behavioural responses to different parasitoid species

#### 4.1 Chapter 4 outline

Chapter 4 investigated differences in Argentine stem weevil (ASW) behavioural responses to three parasitoid species that vary in their extent of interaction with the weevil. This experiment used microcosms with *Lolium perenne* where the frequency of weevil behaviour on entire plants and sections of plants were measured. This work showed that ASW may have specific responses to coevolved parasitoids and was pre-adapted to respond to novel parasitoids in the *Microctonus* genus.

Hypothesis 5: The ASW would have stronger avoidance responses to the parasitoid, *Microctonus hyperodae*, where asymmetric selection has occurred in the weevil's favour compared to the novel interaction with *Microctonus aethiopoides* where the weevil may have pre-adapted responses and there would be no response to an aphid-specific parasitoid where no interaction has occurred.

#### Flow diagram outlining Chapter 4: Sensitivity of Argentine stem weevil behavioural responses to different parasitoid species



## 4.2 Abstract

Classical biological control can create novel host-parasitoid associations where hosts may have different behavioural responses in new parasitoid interactions compared to their coevolved parasitoids. The sensitivity of avoidance behaviours of the pastoral pest, Argentine stem weevil (*Listronotus bonariensis*) (ASW) were compared in the presence of parasitoid species that have different extents of association with the weevil. This was conducted in a laboratory microcosm experiment with a randomised spatial block design. The hypothesis was that the long established host-parasitoid association with asymmetric selection in ASW's favour would elicit the most sensitive avoidance behaviours when exposed to *Microctonus hyperodae*. There would be some behavioural relationship with the ASW in a novel host-parasitoid association with another *Microctonus* parasitoid, *M. aethiopoulos*, due to pre-adaptation of responses and no behavioural sensitivity to a parasitoid (*Aphidius colemani*) that has no prior association with ASW. This investigation did indeed show that the ASW had a significantly higher frequency of crouching behaviour when exposed to *M. hyperodae* compared to in the presence of *M. aethiopoulos* and *A. colemani*. This is the first evidence that the ASW actively uses crouching as a defensive behaviour. *M. aethiopoulos* did elicit an intermittent but significant reduced feeding response from the ASW. This may indicate that there was some extent of pre-adaptation in the ASW's favour with responses regarding *M. aethiopoulos*. These findings show that ASW has specific behavioural responses to its coevolved parasitoid and that ASW may have pre-adapted and generalised defensive responses to parasitoids that have similar traits.

## 4.3 Introduction

Coevolution in insect host-parasitoid associations is a key evolutionary phenomenon in biological control that can involve host behavioural defensive traits (Godfray 1994; Kraaijeveld et al. 1998; Andersen & Mills 2018; Ingerslew & Finke 2018) and is often considered a close reciprocal arms race between two species with mutual selection pressures (Kraaijeveld et al. 1998; Lapchin 2002; Kraaijeveld & Godfray 2009). However, many host-parasitoid interactions involve asymmetric selection, where hosts evolve in response to parasitoids but this selection pressure is not reciprocal with the parasitoid (Brodie & Brodie 1999; Abrams 2000; Lapchin & Guillemaud 2005; Casanovas et al. 2018). This is because either there is prey density compensation (Abrams 2000) or the parasitoid does not have the same adaptive potential (Holt & Hochberg 1997; Hufbauer & Roderick 2005; Goldson et al. 2014; Casanovas et al. 2018). Furthermore, diffuse selection pressure often occurs where insect hosts are simultaneously responding to multiple species such as a guild of natural enemies including parasitoids (Kraaijeveld & Godfray 1999; Steffan & Snyder 2010; Thompson 2014; Ingerslew & Finke 2018). Hosts may have defensive responses that are pre-adapted to be effective when encountering novel natural enemies that have similar traits to those coevolved with the host (Sih et al. 2010; Ehlman et al. 2019) such as predator avoidance behaviour (Sih & Christensen 2001;

Ehlman et al. 2019) and immune encapsulation (Goldson et al. 1992; Koyama & Majerus 2007; Henry et al. 2010; Aya et al. 2019). Host responses may be more effective if the host has a history of diverse and abundant natural enemies but hosts can be more susceptible to novel natural enemies if they have evolved in situations that they can easily identify as dangerous (Ehlman et al. 2019).

The plasticity of host responses is therefore important in host-parasitoid interactions where narrow sensitivity allows the host to optimise defensive traits against particular parasitoid species or genotypes (Kraaijeveld et al. 1998; Lapchin 2002). In contrast, generalist responses enable the host to maintain some fitness when a wide range of natural enemies are encountered (Kraaijeveld et al. 1998; Lapchin 2002). Host response sensitivity is determined by the extent of asymmetric selection (Abrams 2000; Lapchin & Guillemaud 2005), and pre-adaptation (Sih et al. 2010; Ehlman et al. 2019), how applicable each behavioural response is to different natural enemies (Steffan & Snyder 2010; Ingerslew & Finke 2018; Humphreys & Ruxton 2019) and the fitness consequences of the response (Lapchin 2002; Kraaijeveld et al. 2003; Hufbauer & Roderick 2005; Lynch et al. 2016). However, if non-antagonistic animals share the same traits as natural enemies such as species in the same predator guild as natural enemies but that do not attack the host (Fill et al. 2012), then these low risk encounters may trigger a strong defensive behavioural response by the host (Eubanks & Finke 2014). Host responses to a high likelihood of attack are usually risky such as hosts dropping off a plant (Steffan & Snyder 2010; Ingerslew & Finke 2018; Humphreys & Ruxton 2019) and have higher fitness costs compared to responses exhibited when there is a moderate risk of attack (Yager 2012; Ingerslew & Finke 2018) such as moving to a less preferred part of the plant (Costamagna et al. 2013). The magnitude of the behavioural response is governed by the cues used by the host to detect natural enemies. These cues are often general or indirect (Thomas 1950; Tautz & Rostás 2008; Ingerslew & Finke 2018), and are predominantly olfactory (Yager 2012; Hermann & Landis 2017) and to a lesser extent visual (Shaw 1993; Hermann & Landis 2017) and vibratory (Cocroft & Rodríguez 2005; Tautz & Rostás 2008). Understanding the extent of asymmetric selection and pre-adaptation in host-parasitoid associations is essential for classical biological control as these may lead to increased host behavioural resistance, resulting in biological control decline as observed in New Zealand pasture with the Argentine stem weevil (*Listronotus bonariensis* Kuschel) (ASW) (Tomasetto et al. 2017b).

