WESTERN SYDNEY
UNIVERSITY

Hawkesbury Institute
for the Environment

How the soil environment affects root feeding scarabs with particular emphasis on the canegrub

By

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BSc (Hons)

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Hawkesbury Institute for the Environment

Western Sydney University

Dec 2016

Declaration of Authenticity

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Acknowledgements

This work was undertaken as part of a PhD research scholarship funded by Sugar Research Australia Limited (SRA) and the Hawkesbury Institute for the Environment (HIE), Western Sydney University. Thank you to all the students and staff at HIE and at SRA for making me feel so welcome and for all the support and advice throughout my PhD.

My PhD supervisor, Scott Johnson, deserves the greatest thanks for his advice, support and guidance throughout my PhD, for which I am extremely grateful. My sincerest thanks go to my secondary supervisor Jeff Powell who has also provided invaluable guidance and advice; I would also like to give a special thank you to the fungal ecology lab group at HIE for welcoming me as one of their own. I also thank Peter Allsopp and Nader Sallam for their invaluable feedback and support, and for providing crucial logistical support throughout this project.

I would like to thank Kirk Barnett, Andrew Gherlenda, Tim Sutton, James Ryalls, Ashley Montagu, Jessica Rigg, Sarah Facey and Sachin Chavan for their support and friendship. My partner Ryan Lipman deserves the utmost gratitude for his steadfast understanding and encouragement.

I would lastly like to thank my supervisor at the University of St Andrews, Jane Wishart, who first sparked my interest in ecological research.

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Abstract

The interactions between insect herbivores and their host plants have fascinated scientists for generations. There is a vast diversity of these insects, which have a variety of different feeding strategies and diet breadth. Many have become significant pests of managed ecosystems, such as forest and crop production systems. The majority of research has focussed on aboveground insect defoliators rather than root feeding insects. This is surprising as root feeding insects are some of the most economically damaging and difficult to control. Several scarab species (Coleoptera: Scarabaeidae) are among the most significant insect pests to agriculture, particularly during the root feeding larval stages.

The soil environment in which scarab larvae feed and develop involves several abiotic and biotic factors which can be highly influential in shaping the relationship between these root feeders and their host plants. Soil nutrients including nitrogen (N), phosphorus (P) and potassium (K), along with soil moisture, are important to plant growth and quality, and thereby are important to the belowground insects which feed on their roots. Silicon (Si) is known to impact plant growth, but can also act as a plant defence mechanism against insect herbivores, although this remains untested in root feeding insects. Biotic soil influences include arbuscular mycorrhizal (AM) fungi, which associate with the majority of land plants, and can alter plant quality and defences, while negatively impacting root herbivore performance, although the mechanisms remain unclear. This work addresses how these different factors within the soil environment affect root feeding scarab larvae. This is initially investigated at the community level, then subsequently using the grass crop plant sugarcane, *Saccharum* species hybrids, and the canegrub, *Dermolepida albohirtum*.

Recent literature on the ecology of scarab larval pests within Australasia and the effects of different soil factors on scarab larvae are synthesised in chapters one and two, respectively. These chapters highlight the paucity of knowledge surrounding the ecology of root feeding scarab larvae and the importance of factors such as soil nutrients (N,P,K and Si) and microbial communities (AM fungi) on plant-herbivore interactions belowground.

Chapter three investigates how fertilisation and irrigation practices, which alter soil nutrients (N,P,K) and moisture, impact host plant communities, scarab larval communities and their natural enemies (entomopathogenic nematodes). Scarab larval communities were positively affected by fertilisation, increasing their abundance by 52%. While irrigation did not impact scarab communities, there was an increase in entomopathogenic nematode presence by 78%, suggesting scarab populations were suppressed by their natural enemies.

Chapters four and five focus on the effects of Si on sugarcane and the canegrub. Specifically, chapter four investigates previous observations of positive responses by root feeding insects to phenolic compounds and a suggested trade-off between carbon and Si based plant defences. Canegrub performance positively correlated with root phenolics, while correlating negatively with root Si. A negative correlation between phenolics and Si suggested positive responses by root feeding insects to high phenolic concentrations may be a response to low Si concentrations. This was the first example of plant Si negatively impacting a root feeding insect. Chapter five looks at the impacts of silicon on sugarcane and canegrub performance under ambient and elevated atmospheric carbon dioxide concentrations (eCO_2). Elevated CO_2 decreased sugarcane root nutritional value while increasing canegrub growth rate and root consumption by 116% and 57%, respectively. Silicon decreased performance of the canegrub under both ambient and eCO_2, highlighting the potential role of Si in future pest management strategies.

Chapter six investigated the impacts of two AM fungal communities on sugarcane and canegrub performance within different soil types, known to have different concentrations of Si. Both AM communities had the same effect on sugarcane and canegrub responses. Arbuscular mycorrhizal fungi promoted sugarcane growth and photosynthesis by 81% and 39%, respectively, while also increasing root Si concentrations, but only in soil with low Si concentrations. Similarly, AM fungi decreased canegrub performance, but only within the low Si soil. This suggested that AM fungi promote Si accumulation within Si depleted soil environments, negatively impacting canegrub performance. Chapter seven concluded this research, building on the observations from chapter five, by directly testing the effects of Si and AM

fungi on plant growth alongside their impacts on canegrub performance, root consumption and immune function within two different sugarcane varieties. Si decreased canegrub performance and consumption, while AM fungi decreased canegrub performance when Si was not applied and only on one plant variety. AM fungi increased canegrub immune function by 62%, a response that was not explainable by any measured plant trait. Canegrub immune function negatively correlated with canegrub mass, suggesting a trade-off between growth and immunity.

The results of this PhD research contribute to the understanding of (a) the impacts of management practices altering soil factors such as N,P,K and moisture on belowground pest communities; (b) the potential trade-off between carbon and Si in plants and the impacts of Si on root feeding insects; (c) the importance of plant Si defences against root feeding insects under climate change; (d) the interactions between AM fungi, host plants and their root feeding insects and the importance of soil Si availability to this relationship; (e) the role of AM fungi and Si on the growth and immunity of root feeding insects.

This research has shown how the impacts of common agricultural management practices can potentially exacerbate scarab pest problems. This work has demonstrated that Si and AM fungi can promote plant growth and reduce canegrub performance, although the effects of AM fungi can be context dependent, specifically on soil Si availability and plant variety. In terms of applied implications, this suggests that future pest management strategies should look to exploit plant Si defences through targeted application of Si fertiliser in Si depleted soils. Practices that encourage native AM communities also hold potential in reducing soil pest persistence, though mechanisms including increased Si uptake, or perhaps even through direct interactions with soil insects.

Preface

This thesis comprises original research conducted by myself with guidance from my supervisors, Scott N. Johnson (primary supervisor) and Jeff R. Powell, as well as external advice from Peter G. Allsopp and Nader Sallam based at Sugar Research Australia Limited. I conceptualised the research project together with my supervisory panel. I have conducted all the data collection, analyses, interpretation and illustrations present in this thesis. I have written this thesis and all publications therein with guidance from the supervisory panel.

This thesis is a standard thesis and not a thesis by publication. However, it consists of a review chapter and five stand-alone experimental chapters that have been written in a format appropriate for peer-review journals. Chapters have either been published (chapters 2 to 5), are under review (chapter 6) or are to be submitted for publications (chapter 7) in peer-reviewed journals. Each research chapter (chapters 3 to 7) is self-contained and thus some repetition will be present.

Chapter 1: General introduction

1.1 Plant–herbivore interactions

The interactions of insect herbivores with plants has fascinated scientists for generations, something that is unsurprising considering at least one–half of the estimated 2 – 10 million described species of insects are herbivores, feeding on plant material (Speight *et al.* 1999; Price *et al.* 2011). The seminal paper by Ehrlich and Raven (1964) initially brought to light the hypothesis of 'coevolution' between insect herbivores and their host plants, where plant defences against herbivores were met with counter adaptations in insects, leading to extensive adaptive radiation. Today there is vast diversity in insect feeding strategies, diet breadth and feeding guilds (Price *et al.* 2011). There is also vast diversity in plant morphology, physiology and life strategies that maximise plant fitness when under attack from insect herbivores. These plant defences can be constitutive defences or induced defences, ranging from leaf toughness (Raupp 1985) to herbivore induced plant volatiles that attract the natural enemies of the attacking insect (Rasmann *et al.* 2005). Indeed, the vast range of diets and feeding strategies employed by herbivorous insects has meant that many have become significant pests of managed ecosystems. It has been estimated that crop losses from insect herbivores would be enough to feed more than one billion people (Birch, Begg & Squire 2011). With an increasing global population, food security is rapidly rising up the global agenda (Tilman 1999). Understanding plant–herbivore interactions is an important cornerstone to successfully working towards ecologically and economically sustainable food production in the future.

1.2 Belowground herbivory

The majority of studies into plant–herbivore interactions have focussed on aboveground rather than belowground herbivory (Brown & Gange 1990; Hunter 2001a), but recognition of the ecological importance of root feeding insects is growing. Root feeding insects are some of the most difficult to control with plants not able to tolerate root herbivory to the same extent as aboveground herbivory, as root herbivores are persistent, inflicting damage to plants for months or years at a

time (Blackshaw & Kerry 2008; Johnson, Erb & Hartley 2016). Belowground pests also remain unnoticed until symptoms of damage are visible aboveground, by which time critical damage has already been inflicted. As a result, many agricultural practitioners prophylactically apply expensive control measures, such as insecticides, with damaging consequences for the environment (Douglas, Rohr & Tooker 2015). As such, root herbivores can be some of the most economically damaging pests. For example, costs of damage and control measures from the western corn root worm (*Diabrotica virgifera virgifera*) exceed US$1 billion annually in the USA (Gray *et al.* 2009).

The soil environment imposes distinct pressures on plants and as such there are stark differences between plant responses to aboveground and belowground herbivory (reviewed in Johnson, Erb & Hartley 2016). Root herbivory, for example, can decrease water and nutrient uptake from the soil which can reduce rates of photosynthesis (Zvereva & Kozlov 2011). Contrastingly, shoot herbivory typically increases photosynthesis (Zvereva & Kozlov 2011; Johnson, Erb & Hartley 2016). Similarly, root feeding insects exhibit a number of life history traits that distinguish them from aboveground herbivores. Root herbivores tend to be longer lived than above ground herbivores, for example scarab larvae can be feeding and developing in the soil for two years. Also, belowground herbivores are in continuous contact with the soil environment and cannot readily relocate away from detrimental abiotic stresses as rapidly as aboveground herbivores (Barnett & Johnson 2013). The impacts of abiotic factors on several key root feeding insects are reviewed in detail in chapter 2.

This continuous contact with the soil means root feeding insects are also in direct contact with microbial communities, many of which have interactions with insect host plants which range from beneficial to pathogenic/parasitic (Edwards *et al.* 2015). In sharing the same physical environment, these microbial communities are likely to impact on plant–herbivore interactions belowground more extensively than they do aboveground. It is therefore arguable that soil biotic and abiotic factors have the potential to impact on root feeding insects more significantly than aboveground herbivores. As such, it is important to investigate the impacts of soil biotic factors (e.g. microbial communities), and soil abiotic factors (e.g. soil nutrients) on host plant

growth and chemistry and how this, in turn, impacts the performance and feeding behaviour of root feeding insects. Indeed, these soil factors can be directly affected by agricultural management practices, e.g. irrigation and fertilisation, thereby impacting belowground insect herbivores.

1.2.1 Impact of agricultural management on belowground herbivory

Many agricultural practices interact with factors such as soil moisture and nutrients. Two of the most common practices of managed ecosystems are irrigation and fertilisation with nitrogen (N), phosphorus (P) and potassium (K)-based fertilisers. The impacts of these practices on root feeding scarab larvae are reviewed in more detail within chapter 2. It is a challenge to make generalisations on how irrigation and fertilisation impact belowground herbivores as the optimum soil conditions are frequently species specific and insects are often adapted to their local environment (Ward & Rogers 2007). For example, for many belowground herbivores irrigation has been shown to be beneficial, often by reducing risk of desiccation, which is a common threat to soft-bodied larvae (Potter *et al.* 1996). However, irrigation can also adversely affect root feeding insects, as larval survival has been observed to be reduced in saturated soils as a result of low oxygen and restricted movement (Davidson, Wiseman & Wolfe 1972a; Matthiessen & Ridsdill-Smith 1991).

In contrast to irrigation, the effects of fertilisation are less likely to impact root herbivores directly, as root feeding insects obtain the majority of their nutrients from root tissue (Erb & Lu 2013). The application of N, P, K fertilisers is likely to improve nutritional quality, in particular the concentrations of N in host plants, thereby benefitting root feeding insects. For example, the performance and populations of several belowground herbivores have been observed to increase in response to fertilisation (Wightman 1974; Potter *et al.* 1996; Way *et al.* 2006), while contrastingly, some studies found no effect of fertilisers on belowground insect populations (Prestidge, Zijpp & Badan 1985; Potter *et al.* 1996). Altering the soil nutrients can also impact plant defences against root herbivores (Erb & Lu 2013), for example Hol (2010) found that N, P, K fertilisation decreases alkaloid based defence root chemicals. As such, the host plant mediated impacts of agricultural management on root feeding insects are likely to be multifaceted. The timing of fertiliser application

has also been suggested to be a critical factor to the response of belowground insects (Brown & Gange 1990).

The variability in responses to these common agricultural management practices highlights the importance of local environmental conditions to belowground insect performance and population dynamics. However, these local environmental conditions are also likely to be strongly impacted by global climate change, with predicted changes in global temperatures, increases in atmospheric carbon dioxide (CO_2) concentrations and altered rainfall patterns.

1.2.2 Insect herbivory under climate change

Atmospheric concentrations of CO_2 are rising and are expected to reach approximately 540-958 μmol mol^{-1} by the year 2100 (IPCC 2014). Such changes in the atmosphere will directly impact the physiology and growth of plants, many of which are fed upon by insect herbivores. Elevated atmospheric CO_2 concentrations (eCO_2) can increase plant susceptibility to herbivores due to a breakdown in defences (Zavala *et al.* 2008; Martin & Johnson 2011), as well as other chemical changes (Guo *et al.* 2014). Elevated CO_2 also causes suppression of the jasmonic acid pathway, which then limits induced defences of plants against chewing herbivores (Ode, Johnson & Moore 2014). Plant nutritional value is also altered in response to eCO_2, as the net carbon (C) uptake of host plants increases as atmospheric CO_2 concentrations increase, diluting plant N concentration (Stiling & Cornelissen 2007; Robinson, Ryan & Newman 2012). Nitrogen is typically a limiting factor in insect herbivore diets, and an excess of C relative to N often causes compensatory feeding in many chewing insects as they attempt to acquire adequate nutrition (Stiling & Cornelissen 2007; Johnson & McNicol 2010; Johnson, Lopaticki & Hartley 2014). These changes in plant chemistry could increase plant susceptibility to insect herbivores, which could potentially lead to increases in damage to agricultural systems under eCO_2 as crops struggle to tolerate an increase in herbivory. While there is evidence in some plants that increases in host plant biomass in response to eCO_2 may be able to compensate for any increase in herbivory (McKenzie *et al.* 2016), the overall effects of eCO_2 on crop damage by insect pests will depend on the system, as plant and insect responses to eCO_2 are variable (Hunter 2001b). Nevertheless, it

would be negligent not to prepare for the possibility of increased crop losses from insect pests under eCO_2.

Considering the significant impacts of root feeding insects on plant productivity and yield of agricultural systems (Hunter 2001a; Blackshaw & Kerry 2008), it is important to understand the response of belowground herbivores to eCO_2. Yet relatively few studies focus on root feeding insects despite their impacts on ecosystem functioning and damage to crops (Staley & Johnson 2008). It is important that attention is paid to how plant–insect relationships will be impacted by eCO_2, especially in the context of novel control strategies that may remediate any adverse effects of climate change on plant susceptibility. Indeed, one possible avenue of research that holds promise in this area is plant silicon.

1.3 Silicon in plants

Soil nutrients such as N, P and K are critical to plant growth. Indeed, these nutrients are taken up by plant roots and can alter host plant quality for insect herbivores, impacting their performance and behaviour (Prudic, Oliver & Bowers 2005; Krauss *et al.* 2007). Silicon (Si) is one of the most abundant elements within the Earth's crust, and almost all plants take up Si from the soil to some extent (Epstein 2009). Plants take up Si as silicic acid (H_4SiO_4), where is it deposited within plant tissue as SiO_2, commonly known as phytoliths or silica bodies (Ma & Yamaji 2015). The concentration of Si found in plant shoots varies from 0.1% to 10% on a dry mass basis (Ma, Miyake & Takahashi 2001). Although this can vary both between and within species (Soininen *et al.* 2013), certain generalisations can be made, for example Poales are typically Si accumulators (Ma, Miyake & Takahashi 2001). Recent work into the molecular transport of Si has found several key transporters present within Si accumulating plants (Ma & Yamaji 2015)

1.3.1 Evolution of plant silicon defences

The selection pressures that have led to the silicification of some plant taxa has recently been debated within the literature (Cooke & Leishman 2011; Katz 2015; Strömberg, Di Stilio & Song 2016). It is hypothesised that high Si accumulating plants can, to some extent, substitute C for Si for functions such as structural support or

defences (McNaughton *et al.* 1985; Schoelynck *et al.* 2010; Schaller, Brackhage & Dudel 2012). From an evolutionary perspective, by the time of the appearance of vascular plants, Si had already been incorporated into plant tissue (411 - 407 Ma) (Trembath-Reichert *et al.* 2015). A major hypothesis is that the functions of Si did not become critical until the Oligocene when global atmospheric CO_2 concentrations were in decline (Craine 2009). Therefore the ability to use Si for support or in place of C based defences during this time may have facilitated the expansion of the grasses (Poaceae), which are typically high in Si. Other hypotheses have associated the radiation of the grasses with the evolution of hypsodont teeth, that can withstand the abrasive nature of a high Si diet (Stebbins 1981; McNaughton *et al.* 1985). This has led to the common idea that grasses and herbivores were part of a co-evolutionary arms race as grassland ecosystems spread across the continents. However, a recent review of the phylogenetic and fossil evidence by Strömberg *et al*. (2016) concluded there was no correlation between large grass eaters, grass dominance and Si content in grasses. Instead, it was suggested that insect herbivory during the Cretaceous may have constituted selection pressure for Si accumulation in grasses (Prasad *et al.* 2005; Strömberg, Di Stilio & Song 2016). Regardless of the selection pressures behind the persistence of plant Si as a functional trait, the importance of Si to many aspects of plant ecology is becoming clear.

1.3.2 Silicon as a defence against herbivores

Although the effects of Si on the growth and yield of many crops has been known for years, the importance of Si to plant ecology is only now being fully recognised (Cooke, DeGabriel & Hartley 2016). Plant Si has been shown to alleviate a number of abiotic plant stresses such as heat stress (Agarie *et al.* 1998), water stress (Ma, Miyake & Takahashi 2001) and heavy metal toxicity (Neumann & zur Nieden 2001). Biotic stresses such as bacterial and fungal damage are known to be reduced by Si (Chérif, Asselin & Bélanger 1994); this can be as a mechanical barrier to penetration and through induction of defensive plant phytochemicals such as phytoalexins (Rodrigues *et al.* 2003; Rémus-Borel, Menzies & Bélanger 2005). The efficacy of Si as a defence against insect herbivores is well reported (Massey, Ennos & Hartley 2006; Kvedaras & Keeping 2007; Massey & Hartley 2009; Korndörfer, Grisoto & Vendramim 2011).