In the case of ASW, it is a major pest in New Zealand pasture that had previously been successfully suppressed by its coevolved parasitoid wasp, *Microctonus hyperodae* Loan in a classical biological control programme (Goldson et al. 1998; Barker & Addison 2006; Ferguson et al. 2019). However, there has been a gradual decline in ASW parasitism rates over the last 20 years in most of New Zealand (Tomasetto et al. 2017b), hypothesised to be due to the contemporary evolution of enhanced avoidance behaviour of the ASW (Goldson et al. 2015; Goldson & Tomasetto 2016) as

indicated by the results of Chapter 3. This suggests that since *M. hyperodae* became established there has been a shift in the host-parasitoid asymmetric selection in the ASW's favour, where the latter has developed counter-resistance under extremely high selection pressure, to which *M. hyperodae* currently cannot adequately respond (Goldson et al. 2014; Tomasetto et al. 2017b; Casanovas et al. 2018). Several contributing factors are probably affecting the asymmetric selection in this host-parasitoid association. Among others, these factors include low diversity of ASW natural enemies (Barker et al. 1989; McNeill et al. 2002; Vink & Kean 2013; Tomasetto et al. 2017b) and a homogeneous pastoral agroecosystem where there are few temporal and spatial refuges (Holt & Hassell 1993; Tomasetto et al. 2017b). These factors severely limit biotic diffuse selection pressure from natural enemies and host plants in a novel environment for the ASW and *M. hyperodae*. Furthermore, there is no close reciprocal coevolutionary arms race between the sexual ASW and *M. hyperodae* (Goldson et al. 2014) which is parthenogenetic in New Zealand (Goldson et al. 1990). The ASW has a much higher adaptive capacity through sexual reproduction (Goldson et al. 2014; Tomasetto et al. 2017b) and is much more genetically diverse than previously thought (Jeanne Jacobs, pers. comm.), which is probably due to multiple unknown introductions. Conversely, *M. hyperodae* has undergone a severe bottleneck when introduced to New Zealand in 1991 (Goldson et al. 1993). *M. hyperodae* can gain new genetic material only by mutation and potentially horizontal gene transfer (Goldson et al. 2014; Tomasetto et al. 2017b). Casanovas et al. (2018) modelled the potential development of resistance in different beetle host-parasitoid associations including the ASW and *M. hyperodae* system using field-based parameter values, and investigated the impact of the asexual or sexually reproduced parasitoids. Their model independently replicated the same pattern of ASW parasitism decline (although development of resistance was slower than in the field) and found that unless parasitoids have at least three times the genetic diversity of the host, then some level of host resistance would develop (Casanovas et al. 2018). The combination of these factors has provided the opportunity for the ASW to develop resistance against a single, very strong selection pressure (Holt & Hochberg 1997) exhibited by *M. hyperodae* in a homogenous agroecosystem with relatively low disturbance (Tomasetto et al. 2017b).

In the New Zealand pastoral agroecosystem, ASW has a novel host-parasitoid association with two strains of *Microctonus aethiopoides* Loan (McNeill et al. 2002; Goldson et al. 2015). The sexual Moroccan strain of *M. aethiopoides* was introduced in 1982 to suppress the lucerne weevil (*Sitona discoideus* Gyllenhal), a Mediterranean pest of lucerne (*Medicago sativa* L.) (Stufkens et al. 1987), of which ASW is a rare host in pasture with up to c. 10 % parasitism in February to December (McNeill et al. 2002). The more widespread parthenogenetic Irish strain of *M. aethiopoides* was introduced in 2006 to suppress the clover root weevil (*Sitona obsoletus* Gmelin) (Gerard et al. 2011), a pest of Palaearctic origin (Bright 1994) that can devastate white clover (*Trifolium repens* L.) in productive

pastures (Gerard et al. 2011). Irish *M. aethiopoides* is thought to primarily parasitise ASW when there is a shortage of available *S. obsoletus* (Goldson et al. 2015) such as in October to November (Gerard et al. 2012). The different extents of selection pressure *Microctonus* parasitoids exhibit on ASW in New Zealand provides an opportunity to investigate how the degree of interaction may affect the sensitivity of behavioural avoidance responses thought to be responsible for the ASW parasitism decline (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b).

This chapter aimed to investigate the sensitivity of ASW's avoidance behaviours to different parasitoid species. The hypothesis was that ASW would have stronger avoidance responses to the parasitoid, *M. hyperodae*, where asymmetric selection has occurred in the weevil's favour compared to the potential pre-adaptation of weevil avoidance behaviour in the novel interaction with *M. aethiopoides*, whereas there would be no weevil response to an aphid-specific parasitoid where no interaction has occurred. This evolutionary investigation will provide insight into the scope of behavioural mechanisms involved in the observed parasitism decline and may inform the future selection of biological control agents.

#### **4.4 Methodology of the Behavioural Sensitivity Experiment where Argentine stem weevil responses to different parasitoid species were measured**

##### **4.4.1 Insect sampling and colony maintenance**

Adult ASW were collected with a suction sampler (inverted leaf blower) using the same protocol as described in Chapter 2 (see Section 2.4.1.1) in January 2018 when large quantities of individuals were being sampled in the Lincoln area for other experiments. Additional ASW and *S. obsoletus* sampling took place during March - May 2018 to gain adult *M. hyperodae* and Irish *M. aethiopoides* to be used in the experiment. The Irish strain of *M. aethiopoides* was used because it shares the same simplified pasture-clover habitat as ASW and *M. hyperodae* and therefore is likely to have the most interaction with ASW. This is in contrast to the Moroccan strain of *M. aethiopoides* which occurs only in the same area as lucerne. A third parasitoid species, *Aphidius colemani* Viereck, which is aphid-specific, was used as a presumed negative control as it is unlikely that any interaction has occurred between this parasitoid and ASW. Bioforce Limited provided *A. colemani* as pupae, which were stored under the same conditions as *Microctonus* pupae as described in Chapter 2 (see Section 2.4.1.1). *A. colemani* is a sexually reproducing species, therefore females were exposed to males in Petri dishes with a damp dental wick for 12 h prior to the females been used in the Behavioural Sensitivity Experiment.

#### **4.4.2 Behavioural Sensitivity Experiment methodology**

This no-choice experimental design comprised a spatial randomised block design with 16 replicates and three parasitoid treatments with Lincoln ASW and nil-endophyte diploid *Lolium perenne* (cv. Grasslands Samson) as the host plant. Parasitoid treatments were Lincoln *M. hyperodae*, Lincoln Irish *M. aethiopoides* and Bioforce *A. colemani*. Behavioural data recording was conducted on two dates over eight blocks on May 18 and eight more blocks on May 22 2018. To standardise and simplify the experiment as much as possible, all ASWs used in the experiment were starved for three days prior to the experiment starting and only female parasitoids that were  $\leq 5$  days old and diploid *L. perenne* were used (refer to Section 2.4.1.2). The experimental jar microcosm set-up and protocol is described in Section 2.5.1.2. On the day of the experiment, 10 starved adult ASWs were added to each replicate 2 h before the onset of night. One parasitoid was added to each replicate in a randomised order 1.5 h after the ASWs and around 30 minutes before data recording began at the onset of night. Behavioural data recording from each replicate were recorded in the same order for four consecutive 35-60 minute intervals during the night using red light from a head torch. ASW behaviours that could be either parasitoid avoidance behaviour or vulnerable behaviour (Gerard 2000; Phillips 2002) on each 50 mm high plant section (high, middle and low) was recorded. These behaviours are defined in Section 2.4.1.4. Insect and plant individuals were used only once.

#### **4.4.3 Statistical analysis of Behavioural Sensitivity Experiment results**

The Behavioural Sensitivity Experiment used the same statistical analysis as described in Section 3.3.4 using the statistical programme Rstudio 1.1.447 (RCoreTeam 2018). Untransformed data was used as transformations were not necessary for analyses and P-values were used to determine significant differences (refer to Section 2.4.1.7). Only crouching, feeding and on-plant ASW behavioural data were reported because the other recorded behaviours had a lack of data or had no significant differences. Behavioural analysis included a generalised linear mixed effects model with penalised quasi-likelihood (GLMMPQL) with a binomial distribution (Venables & Ripley 2002; Bates et al. 2015; Pinheiro et al. 2018), a generalised linear model (GLM) with a binomial distribution (RCoreTeam 2018) and a Log-linear analysis with a Poisson distribution (RCoreTeam 2018). For consistency with the previous chapters, significant differences in all tables are indicated by the same superscript numbers beside values (refer to Section 2.4.1.7).

## 4.5 Results of the Behavioural Sensitivity Experiment comparing Argentine stem weevil responses to three parasitoid species

### 4.5.1 Crouching responses of Argentine stem weevil in the presence of three parasitoid species in the Behavioural Sensitivity Experiment

The GLMMPQL analysis revealed that crouching behaviour on entire plants was significantly higher when ASWs were exposed to *M. hyperodae* (7.4 %) compared to *M. aethiopoides* and *A. colemani* (< 3.5 %,  $p < 0.032$ ) (Figure 4.1). This was particularly the case on the lowest plant section where crouching behaviour was significantly higher when ASWs were exposed to *M. hyperodae* (3.4 %) compared to the *A. colemani* treatment (1.0 %,  $p = 0.036$ ). When ASW crouching behaviour was compared between parasitoid treatments at each time step with the GLM analysis, there was a significantly higher proportion of weevils crouching on the entire plant when exposed to *M. hyperodae* (10.6 %) compared to *M. aethiopoides* (4.4 %,  $p < 0.04$ ) and *A. colemani* (3.3 %,  $p = 0.018$ ) in the last time step only. There were no other significant differences in crouching behaviour between and within the parasitoid treatments in the other time steps.

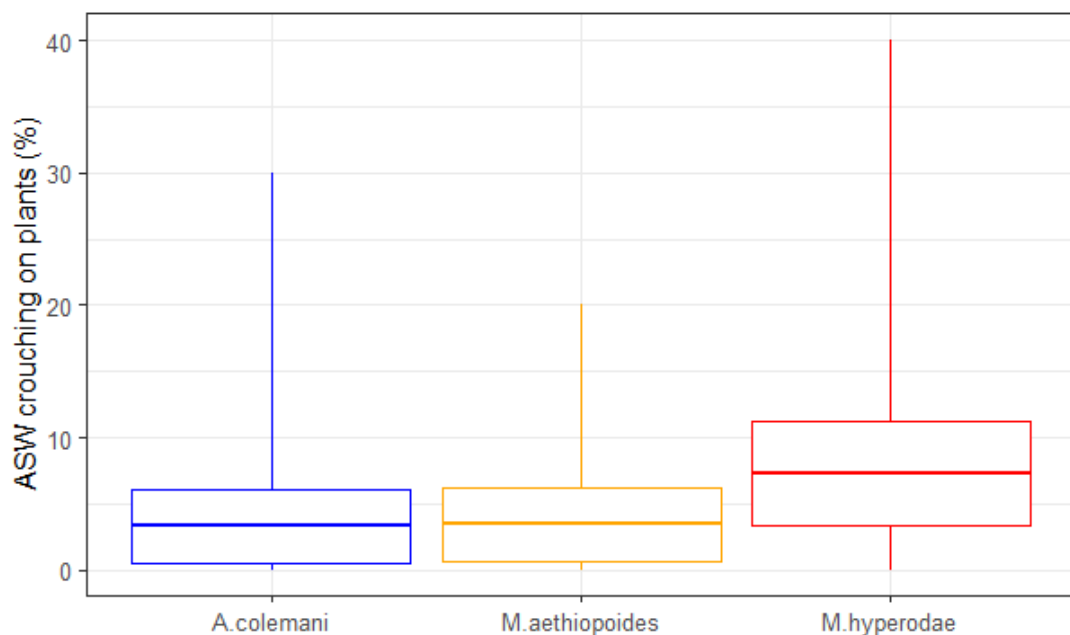


Figure 4.1 Mean percentage of Argentine stem weevils crouching on diploid *Lolium perenne* in the presence of either *Aphidius colemani*, *Microctonus aethiopoides* or *Microctonus hyperodae* parasitoids in the Behavioural Sensitivity Experiment. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a binomial distribution,  $n = 16$ .