These Si based defences are usually deployed as abrasive phytoliths, which are solid bodies that form when silicic acid (H_4SiO_4), taken up by plant roots, precipitate as silica (SiO_2). These distinct opaline phytoliths can be deposited almost anywhere within the plant tissue, both between and within epidermal and vascular plant cells (Strömberg, Di Stilio & Song 2016) increasing the overall plant toughness. This silicification has been shown to increase insect mandibular wear (Massey & Hartley 2009) and to reduce the palatability and the digestibility of the plant tissue (Massey, Ennos & Hartley 2006). This is thought to be through the mechanical protection of the chlorenchyma cells, where insects retrieve a lot of starch and protein (Hunt *et al.* 2008). Additionally, preferential oviposition on leaves with low Si concentrations has been observed (Correa *et al.* 2005), which has been suggested to indicate plant Si could be driving insect behaviours beyond performance and food selection (Cooke, DeGabriel & Hartley 2016).

The majority of research on plant Si interactions with insects has focussed on aboveground herbivores, with few studies investigating the importance of Si within belowground systems (Wieczorek *et al.* 2015), and no studies investigating the impact of Si on root feeding insects. This highlights the need to investigate the role of Si in plant–herbivore interactions belowground. The economic importance of many root feeding insects (Hunter 2001a) and the efficacy of plant Si in reducing insect performance and consumption (although untested in belowground systems) highlights the potential of Si to be used in novel pest management strategies. Interestingly, host plant Si uptake has been shown to be increased by arbuscular mycorrhizal (AM) fungi (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996), obligate symbiotic fungi that colonise the roots of most terrestrial plants.

1.4 Arbuscular mycorrhizal fungi

The majority of vascular plants in terrestrial systems associate with AM fungi (Smith & Smith 2011). This association can be mutualistic and is generally based on the bidirectional transfer of C from the host plant and soil nutrients from the fungus, such as P and N (Smith & Read 2010). The degree to which this ancient relationship is mutualistic is often determined by plant and fungal community identities as well as environmental factors such as soil type and nutrient availability (Jones & Smith 2004).

Moreover, the species composition of AM fungi can also be influenced by host plant identity and local abiotic soil characteristics (Oehl *et al.* 2010; Davison *et al.* 2015). Aside from increased nutrient uptake, mycorrhizal plants often exhibit a number of other traits such as improved water uptake, increased growth and increases in rates of photosynthesis (Smith & Read 2010). Indeed, AM fungi are known to initiate changes in plant defence pathways and chemicals (Jung *et al.* 2012), with different mycorrhizal species differentially impacting host plant defences and also insect herbivore performance (Bennett & Bever 2007). The response of insects to AM fungi can also be dependent on whether single or multiple AM species are associated with the host plant (Currie, Murray & Gange 2011), highlighting the importance of a community approach (Gehring & Bennett 2009). Nevertheless, the response of aboveground insect herbivores to AM plants is highly variable (see Koricheva, Gange & Jones (2009), and references therein) and the mechanisms remain unclear (Bennett, Alers-Garcia & Bever 2006).

The majority of research on interactions between AM fungi and insect herbivores has been on aboveground insects, while relatively few have investigated how AM fungi affect root herbivore performance (see Johnson & Rasmann (2015) and references therein). Of these handful of studies, almost all, except one (Currie, Murray & Gange 2011), found that AM colonisation of the host plant negatively impacted root herbivores, suggesting a significant role of AM fungi in plant defences. The mechanisms behind these negative effects on root feeders is not known (Gange 2001; Johnson & Rasmann 2015), although could be associated to mycorrhizal induced alteration of root defence chemicals (Morandi 1996). As mycorrhizal plants are known to exhibit increased uptake of Si (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996), this highlights the possible role of Si within the relationship between AM fungi and root feeding insects. The obscurity surrounding the mechanisms behind AM interactions with root herbivores calls for future research to investigate this complex relationship.

The overall positive impacts on host plants coupled with the negative impacts on belowground herbivores suggest possible agricultural applications for AM fungi. While AM inocula have been commercially available for several years, their efficacy

in the field is highly variable (Berruti *et al.* 2013) and context dependent, as the existing microbial communities can determine the competitive success of AM inocula (Hartley & Gange 2009). This indicates context specific experimental investigations may be required to discover the persistence of commercial AM inocula in the field, and their efficacy in promoting plant productivity and defences/tolerance to insect herbivores.

1.5 Root feeding scarab larvae

There are over 31,000 described species of scarab beetles worldwide (Coleoptera: Scarabaeidae) (Hangay & Zborowski 2010). Many of these scarabs have become destructive pests of grassland and agro-ecosystems (Potter & Braman 1991). This problem is exacerbated by agriculture, where there has been large scale transition of grasslands into arable crop production systems, or of forests and woodlands into pastures. Throughout Australia there are several examples of native scarab larvae, such as Christmas beetles (*Anoplognathus* spp.) or the dusky pasture scarab (*Sericesthis nigrolineata*), that have become destructive pests of pasture and crops. The larvae of the Greyback cane beetle (*Dermolepida albohirtum* (Waterhouse)), colloquially known as canegrubs, is another example within Australia of particular economic significance.

1.5.1 The canegrub

The canegrub is a long standing pest of sugarcane crops (*Saccharum* spp. hybrids) across Queensland, Australia. These insects are native to Australia, originally feeding on the roots of native grasses (Allsopp 2010).

The greyback cane beetle normally has a one year lifecycle and, similar to the lifecycle of other scarabs, the period spent as adults is relatively short. Eggs are normally laid in early summer, which tends to be two weeks after the adults have emerged (Logan & Kettle 2002). The first instar duration is typically four weeks with larvae being found in the soil throughout summer and sometimes into early autumn. The second instar tends to last around four or five weeks, and the third instar lasts around seven months (Sallam 2011). The development time is influenced by several environmental factors such as soil texture, temperature and moisture (Illingworth & Dodd 1921;

Sallam 2011). Population reports indicate larval numbers per plant can vary from three to 15 or more (Sallam 2011). Larvae inflict the most crop damage during their third instar as this is when the insects are at their most voracious.

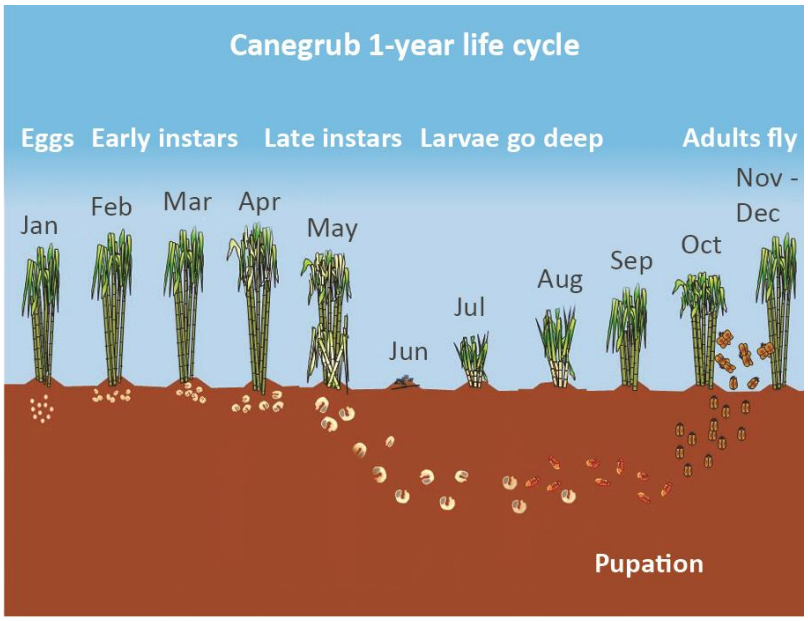

Figure 1-1. Canegrub (*Dermolepida albohirtum*) one year lifecycle from January through to December. Reproduced with permission from Sugar Research Australia Limited.

With the introduction and establishment of sugarcane to Australia throughout the late 1700s and 1800s, many of the original forests and floodplains were replaced by sugarcane fields. This provided a host plant for canegrubs that produced large quantities of roots with relatively high nutritional quality. This, coupled with the loss of environments that promote canegrub natural enemies and pathogens, resulted in the significant pest problem faced by the Australian sugar industry today (Robertson *et al.* 1995; Allsopp 2010). The sugar industry suffers losses up to $AU 40 million annually as a result of damage inflicted by canegrub outbreaks (Chandler 2002; Allsopp 2010). The biology and ecology of the canegrub is discussed further in chapter 2.

1.6 Sugarcane

The term 'sugarcane' is usually in reference to five species from the *Saccharum* genus, which is a member of the grass family Poaceae. These species are *S. barberi*, *S. robustum*, *S. sinense*, *S. spontaneum* and *S. officinarum* (Stevenson 1965).

These *Saccharum* species are tall perennial grasses that are native to warm temperate and tropical regions of South Asia and Melanesia. All sugarcane species are able to interbreed, and cultivated sugarcane for the production of sugar are complex hybrids. It is thought that sugarcane was first cultivated in New Guinea, where *S. officinarum* originates, around 6000 BC (Deerr 1949). Around 70% of sugar produced around the world comes from *S. officinarum* and the hybrids from this species (Cope, Nesbitt & Johnson 2016).

Sugarcane was first introduced to Australia on the arrival of the First Fleet in 1788, but it was not until the late 1800s that the first viable cane plantation was established near Brisbane, Queensland. Today, sugarcane is grown along the east coast of Queensland, as far south as northern New South Wales. Sugarcane is typically grown by replanting part of a mature plant stalk, where growers cut the stalk into lengths of about 40 cm, these are called 'setts' (Canegrowers Australia 2010). A crop of sugarcane will take between 9 and 16 months to grow, although this is variable depending on climate as crops in New South Wales can take as long as 24 months due to lower temperatures. Once mature, the canes are harvested, leaving the underground stalk material, the 'stool', to allow for regrowth. A typical crop cycle consists of one planted crop and then three to four 'ratoon', or regrowth, crops (Canegrowers Australia 2010).

Since the 1800s, crop losses from root feeding insects have been well documented, with significant research on the biology of these pests, and on biological, chemical and cultural controls (Robertson *et al.* 1995).

Sugarcane is known to be highly responsive to AM fungi, increasing yield, particularly when soil nutrients are low (Kelly *et al.* 1997, 2001; Magarey, Bull & Reghenzani 2005). Moreover, sugarcane is a Si accumulating plant, and Si has been shown to negatively impact aboveground insects pests of sugarcane, such as the caneborer (*Eldana Saccharina*) (Kvedaras & Keeping 2007; Keeping, Kvedaras & Bruton 2009). As such, this system provided a good model to investigate the impacts of abiotic and biotic soil factors, specifically soil Si and AM fungi, on host plant interactions with a root feeding insect, which is the main theme of this thesis.

1.7 Thesis overview

The overall aim of this thesis is to examine the impacts of different soil abiotic and biotic factors on grasses and their belowground scarab herbivores, with a focus on sugarcane and the canegrub. The impacts of different abiotic (e.g. soil moisture, soil nutrients) and biotic (AM fungi) soil factors on scarab populations and performance are initially reviewed in chapter 2. This is then investigated further by experimentally assessing how altering soil moisture and nutrients (N, P and K) in a managed system impacts scarab populations and their natural enemies (entomopathogenic nematodes) within chapter 3. From here this work focuses on sugarcane, a grass crop with a damaging scarab pest (the canegrub) as our model system.

Plant Si, taken up from the soil as silicic acid, has been identified as an effective plant defence against insect herbivores aboveground but is yet to be investigated in belowground systems. A main objective was to examine the impacts of plant Si defences on canegrub performance, while also assessing the role of phenolics as trade-offs had previously been observed in Si accumulating plants (chapter 4). Considering the possible impacts of climate change on plant-insect herbivore interactions we also looked to assess the role of Si defences belowground under elevated atmospheric CO_2 concentrations (chapter 5). The mechanisms behind the negative impacts of AM fungi on root feeding insects are yet to be determined, yet AM fungi are known to increase Si uptake in some plants. This work aimed to identify impacts of AM fungi on canegrub performance with a view to highlight the potential role of Si underlying their plant-mediated interactions with root feeding insects (chapter 6). The results of chapter 6 were developed further by directly testing the impacts of AM fungi and Si on sugarcane alongside canegrub performance and immunity (chapter 7). The main factors investigated within each thesis chapter are outlined in Fig. 1-2. There are numerous soil organisms and processes, other than those which form the work reported in this thesis, which interact with plant roots and root feeding insects. As such, it is important to recognise that these experiments, which focus on particular test systems, provide a partial insight into the broader ecology and complex interactions between root feeding scarabs and their host plants.

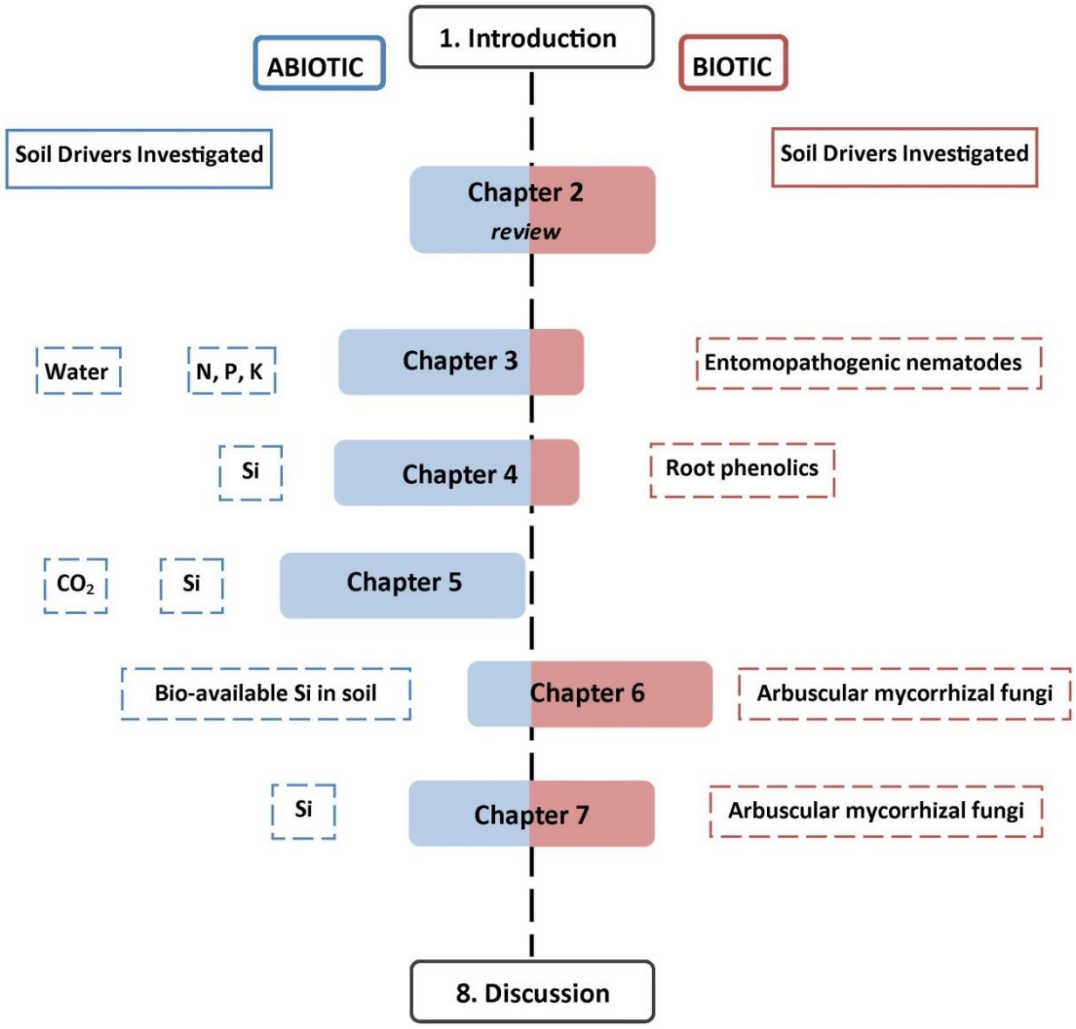

Figure 1-2. Outline of thesis chapters 1 to 8. Experimental chapters 3 to 7 are shown with the main abiotic factors investigated (blue) on the left and main biotic factors investigated (red) on the right.

1.8 Thesis outline

This general introduction has covered what is largely known regarding the interactions between different soil factors of interest and root feeding scarab larvae, setting the context for the thesis.

Chapter 2 reviews current knowledge of the biology and ecology of significant scarab species across Australasia, including the canegrub. There is a focus on scarab interactions with abiotic factors such as soil moisture, temperature and nutrients, as well as biotic factors such as soil microbial pathogens and host plant symbionts. This is synthesised with a view to highlight how these ecological interactions can be exploited and applied in agriculture for improved pest management alongside suggestions for future research directions. This review entitled 'Belowground ecology

of scarabs feeding on grass roots: current knowledge and future directions for management in Australasia' (Adam Frew, Kirk Barnett, Uffe N. Nielsen, Markus Riegler and Scott N. Johnson) was published in *Frontiers in Plant Science*, vol. 7: 321, on 22 March 2016.

Chapter 3 investigates the impacts of the common management practices of irrigation and fertilisation on the belowground populations of scarab larvae. This investigation focuses on the understory grass communities of a eucalypt plantation (Fig. 1-3), the scarab populations and the populations of scarab natural enemies, entomopathogenic nematodes (EPN). This research entitled 'Do eucalypt plantation management practices create understory reservoirs of scarab beetle pests in the soil?' (Adam Frew, Uffe N. Nielsen, Markus Riegler and Scott N. Johnson) was published in *Forest Ecology and Management*, vol. 306: 175-180, on 15 October 2013.

Figure 1-3. Hawkesbury Forest Experiment comprising 160 *Eucalyptus saligna*. Image supplied by Adam Frew.

Chapter 4 examines the role of C based and Si based compounds (Fig. 1-4) in plant defences against root feeding insects. Plants with high concentrations of phenolics have been observed to promote root herbivore performance, which is contrary to predictions. Taking into account the observation that many Si accumulating plants exhibit 'trade-offs' between C and Si based compounds, this work investigates the impacts of Si and phenolic compounds in sugarcane on canegrub performance. This research entitled 'Trade-offs between silicon and phenolic defences may explain

enhanced performance of root herbivores on phenolic-rich plants' (Adam Frew, Jeff R. Powell, Nader Sallam, Peter G. Allsopp and Scott N. Johnson) was published in *Journal of Chemical Ecology*, vol. 42: 768-771 on 1 August 2016.

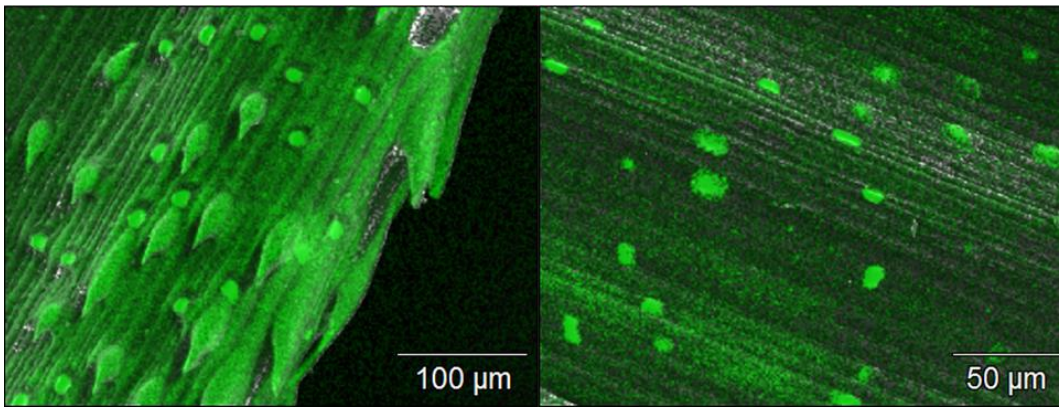

Figure 1-4. Scanning electron microscopy images of silica (SiO_2) phytoliths in *Deshampsia cespitosa* (left) and *Festuca rubra* (right) plant tissue. Reproduced with permission from Professor Sue Hartley, University of York.