#### 4.5.2 Feeding responses of Argentine stem weevil in the presence of three parasitoid species in the Behavioural Sensitivity Experiment

The GLMMPQL analysis indicated that ASW feeding behaviour on entire plants in the presence of *M. hyperodae* (22.5 %) was significantly reduced compared to in the presence of *A. colemani* (35.5 %,  $p < 0.015$ ). There were no significant differences between the proportion of ASWs feeding in the presence of *M. aethiopoides* (30.2 %) and the other parasitoid treatments (Figure 4.2).

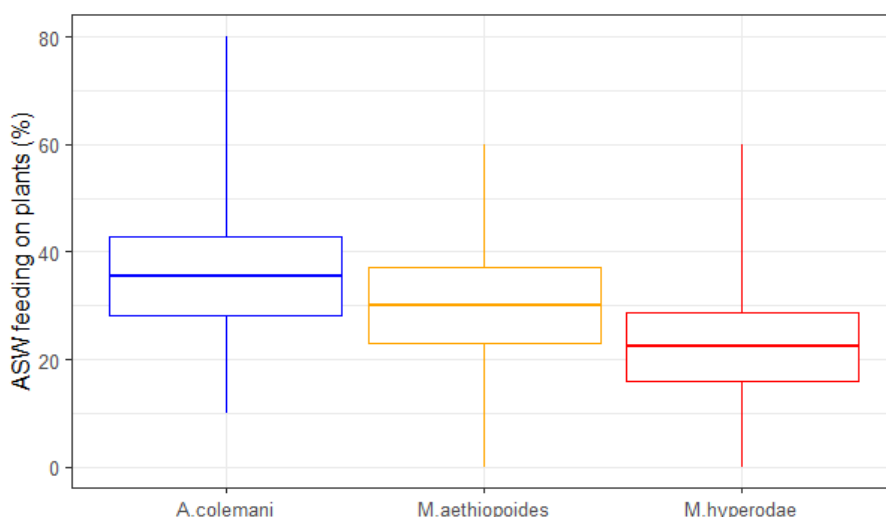


Figure 4.2 Mean percentage of Argentine stem weevils feeding on diploid *Lolium perenne* in the presence of either *Aphidius colemani*, *Microctonus aethiopoides* or *Microctonus hyperodae* parasitoids in the Behavioural Sensitivity Experiment. The boxplot is modified to show the mean (median line), 95 % CL (upper and lower box) and the range (tails) with a binomial distribution,  $n = 16$ .

When ASW feeding behaviour was compared between parasitoid treatments at each time step in the GLM analysis, there were significantly fewer ASWs feeding on the entire plant when exposed to *M. hyperodae* compared to *M. aethiopoides* and *A. colemani* in multiple time steps (Table 4.1). Similarly, there were significantly fewer ASWs feeding when exposed to *M. aethiopoides* ( $\leq 29$  %) compared to *A. colemani* in two intermittent time steps ( $\geq 37$  %,  $p < 0.038$ ). Supporting significant differences for the above findings can be viewed in Table 4.1. The only reoccurring differences in within-plant ASW feeding distributions with the Log-linear analysis was in the first two time steps. There were more ASWs feeding on the lowest plant section when exposed to *M. aethiopoides* ( $\geq 29$  %,  $p < 0.007$ ). When exposed to *A. colemani*, there were fewer ASWs feeding on the middle plant section ( $< 13$  %), compared primarily with the lowest section ( $> 23$  %,  $p < 0.039$ ) but these patterns did not persist in the later time steps.



Table 4.1 Read table horizontally: Observed mean percentage of Argentine stem weevils feeding on plant sections (50 mm height) compared between parasitoid treatments at each time step in the Behavioural Sensitivity Experiment. Feeding behaviour in each plant section within each time step was analysed separately using a GLM (binomial distribution). The same superscript number beside values in the same row indicates significant differences ( $P \leq 0.05$ ,  $n = 16$ ) (refer to Section 2.4.1.7). Colours; entire plant = green; highest plant section = grey; middle plant section = light brown; lowest plant section = dark brown; *Aphidius colemani* = blue; *Microctonus aethiopoides* = orange; *Microctonus hyperodae* = red; parasitoid absent (control) = purple; parasitoid present = black.

Time step 1				
Parasitoid treatments		<i>Aphidius colemani</i>	<i>Microctonus aethiopoides</i>	<i>Microctonus hyperodae</i>
Plant section	Entire plant	40 <sup>1</sup>	29 <sup>1</sup>	30
	Highest	12 <sup>1</sup>	4 <sup>1,2</sup>	9 <sup>2</sup>
	Middle	8	7	5
	Lowest	20	18	16
Time step 2				
Parasitoid treatments		<i>Aphidius colemani</i>	<i>Microctonus aethiopoides</i>	<i>Microctonus hyperodae</i>
Plant section	Entire plant	37 <sup>1</sup>	34 <sup>2</sup>	24 <sup>1,2</sup>
	Highest	15 <sup>1,2</sup>	4 <sup>1</sup>	8 <sup>2</sup>
	Middle	7	9	6
	Lowest	16	21 <sup>1</sup>	11 <sup>1</sup>
Time step 3				
Parasitoid treatments		<i>Aphidius colemani</i>	<i>Microctonus aethiopoides</i>	<i>Microctonus hyperodae</i>
Plant section	Entire plant	37 <sup>1,2</sup>	23 <sup>1</sup>	20 <sup>2</sup>
	Highest	11 <sup>1</sup>	5 <sup>1</sup>	9
	Middle	11 <sup>1</sup>	7	3 <sup>1</sup>
	Lowest	15 <sup>1</sup>	11	8 <sup>1</sup>
Time step 4				
Parasitoid treatments		<i>Aphidius colemani</i>	<i>Microctonus aethiopoides</i>	<i>Microctonus hyperodae</i>
Plant section	Entire plant	28 <sup>1</sup>	35 <sup>2</sup>	16 <sup>1,2</sup>
	Highest	6	11 <sup>1</sup>	4 <sup>1</sup>
	Middle	9 <sup>1</sup>	11 <sup>2</sup>	4 <sup>1,2</sup>
	Lowest	13	13	8

#### 4.5.3 Argentine stem weevil responses 'on-plant' in the presence of three parasitoid species in the Behavioural Sensitivity Experiment

There were no significant on-plant results from the GLMMPQL or GLM analysis. However, the Log-linear analysis indicated that within-parasitoid treatments there were some limited ASW within-plant distribution patterns. In time step 1, ASWs in all parasitoid treatments had a higher proportion of ASWs on the lowest plant section, but this distribution only persisted for multiple time steps in the *M. aethiopoides* treatment ( $> 33\%$ ,  $p < 0.043$ ). There were no other significant differences within parasitoid treatments.

## 4.6 Chapter 4 discussion of the sensitivity of Argentine stem weevil behavioural responses to different parasitoid species

### 4.6.1 Main findings

This work investigated the sensitivity of avoidance behaviours by Argentine stem weevil (ASW) towards different parasitoid species where the weevil either had undergone presumed asymmetric selection pressure, had pre-adapted avoidance behaviours or no interaction with the parasitoid. As hypothesised, the extent of the ASW's behavioural responses followed a gradient along the frequency of interaction with the parasitoid species with avoidance responses most sensitive in the presence of *M. hyperodae*, illustrated by the observed increased crouching response (see Section 4.5.1; Fig. 4.1) and the reduced feeding response (Fig. 4.2; Table 4.1). Although subtle, this is the first evidence that the crouching behaviour may actively be used as a defensive trait and is further discussed in Section 4.6.2.

ASW's sensitive response to *M. hyperodae* is presumed to be due to the well-established host-parasitoid association (Ferguson et al. 2019) where several potential avoidance behaviours (Barratt et al. 1996; Gerard 2000; Phillips 2002) presumably developed under intense *M. hyperodae*-derived asymmetric selection pressure (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b). The ASW also showed some avoidance responses to the Irish strain of *M. aethiopoides* in the form of inconsistent reduced feeding (see Section 4.5.2; Fig 4.2; Table 4.1) and within-plant distribution (see Sections 4.5.2-3). These findings suggest that the ASW does attempt to respond to *M. aethiopoides* to some extent and may indicate pre-adapted avoidance behaviours, which is discussed further in Section 4.6.3. This could occur because ASW's avoidance behaviours have presumably become more sensitive in respect to the asymmetric selection pressure caused by *M. hyperodae* (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b) and both parasitoids belong to the same genus and have similar characteristics (Shaw 1993; Barratt et al. 1996; Phillips 2002). Unlike when exposed to *Microctonus* spp., ASW had little response to the aphid-specific *A. colemani* where there is no host-parasitoid association. This further suggests that ASW's behavioural response sensitivity to parasitoids is dependent on the extent of asymmetric selection caused by reoccurring interactions and that ASW can detect cues from different *Microctonus* parasitoids to varying degrees.

#### 4.6.2 Crouching behaviour

The increased crouching response by ASW in the presence of *M. hyperodae* on the host plant is the first evidence of the weevil using this behaviour for defence and is a specific response to *M. hyperodae* (see Section 4.5.1; Fig 4.1). This crouching response reflects the relatively high level of asymmetric selection that the ASW has probably undergone in response to *M. hyperodae* and was first hypothesised by Phillips (2002) where *M. hyperodae* did not successfully oviposit in crouching ASWs in Petri dishes. Similarly, *M. hyperodae* has rarely attempted to oviposit in motionless individuals of a different weevil species in the same genus, *Listronotus oregonensis* LeConte (Cournoyer & Boivin 2005). Similar motionless defensive behaviour against natural enemies has been used widely by other arthropods such as caterpillars (Freitas & Oliveira 1996; Bächtold et al. 2012), ants (Bengston & Dornhaus 2015), sawfly larvae (Boevé & Müller 2005), aphids (Gottlieb et al. 2017; Meresman et al. 2017) and spiders (Garcia & Styrskt 2013). Although this crouching behaviour appears to be a highly effective ASW defensive behaviour, this response has not increased in frequency in previous experiments when exposed to *M. hyperodae* (Gerard 2000; Phillips 2002). This suggests that an increased crouching response is difficult to detect and does not often occur (Fig. 4.1). For instance, when analysed by time step, this response occurred only in the last time step in the presence of *M. hyperodae* (refer to Section 4.5.1). Although increased crouching behaviour does not require the ASW to move to a different section or off the host plant, it may be infrequent because of unknown associated costs such as less feeding. The current finding may be an indication that ASW is adapting its behavioural response to the New Zealand *M. hyperodae* host-parasitoid association, as behavioural shifts generally require very small genetic changes compared to a physiological response (Lefèvre et al. 2012; Lynch et al. 2016).

#### 4.6.3 Behavioural responses compared between *Microctonus* parasitoids

Unlike the specific *M. hyperodae* crouching response discussed above, ASW responded with reduced feeding to both *M. hyperodae* and the Irish strain of *M. aethiopoides* to different extents (Section 4.5.2; Fig. 4.2), reflecting the different evolutionary history between the weevil and these parasitoids. ASW's reduced feeding response was most prevalent with *M. hyperodae* (Fig. 4.2) which is supported by previous work (see Chapter 2-3) (Barratt et al. 1996; Gerard 2000) and reflects the presumed coevolved host-parasitoid association prior to the introduction of *M. hyperodae* into New Zealand. ASW also significantly responded with an intermittent reduced feeding to *M. aethiopoides* (Table 4.1) which is a novel host-parasitoid association that has occurred at low levels since 2006 with the Irish strain of *M. aethiopoides* (Gerard et al. 2012) used in the experiment and prior to 1995 for the Moroccan strain of *M. aethiopoides* (McNeill et al. 2002). ASW exposed to *M. aethiopoides* also had within-plant distribution responses that did not occur with the *M. hyperodae* treatment. These included having more ASW individuals on the lowest plant section and feeding (refer to

Sections 4.5.2-3). These behavioural findings indicate that ASW may have pre-adapted behavioural responses to *M. aethiopoides*. This is probable as *M. aethiopoides* has similar traits to *M. hyperodae* such as oviposition behaviour (Barratt et al. 1996; Phillips 2002) and morphology (Shaw 1993). Furthermore, similar ASW responses likely reflect the same unknown cues which the weevil uses to detect the parasitoids (Sih et al. 2010). Pre-adaptation is common in novel natural enemies (García-Robledo & Horvitz 2011; Jeffs & Lewis 2013; Colares et al. 2015) but there is substantially less literature on hosts having defensive pre-adaptation to novel natural enemies (Sih et al. 2010; Ehlman et al. 2019).