Chapter 5 investigates the impacts of Si and eCO_2 on sugarcane growth and defences against the canegrub (Fig. 1-5). Specifically this study looks at how eCO_2 and soil silicon supplementation alters host plant photosynthesis, nutritional value and defence chemistry alongside plant mediated effects on canegrub growth rates and root consumption. This research entitled 'Increased root herbivory under elevated atmospheric carbon dioxide concentrations is reversed by silicon-based plant defences' (Adam Frew, Peter G. Allsopp, Andrew N. Gherlenda and Scott N. Johnson) was published in *Journal of Applied Ecology*, doi: 10.1111/1365-2664.12822, on 9 November 2016.

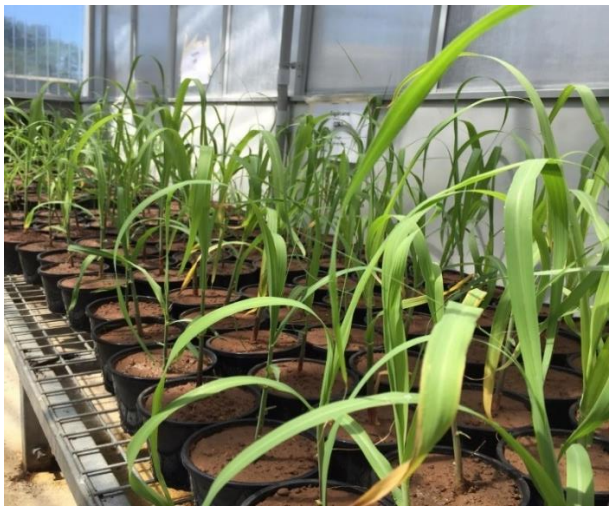

Figure 1-5. Sugarcane (*Saccharum* species hybrid) variety Q200 growing in a glasshouse. Image supplied by Adam Frew

Chapter 6 investigates the impact of different AM fungal communities on sugarcane growth and defences against the canegrub within two different soil types, known to differ in their Si concentrations (Fig. 1-6). Specifically this work looks at how AM fungi may differentially impact sugarcane growth, nutritional value and defences depending on soil conditions, and how this impacts canegrub performance. This research entitled 'Arbuscular mycorrhizal fungi promote silicon accumulation in plant roots with negative impacts on root herbivores' (Adam Frew, Jeff R. Powell, Peter G. Allsopp, Nader Sallam and Scott N. Johnson) is currently under review.

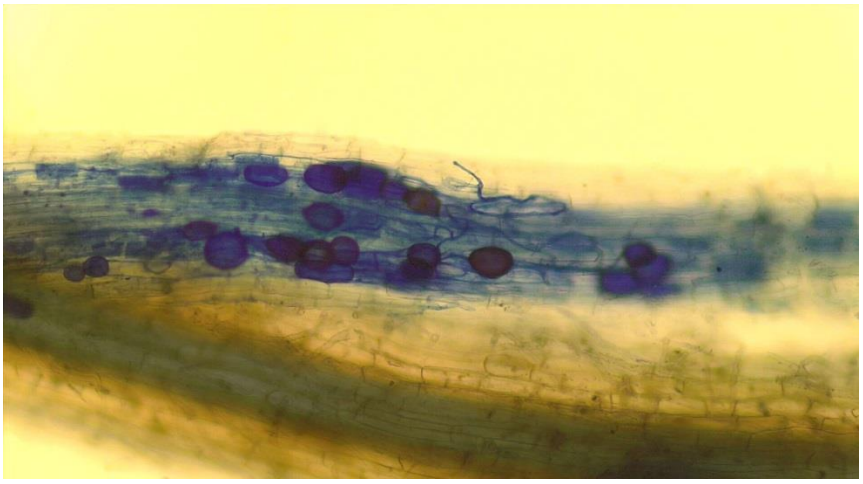

Figure 1-6. Arbuscular mycorrhizal fungi within stained sugarcane (*Saccharum* species hybrid) roots. Image supplied by Adam Frew.

Chapter 7 looks to directly test the effects of AM fungi and Si supplementation on different sugarcane varieties and the impacts on canegrub performance and immunity (Fig. 1-7). Specifically this study investigates how these treatments impact on the growth and chemistry of sugarcane and how these treatments alone or interactively affect canegrub performance and immune system function. This research entitled 'Arbuscular mycorrhizal fungi stimulate immune function whereas silicon diminishes growth in a soil dwelling herbivore' (Adam Frew, Jeff R. Powell, Ivan Hiltpold, Peter G. Allsopp, Nader Sallam and Scott N. Johnson) will be submitted for publication in January 2017.

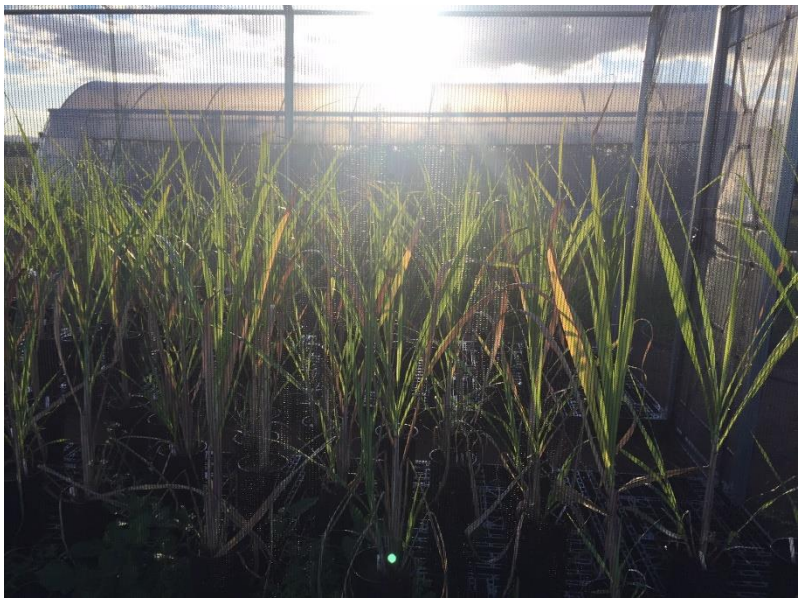

Figure 1-7. Sugarcane (*Saccharum* species hybrids) varieties Q200 and Q240 growing in a shade house. Image supplied by Adam Frew.

Chapter 8 synthesises the key findings of this research within the framework of the central theme of soil abiotic and biotic factors impacting root feeding scarab larvae, with emphasis on Si, AM fungi the canegrub. The wider ecological implications of the findings are discussed alongside their applied potential in the field with a view to novel pest management strategies.

Chapter 2: Belowground ecology of scarabs feeding on grass roots: current knowledge and future directions for management in Australasia – review.

Published as Frew *et al*. 2016, *Frontiers in Plant Science*, **7**, 321

2.1 Abstract

Many scarab beetles spend the majority of their lives belowground as larvae, feeding on grass roots. Many of these larvae are significant pests, causing damage to crops and grasslands. Damage by larvae of the greyback cane beetle (*Dermolepida albohirtum*), for example, can cause financial losses of up to AU$40 million annually to the Australian sugarcane industry. We review the ecology of some scarab larvae in Australasia, focussing on three subfamilies; Dynastinae, Rutelinae and Melolonthinae, containing key pest species. Although considerable research on the control of some scarab pests has been carried out in Australasia, for some species, the basic biology and ecology remains largely unexplored. We synthesise what is known about these scarab larvae and outline key knowledge gaps to highlights future research directions with a view to improve pest management. We do this by presenting an overview of the scarab larval host plants and feeding behaviour; the impacts of abiotic (temperature, moisture and fertilization) and biotic (pathogens, natural enemies and microbial symbionts) factors on scarab larvae and conclude with how abiotic and biotic factors can be applied in agriculture for improved pest management, suggesting future research directions.

Several host plant microbial symbionts, such as arbuscular mycorrhizal fungi and endophytes, can improve plant tolerance to scarabs and reduce larval performance, which have shown promise for use in pest management. In addition to this, several microbial scarab pathogens have been isolated for commercial use in pest management with particularly promising results. The entomopathogenic fungus *Metarhizium anisopliae* caused a 50% reduction in cane beetle larvae while natural enemies such as entomopathogenic nematodes have also shown potential as a biocontrol.

Continued research should focus on filling the gaps in the knowledge of the basic ecology and feeding behavior of scarab larval species within Australasia. This should include host plant preferences and behavioural cues which could be utilised in pest management, for example, in trap crops. The direction of future research in biocontrol strategies should focus on identifying naturally occurring, locally adapted pathogens, if they are to achieve high efficacy in the field.

2.2 Introduction

Worldwide there are over 31,000 species of scarab beetles (Coleoptera: Scarabaeidae)(Jameson 2015) and within Australia alone there are well over 2,200 described species (Hangay & Zborowski 2010). These scarabs can be found across tropical, subtropical and temperate regions of Australia and New Zealand in a broad range of ecosystem types including agroecosystems (Allsopp 2010). Many scarabs have become destructive pests of grasslands as root feeders (Potter & Braman 1991). There are also instances where introduced plant species have become the preferred host to a number of native scarabs such as greyback cane beetle larvae (*Dermolepida albohirtum* Waterhouse, subfamily: Melolonthinae) feeding on sugarcane (*Saccharum* spp.). Moreover, the problem of such species becoming pests has been exacerbated by agriculture (Robertson *et al.* 1995), such as large-scale transition of grassland into arable crop production, or of forests and woodlands into pastures. Crop losses due to scarab larval damage for sugarcane in Australia alone can result in losses up to AU$40 million annually (Chandler 2002). Historically, this problem has been addressed by using chemical pesticides, which can have serious collateral effects on non-target organisms and the environment (Jackson & Klein 2006). As such, alternative management strategies are being continually investigated (Goldson *et al.* 2015).

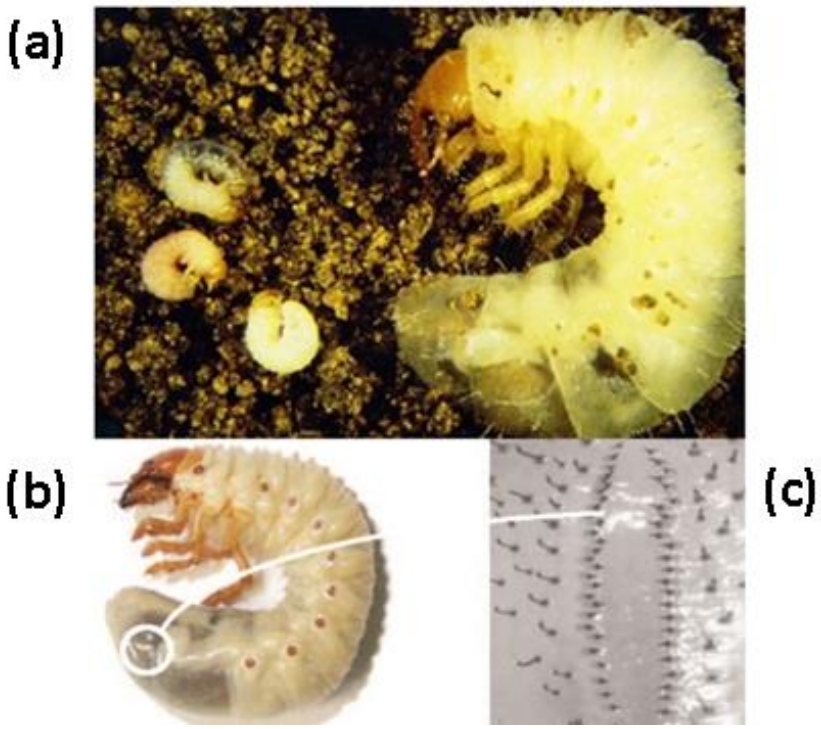

Figure 2-1. Scarab larvae: **(a)** African black beetle larvae *Heteronychus arator*, **(b)** greyback cane beetle larva *Dermolepida albohirtum*, **(c)** close-up of hair pattern (raster) used to identify greyback cane beetle larvae. Images supplied by Western Australian Department of Agriculture and Food (African black beetle) and Sugar Research Australia (greyback cane beetle larva).

Understanding the biology and behaviour of scarab larvae, including their interactions with host plants and the soil environment (or rhizosphere) is an essential component to enabling effective management and control, both in Australia and at a global scale. There are numerous studies on these larvae within Australasia, some of which have elucidated core biology, behaviour and even responses to future environment such as climate change (Johnson, Lopaticki & Hartley 2014). However, for many scarab species this work was carried out some time ago, while for others the majority of their ecology has yet to be described. This is partly due to their soil dwelling habit which has made culturing and experimentation particularly challenging. It is therefore timely to synthesise the fragmented information available on this group of root feeding pests in Australasia. In this review we identify where knowledge is lacking, highlight promising research avenues into pest management, to suggest where continued research should be focussed. In particular, this review focusses on belowground influences which impact larval development and survival.

Edaphic variables such as soil moisture and temperature alongside biotic interactions with microbiota both in the soil and with host plants show most promise for improved current pest management.

Figure 2-2. Third instar larva of the greyback cane beetle (*D. albohirtum*). Image supplied by Adam Frew.

We concentrate on three subfamilies belonging to the family Scarabaeidae: Dynastinae (e.g. African black beetle *Heteronychus arator* Fabricius and Argentine scarab *Cyclocephala signaticollis* Burmeister), Rutelinae (e.g. Christmas beetles *Anoplognathus* spp. Leach) and Melolonthinae (e.g. dusky pasture scarab *Sericesthis nigrolineata* Boisduval and greyback cane beetle *D. albohirtum*). Within these subfamilies we focus on the key pest species/genera examples mentioned, while including any relevant information from other species within the subfamilies. The redheaded cockchafer, *Adoryphorus couloni* Burmeister (subfamily: Dynastinae) is also a significant pasture pest within Australia and was comprehensively reviewed recently (Berg *et al.* 2014). Hence, we do not include this species within the review. Within the three subfamilies we specifically focus on:

- Host plants and feeding behaviour
- Abiotic soil factors (temperature, moisture and fertilisation)
- Biotic soil factors (pathogens, natural enemies and symbionts)
- Applied perspectives
- Future directions

2.3 Host plants and feeding behaviour

While the majority of scarabs are grass root feeders in their larval stages (Figure 2-1 and 2-2) (Goodyer & Nicholas 2007), some larvae feed on organic matter in the soil litter (Jackson & Klein 2006). For some pest scarab species, feeding ecology has been documented relatively well. Across the subfamilies discussed here the most damaging and voracious feeding occurs during the third instar, therefore the timing of development of pest scarab larvae is important to consider from a pest management perspective (Figure 2-3). Indeed, the ability of all scarab larvae to locate suitable hosts is equally as important as the nutritional value of the host plant. Carbon dioxide emission by the host plant is an important root exudate that plays a role in host plant location by root herbivores (Johnson & Gregory 2006); however, other volatile root exudates are clearly critical in host plant location by scarab larvae (Eilers *et al.* 2012). The topic of host plant location by root feeders was reviewed by Johnson & Gregory (2006) and revised by Johnson & Nielsen (2012), and we will not discuss this in detail here. Here we will present what is known regarding the feeding behaviour of some of the key species from within Dynastinae, Rutelinae and Melolonthinae.

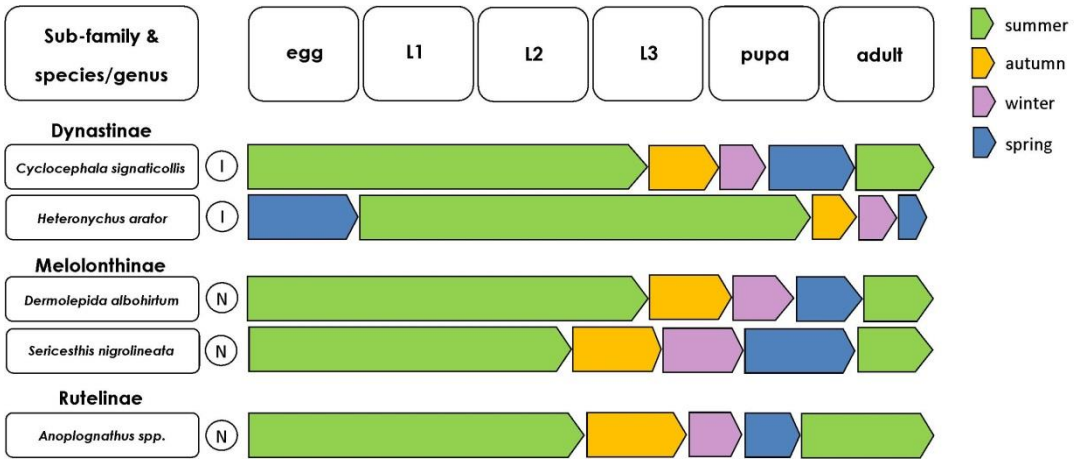

Figure 2-3. Seasonal occurrence of scarab life stages for each of the key scarab pest species. Such information can help to design life-stage specific, targeted pest control programs. Colour of arrows indicates the season in which each scarab life stage typically occurs (within Australia and New Zealand). Circles with 'I' indicate species invasive to Australasia, circles with 'N' indicate species native to Australasia.

2.3.1 Dynastinae

The African black beetle has been described as a sporadic pest of pastures and crops across New Zealand and Australia (Matthiessen & Ridsdill-Smith 1991). Plant species composition influences the distribution of the African black beetle across the landscape (King and Kain, 1974; King et al., 1982). The larvae seem to have reduced performance on species such as *Medicago sativa* (King *et al.* 1975) and tend to avoid feeding on *Trifolium repens* (Sutherland & Greenfield 1978), which is due, at least in part, to the feeding deterrents medicarpin and vestitol present in the roots (Russell *et al.* 1982). That said, larvae will eat *T. repens* roots if given no other choice (King, Mercer & Meekings 1981b). Despite this, *T. repens* is a common food source for other scarab larvae such as *Costelytra zealandica* White (subfamily: Melolonthinae) (King, Mercer & Meekings 1981c; Russell *et al.* 1982; Prestidge, Zijpp & Badan 1985).

By contrast, the grasses *Lolium perenne* and *Paspalum dilatatum* have been shown to be a preferred food choice of pasture grass species (King 1977; King, Mercer & Meekings 1981c). King (1977) found that African black beetle larval mass gain was greater on *L. perenne* when compared with *T. repens* and *Lotus pedunculatus*, but also that organic matter in the soil stimulated this feeding and increased weight gain. The organic content of the soil acting as a feeding stimulant has therefore been suggested as having implications for damage in soil with high peat content (Bell *et al.* 2011). Indeed the African black beetle is a significant pest of *L. perenne* pastures, both as larvae and adults, feeding on aboveground and belowground portions of the plant respectively (Popay & Bonos 2008). The endophytic fungus *Neotyphodium lolii*, forms a mutualistic relationship with *L. perenne* (Raman, Wheatley & Popay 2012). Feeding by adult African black beetles is well documented to be deterred by *N. lolii* infected *L. perenne* (Popay & Baltus 2001), which has been attributed to the presence of alkaloids (Thom *et al.* 2014). More recently, Qawasmeh, Raman & Wheatley (2015) found that different strains of *N. lolii* had an impact on the aboveground volatile profile of *L. perenne* and the attractiveness of this host plant to adult African black beetles.

The majority of research into endophyte induced protection has focussed on aboveground herbivores (Popay and Baltus, 2001). One study on a specific *N. lolii*

strain noted that the African black beetle larvae were observed to have a reduced occurrence in *N. lolii* infected grasses (Hume *et al.* 2007). More recently, another study has found changes in the root volatile profile in response to *N. lolii* infection and found decreased attraction to *C. zealandica* larvae belowground (Rostás, Cripps & Silcock 2015).

Considering damage can be significant, more research focussing on the efficacy of *N. lolii* strains in deterring African black beetle larvae would be the logical next step. In the field, replacing turfgrass or pasture with *N. lolii* infected *L. perenne* could convey protection against African black beetle adults at the very least, perhaps reducing oviposition, and indeed may deter all alkaloid sensitive insect herbivores (see 'Applied perspectives' section 2.6).

The feeding behaviour of Argentine scarab larvae has not received significant attention in the literature despite its pest status on turf and pastures (Carne 1957a). Within Argentina, the larvae are known as pests particularly of potato crops (Berón & Diaz 2005), but are known to feed on roots of flax, lucerne, sunflower and carrot crops as well (Mondito *et al.* 1997). In Australia, however, the larvae feed mainly on grass roots. Carne (1957a) noted that the larvae were found in the greatest numbers in grasslands with *Cynodon dactylon* and *P. dilatatum*. It was also noted that this scarab could successfully develop on a diet composed solely of decomposing organic matter; however, the abundance found in pastures indicates some of their nutrient requirements are derived from grass roots. It is evident the Argentine scarab larvae feed on both organic matter and actively on grass roots but other than a few studies no other feeding behaviour investigation has been carried out on the Argentine scarab in Australian grasslands. The lack of context specific studies on the larval feeding preferences of this scarab species, alongside the efficacy of management practices, calls for initial host preference studies to be conducted before any control initiatives can effectively be researched and applied.

2.3.2 Rutelinae

The feeding behaviour of adult *Anoplognathus* spp., which consume the leaves of eucalypts, is addressed well within the literature (Carne, Greaves & McInnes 1974;

Edwards, Wanjura & Brown 1993; Steinbauer & Wanjura 2002; Johns, Stone & Hughes 2004; Steinbauer & Weir 2007), in contrast to the information on larval feeding behaviour, which is relatively scarce.

Anoplognathus larvae are known to feed on organic matter in the soil, grass roots and crop roots (Carne 1957b; Sallam 2011). Some species within the genus, such as *Anoplognathus montanus,* will commonly feed on rotting organic material such as timber, but will also feed on the finer roots of eucalypts (Carne 1957b). Carne *et al*. (1974) stated that larvae of *Anoplognathus* feed primarily on organic matter in the soil and tend not to seek out plant roots. While Davidson & Roberts (1968a) confirmed this, they nonetheless stated that the organic matter they feed on is composed mainly of plant roots. Here, they also found that when Christmas beetle larvae fed on the grasses *Phalaris tuberosa* and *T. repens*, they often failed to reach pupation, which could be due to secondary metabolites in the plant. In a further study that year, it was found that Christmas beetle larvae avoided feeding on *T. repens* altogether (Davidson & Roberts 1968b), a behavior also exhibited by African black beetle larvae.

The larvae of *Anoplognathus* spp. have been reported as pests of sugarcane, although only when numbers are high (Samson, Sallam & Chandler 2013). Significant damage to pastures by Christmas beetle larvae is well known, particularly by the third instar (Urquhart 1995). Feeding populations of larvae can be influenced by aboveground herbivores. A study by Roberts & Morton (1985) investigated the effects of grazing pressure on the biomass of *Anoplognathus* spp. larvae, and found that larval abundance peaked under low to intermediate grazing pressure. Therefore, low pasture damage by larvae may be exacerbated by moderate grazing of livestock aboveground.

2.3.3 Melolonthinae

The greyback cane beetle is a long standing pest within sugarcane and the larvae can cause devastating damage to crops (Chandler 2002). Initial uncertainties regarding larval feeding of mainly organic material in the soil (Illingworth & Dodd 1921) were resolved as a result of compelling evidence for grass roots as the main resource

(Sallam 2011). Root feeding was shown by Logan & Kettle (2002) who investigated the effect of food type on the survival and development of first instar greyback cane beetle larvae. Larval survival and development was highest in treatments with grass seedlings and lowest in soil alone. This result was confirmed by a second experiment using sugarcane, Guinea grass (*Panicum maximum*), cane trash (mulch), and a soil only environment, where larval survival and mass was lowest in the soil only treatment and highest when cane or grass were available (Figure 2-4).

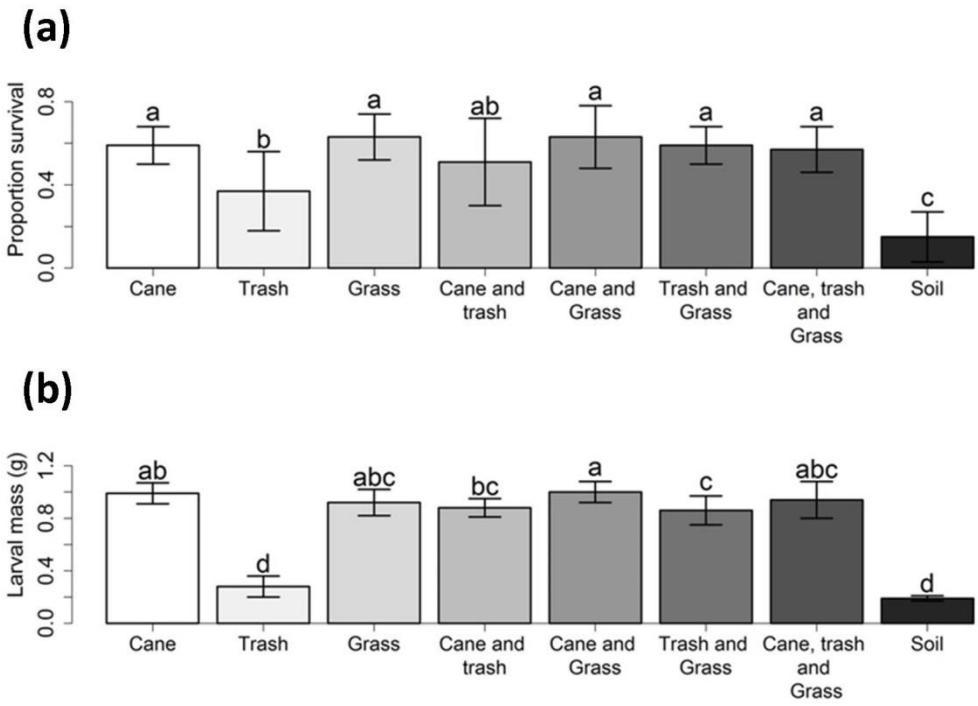

Figure 2-4. Survivorship and mass of early instar larvae of *D. albohirtum*. **(a)** Mean proportion survival (SD), and **(b)** mean larval mass in grams (SD), of larvae after 4 weeks in bins with food composed of either sugarcane, Guinea grass, cane trash, combinations of two or three of these, or none of these. Different letters indicate significant effects of treatments. Adapted from Logan and Kettle (2002).

In Australia, cane beetles are the major pests to the sugar industry (McLeod, McMahon & Allsopp 1999; Horsfield *et al.* 2008), as a result there have been several studies into pest management and environmental conditions that may impact on larval induced damage to sugarcane (Robertson *et al.* 1995; Robertson & Walker 2001; Chandler 2002). Coupled with the development of pest management strategies, Allsopp (1991) investigated feeding stimulants of greyback cane beetle larvae, which could be used to enhance the efficacy of larval baits. Larvae showed a

strong feeding response to fructose and sucrose. Both sucrose and fructose, along with glucose, are the most abundant sugars found in sugarcane, and are both at higher concentrations in the lower stem of sugarcane compared with the roots (Meade & Chen 1977).

Estimates of population size and density within sugarcane fields vary from three or four larvae per cane plant (Ward & Robertson 1999) to numbers of 15 per plant, or more (Jarvis 1933; Sallam 2011). Some Melolonthinae larvae have shown specific soil type preferences. A study by Cherry & Allsopp (1991) found distinct soil type preferences between different species, with some larval populations of some species positively correlated with clay and silt, and negatively with sand content, while other species showed opposing correlations. Yet for other species, such as the greyback cane beetle, soil type has little influence on the distribution (Robertson & Walker 2001). Overall there is no 'one soil type fits all' for scarab species as studies have shown species specific preferences (Gordon & Anderson 1981; Cherry & Hall 1986).

Studies conducted into the feeding behaviour of dusky pasture scarab larvae have focussed on climatic and abiotic influences rather than host preference. The larvae can feed and survive in soil in the absence of plant roots (Ridsdill-Smith, 1975; Porter, 1980), however it is not clear if they are able to develop into adults on soil organic matter alone. The feeding behaviour, and relative consumption of food is largely influenced by temperature (Davidson, Wiseman & Wolfe 1972a; Ridsdill-Smith, Porter & Furnival 1975; Cairns 1978) and under field conditions there is often a seasonal pattern of larval feeding as a result of local temperatures. Ridsdill-Smith, Porter & Furnival (1975) carried out an investigation into the feeding behaviour of dusky pasture scarab larvae using slices of carrot under different temperatures. It was found that the larval consumption of food peaked at 30°C while, interestingly, the efficiency of conversion of ingested food (which accounts for larval growth and the mass of food consumed) peaked at a temperature of 14°C. This study would have been far more valuable, had the larvae been fed on living roots, or a variety of food sources. In a further study by Ridsdill-Smith (1977) it was found that the feeding of dusky pasture scarab larvae declined when the population densities were high, although this was likely a result of a lack of young living roots. This was confirmed by

Ridsdill-Smith & Roberts (1976), who also showed that larval growth reduced as density increased, which was also likely to be due to a limited food supply. The study also suggested that the larvae preferred to feed on younger roots.

One recent study by Johnson, Lopaticki & Hartley (2014) provided evidence of compensatory feeding by the dusky pasture scarab larvae under elevated atmospheric CO_2 (eCO_2) on *Microlaena stipoides,* a C_3 grass. Despite this increased feeding, the performance of the dusky pasture scarab was much lower under these eCO_2 conditions, which was likely due to a reduction in the root nitrogen concentrations. Interestingly, under ambient CO_2, larvae consumed 48% more material from *M. stipoides* than from *Cymbopogon refractus*, a C_4 grass. Generally, C_3 grasses are thought to be more susceptible to herbivory than C_4 grasses (Caswell *et al.* 1973). More studies of this type are necessary to elucidate the relationship between scarabs and their host plants, particularly when considering changes in feeding behaviours as a result of climate change. It can be concluded from these studies that the feeding behaviour of the dusky pasture scarab larvae is strongly influenced by abiotic factors such as temperature and, indirectly, atmospheric CO_2. As such, future investigations should investigate host plant preferences alongside abiotic and biotic interactions, including changes in atmospheric CO_2 concentrations.

2.4 Abiotic soil factors

Abiotic factors have been seen to have a strong influence on insect pests of Australasia (Powell *et al.* 2003). All root feeding insects respond directly to their immediate physical and chemical environment (Barnett & Johnson 2013). Here, we review some significant abiotic factors impacting on scarab larvae: temperature, moisture and fertilisation. We focus on species within Dynastinae, Rutelinae and Melolonthinae found in Australasia. We also draw on studies of other species within these subfamilies outside Australasia to indicate the general impact of abiotic rhizospheric factors on scarab larvae. These factors are considered with a view to highlight where agricultural practices could be modified to reduce damage by scarab larvae (discussed in more detail in 'Applied perspectives' section 2.6).

2.4.1 Temperature

The temperature of the soil can impact significantly on scarabs, particularly in the egg and early larval stages. For example, temperature has been seen to have an impact on population fluctuations of the African black beetle (East, King & Watson 1981; King, Mercer & Meekings 1981a). Despite this importance, few studies have focussed on the temperature preferences for oviposition by scarab females.

Regarding larval stages, a single exposure of 35°C for 24 hours has been shown to kill 100% of first instar larvae of *Anoplognathus* spp. and the dusky pasture scarab, while around 62% survive when exposure to such temperatures is only for 12 hours (Davidson, Wiseman & Wolfe 1972b). Within the same study, second instar larvae showed a higher tolerance for high temperatures, for example at 37.5°C, 73% of first instar larvae died while only 40% of second instar died. Regarding the lower temperature threshold it is generally understood that at low temperatures (below 16°C) scarab eggs will take longer to hatch and larvae will take longer to develop (Davidson, Wiseman & Wolfe 1972a). This relationship between temperature and development was investigated in greyback cane beetle pupae (Logan & Kettle 2007), where the minimum and maximum time for pupal development was found to be 26 days at 30°C and 75 days at 18°C, respectively. The low temperature threshold, at and below which no development occurs was 12°C. There are several studies showing the influence of temperature on the growth and development of the dusky pasture scarab (Davidson, Wiseman & Wolfe 1972a; Ridsdill-Smith 1975; Ridsdill-Smith, Porter & Furnival 1975; Cairns 1978). The relative growth rate of these larvae was found to have lower and upper temperature limits of 5°C and 32°C, respectively, with optimum growth occurring around 17.5°C (Ridsdill-Smith, Porter & Furnival 1975). One study on *Rhizotragus majalis* Razoumowsky (subfamily: Melolonthinae), indicated that later instar larvae have much greater mobility and therefore older scarab larvae are likely to be less susceptible to temperature stress through avoidance behavior (Villani & Nyrop 1991). This was confirmed by Zhang *et al*. (2003) who confirmed higher mobility in second and third instars by monitoring their acoustic sounds, which also increased with soil temperature, while below 9°C sound production fell to a minimum. Overall, temperature plays an important role in the

survival, and the rate of development of scarab larvae. Generally, larval growth rate increases with temperature, where upper limits tend to be between 35-40°C, and as temperatures drop to 16°C or below, development is significantly reduced. First instar larvae tend to be the most sensitive to temperatures stress, while scarab eggs and later instar larvae are more tolerant.

These larval responses to temperature indicate how significant climate can be to larval populations. Indeed, high temperatures at a particular time of development can have particularly large impacts on greyback cane beetle populations. Horsfield *et al*. (2008) analysed larval damage records and climatic averages from 1989 to 2003 and showed that prolonged hot and dry conditions during the late spring can limit population numbers by impacting on emergence, as well as synchrony of emergence with feeding, mating and egg laying. Conversely, milder and wetter spring season can promote adult emergence and the ability of the adults to successfully feed, mate and lay eggs. This would directly impact on successive larval populations and therefore damage to cane the following year.

2.4.2 Moisture

Soil moisture is often referred to as the most important property that affects the development and survival of scarab larvae belowground (Brown & Gange 1990; Barnett & Johnson 2013). Indeed, eggs of many scarab species must absorb water before hatching (Potter 1983), hence the availability of water in the soil can be critical to scarab population dynamics. Soil moisture is also the factor best examined in the literature with regards to female oviposition in scarabs (Potter 1983; Cherry, Coale & Porter 1990; Allsopp, Klein & McCoy 1992; Logan 1997). Several studies have shown different optimal soil moisture conditions for maximum oviposition. Some Melolonthinae scarabs are known to oviposit in soils around field capacity (-74 kPa) (Logan 1997), while others within the same subfamily prefer a range between field capacity and dry soil near wilting point (-1500kPa) (Logan 1997). Ward & Rogers (2007) carried out a study on soil moisture ovipositional preferences in four Melolonthinae scarabs found in Australia, including the greyback cane beetle. It was concluded that those species adapted to the semi-arid tropics, where rainfall is unreliable, have little or no preferences observed beyond a reduction in oviposition

in very dry soil (-1500 kPa). However, in subtropical and temperate (with less seasonal rainfall) adapted species there were clear preferences for drier soils (-1000 kPa). This suggests that the climates in which key/target pest species have originated and are adapted to, must be considered in attempts to manage populations. It also indicates that for those tropically adapted species, moisture control as a form of pest management may not be the way forward, as their ovipositional preferences are likely to be driven by factors other than soil moisture.

Moisture content of the soil can directly impact on scarab larvae populations. African black beetle populations, for example, have been shown to be suppressed in regions with early summer rainfall (Matthiessen & Ridsdill-Smith 1991) as first instar larvae are more moisture sensitive than egg stage or later instars (King 1979; King, Mercer & Meekings 1981a). In periods of seasonal drought, the larval populations are no longer suppressed by the normally high moisture content, resulting in damaging outbreaks (Matthiessen & Ridsdill-Smith 1991). Whether these population responses would be the same in different soils is uncertain. Matthiessen (1999) showed that soil type had a significant impact on African black beetle larval survival, and that this factor interacted with soil moisture, where larval survival was higher under regular watering treatments compared with no watering, but only in some soil types. With these studies in mind, investigations are necessary to elucidate the interaction between soil moisture and soil texture, where larval populations are monitored under different common soil types in the field, under a range of soil moisture treatments. Future work should also include extreme climate events, such as drought and flooding, as the frequency of such events are predicted to increase in the future (IPCC 2014). This way, we can gain a better picture of how belowground scarab pest status will change in the future.

Several studies have reported responses from other scarabs to soil moisture. For example, within the genus *Cyclocephala*, larvae are significantly more abundant and also have higher mass in irrigated, compared to non-irrigated plots (Potter *et al.* 1996). Survival of dusky pasture scarab larvae have been shown to be optimal between -100 to -150 kPa, while in saturated soils, larval survival is negatively proportional to the length of exposure (Davidson, Wiseman & Wolfe 1972a). Indeed,

studies involving *R. majalis*, have shown that larvae move quickly towards the surface when the moisture content of the soil is increased, yet little movement is exhibited in response to drought conditions (Villani & Wright 1988).

Changes in soil moisture will also impact the host plants of scarab larvae. In addition to this, the diffusion of plant root volatiles is reduced in high soil moisture, however some moisture is required to prevent total vertical diffusion (Hiltpold & Turlings 2008). Indeed, natural enemies of scarab larvae, such as entomopathogenic nematodes (EPN), are more effectively recruited by plant volatiles and have higher virulence in soils with high moisture content (Grant & Villani 2003). Therefore future studies into the effects of different soil moisture contents within a variety of soil types, would also benefit to consider how the natural enemies and pathogens respond under these conditions. This way a more holistic and ecologically relevant picture can be constructed.

2.4.3 Fertilisation

The response of soil dwelling root feeders to fertilisation has received some attention within the literature. Frew *et al*. (2013) found that the application of nitrogen (N), phosphorus (P) and potassium (K) fertilisers promoted more nutritionally superior grass species, which in turn increased abundance of dusky pasture scarab larvae. However, Potter *et al.* (1996) investigated the effects of different agricultural practices on scarab populations over three years and found no significant effect of N, P, K fertiliser on *Cyclocephala* spp. density or growth. Radcliffe (1970) added organic (cow dung) fertiliser to the soil and found that this lessened the damage to grass roots by *C. zealandica.* This may have been where the larvae switched from feeding on the grass roots to the increased provision of organic matter in the soil, or the addition of excess organic matter may have contributed to better compensatory root growth in response to damage, or a combination of both. In the same study it was found that larvae development was more advanced when treated with N fertiliser (Radcliffe 1970). It has also been shown that the addition of organic fertiliser increases the mass gain of *C. zealandica* larvae (Wightman 1974). In contrast to these findings, other studies on *C. zealandica* have shown the addition of N fertilisers has had no effect on larval feeding and survival (Prestidge, Zijpp & Badan 1985) or

population density (Prestidge & East 1984), with similar responses found with *Popilla japonica* Newman (subfamily: Rutelinae) to the application of N, P, K fertiliser (Crutchfield, Potter & Powell 1995). Other root feeding insects have been shown to respond positively to the addition of N fertiliser, such as the rice weevil larvae (*Lissorhoptrus oryzophilus* Kuschel (Curculionidae, Erirhininae)) and the western corn rootworm (*Diabrotica virgifera virgifera* LeConte (Chrysomelidae, Galerucinae)) (Spike & Tollefson 1988). In the comprehensive review of belowground herbivores by Brown & Gange (1990), it was suggested that the timing of fertilisation is important to the effect on the root feeding larvae. They suggested that if N fertiliser is applied before larvae are present then this promotes root growth, which in turn gives a greater food supply to larvae, while if fertiliser is added after larval establishment then the damage to grasses is less (Spike & Tollefson 1988).