#### **4.6.4 Limitations**

This work investigated whether ASW behavioural responses differed when exposed to parasitoids that had different degrees of interaction with the ASW. This was possible because the evolutionary history was known for the host-parasitoid associations. However, parasitoids only from the *Microctonus* genus are known to attack ASW to varying extents in New Zealand, which prevented weevil response comparisons with parasitoids from other genera that can also parasitise the weevil. A third *Microctonus* parasitoid, the New Zealand endemic *Microctonus zealandicus* Shaw, which seldom attacks ASW (Stephen Goldson, pers. com.) may have been useful as a treatment reflecting a very low level of interaction. However, logistically this was not possible given the rarity of this parasitoid species as well as there already being a similar treatment that reflected no interaction with the ASW in the form of *A. colemani*.

#### **4.6.5 Future research and conclusions**

Future research should aim to identify what cues the weevil uses to detect *M. hyperodae* which would allow the investigation of manipulating the cues, such as through artificial technology, to reduce ASW damage and may be one angle pursued to mitigate the impact of the ASW parasitism decline. Furthermore, the methodology and technology produced in this potential work will be applicable to other biological control systems. ASW behavioural responses and the cues that ASW uses to detect parasitoids may vary depending on the ASW populations and parasitoid 'ecotype' or strain within New Zealand, given that ASW certainly differ in the strength of behavioural responses to *M. hyperodae* such as weevils from the Waikato compared to Otago (see Chapter 3). This is an interesting area of investigation as literature is often from the parasitoid's perspective (Vinson 1976; Godfray 1994; Cournoyer & Boivin 2005; González et al. 2018) rather than the host's (Hermann & Thaler 2014). The detection of parasitoid cues strongly relates to the genetic diversity between ASW and *M. hyperodae* populations and their recent evolutionary history. Understanding how pests detect and then respond to different natural enemies as well as how these responses change over time is essential for continued pest management.

## Chapter 5

### Thesis discussion

#### 5.1 Maintaining successful biological control

Successful classical biological control is worth billions of dollars in pest management annually (Losey & Vaughan 2006) with benefit:cost ratios ranging from 5:1 to >1000:1 (Naranjo et al. 2015). For example the suppression of clover root weevil (*Sitona obsoletus* Gmelin) by *Microctonus aethiopoides* Loan from Ireland in New Zealand pasture has over 80 % parasitism and saves \$156.5 million p.a. (Hardwick et al. 2016). However, most classical biological control introductions fail due to a lack of comprehensive understanding of how pests and biological control agents associate in exotic ecological communities and environments (Stiling 1993). Approximately only 10 % of classical biological control introductions lead to successful pest management (Greathead & Greathead 1992; Gurr et al. 2000; Cock et al. 2016). Considering how vital biological control is for agriculture around the world (Losey & Vaughan 2006; Naranjo et al. 2015), there would be devastating social and economic consequences if a proportion of that 10 % of successful classical biological control were to begin to fail due to the development of pest resistance in the prey or host (Goldson et al. 2014).

Maintaining current biological control programmes in New Zealand's pastures is essential as this is the largest agricultural sector in the country and has an estimated \$19.6 billion p.a. production value (Anon. 2016). This pastoral productivity relies on the biological control of key pests of *Lolium* ryegrass and clover species, such as the Argentine stem weevil (*Listronotus bonariensis* Kuschel) (ASW) (Ferguson et al. 2019). Successful classical biological control programmes are generally assumed to be self-perpetuating and evolutionarily stable (Holt & Hochberg 1997), resulting in limited continued monitoring of and research into those systems. However, contemporary evolution of pest resistance in biological control can occur (Hufbauer & Roderick 2005), although rarely with insects (Pascoal et al. 2014; Mills 2017; Tomasetto et al. 2017b). Despite this, contemporary evolution of pest resistance is currently the most parsimonious explanation for the ASW biological control decline (Goldson et al. 2014; Tomasetto et al. 2017b). The driving mechanism of this decline in biological control was hypothesised to be enhanced avoidance behaviour to the asexual *Microctonus hyperodae* Loan where intense asymmetric selection pressure (defined in Section 4.3) acted upon existing ASW behavioural responses in the dominant pasture types (Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b) (refer to Chapter 2-3). This thesis investigated whether there is any behavioural evidence for the above hypothesis by establishing whether avoidance behaviour by the ASW could be detected and whether this behaviour differed between plant types (see Chapter 2) and between regional populations (see Chapter 3) that reflected the current parasitism rates on those

hosts and in those locations. Furthermore, ASW behavioural responses were compared between different parasitoid species to determine the parasitoid specificity of weevil behavioural responses (see Chapter 4).

## 5.2 Plant-mediated responses

This work determined that ASW avoidance behavioural responses to *M. hyperodae* differed depending on the *Lolium* plant type, which reflected recent parasitism rates observed on these host plants (Goldson et al. 2015; Goldson & Tomasetto 2016). The strongest response was observed on the diploid hybrid (diploid *L. perenne* x diploid *L. multiflorum*), with prompt plant abandonment, while there was a similar but delayed response on diploid *Lolium perenne* L. and no such response on tetraploid *L. multiflorum* Lam. A reduced feeding response occurred on all host plants but was strongest on the hybrid (see Chapter 2). Notably, the plant abandonment findings reflect the considerable drop in recent parasitism rates on the diploid hybrid and *L. perenne* compared to the 1990s but not on tetraploid *L. multiflorum* (Goldson & Tomasetto 2016). This supports the hypothesis that the ASW developed enhanced avoidance behaviours on the dominant pastures in the field (Goldson et al. 2015), particularly on the hybrid whereas the behavioural evidence is less convincing for *L. perenne*, these aspects are further discussed in Chapter 2. The results imply that plant traits and ploidy have a major role in affecting ASW behavioural responses to *M. hyperodae* as previously hypothesised (Goldson et al. 2015; Popay et al. 2017; Tomasetto et al. 2017a; Tomasetto et al. 2017b). Plant traits that might be important facilitators of ASW behaviour include the size of plant cells (Charlton & Stewart 2000), plant architecture (Tomasetto et al. 2017a), fibre content (cellulose and lignin) (Goldson 1982; Barker 1989) and secondary metabolites (Dyer et al. 2004; Smilanich et al. 2009; Kelly & Bowers 2018). Understanding what specific plant traits make the ASW and other pests vulnerable to parasitism and how to implement them can be applied to plant breeding to produce new *Lolium* cultivars that affect host feeding behaviour, provide stronger cues to improve parasitoid searching efficacy and reduce host immunological responses (Stenberg 2017; Kelly & Bowers 2018). Many of the traits involved in indirect plant defences have been lost during the domestication process or are ineffective in novel associations. However, these traits can now be reintroduced using wild relatives and manipulated in plant breeding (Stenberg et al. 2015; Stenberg 2017).

## 5.3 Regional Argentine stem weevil behavioural responses and the effect of climate

Although the ASW parasitism rates have a similar pattern of decline in most of New Zealand and this began to occur approximately 7 years (c. 14 weevil generations) after *M. hyperodae* was first released (Tomasetto et al. 2017b), the extent of decline varies substantially between regions (Goldson et al. unpublished data). Chapter 3 investigated whether the extent of current ASW

behavioural responses reflected the parasitism history and recent parasitism rates of different regional populations as evidence of potential contemporary evolution. ASW avoidance responses did differ substantially between regional populations where Waikato (Ruakura) weevils had the strongest reduced feeding and plant abandonment responses to *M. hyperodae*, reflecting the 81 % decline in field parasitism rates (Goldson et al. unpublished data). ASWs from populations with a moderate parasitism decline of 40 % (Goldson et al. unpublished data) had moderate to weak delayed feeding responses whereas ASW populations that have always had very low parasitism (Ferguson 1997) (Goldson et al. unpublished data) had a very weak delayed feeding response (Invermay weevils) or no response (Waihi weevils) to *M. hyperodae*. These results are the first evidence supporting the hypothesis that the ASW behaviour is the main mechanism for the parasitism decline (see Chapter 3). Furthermore, this may be the first evidence of contemporary evolution of pest resistance in the form of a behavioural mechanism in a once-successful insect-insect classical biological control programme (Goldson et al. 2014; Goldson et al. 2015; Goldson & Tomasetto 2016; Tomasetto et al. 2017b).

Further investigation is required to understand why there were strong ASW behavioural responses and a decline in weevil parasitism in some regions but not others. However, there is preliminary evidence by Goldson et al. (unpublished) which suggests that the regional differences in ASW behaviour and parasitism decline are due to the extent of *M. hyperodae* activity within each region, as parasitism was considered to be the main selection pressure instigating the contemporary evolution. This *M. hyperodae* activity is driven by the number of degree-days (DD) above an activity threshold of 10 °C (Goldson & Proffitt 1990) during January-June 1994-1999 when peak parasitism was occurring (Goldson et al. 1998; Barker & Addison 2006). There was a strong linear relationship between proportion of parasitism decline and the number of DD in which adult *M. hyperodae* was active. The Waikato region, represented by Ruakura (large parasitism decline), had a third more DD than the region of Canterbury, represented by Lincoln (moderate parasitism decline) and two thirds more DD than the region of Otago, represented by Invermay (no parasitism decline). Similarly, Canterbury (Lincoln) had a third more DD than Otago (Lincoln) (Goldson et al. unpublished data). This is because temperature determines the number of *M. hyperodae* generations, the amount of time adult *M. hyperodae* are active, the number of interactions that the ASW has with *M. hyperodae* and how many adults emerge from post-diapause host weevils (Goldson & Proffitt 1990; Barlow et al. 1993; Goldson et al. 1995; Goldson et al. 1998).

This suggests that *M. hyperodae* activity is the main selection pressure driving the contemporary evolution of enhanced behavioural avoidance and that climate is probably responsible for low parasitism rates in some regions such as Otago (Ferguson 1997). This is somewhat at odds with previous findings that did not find any effect of climate on the parasitism decline (Tomasetto et al. 2017b; Tomasetto et al. 2018b). However, these studies were analysed with pooled data from many

locations that were at times data deficient (Tomasetto et al. 2017b) or investigated the slope of decline (Tomasetto et al. 2018b) rather than the difference between current ASW parasitism rates and those at peak parasitism in key localities as used by Goldson et al. (unpublished data). Furthermore, mean temperatures throughout each year were used in previous research (Tomasetto et al. 2017b; Tomasetto et al. 2018b) rather than the number of DD when most *M. hyperodae* adults are active. In addition, Tomasetto et al. (2018b) found that there was a 1-year time lag in Lincoln compared to Ruakura in the development of resistance although *M. hyperodae* adults were released at the same time which supports the findings of Goldson et al. (unpublished). Although the preliminary investigation by Goldson et al. (unpublished) suggests that climate plays an important indirect role in the development of the ASW's contemporary evolution, it is unlikely that climate change has been an important factor. This is because of the relatively short temporal scale of ASW exposure to *M. hyperodae* prior to the parasitism decline. However, climate change may further enhance the contemporary evolution of ASW resistance in the future as increased temperature will result in higher levels of insect activity (Gerard et al. 2013). The underlying genetic mechanism(s) is currently unknown for enhanced avoidance behaviour. The ongoing work to identify the underlying genetic mechanism(s) is crucial to understanding the genetic nature of the ASW resistance and would be the first credible example in the literature of a previously successful insect host-parasitoid biological control programme failing due to contemporary evolution.