It is known in some plants that when N is limiting in the soil, plant defence investment increases in the leaves (Schmelz *et al.* 2003; Chen, Ruberson & Olson 2008). Low soil N content could similarly affect root defence investment allocation, thereby impacting the root feeding scarab beetle larvae populations. It has been suggested that fertilisation may cause a reduction in the defensive root compounds (Hol 2011; Erb & Lu 2013). These may be direct secondary defences affecting scarab feeding or performance, or indirect defences involving recruitment of natural enemies such as EPNs (see section 2.5.1 on 'Pathogens, natural enemies and symbionts'). Such plant responses to fertilisation addition could be linked to arbuscular mycorrhizal (AM) fungal associations. AM associations have been shown to increase induced plant defence responses (Pozo & Azcón-Aguilar 2007), but root colonisation by AM fungi is known to be reduced when soil nutrients (particularly P and N) are high (Vannette & Hunter 2009; Smith & Read 2010). Therefore any decrease in plant defences in response to high N, could be mediated by limited AM colonisation.

Overall, the literature is not consistent regarding the impact of fertilisation on scarab larvae and similar species, although both positive and null effects seem to be the most common responses reported. Any positive effect is likely to be due to an increase in organic matter for younger instar scarabs to ingest and an increase in the nutritional value of host plant species. An increase in nutrient availability may also

result in an increase in the tolerance of the host plant to herbivory, although this is likely to be dependent on the nutrient and specific herbivore in question (Wise *et al.* 2007). This may also impact on important microbial plant associations in the soil (Smith & Read 2010), which can indirectly impact on herbivores (Bennett & Bever 2007; Biere & Bennett 2013). Therefore soil fertility may promote root feeding scarabs, but also may increase plant tolerance to herbivory as well as benefit the natural enemies of scarabs belowground. Continued research should aim to include as many contributing factors to plant–insect interactions within the soil (such as AM fungi and EPNs) as possible, as these are likely to produce outcomes more relevant in the field.

2.5 Biotic soil factors

2.5.1 Pathogens, natural enemies and symbionts

Scarabs have a number of natural enemies and insect pathogens that threaten their survival. Scarab larvae have evolved within the soil environment, which naturally brings them in close contact with numerous soil organisms and microbiota, some of which are pathogens (Jackson & Klein 2006). Here we discuss some pathogens and natural enemies that have been identified to hold potential as biocontrol agents against scarab larval pests in the field.

Entomopathogenic fungi are ubiquitous in soils, particularly those within the genera *Metarhizium* and *Beauveria.* Greyback cane beetle larvae are easily infected by the entomopathogenic fungus *Metarhizium anisopliae.* The impact of this naturally occurring fungus on the larval populations is not density dependent and as such has been shown to account for a fixed mortality rate, regardless of the population density, while the spores are known to be resistant to many agricultural practices (Sallam, Bakker & Dall 2003; Sallam *et al.* 2007). This fungus has been isolated and commercialized as BioCane^{TM} and used as a fungal biocontrol that in trials has shown more than 50% control of the canegrub after six months of a single application (Logan *et al.* 2000). Interestingly, Berón & Diaz (2005) carried out susceptibility trials of the Argentine scarab larvae to different strains of *M. anisopliae*. All strains showed low virulence against the larvae, possibly due to the lack of host specificity to the

Argentine scarab. However, a particular strain of the entomopathogenic fungus *Beauveria bassiana* did show up to 70% mortality in Argentine scarab larvae. The differences in virulence of *M. anisopliae* towards different scarab species larvae shows how the insect response to microbial pathogens can often be species specific, and can vary significantly. Another *Beauveria* sp. that has shown success as a biocontrol is *B. brongniartii*, which has been successful acting against a broad range of hosts. Some native strains have been isolated from *Melolontha melolontha* Linneaus (subfamily: Melolonthinae) and used as pest controls across Europe with good success (Dolci *et al.* 2006). Similar work with *Beauveria* strains isolated from Madagascar and Turkey have also seen success (Maurer *et al.* 1997; Sevim *et al.* 2010). These are further examples of successful isolation and application of naturally occurring scarab pathogens.

A significant pathogenic microorganism, particularly noted in efficacy against the greyback cane beetle larvae, is the protozoan *Adelina* sp. which is a density dependent pathogen (Robertson *et al.* 1998). High *Adelina* incidence causes a drop in the larval population which in turn impacts on the *Adelina* incidence in the soil. Interestingly, Sallam, Bakker & Dall (2003) found that *Adelina* incidence was higher in soil with grass cover compared to bare soil areas, which could be due to higher moisture retention and cooler temperatures. Responses such as these should be taken into account when managing larval populations in agriculture to optimise natural pathogen efficacy.

Within New Zealand, the bacteria *Serratia entomophila* and *Serratia proteamaculans* were isolated from *C. zealandica* as the cause of amber disease, which leads to the cessation of feeding of the scarab grub resulting in eventual death (Hurst, Glare & Jackson 2004). These bacteria were developed as biopesticides against scarabs and have been used for almost twenty years as biocontrol agents. These are further examples of microbial pathogens adapted to their host, and their host range which were used to great success as a control method of scarabs (Hurst *et al.* 2000).

There are a number of viruses that infect scarabs, such as pox viruses and iridescent viruses; however little research has been done on their potential as biocontrols, and

their presence and effect on scarab populations under natural conditions has not yet been documented (Jackson & Glare 1992). Damage by the Dynastinae scarab larvae within the genus *Oryctes* has been successfully mitigated via the *Oryctes* virus (Huger 2005), which is a unique virus, in that it was identified as the first rod-shaped, non-occluded insect virus, and is highly infectious. It has been isolated, purified and used in pest control for over 10 years, but it has low success on any species outside of the target scarab genus *Oryctes* (Huger 2005). Current research is focussed on selecting strains of the virus for greatest persistence in the environment.

One of the major natural enemies of scarabs are EPNs, which are internal parasites of scarabs. They do not act alone, but rather it is their association with entomopathogenic bacteria that kill the scarab hosts. *Steinernema* and *Heterorhabditis* are the two genera of EPNs and there are a number of species within both genera that infect scarabs (Klein 1993). The EPNs kill the larvae via their symbiotic bacteria *Xenorhabdus* spp.. Several species have been isolated from scarab grubs, such as *Steinernema glaseri*, *S. anomaly, Heterorhabditis megidis,* and several different strains of *S. carpocapsae* and *H. bacteriophora* (Klein 1993), and their potential to control scarab larvae populations is being investigated. Some nematodes have shown success in laboratory and field trials against scarab larvae, with particular interest in *Steinernema scarabaei* as an effective control against a range of scarabs dominant in North America and Asia (Stock & Koppenhöfer 2003). However, other efforts to use EPNs in the field have not been successful, which have been attributed to a lack of understanding of the nematode *Beauvieria* bacterium complex and differences in target species susceptibility, biology or behaviour (Klein 1993; Georgis *et al.* 2006). Recently Wu *et al*. (2014) tested and compared the virulence of four EPN species and their interactive effects with entomopathogenic fungi against the scarab larvae of *Cyclocephala lurida* Bland (subfamily: Dynastinae). They concluded that the impact of *H. bacteriophora* alone or in combination with the fungal pathogens was comparable to that of an imidacloprid insecticide against the larvae. This indicates the potential EPNs have as biocontrols and that further work is warranted to fully elucidate the interaction between natural enemies, pathogens and host. Plants can recruit EPNs via attractive volatile signals as a natural defense strategy (Grewal *et al.*

1994; Rasmann *et al.* 2005). It has been shown that EPNs can be selectively bred for enhanced responsiveness to these volatile cues (Hiltpold *et al.* 2010), meaning that improved efficacy of commercial EPN use is still ongoing and holds great potential as a biological control method of scarabs in agriculture and industry.

Finally, diverse communities of endosymbiotic bacteria that assist with the digestion of plant material, particularly cellulose and hemicelluloses, live within the hindguts of scarab larvae (Cazemier *et al.* 2003; Huang *et al.* 2010). Pittman *et al*. (2008a) found that there were species within the bacterial community of the greyback cane beetle larvae hindgut that were consistently found within the larvae across their geographical distribution. These bacteria were successfully transformed and reintroduced into the hindgut of the larvae, which indicates they are strong candidates to control the populations of greyback cane beetle larvae through the expression of anti-feeding compounds within the larval gut (Pittman *et al.* 2008b). Non-resident bacteria are normally not useful in such paratransgenic control methods because they are unable to remain established within the gut (Chapco & Kellin 1994). Therefore the discovery, successful transformation and establishment of these candidate bacteria within the greyback cane beetle larval gut provides good grounding for the future development of paratransgenic control methods of the larvae.

2.6 Applied perspectives

We have discussed the impacts of some abiotic and biotic factors within the soil environment that impact on scarab larval populations. Many agricultural practices interact with these factors within the soil, and could potentially mitigate or exacerbate scarab damage to grasses and crops (Barnett & Johnson 2013) (see Figure 2-5 for a summary of key interactions within an applied context).

Scarab larvae have been shown to respond to the application of fertilisers (Wightman 1974; Frew *et al.* 2013). However it is important to note that AM–plant associations can be negatively impacted by fertilisation (Smith & Read 2010). Therefore, the application of N, P, K fertiliser, particularly to newly establishing crops or pastures should be kept to a minimum, to minimise any positive impacts on scarab populations

and to ensure effective AM colonisation to enhance grass productivity and defences. The addition of mulch, is commonly used to conserve moisture and generally improve soil fertility, and therefore could reduce the priming of plant defences to herbivores by reducing AM colonisation (Grant *et al.* 2005; Smith & Read 2010).

Mulch also affects temperature, which in turn may influence scarab beetle larvae. Different types of mulch have been shown to have different effects on the temperature of the soil (Ramakrishna *et al.* 2006). For example, polythene mulch has been shown to increase soil temperature by 6^{o}C, while straw mulch also increased soil temperature, but to a lesser extent (Ramakrishna *et al.* 2006). Contrastingly, a study by Lal (1974) found that mulch consistently decreased the maximum soil temperature across a range of depths (5, 10 and 20 cm), with the biggest difference of 8°C, seen at 5cm below the soil surface. Tillage is another agricultural practice which has been shown to affect soil temperature (Griffith *et al.* 1973; Malhi & O'Sullivan 1990; Licht & Al-Kaisi 2005). Conventional tillage increases top soil temperatures by 2.8°C compared with no tillage (Malhi & O'Sullivan 1990), although smaller increases in temperature of 1.9°C have also been reported (Licht & Al-Kaisi 2005). Higher soil temperatures (depending on climatic conditions) reduce greyback cane beetle populations (Horsfield *et al.* 2008), and first instar larvae of the dusky pasture scarab have been found to be the most temperature sensitive (Davidson, Wiseman & Wolfe 1972b). However, other common practices such as irrigation are known to lower soil temperatures by up to 3.8°C (Wang *et al.* 2000).

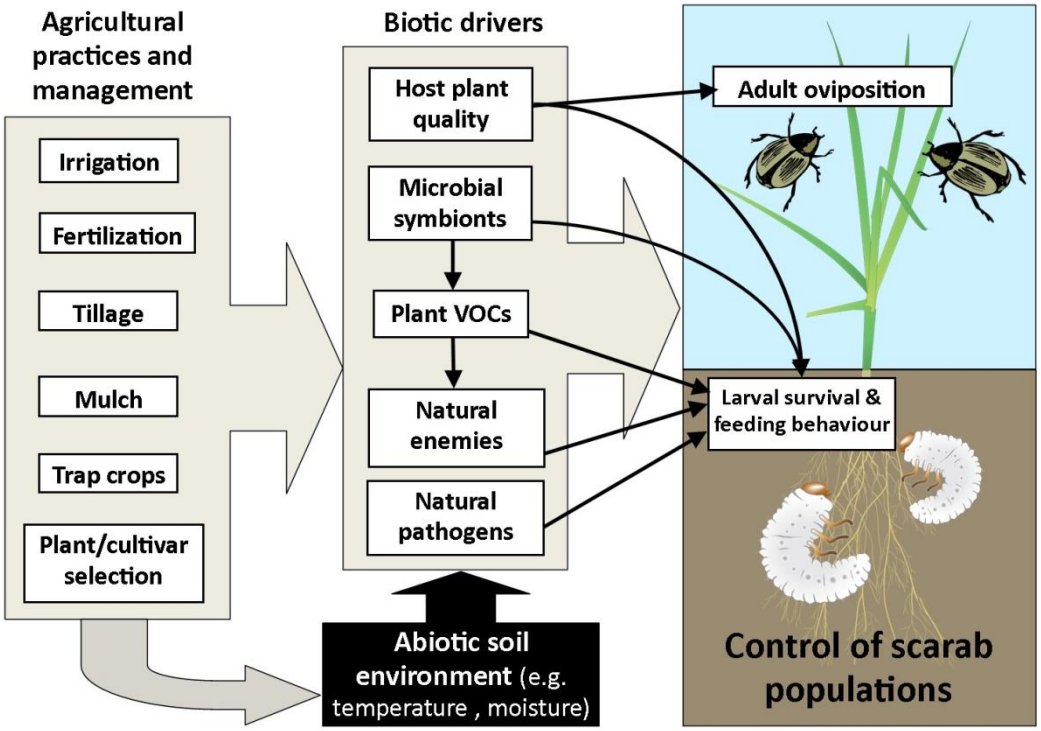

Figure 2-5. Diagram of agricultural practices and management factors that impact on plant and soil factors (abiotic and biotic), which in turn can influence oviposition by adults together with larval survival and feeding behaviour. Arrows indicate key linkages between interacting factors.

Taking these effects into account, the timely refrain from irrigation alongside the application of polythene or straw mulch coupled with tillage, for example, could raise soil temperature sufficiently to impact on larval populations. However, limiting soil moisture could decrease the efficacy of EPN populations within the soil at controlling scarab populations. The effects of raising temperatures in this manner on crop health and yield, however, should also be investigated.

The effects of other land management practices on scarab larvae populations have been reported such as the study by Potter *et al.* (1996), who found that intense mowing of grasses and the addition of aluminum sulfate treatments significantly decreased populations of *Cyclocephala* spp., as well as the average larval mass. This study, however, only was done within one soil type, which is a critical factor (Cherry & Allsopp 1991; Matthiessen 1999), and scarab responses may differ under different soils.

Many crops have irrigation systems in place to ensure sufficient water is supplied, which can lead to very different soil conditions compared to natural systems. Mulch, as discussed, is commonly used in agriculture to conserve moisture and increase fertility of soil, and so it naturally follows that in mulched systems, moisture retention of the soil will be higher (Moody, Jones & Lillard 1963; Lal 1974; Ramakrishna *et al.* 2006). Host plant location by larvae beneath the soil surface could be improved under these moist soil conditions due to the fluid dynamics of root exudates (Gouinguené & Turlings 2002; Hiltpold & Turlings 2008). However, at the same time, natural enemies such as EPNs will also benefit from this phenomenon as it has been shown across several species that EPN virulence increases with soil moisture content (Kung, Gaugler & Kaya 1991; Grant & Villani 2003; Frew *et al.* 2013). Therefore, as practices such as fertilisation may decrease EPN attracting volatiles while irrigation enhances EPN mobility and survival, effective strains of host specific EPNs should be applied to pastures or crops requiring little fertilisation alongside ample irrigation to effectively repress scarab larval populations.

Other soil antagonists can be impacted by land use practices. For example, larvae of the scarab *Ataenius spretulus* Haldeman (subfamily: Aphodiinae) were found, within a golf course environment, to be in greater abundance where the turf had been mowed to fairway height (1.6cm), compared with turf mowed to rough height (5.1cm). This correlated with the number of larvae found to be infected with a bacterial pathogen, *Bacillus* sp., where 68% of larvae were infected in the turf mowed to rough height, compared to 34% of larvae infected in turf mowed to fairway height. In addition to this, *Anoplognathus* spp. and *Sericesthis* spp. larval populations have been shown to peak under moderate grazing pressure, yet were lowest under high intensity grazing (Roberts & Morton 1985). These findings alone are unlikely to have a direct applied significance to all scarab larval pest management. However, they may provide critical information for other managed grassland systems, where decreasing regular mowing or allowing high intensity grazing may mitigate larval infestations in future years. Common practices such as mowing should be investigated for their impacts on critical soil abiotic factors such as moisture alongside scarab larval populations and their interactions with natural pathogens.

In direct attempts to mitigate damage caused by insect herbivores, the 'push-pull' system is a method which aims to utilise repellant or unattractive plants while simultaneously using attractive yet less valuable plants to attract pests away from valuable crops or pastures (Pickett *et al.* 2014). A similar system could be utilised against scarab larval pests. For example, where African black beetle populations are problematic, the use of *T. repens* and *N. lolii* infected *L. perenne* could be used as a repellant (the former of which may also be effective against Christmas beetle larvae (Davidson & Roberts 1968a)), while *L. perenne* and *P. dilatatum* could be utilized within 'trap crops', particularly as areas with *P. dilatatum* are also preferred sites for oviposition. The use of the endophyte *N. lolii* should also be utilised in the form of replacing or incorporating endophyte infected *L. perenne* into managed pastures. Indeed, *P. dilatatum* could also be useful, alongside *C. dactylon* in 'trap cultures' for other Dynastinae species such as the Argentine scarab (Carne 1957a). It has been suggested, however, that the efficacy of 'push-pull' systems would be improved if a better understanding of the mechanisms were obtained, for example the specificity and distance ranges of plant volatile cues (Eigenbrode *et al.* 2016).

In the end, where effective biocontrol methods are commercially available, these should be employed in conjunction with the use of agricultural and land-use practices, such as irrigation and mowing (where applicable) to create optimal conditions for efficacy and infectivity. Where scarab plant host preferences are known (for feeding or oviposition), these can be employed in 'push-pull' strategies, to limit larval populations in areas of interest. Where either of these are unavailable or remain unknown, such is the case for some of our focal species, timely utilisation of certain land-use practices can be applied to create poor conditions for the scarab populations (e.g. during the first instar, when larvae are most vulnerable to temperature stress). Indeed, in either situation, encouragement of natural beneficial soil microbes (such as AM fungi) should also be applied. However, as there are gaps in the knowledge for ecology of many scarab species, the direction of future research is of primary importance in improving strategies to limit pest scarab larvae in grasses across Australasia.

2.7 Future directions

2.7.1 Basic ecology

Some of the work on the basic ecology of scarab larval pests to grasses was carried out over 20 years ago (Carne 1957a; Carne & Chinnick 1957; Ridsdill-Smith 1975), with little research on particular species since. It is our belief that for those species where there remains some paucity of knowledge in their basic ecology, feeding trials looking at host preference alongside population monitoring under different conditions (this includes monitoring of abiotic factors and microbial sampling) should be prioritised. With this knowledge, more effective implementation of strategies such as 'push-pull' systems or other agricultural practices that suppress scarab beetle populations can be applied within context. This means management systems could take into account species specific responses, accounting for local abiotic and biotic interactions.

2.7.2 Volatile cues

The effectiveness of classic pest management strategies such as 'push-pull' systems have recently been criticised, particularly for focussing too much on long-range effects, and should consider all cues that can work synergistically (Eigenbrode *et al.* 2016). Indeed we would concur with this framework for application to belowground pests, but such behavioural cues would first require investigation. We recommend that future research should investigate olfactory cues of pest larvae and their natural enemies belowground to plant roots, and how these may interact with common agricultural and land-use practices. Experiments such as those carried out by Rasmann *et al*. (2005) using six-arm olfactometers are an ideal starting point to determine attractiveness of plant species to scarab larval pests and/or their natural enemies.

2.7.3 Pathogens and microbes

Biocontrol of scarab pests has been particularly successful where a naturally occurring pathogen is identified, isolated and then applied within its naturally occurring range (Maurer *et al.* 1997; Hurst *et al.* 2000; Sallam, Bakker & Dall 2003; Dolci *et al.* 2006; Sallam *et al.* 2007; Sevim *et al.* 2010). Hence, knowledge of

belowground community composition is important if native microbes or EPNs are to be utilised in the control of insect pests in the soil. Using methods similar to that of Sevim *et al*. (2010), the presence of naturally occurring scarab pathogens could be identified using a baiting method (Zimmermann 1986). The pathogen can then be isolated from infected larvae and the DNA sequenced; effective isolates can then be used in bioassays to test pathogenicity against the target pest species. We recommend the isolation, identification and ultimately the application of natural pathogens, where possible. The persistence of scarab pathogens in the soil indicates some level of evolutionary success, which should be exploited in efforts to control problematic species.

2.8 Concluding remarks

Here, we have presented information on several key scarab larval species within three subfamilies, known to cause significant damage to grasslands and crops within Australia and New Zealand. While the ecology of some species has been well researched, information on others, including the Argentine scarab, has not been described in any detail. The feeding behaviour and general ecology has been investigated for species such as African black beetle larvae and greyback cane beetle larvae. These pests have had significant attention as a result of their impact on agriculture, and control methods such as the application of natural pathogens, or the application of host plant endophytes have shown noteworthy promise. Although our knowledge is somewhat limited for some species, there is good evidence that changes in management can potentially have a large impact in limiting damage to crops and grasslands. Overall it seems clear that in terms of improved pest management of scarab larvae, it does not make sense to run before we can walk. Immediate research concerns should lie with filling gaps in the ecology of scarab species within Australasia. This should include assessing population dynamics, interactions and influences with abiotic factors within the local environment. In addition to this, successful biocontrol strategies, both within and outside Australasia, have utilised naturally occurring pathogens and natural enemies, which are adapted to their host and local environment. Therefore, similar strategies need to be central to future biocontrol research on Australasian scarab pests. Overall, pest management

strategies that are applied within context would be more effective with an improved fundamental ecological understanding of key scarab pests.

2.9 Acknowledgements

This work was undertaken as part of a research project funded by the Hawkesbury Institute for the Environment (HIE) and Sugar Research Australia. It is produced as part of a series of articles from the ninth Australasian Congress of Grassland Invertebrate Ecology and with sponsorship from HIE. The authors would like to thank the reviewers for their invaluable comments.

Chapter 3: Do eucalypt plantation management practices create understory reservoirs of scarab beetle pests in the soil?

Published as Frew *et al*. 2013, *Forest Ecology and Management*, **306**, 275-280

3.1 Abstract

Eucalypt management practices can affect the population dynamics of defoliating insects. To date, research has focussed on how these practices alter eucalypt physiology and chemistry, which in turn affect canopy herbivores. Management practices such as irrigation and fertilisation, however, could also shape the understory plant community and potentially improve habitats for grass root-feeding scarab beetle larvae that later can become defoliators as adults. Using a large scale factorial field experiment comprising 2560 *Eucalyptus saligna,* we investigated the effects of irrigation and fertilisation on the understory ecology of a eucalypt plantation. We specifically focussed on grass communities and populations of scarab beetles and their natural enemies (entomopathogenic nematodes, EPNs). Irrigation and fertilisation increased grass coverage by 40% and 42%, respectively, and affected grass species composition. In particular, fertilisation favoured colonisation with C_3 grasses (e.g. *Microlaena stipoides*) that have higher nitrogen concentrations over lower quality C_4 grasses (e.g. *Setaria incrassata*). Fertilisation increased the nitrogen concentration of grasses by 30% on average. Scarab abundance increased by 52% in fertilised plots, potentially due to higher nutritional quality of host plants and the dominance of nutritionally superior species. Irrigation increased soil water content, but did not promote scarab larvae abundance. The presence of EPNs, however, was 78% higher in irrigated plots, which suggests scarab larvae populations may have been controlled by EPNs. This study illustrates how plantation management practices can affect understory communities of both plants and soil invertebrates with potential for creating 'reservoirs' of scarab beetle pests.

3.2 Introduction

Timber plantations in Australia have increased in area by 51% over the last 10 years, with eucalypt plantations accounting for the biggest increase, now covering 0.99 million hectares (49% of total plantation area) (Gavran & Parsons 2010). Moreover, eucalypts are becoming increasingly conspicuous as the most widely planted hardwood species in the world (Turnbull 1999; Paine, Steinbauer & Lawson 2011). Defoliating insects can cause significant damage to eucalypt plantations (Ohmart & Edwards 1991; Paine, Steinbauer & Lawson 2011). Even partial defoliation can decrease productivity and growth of eucalypts (Quentin *et al.* 2011). In addition to reducing transpiration rates of leaves, defoliators often render trees more susceptible to other pests and pathogens (Mackay 1978) and frequently play a central role in plantation dieback (Jurskis 2005). Amongst the defoliators, several species of scarab beetles (Coleoptera: Scarabaeidae) are known to sporadically cause severe damage and can even lead to widespread tree mortality (Browne 1968; Ohmart & Edwards 1991; Paine, Steinbauer & Lawson 2011).

Many of these scarab beetles feed on grass roots during their soil dwelling larval development before switching to eucalypts as adults. While eucalypt species vary in their susceptibility to these scarabs (Pryor & Johnson 1981), several scarab species are considered significant pests of eucalypts. For example, *Sericesthis* spp. alongside the Christmas beetles (*Anoplognathus* spp.) are common defoliators and are a particular problem in several important plantation eucalypt species (Johns, Stone & Hughes 2004). In addition, the African black beetle (*Heteronychus arator*) is regarded as the most damaging invasive insect pest of Australian eucalypt plantations (Paine, Steinbauer & Lawson 2011), where the adults feed at the base of young trees, killing them by removing their bark.

Because defoliating insects can cause widespread damage in both hardwood and softwood timber plantations, some researchers have examined how management practices might affect their ecology and population dynamics (Ciesla 2011). In particular, the roles of irrigation and fertilisation have been investigated to determine whether these exacerbate pest problems (Nowak & Berisford 2000; Coyle 2002; Coyle, Nowak & Fettig 2003; Coyle, Booth & Wallace 2005; Paine & Hanlon 2010).

These studies focussed on how management practices affect defoliating insects via changes in tree physiology and chemistry (Hopmans *et al.* 2008; Paine & Hanlon 2010; Edenius *et al.* 2012). Irrigation and fertilisation, however, may also affect understory plant communities and alter soil conditions, and through this influence the abundance and diversity of insect pest species. In addition to neighbouring pastures (Landsberg & Wylie 1988), the grassy understories of eucalypt plantations are prime habitats for scarab beetle larvae, many of which respond to biotic and abiotic changes in their environment (Villani & Wright 1990; Villani *et al.* 1999). The effects of management on the understory may therefore create 'reservoirs' of insect pests, particularly the root feeding scarab beetle larvae, which then emerge as adult defoliators in spring–summer (Paine, Steinbauer & Lawson 2011). Scarab beetles species have overlapping perennial generations with larvae found in the soil all year around.

Fertilisation and irrigation potentially affect root feeding insects in different ways (Johnson & Murray 2008). Soil moisture is often the most important property to affect root herbivores (Brown & Gange 1990). For instance, if the soil is dry then there is risk of larval desiccation (Johnson *et al.* 2010a), and if there is insufficient rainfall throughout spring and summer adults are often unable to emerge from the pupal stage and die (Goodyer & Nicholas 2007). Conversely, reduced soil moisture has led to scarab outbreaks in New Zealand through improved survival (King, Mercer & Meekings 1981c). In addition, the application of fertiliser can increase the weight gain of larvae (Wightman 1974), presumably because higher quantities of nutrients in the soil improve the nutritional quality of the roots. While valuable for indicating the likely effects of fertilisation and irrigation on root-feeding insects, these laboratory studies did not realistically replicate plantation conditions.

Using a large scale factorial field experiment comprising 2560 *Eucalyptus saligna* over an area of 5 ha, this study investigated the effects of irrigation and fertilisation on the understory ecology of a eucalypt plantation, focussing on grass communities, soil-dwelling populations of scarab beetle larvae and their natural enemies, entomopathogenic nematodes (EPNs). *Eucalyptus saligna* is a close relative of *Eucalyptus grandis*, a species that is highly susceptible to scarab beetle attack

(Carnegie *et al.* 2008). We aimed to characterise how these factors affected grass communities (species and understory coverage), grass nutritional quality (carbon and nitrogen concentrations), canopy coverage, soil water content, together with the abundance of scarab larvae and EPNs. We hypothesised that larval abundance will be promoted by both irrigation and fertilisation through reduced beetle desiccation and increased grass quantity (coverage) and quality (nitrogen concentration), respectively. We further hypothesised that EPN abundance would be positively affected by irrigation and fertilisation as a consequence of greater host abundance.

3.3 Materials and methods

3.3.1 Site description – Hawkesbury forest experiment

The field site (5 ha) was converted from a native grassland to an enclosed paddock in 1997. The site is situated on an alluvial floodplain near the Hawkesbury River in western Sydney (Australia) at 25 m elevation (33°36'40''S, 150°44'26.5''E). The soil – described fully in Barton *et al*. (2010) – is of the Clarendon Formation type (Isbell 2002), an alluvial formation of low-fertility sandy loam soils (top 70 cm) with low organic matter content (0.7%), moderate to low-fertility (available P, 8 mg kg^{-1}; exchangeable cations: K 0.19; Ca 1.0; Mg 0.28 mEq, 100 g^{-1}) and low water holding capacity. In March 2007 the site was cleared of vegetation with glyphosate herbicide (Roundup™, Scotts Australia Pty Ltd., Baulkham Hills, NSW, Australia) treatment before planting 2560 *E. saligna* (details below) in April 2007 at a density of 1000 trees ha^{-1} (2.6 × 3.85 m tree spacing). At planting, trees were supplied with insecticidal imidacloprid tablets (Initiator™, Bayer Crop Science, East Hawthorn, VIC, Australia). These tablets also contained nutrients (N, P, K, Mg) to promote initial plant growth. At this point, the site was free of understory plants. From November 2008, no pesticides or herbicides were applied and natural grass colonisation between trees was allowed. By December 2011 the grasses at the site were dominated by African love-grass (*Eragrostis curvula*), weeping meadow grass (*Microlaena stipoides*) and couch grass (*Elymus repens*). Other grass species were present in smaller quantities, including summer grass (*Digitaria sanguinalis*), pigeon grass (*Setaria incrassata*), windmill grass (*Chloris truncata*) and cocksfoot grass (*Dactylis glomerata*).

3.3.2 Treatments

Sixteen plots, each containing 160 trees in 10 rows of 16 *E. saligna* (provenance Styx River, NSW; seedlot 20752 CMA from the Australian Tree Seed Centre, Clayton South, VIC, Australia) were designated for irrigation and fertilisation treatments using a 2 × 2 factorial design, such that four plots received both irrigation and fertilisation, four received just irrigation, four were fertilised only and the remaining four were left untreated. Treatments were initiated when the trees were planted in April 2007. Irrigation treatments were applied every four days throughout the year using an *in situ* irrigation system that delivered water evenly throughout the plot via 65 spray heads, equivalent to 15 ml of rainfall (24,000 L per plot year^{-1}). Fertilisation treatments were also applied every four days between September and April at a rate of 150 kg N ha^{-1} (Nutrifeed19 and Liquid N, Amgrow Fertilisers, Lidcome, NSW, Australia). At the time of this study (January 2012) trees typically ranged in height, being 12.9 ± 0.27 m, 12.8 ± 0.29 m, 16.9 ± 0.35 m and 17.4 ± 0.32 m for control, fertilised, irrigated and both irrigated and fertilised trees, respectively. The diameter of trees at 65 cm from the ground was 15.3 ± 0.32 cm, 15.2 ± 0.63 cm, 18.1 ± 0.64 cm and 19.5 ± 0.59 cm for these same trees (B. Amiji, personal communication).

3.3.3 Soil and grass sampling

The sampling was performed over two weeks during January 2012 when the plantation understory had become properly established (c. three years after the last applications of insecticides and herbicides). Larval densities had been observed to be greatest during January (G. Lopatiki, personal communication), and the new generations of larvae laid from eggs in the previous spring were sufficiently large to distinguish and recover from the soil. Four 1 m^2 sample locations from each plot were selected at random. Soil moisture was measured in each sample location using a moisture probe – three readings were taken and averaged. Grass coverage of the 1 m^2 sample areas was estimated using a 1 m^2 quadrat split into a grid of 100 sections (10 × 10 cm each). Grass and soil were taken from a 20 cm × 20 cm area in the centre of the 1 m^2 sample area, to a depth of 10 cm (c. 4 L). Grass and soil were separated and examined by hand for presence of beetle larvae, which were counted and identified to species level for scarabs and to family level for other beetles (Lawrence

et al. 2000). A 1 kg sub-sample of soil was taken for nematode extraction (see below). The grasses were snap frozen and stored at −20 °C, freeze dried and ground before analysing shoots for carbon and nitrogen concentrations using a LECO TruSpec® CHN analyser. Our previous work indicated that shoot and root C and N concentrations are highly correlated in these grass species (S.N. Johnson, personal communication). Shoot concentrations were therefore used as an approximation of how treatments were affecting root C and N concentrations.

3.3.4 Entomopathogenic nematode extraction

We used the *Galleria mellonella* baiting method of Bedding and Akhurst (1975) for detecting the two types of EPNs: steinernematids and heterorhabditids. Two larvae (c. 15 mm long) were placed in a container with 1 kg soil for each of the soil samples collected. To minimise any differences in soil moisture between samples and to reduce potential impacts this may have on EPN extraction, soil samples were not taken on days when the irrigation treatment was applied. The soils were incubated at 25 °C for two days before the larvae were extracted, dissected and examined under a microscope (Olympus CKX41 at × 100 magnification) for presence of EPNs. One soil sample from each plot was used to measure EPN density. All EPNs in each *G. mellonella* cadaver were counted using a 1 mm^2 grid. The *G. mellonella* larvae used were from an established culture at the Hawkesbury Institute for the Environment, UWS.

3.3.5 Estimation of canopy cover

The canopy cover was estimated using a modified protocol taken from Macfarlane *et al*. (2007). Using a digital camera, photographs of the canopy over each plot were taken from the four sides of the plot. Canopy coverage for each plot was estimated by quantifying the proportion of sky in each photograph using Adobe Photoshop® 7.0 (San Jose, CA, USA) and taking an average of the four values from each plot.

3.3.6 Statistical analysis

Two way analysis of variance (ANOVA) with irrigation, fertilisation and an interaction of the two included as fixed effects were applied to analyse grass coverage, soil moisture, canopy cover, grass C and N, larval abundance and EPN abundance. Plot

number was included as a block term in each test, with sample number nested within each block. Chi squared tests were used to analyse whether irrigation and fertilisation affected the incidence of different grass species in each plot. For EPN and scarab larval abundance, plant and soil responses were initially included as covariates but sequentially dropped if non-significant to provide the most parsimonious model. All analyses were conducted on untransformed data unless otherwise stated in figure and table legends using Genstat (version 14, VSN International, UK).

3.4 Results

3.4.1 Understory responses

Although irrigation and fertilisation affected the growth of *E. saligna*, (see section 3.3.2), no treatment affected canopy cover (statistical results shown in Table 3-1), data not shown. The understory was, however, significantly affected by both treatments (Fig. 3-1). In particular, fertilisation without irrigation promoted the C_3 species weeping meadow grass (*M. stipoides*) (X_1^2=4.00, P = 0.045) and led to the exclusion of the C_4 species pigeon grass (*S. incrassata*) (Fig. 3-1a). Couch grass (*E. repens*) was the dominant species at the site.

Table 3-1. Two way ANOVA results for canopy and understory responses to irrigation and fertilisation treatments. Statistically significant responses (P < 0.05) highlighted in bold.

	Figure reference	Factors					
		Irrigation		Fertilisation		Irrigation x Fertilisation	
		$F_{1,12}$	P	$F_{1,12}$	P	$F_{1,12}$	P
% grass coverage	3-1b	**7.42**	**0.018**	**8.16**	**0.014**	1.33	0.272
Soil Moisture*	3-1c	**12.54**	**0.004**	0	0.95	0.02	0.883
Canopy Cover	–	3.3	0.094	1.99	0.183	1.28	0.28
Grass Nitrogen	3-2a	1.9	0.194	**11.99**	**0.005**	3.31	0.094
Grass Carbon	3-2b	1.48	0.248	**5.32**	**0.04**	0.86	0.371
Grass carbon:nitrogen ratio	3-2c	2.54	0.137	**4.9**	**0.047**	3.38	0.074

* Root square transformed

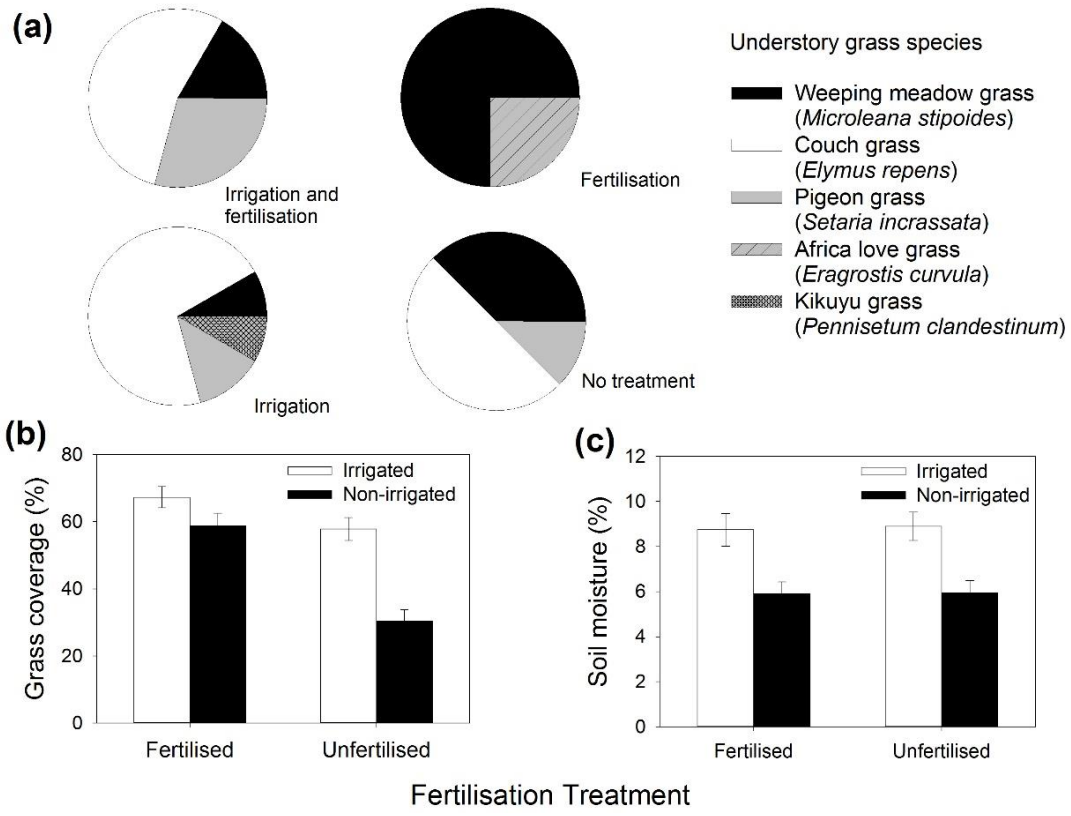

Figure 3-1. Effects of irrigation and fertilisation on **(a)** species composition of understory grasses, **(b)** grass coverage and **(c)** soil moisture (mean ± standard error shown, *N* = 32).