#### **5.4 Argentine stem weevil behavioural responses to different parasitoid species**

The differing sensitivity of the ASW's behavioural responses to different parasitoid species reflects the phylogenetic origin of the parasitoids and the history of association between the ASW and parasitoid species. The ASW had a specific crouching response to *M. hyperodae* that that may have originated in South America and asymmetric selection has acted on due to high parasitism pressure (refer to Chapter 4). The ASW also had a reduced feeding response to *M. aethiopoides* and is probably an illustration of trait pre-adaptation (refer to Chapter 4) as this commonly facilitates novel host-parasitoid associations in biological control (Price et al. 1980; Poulin 2011; Abram et al. 2019). This may also be an example of a generalised response to *Microctonus* parasitoids as this genus is diverse in South America (Craig Phillips, pers. comm.). These findings have implications for future biological control of the ASW as stronger host-parasitoid association may occur with particular ASW and *M. aethiopoides* populations. However, understanding the genetic diversity and adaptive capability of both these species is ongoing (Jeanne Jacobs; Peter Dearden, pers. comm.) and is still in its infancy. Furthermore, there may be potential to introduce additional *M. aethiopoides* (and *M. hyperodae*) genotypes or strains to reduce the impact of the reduced parasitism by the current *M. hyperodae* genotypes.



## 5.5 Implications

The current situation of the ASW's biological control decline and probable contemporary evolution emphasises the imminent need to maintain successful biological control programmes through long-term monitoring and understanding the capability of pests to develop resistance to biological control agents. This involves filling the knowledge gaps of pest and agent genetic diversity, available variation in pest susceptibility (variation for selection to act upon) and natural enemy diversity (potential for diffuse selection (defined in Section 4.3)) (Tomasetto et al. 2017b). Overcoming the avoidance behaviour by the ASW as shown in the previous chapters is required to restore biological control efficacy. Achieving this may involve increasing *M. hyperodae* adaptive capacity and the diversity of ASW natural enemies. Introducing new strains of *M. hyperodae* or related parasitoids may tip the asymmetric selection in the parasitoid's favour and create diffuse selection to prevent further contemporary evolution of resistance by the ASW. There is also the possibility of making *M. hyperodae* reproduce sexually (Peter dearden, pers. comm.) which would substantially improve the parasitoid's adaptive potential by creating a conventional coevolutionary arms race between the ASW and *M. hyperodae* where the parasitoid can respond to the ASWs' advances in behavioural avoidance and other potential defences (Casanovas et al. 2018). This is possible because sexual *M. hyperodae* strains exist but not in New Zealand as four males emerged in quarantine (Goldson et al. 1990). Furthermore, the activation and suppression of genes related to sexual reproduction in *Microctonus* parasitoids is under investigation (Peter Dearden, pers. comm.). The idea is that this genetic manipulation would allow *M. hyperodae* populations to counter-coevolve with the ASW's adaptation. However, this requires a detailed understanding of the genetic diversity of *M. hyperodae*, which is currently unknown but is under investigation by collaboration between the Bio-Protection Research Centre, AgResearch and the University of Otago.

A major implication that this work has highlighted is the threat of resistance developing in other successful biological control programmes such as with the *S. obsoletus*, that causes \$156.5 million p.a. of damage in New Zealand after biological control by the Irish strain of *M. aethiopoides* (Hardwick et al. 2016). This is a very similar classical biological control system to *M. hyperodae* (Goldson et al. 2014) where the Irish strain of *M. aethiopoides* has been extremely successful since its introduction in 2006 (Gerard et al. 2010; Gerard et al. 2012; Ferguson et al. 2019) creating a strong and consistent selection pressure (Goldson et al. 2014). However, unlike *Lolium* pasture, clover has only the Irish strain of *M. aethiopoides* and limited cultural control to suppress *S. obsoletus* (Bell et al. 2004; Ferguson et al. 2019). It took at least 7 years (c. 14 generations) before parasitism by *M. hyperodae* started to decline (Tomasetto et al. 2017b). This suggests that parasitism decline or other indications of resistance could be imminent with *S. obsoletus* if contemporary evolution is occurring. Considerable resources need to be urgently committed to understanding the genetic

diversity and other factors affecting potential resistance of *S. obsoletus* and other successful biological control programmes as the loss of biological control could severely damage New Zealand's improved pastures. It is probable that contemporary evolution of insect pest resistance is occurring undetected elsewhere in the world due to a lack of long term monitoring. Also, knowing the adaptive capacity of pests and natural enemies is important as is identifying knowledge gaps in situations where resistance may develop. This threat to successful and future biological control requires urgent attention in which this thesis and the ongoing investigation of the ASW's parasitism decline will provide crucial insight.

## 5.6 Conclusions

The PhD explored the behavioural responses of the ASW when exposed to *M. hyperodae* that are probably responsible for the observed parasitism decline and collapse in biological control due to the potential contemporary evolution of enhanced avoidance behaviours. This may be the first published example of contemporary evolution of pest resistance in an insect host-parasitoid biological control system if future work can identify the underlying genetic mechanisms. The investigations here revealed behavioural mechanisms that explain previous observations of ASW parasitism rates differing depending on the *Lolium* host plant. Also that the strength of the avoidance behaviours differed substantially between ASW populations from different regions, reflecting the parasitism history and intensity of each region (Goldson et al. unpublished). The immense behavioural variation between ASW populations from the Waikato region compared to those from Canterbury and Otago suggests these populations have far greater genetic diversity than previously thought (Williams et al. 1994) and is supported by preliminary genome-by-sequencing results (Jeanne Jacobs pers. comm.). These findings emphasise the major role that non-consumptive effects (defined in Section 2.3) in the form of behavioural responses have in biological control, which should be comprehensively studied during pre-release assessments and post-release monitoring. Avoidance behaviours and the cues used to detect pests' natural enemies and hosts need to be exploited to improve future plant protection by enhancing consumptive and non-consumptive effects e.g. (Lamy et al. 2017; Wäckers et al. 2017; Bouagga et al. 2018; Ingerslew & Finke 2018). This work highlighted the importance of maintaining biological control rather than let it breakdown (Hufbauer & Roderick 2005; Mills 2017; Tomasetto et al. 2017b) and that there is a great need to pre-emptively identify the capability of key pests to develop resistance to biological control agents (Tomasetto et al. 2017b). Furthermore, effective methods to restore biological control efficacy that are economically viable need to be developed. Maintaining successful biological control is essential for the food security of the increasing global human population (Naranjo et al. 2015; Hajek & Eilenberg 2018; Pretty et al. 2018) and understanding insect behavioural interactions of economic importance will contribute significantly to this.

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