Grass coverage was significantly increased by both fertilisation and irrigation treatments (Fig. 3-1b, Table 3-1) whereas irrigation increased soil moisture (Fig. 3-1c, Table 3-1). Fertilisation increased nitrogen concentrations in grasses (Fig. 3-2a, Table 3-1). The carbon concentration of grasses was unaffected by irrigation or fertilisation (Fig. 3-2b, Table 3-1) and the carbon to nitrogen ratio decreased with fertilisation as a result (Fig. 3-2c, Table 3-1).

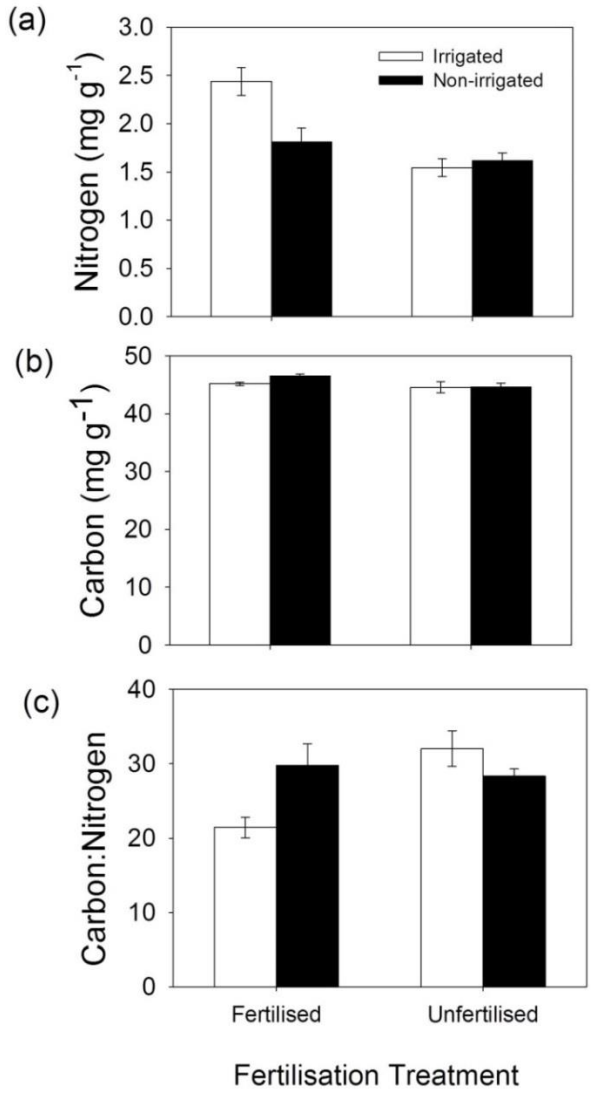

Figure 3-2. Foliar **(a)** nitrogen, **(b)** carbon concentration of grasses and **(c)** carbon : nitrogen ratio under irrigation and fertilisation treatments. Mean ± standard error shown, *N* = 32.

3.4.2 Larval scarabs and EPNs

Scarab larvae constituted the majority (75%) of root feeding insects recovered, with smaller numbers of click beetles (Elateridae; 17%), bess beetles (Passalidae; 5%) and ground beetles (Carabidae; 3%). Scarab larvae were dominated by *Sericesthis nigrolineata* (57%) and *Sericesthis geminata* (36%) with a small number of *Scitala sericans* (7%).

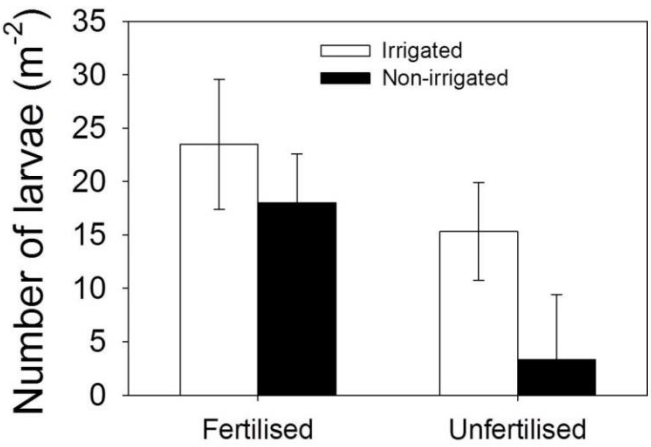

Figure 3-3. Number of larvae found under the different irrigation and fertilisation treatments (mean ± standard error, adjusted for covariance, *N* = 32).

There were no differences in the scarab species composition between plots and so the population was considered collectively. Despite the number of scarab larvae being lower in non-irrigated plots (Fig. 3-3), this was not statistically significant ($P = 0.155$). However, fertilisation significantly increased larval abundance (Fig. 3-3, Table 3-2). Grass coverage was a significant covariate in the model ($F_{1,11} = 8.57$, $P = 0.014$). Irrigation significantly increased the presence of EPNs in plots (Fig. 3-4a, Table 3-2), and also increased EPN density (Fig. 3-4b, Table 3-2) within plots, but fertilisation had no effect (Table 3-2).

Table 3-2. Two way ANOVA results for the larval and EPN responses to irrigation and fertilisation treatments. Statistically significant effects ($P < 0.05$) highlighted in bold.

	Figure reference	Degrees of freedom	**Factors**					
			Irrigation		Fertilisation		Fertilisation x Irrigation	
			F	*P*	F	*P*	F	*P*
Larvae*	3-3	1, 111	2.34	0.155	**6.44**	**0.028**	0.83	0.381
EPN presence (all plots)	3-4a	1, 124	**6.54**	**0.012**	0	0.983	1.15	0.222
EPN density (sub-sample)	3-4b	1, 14	**8.53**	**0.013**	0.07	0.789	0.98	0.343

* Log+1 transformed

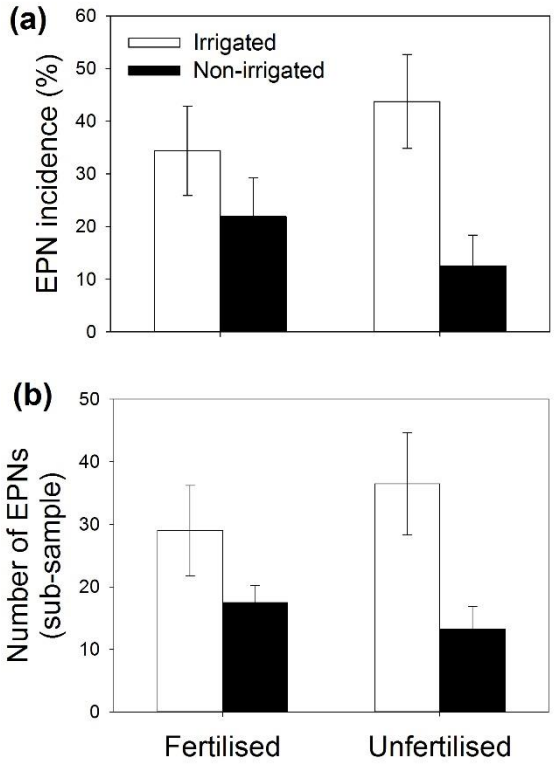

Figure 3-4. The effects of irrigation and fertilisation on **(a)** incidence and **(b)** density of EPNs in the soil (number recovered from two *G. mellonella* larvae). Mean ± standard error shown, *N* = 32 and 16, respectively.

3.5 Discussion

Scarab beetles have been regarded as pests for some time, yet little attention has been given to how plantation management affects these insects within plantations (Jackson & Klein 2006). The aim of this study was to determine the effects of eucalypt plantation management practices on the understory ecology and specifically the scarab beetle populations, under realistic field management conditions. We found that fertilisation promoted scarab larval populations in the soil. Interestingly the fertilisation treatment was the only treatment in which couch grass (*E. repens*) was absent, and African lovegrass (*E. curvula*), was present. However, in particular, fertilisation promoted the growth of weeping meadow grass (*M. stipoides*), which is one of the more nutritious grass species found within the area (Chivers & Aldous 2005) and increased nitrogen concentrations in grasses overall. These changes potentially explain the higher abundance of scarab beetle larvae in the fertilised

plots. While irrigation also promoted scarab abundance, this was not to the same extent and was possibly limited by the higher incidence of EPNs in irrigated plots.

3.5.1 Fertilisation and soil fauna

Previous studies in pasture systems have shown that the addition of fertilisation increases scarab abundance (Wightman 1974), so it seems likely that the scarab larvae in the present study benefited from the qualitative (e.g. N concentration) and quantitative (e.g. shifts to C_3 species) improvements in the plant community. Although it is important to note that African lovegrass, a C_4 grass, constituted a proportion of the fertilised plots, these were dominated by weeping meadow grass, a C_3 plant. Many insects are nitrogen limited since concentrations are higher in the insect body than their host plants (McNeill & Southwood 1978; Mattson 1980; White 1993), which are particularly low in the roots (Brown & Gange 1990). Improvements in root nitrogen concentrations therefore seem likely to have beneficial effects in this instance. Moreover, the promotion of C_3 species such as *M. stipoides* might also benefit scarab larvae. C_3 grasses usually have higher nitrogen concentrations and are less tough than C_4 grasses (Barbehenn, Karowe & Spickard 2004), so might be more favourable hosts for root-feeding scarabs, as has been demonstrated for aboveground herbivores in the framework of the C_3–C_4 hypothesis (Caswell *et al.* 1973; Scheirs, De Bruyn & Verhagen 2001).

An increase in plant nitrogen concentrations may also, however, affect plant defences, including direct (Stout, Brovont & Duffey 1998) and indirect defences which underpin recruitment of natural enemies of shoot herbivores (Ibrahim *et al.* 2008). The effects of fertilisation on root defences are largely unknown however (Erb & Lu 2013). One of the few studies to address this showed a significant decrease in production of root pyrrolizidine alkaloids following fertilisation, although this was not in a grass species (Hol 2011). The effects of fertilisation on root defences, both direct and indirect, may thus be minimal (as hypothesised by Erb and Lu (2013)). We found that fertilisation did not increase EPN numbers, which suggests that indirect defences (e.g. EPN recruitment) were not increased with fertilisation in this system.

Higher scarab abundance in fertilised plots may have arisen by improved larval survival and performance, but also in the case of *Sericesthis* spp., through preferential oviposition by maternal insects on higher quality plants that favour offspring. This may be particularly true when scarab beetle larvae have limited capacity to relocate within the soil (Johnson *et al.* 2006). Indeed, studies with scarabs in other systems have demonstrated similar ovipositional preferences (Allsopp, Klein & McCoy 1992; Logan 1997), so it seems likely that higher larval abundance arose through better offspring performance and maternal preferences.

3.5.2 Irrigation and soil fauna

In the present study, we hypothesised that irrigation would also promote scarab abundance. The moisture content of the soil is important for the survival of beetle larvae; if the soil is dry then there is risk of desiccation, but if it is saturated and anaerobic then often the larvae die of asphyxiation (Campbell 1937). Indeed, many species of scarab eggs must absorb water before hatching (Potter 1983), and hence the availability of water in the soil can be critical to scarab survival. We found that while scarab beetle larvae were more abundant in irrigated plots, the effects of irrigation were weaker than fertilisation. Moreover, EPNs abundance was positively influenced by irrigation and we propose that this reduced scarab numbers. Similar to our findings, studies in other systems have found that increasing soil moisture levels increases the persistence of EPNs, which ultimately reduces root herbivore abundance (Preisser, Strong & Diehl 2004). Increased soil moisture probably benefits EPNs in several ways, including reduced desiccation, improved motility and enhanced efficacy of chemical signalling from the damaged roots (Grant & Villani 2003). In the latter case, studies have shown that moderately humid soils favour EPN recruitment to plants under attack because of enhanced diffusion of recruitment volatiles (Hiltpold & Turlings 2008). Therefore, it is possible that irrigation increases the effectiveness of grass chemical defences via EPN recruitment and virulence. In this respect, this study illustrates the importance of assessing natural enemy responses to changes in soil conditions in addition to pest herbivore responses, to make realistic predictions about the net effects of management and environmental changes.

3.5.3 Conclusions

The aim of this study was to investigate how typical management practices of irrigation and fertilisation affect the understory ecology of a eucalypt plantation, focussing on grass communities and populations of scarab beetles. The plantation understory represents just one habitat for scarab larvae that feed on grass roots, with neighbouring pastures also being important sources (Landsberg & Wylie 1988). The obvious proximity of the understory to trees and the fact it is a relatively stable and undisturbed habitat, compared to pastures, make this a potentially important 'pest reservoir', however. Irrigation and fertilisation practices, although directed towards eucalypt growth, also affected the understory. Fertilisation, in particular, was associated with increased scarab populations and could lead to greater defoliation by eucalypt defoliating species such as *Sericesthis* spp., when these insects emerge aboveground. This suggests that plantation management can affect understory ecology and pest prevalence, perhaps more commonly than is thought and also in other systems where understory plants are present such as orchards and oak woodlands that can also experience scarab population outbreaks.

3.6 Acknowledgements

The authors gratefully acknowledge the technical assistance and advice of Burhan Amiji, Gavin McKenzie, Dr Craig Barton and Dr Kaushal Tewari. We are thankful to Jim McNicol of Biomathematics and Statistics Scotland for statistical analysis advice. We thank Ian Faithfull for the identification of scarab beetle larvae. Funding was received from the Hawkesbury Institute for the Environment, University of Western Sydney. The site was established with support from the Australian Greenhouse Office grant 0506/0085 and subsequently by the Commonwealth Department of Climate Change, with additional funding from the NSW Department of Environment and Climate Change (grant T07/CAG/16).

Chapter 4: Trade-offs between silicon and phenolic defences may explain enhanced performance of root herbivores on phenolic-rich plants

Published as Frew *et al*. 2016, *Journal of Chemical Ecology*, **42**, 768-771

4.1 Abstract

Phenolic compounds play a role in plant defence against herbivores. For some herbivorous insects, particularly root herbivores, host plants with high phenolic concentrations promote insect performance and tissue consumption. This positive relationship between some insects and phenolics, however, could reflect a negative correlation with other plant defences acting against insects. Silicon (Si) is an important element for plant growth and defence, particularly in grasses, as many grass species take up large amounts of Si. Negative impacts of a high Si diet on insect herbivore performance have been reported aboveground, but are unreported for belowground herbivores. It has been hypothesised that some Si accumulating plants exhibit a trade-off between carbon based defence compounds, such as phenolics, and Si based defences. Here we investigated the impacts of Si concentrations and total phenolic concentrations in sugarcane roots on the performance of the root feeding greyback canegrub (*Dermolepida albohirtum*). Canegrub performance was positively correlated with root phenolics, but negatively correlated with root Si. We found a negative relationship within the roots, between total phenolics and Si concentrations. This suggests the positive impacts of phenolic compounds on some insects may be the effect of lower concentrations of Si compounds in plant tissue. This is the first demonstration of plant Si negatively affecting a belowground herbivore.

4.2 Introduction

Plants produce many metabolites, some of which play an essential role in defence against herbivores. For example, there are over 9000 phenolic based compounds that are widespread across the plant kingdom in both leaves and roots (Mithöfer & Boland 2012). A recent review in this journal reported several examples of positive

relationships between root herbivore performance and root phenolic concentrations (Johnson & Nielsen 2012). Johnson and Nielsen (2012) suggested these positive responses to phenolic compounds could be correlated to other, unmeasured, plant traits including alternative defences.

The importance of silicon (Si) in plants, particularly within the Poaceae, is now clear (Massey & Hartley 2009; Cooke & Leishman 2011). Silicon is a constitutive defence (Massey & Hartley 2009), though prolonged and intense damage induces an increase in Si (Massey, Ennos & Hartley 2007). Silicon is also involved in other defence responses (see examples in Cooke and Leishman (2011)). As a constitutive defence, the deposition of silica (SiO_2) phytoliths has a negative effect on insect herbivore performance (Massey & Hartley 2009). Several studies suggest that Si accumulating plants partially substitute carbon with Si based defences, which may result in more efficient allocation of carbon (Cooke & Leishman 2011; Schaller, Brackhage & Dudel 2012). Further studies have demonstrated that some Si accumulating plants have lower concentrations of phenolic compounds (Cooke & Leishman 2012).

Despite strong responses from aboveground herbivores, almost no attention has been paid to the efficacy of Si based defences against root feeding insects, even though they cause large losses to crops. For example, greyback cane beetle larvae (*Dermolepida albohirtum*), known as canegrubs, are voracious feeders on roots of sugarcane (*Saccharum* spp.; Poaceae) and are an example of a damaging pest to agriculture. These insects are native to Australia and have become a devastating pest to the Australian sugar industry (Allsopp 2010).

The importance of this pest provided a good model to test the impacts of phenolic compounds and Si compounds in sugarcane roots on the canegrub, and to investigate any relationship between a carbon based defence and Si in a grass-root system.

4.3 Methods and materials

4.3.1 Chambers

We used a glasshouse chamber (3 m × 5 m × 3 m; width × length × height) with UV transparent plexiglass (6 mm thick) walls and roof that was naturally lit throughout

the experiment. Air temperature was regulated at 30°C (±4°C) and fell to 22°C (±4°C) at night. Humidity was controlled at 60% (±6%).

4.3.2 Plant growth and treatments

Thirty sugarcane (*Saccharum* species hybrid) plants were grown from single-eye cuttings from variety Q200. After germination in gamma-irradiated potting mix (Richgro® All Purpose Potting Mix) for three weeks the plants were transferred into 3.7 litre pots using 1:1 soil:potting mix; the soil used was a sandy loam soil from the Hawkesbury region (full details in Barton *et al*. (2010). All pots were randomised weekly within the chamber. All pots had 2 g of Osmocote Controlled Release fertiliser added to ensure no nutrients were limiting plant growth. Half of the plants in each chamber were watered every three days with 100 ml tap water, while the other half received 100 ml of 500 mgL^{-1} soluble silica in the form of $NaSiO_3.9H_2O$. All plants received water regularly as required to ensure healthy growth. After 18 weeks all plants were removed from the pots. The roots were washed and placed in a 40°C oven for 48 hours; these were then weighed, ball-milled, and chemically analysed. One subsample of fresh root material was retained from each plant for insect feeding trials carried out on the same day.

4.3.3 Feeding trials

To assess the impacts of Si and phenolic concentration on the growth and root consumption by the canegrubs, we conducted feeding trials adapted from Massey and Hartley (2009). Individual third instar larvae, starved for 24 hours, were weighed before being placed in a Petri dish (14 cm diameter) with around 5 g of fresh sugarcane root material, taken from the harvested sugarcane plants grown under high and ambient silicon environments. To ensure the root sample was representative of the plant root system, both fibrous and fine roots were taken from the upper, middle and lower regions of the root system. Individual larvae and root sample were randomly allocated, kept at 26°C. Larvae were allowed to feed for 24 hours, after which they were starved for 12 hours to ensure all frass was expelled, before being reweighed. Values of water content, derived from root samples from the same plants, were used when converting fresh mass of roots to dry mass, to

account for any evaporative water loss during the experiment. Sample sizes were slightly unbalanced as some of the plants died prior to harvest.

Relative consumption (RC) is an estimate of the mass of root material ingested over the 24 hour period relative to initial body mass. RC was calculated from: food ingested (mg change in dry mass)/ mean body mass over experimental period (mg fresh mass).

4.3.4 Chemical analysis

All sugarcane root samples were ball-milled to powder and a subsample of approximately 40 mg was analysed for nitrogen and carbon concentrations using an elemental analyser (FLASH EA 1112 Series CHN analyser, Thermo-Finnigan, Waltham, MA USA).

Leaf and root Si concentrations were analysed by pressing 2 g of sample powder into a 3 mm thick, 13 mm diameter pellet with a pressure of 13 bar. Silicon concentration (expressed as percentage dry mass) was analysed from the pellets with an X-ray fluorescence spectrometer (Epsilon 3^x, PANalytical, EA Almelo, The Netherlands), as described in Reidinger *et al*. (2012).

Total phenolic concentrations in the roots was determined as described in Salminen and Karonen (2011), in technical triplicates, using a Folin-Ciocalteu assay with gallic acid monohydrate (Sigma-Aldrich, St. Louis, MO, USA) as the quantification standard.

4.3.5 Statistical analysis

R statistical interface (v3.0.1) was used for all statistical analyses. All correlations among variables were analysed using Spearman's rank correlation test using the 'cor.test' function. Permutational multivariate analyses of variance (PERMANOVA) using the 'adonis' function within R package 'vegan' (Oksanen *et al.* 2015) were used to analyse the sugarcane root chemical responses (total phenolic and Si concentrations) and canegrub responses as the data did not meet the assumptions of ANOVA after transformations were applied.

4.4 Results

There was a positive correlation between the change in mass of canegrubs and the total phenolics of the roots (r=0.58, R^2=0.34, $F_{1, 24}$ = 12.01, P < 0.01) (Fig. 4-1a).

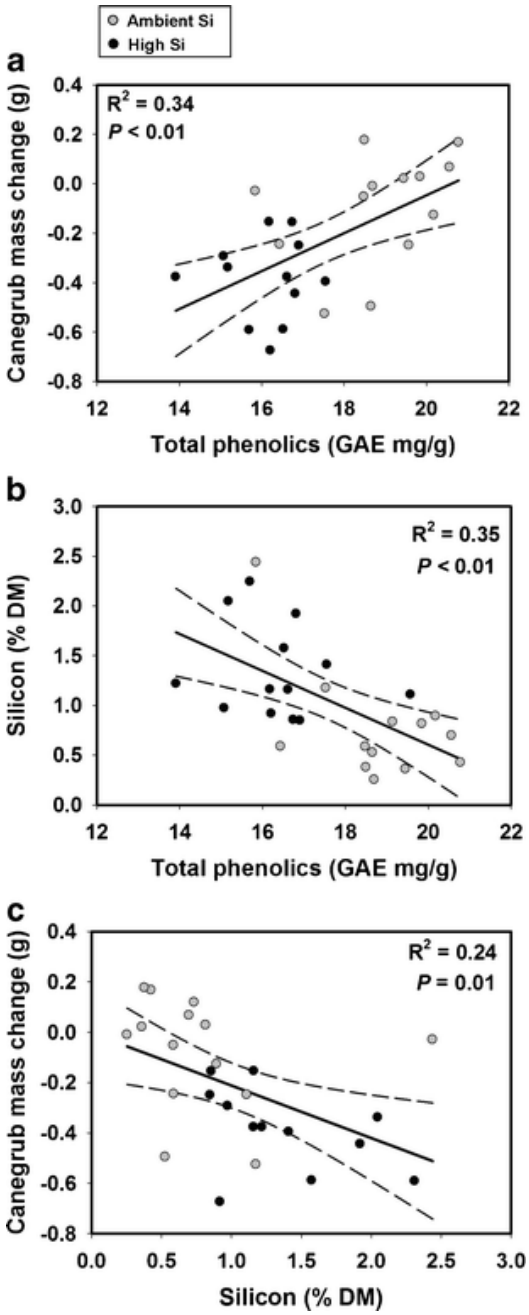

Figure 4-1. Correlations between **(a)** canegrub change in mass (g) and total sugarcane root phenolic concentration (GAE mg/g), **(b)** total sugarcane root silicon concentration (% dry mass) and total sugarcane root phenolic concentration (GAE mg/g) and **(c)** greyback canegrub change in mass (g) and total sugarcane root silicon concentration (% dry mass). Solid lines represent linear regression through all the points. Dashed lines represent 95% confidence intervals. N = 12 for high Si, N = 13 for low Si.

This relationship was somewhat reflected in the positive correlation between relative consumption of the roots by the canegrubs and the root phenolics (r=0.49, R^2=0.25,

$F_{1, 24}$= 7.57, *P*=0.01; data not shown). There was a negative correlation between Si concentrations in the roots and the total phenolic content (*r* = -0.59, R^2=0.35, $F_{1,24}$=12.62, *P* < 0.01), which was the strongest relationship of the variables measured (Fig. 4-1b). Similarly, we found a negative correlation between canegrub change in mass and root Si (*r* = -0.49, R^2=0.24, $F_{1, 24}$ = 7.77, *P* = 0.01) (Fig. 4-1c), which was also reflected in the negative correlation between relative consumption of root mass and root Si (*r* = -0.42, R^2 = 0.18, $F_{1 , 24}$= 5.15, *P* = 0.03; data not shown). There was no significant correlation between the change in mass of canegrubs and the carbon: nitrogen ratio of the root material (*r* = 0.02, R^2 = 0.01, $F_{1, 24}$ = 0.02, *P* = 0.89; data not shown). Likewise, there was no relationship between relative consumption and the carbon: nitrogen ratio of the roots (*r* = 0.03, R^2 = 0.001, $F_{1, 24}$ = 0.02, *P* = 0.88; data not shown).

4.5 Discussion

We found that the performance of the canegrub increases when feeding on sugarcane root material with higher total phenolic concentrations. This finding is consistent with other investigations on belowground herbivores (Johnson & Nielsen 2012). This suggests that some insects are not only able to overcome these defences, but also may also benefit from them. These positive effects may be because phenolic compounds are nutritionally beneficial as they can substitute tyrosine, used for cuticle sclerotisation, with compounds such as gallic acid (Johnson & Nielsen 2012). However, a positive response to phenolics could also be a response to other plant traits. We took into account the carbon:nitrogen ratio of the roots, as this is indicative of plant nutritional quality, but found that this ratio did not correlate with any other variables measured.

Several studies have found that plants with higher Si concentrations have lower phenolic concentrations and it has been proposed that plants may exhibit a trade-off between Si and carbon based herbivore defences (Cooke & Leishman 2012), although this idea remains unexplored in root systems. We found a negative correlation between root total phenolic and root silicon concentrations. We also found a negative correlation between canegrub performance and the Si concentration of root material. The negative impacts of Si on insect herbivore performance via the hard

silica phytoliths in plant tissue are well known (Massey & Hartley 2009). These can act as feeding deterrents, but also reduce digestibility through protection of the parenchyma cells, where insects receive their starch and protein. Plant material with lower Si content potentially allows insects to efficiently utilise the available nutrients, such as nitrogen, due to an increased digestibility of the material (Massey & Hartley 2009).

Taking into consideration the correlative data from our study, along with findings from previous investigations (Schaller, Brackhage & Dudel 2012; Cooke & Leishman 2012), the positive impacts of phenolics on some herbivores may actually reflect the effects of low Si concentrations in the host plant material. Interactions between plant Si and mammalian herbivores belowground have been investigated previously (Wieczorek *et al.* 2015). To our knowledge, this study is the first demonstration of Si negatively affecting an insect root herbivore. Our data led us to hypothesise that root Si could be a potent defence against other belowground herbivores. This finding could have agricultural and ecological implications, which are beyond the scope of this rapid communication, but we hope our report encourages researchers to further explore such relationships belowground.

4.6 Acknowledgements

We thank the teams at Sugar Research Australia and the Hawkesbury Institute for the Environment for their support throughout this project, particularly Andrew Gherlenda, Allen Eaton and Lisa Derby for their help and advice. Funding was provided by Sugar Research Australia (project no.2014/104) and the Hawkesbury Institute for the Environment, Western Sydney University.

Chapter 5: Increased root herbivory under elevated atmospheric carbon dioxide concentrations is reversed by silicon based plant defences

Published as Frew *et al*. 2016, *Journal of Applied Ecology*, doi: 10.1111/1365-2664.12822

5.1 Abstract

Predicted increases in atmospheric concentrations of CO_2 may alter the susceptibility of many plants to insect herbivores due to changes in plant nutrition and defences. Silicon (Si) plays a critical role in plant defence against herbivores, so increasing such Si based defences in plants may help remediate situations where plants become more susceptible to herbivores.

Sugarcane (*Saccharum* spp. hybrid) were subjected to fully factorial treatment combinations of ambient (aCO_2) or elevated (eCO_2) atmospheric CO_2 concentrations; ambient Si or Si supplementation; insect-free or subject to root herbivory by greyback canegrub (*Dermolepida albohirtum*). A glasshouse study was used to determine how these factors affected rates of photosynthesis, growth, chemistry (concentrations of Si, carbon (C), nitrogen (N) and non-structural carbohydrates). Changes in canegrub mass were determined in the glasshouse pot study, together with more detailed assessment of how eCO_2 and Si supplementation affected performance and feeding behaviour (relative growth rate and relative consumption) in a 24-hour feeding efficiency assay.

Elevated CO_2 and Si supplementation increased rates of photosynthesis (+32% and 14%, respectively) sugarcane biomass (+45% and 69%, respectively). Silicon supplementation increased Si concentrations in both leaves and roots by 54% and 75%, respectively. eCO_2 caused root C:N to increase by 12%.

Canegrub performance and consumption increased under eCO_2; relative growth rate (RGR) increased by 116% and larvae consumed 57% more root material (suggestive of compensatory feeding). Silicon application reversed these effects, with large

decreases in mass change, RGR and root consumption (65% less root mass consumed).

Synthesis and applications. Our results suggest future atmospheric carbon dioxide concentrations could lead to increased crop damage by a belowground herbivore. Increasing bioavailable Si in soil stimulated Si based defences which dramatically decreased herbivory and herbivore performance. Our findings suggest future pest management strategies could benefit from characterising deficiencies in bioavailable Si in agricultural soils and targeted application of Si fertilisers. Moreover, future breeding programmes should exploit variation in Si uptake between cultivars to enhance Si uptake in new crop varieties. Silicon based plant defence proved to be highly beneficial for remediating the negative effects of atmospheric change on sugarcane susceptibility to herbivory and could be applicable in other crops.

5.2 Introduction

Achieving sustainable crop production in the face of global climate change is a significant challenge as the world's population increases by 1.2% each year (Baulcombe *et al.* 2009). Increasing populations, together with increasing demand for food, water and energy combine with climate change threatening to create a 'perfect storm' scenario of global events (Beddington 2009). To combat crop losses from insect herbivores, there has been a seven-fold increase in insecticide usage in the last 40 years (Tilman *et al.* 2001). However, the application of insecticides is costly and often environmentally unsustainable (Douglas, Rohr & Tooker 2015), and has led to increased restrictions on pesticide application (Birch, Begg & Squire 2011). Therefore, new methods of crop protection are necessary to ensure sustainable food security under climate change.

Atmospheric concentrations of carbon dioxide (CO_2) are rising and are expected to reach approximately 540-958 μmol mol^{-1} by the year 2100 (IPCC 2014). These changes in the global environment have the potential to irreversibly alter ecosystem processes, but at the very least they will impact directly on the physiology of plants, many of which are fed upon by insect herbivores. In response to elevated atmospheric CO_2 concentrations (eCO_2) many plants increase in susceptibility to

herbivores, which can be due to a breakdown in defences (Zavala *et al.* 2008; Martin & Johnson 2011), as well as other chemical changes such as alterations in the amino acid profile (Guo *et al.* 2014) . Elevated CO_2 also causes suppression of the jasmonic acid pathway, which limits induced defences of plants against chewing herbivores (Ode, Johnson & Moore 2014). The nutritional value of plants is also altered in response to eCO_2, as the net carbon (C) uptake of host plants increases as atmospheric CO_2 concentrations increase; this dilutes plant nitrogen (N) concentration (Stiling & Cornelissen 2007; Robinson, Ryan & Newman 2012). As N is typically a limiting factor in insect herbivore diets, an excess of C relative to N results in compensatory feeding in many chewing insects as they attempt to acquire adequate nutrition (Stiling & Cornelissen 2007; Johnson & McNicol 2010; Johnson, Lopaticki & Hartley 2014). These changes in plant chemistry could increase plant susceptibility to insect herbivores, which could impact on ecosystem function and potentially lead to increases in crop damage under eCO_2 as crops struggle to tolerate an increase in herbivory. While there is evidence in some plants that increases in host plant biomass in response to eCO_2 may be able to compensate for any increase in herbivory (McKenzie *et al.* 2016), the overall effects of eCO_2 on crop damage by insect pests will depend on the system, as plant and insect responses to eCO_2 are variable (Hunter 2001b). Nevertheless, it would be negligent not to prepare for the possibility of increased crop losses from insect pests under eCO_2.

Root feeding insects have significant impacts on plant productivity, significantly reducing the yield of agricultural systems (Hunter 2001a). Predicting the response of these 'hidden' herbivores to eCO_2 is a cornerstone to understanding how future ecosystem functioning will be affected, as well as to achieving sustainable food production. However, few studies focus on root feeding insects (Staley & Johnson 2008). This is problematic because plants often cannot tolerate root herbivory, not only because root feeding causes acute damage but also because many root feeding pests are exceptionally persistent, with damage to plant tissues lasting many months or even years (Johnson, Erb & Hartley 2016). This persistence frequently results in prime agricultural land being taken out of production (Blackshaw & Kerry 2008). It is important that attention is paid to how plant–insect relationships will be impacted

by eCO_2, especially in the context of novel control strategies that remediate any adverse effects of climate change on plant susceptibility.

Plant silicon (Si) offers one avenue as it promotes growth in many plant species, particularly among the Poaceae. Silicon plays a role in both induced and constitutive defences in plants against pathogens and herbivores (Reynolds, Keeping & Meyer 2009; Huitu *et al.* 2014; Van Bockhaven *et al.* 2015). The disposition of silica (SiO_2) phytoliths has considerable negative effects on growth performance and food consumption of insect herbivores (Massey & Hartley 2009), including aboveground pests of sugarcane (Keeping, Kvedaras & Bruton 2009). The evolutionary significance of plant Si was recently highlighted by Cooke & Leishman (2011) as the radiation of the grasses coincided with the evolution of mammalian eudont teeth, indicating possible coevolution between plant silicification and herbivores (McNaughton & Tarrants 1983). Indeed, plant Si concentration can also influence insect ovipositional preferences (Correa *et al.* 2005), which suggests it could play a more significant role in insect population dynamics as well as in insect consumption and performance. As such, Si accumulation has been referred to as a neglected plant functional trait (Katz 2015).

Despite strong responses from many aboveground herbivores, almost no attention has been paid to the efficacy of Si based defences against root chewing insects despite their destructive potential to crops (Hunter 2001a). Considering the evidence that eCO_2 is likely to fundamentally alter insect–plant relationships, there is a need to investigate the role of Si based defences and their impact on belowground root feeders under future atmospheric CO_2 concentrations.

This study, to our knowledge, is the first to test the impacts of Si soil supplementation on a belowground herbivore under eCO_2. Here we examine the responses of sugarcane *Saccharum* spp. hybrid (Poaceae), and the impacts on the performance of larvae of the greyback cane beetle *Dermolepida albohirtum* (Waterhouse), colloquially known as canegrubs.

5.3 Materials and methods

5.3.1 Study system

Canegrubs are key pests of Australian sugarcane. Larvae live and develop belowground in the soil progressing through three larval instars feeding extensively on roots of sugarcane crops. Damage caused by the canegrub can cost the Australian economy up to AU$40 million a year in crop losses (Chandler 2002; Allsopp 2010). Within our study all canegrubs were third-instar, as these are typically the most voracious feeders of roots. We sourced larvae of *D. albohirtum* from sugarcane fields in north-eastern Queensland, Australia.

5.3.2 Chambers

Two glasshouse chambers, one maintained at ambient CO_2 (aCO_2) of 400 µmol mol^{-1} and the other at elevated CO_2 of 640 µmol mol^{-1}, were used. These chambers, 3m × 5m × 3m; width × length × height with UV transparent plexiglass (6 mm thick) walls and roof, were naturally lit throughout the experiment. Daytime air temperature was regulated at 30°C and fell to 22°C (±4°C) at night. Humidity was controlled at 60% (±6%). Carbon dioxide levels were controlled via the monitoring and control system PlantVisorPRO (Carel Industries, Padova, Italy). Carbon dioxide levels within each chamber were monitored by a CO_2 probe (GMP222, Vaisala, Vantaa, Finland), with CO_2 (food grade, AirLiquide, Australia) injected from pressurized cylinders through solenoid valves. Before entering a chamber, CO_2 was passed through a Purafils column to eliminate possible ethylene contamination.

5.3.3 Plant growth, treatments and measurements

Sixty sugarcane plants were grown from single-eye cuttings from a common cultivar within Australia bred by Sugar Research Australia, Q200. These were germinated in trays of gamma-irradiated potting mix (Richgro© All Purpose Potting Mix) for three weeks in the ambient CO_2 glasshouse chamber and received water *ad libitum*. These were then transferred into 3.7 L pots using 1:1 soil:potting mix; the soil used was a sandy loam soil sourced from the Hawkesbury Forest Experiment, which is fully described in Barton *et al*. (2010). All pots received 10 g of NPK fertiliser in the form of Osmocote© controlled-release fertiliser, to ensure essential nutrients were not

limiting plant growth. Throughout the experiment, to reduce any 'chamber effects' associated with using the two chambers, the sugarcane plants were randomly circulated within the chamber every three days, and the chambers were swapped every c.14 days by transferring the plants between the chambers and adjusting the environmental conditions. This does not entirely resolve any pseudoreplication issues, however this approach provides almost identical results to truly replicated glasshouse experiments (Johnson *et al.* 2016b). Half of the plants in each chamber were watered every three days with 100 mL tap water, while the other half received 100 mL of 500 mgL^{-1} soluble silica in the form of $NaSiO_3.9H_2O$ (Cid *et al.* 1990). $NaSiO_3.9H_2O$ is a highly efficient silicon fertiliser in other grass crops (Mecfel *et al.* 2007), and has been used in several previous studies (Reynolds, Keeping & Meyer 2009). Throughout the experiment all plants received tap water as required. Rates of photosynthesis were measured on each plant approximately every three weeks with a Portable Photosynthesis System (LI-6400, Li-COR Inc., Lincoln, USA). Measurements were conducted within the growth chambers. Plants were grown under their respective treatments for 18 weeks before being harvested. Three weeks prior to harvesting the plants, three *D. albohirtum*, all third instar, were weighed and placed in soil of half of the plants in each chamber. At the same time, eight pots with no plants, only soil, were placed into each chamber and grubs were also placed into these pots. Half of these pots in each chamber also received Si solution to account for any direct impacts of the CO_2 and Si treatments on the larvae. After three weeks, all plants were removed from the pots, along with the larvae, which were weighed, and the mean change in mass of the three larvae was recorded as a measure of performance (pot study). The leaves, stems and roots were separated, roots were thoroughly washed, and all plant material was placed in a 40°C oven for 72 hours, and then weighed. One subsample of fresh root material was retained from each plant to be used for feeding efficiency assays.

5.3.4 Feeding assays

To assess the impacts of eCO_2 and Si supplementation on the growth and feeding behaviour of *D. albohirtum* larvae, feeding efficiency assays were conducted adapted from Slansky (1985) and Massey & Hartley (2009). Individual larvae were starved for

24 hours and weighed before being placed in a Petri dish with a known mass of fresh sugarcane root material, taken from harvested sugarcane plants that were grown under factorial treatments of ambient or elevated CO_2 and + or - Si. Larvae were kept at 26°C and allowed to feed for 24 hours, after which time they were starved for a further 12 hours to allow the frass to pass, before being reweighed. Values of water content, derived from root samples from the same plants, were used when converting fresh mass of roots to dry mass. Sample sizes were slightly unbalanced for each treatment as some of the plants died prior to harvest.

- Relative growth rate (RGR) calculates body mass growth relative to initial body mass, and was calculated from: mass gained (g)/initial mass (g)/ time (days).

- Relative consumption (RC) estimates the mass of root material ingested over the 24-h period relative to initial body mass and was calculated from: food ingested (mg change in dry mass)/ mean body mass over experimental period (mg fresh mass).

5.3.5 Chemical analysis

All dry plant leaf, root and stem samples were ball milled and a subsample of approximately 40 mg was analysed for nitrogen and carbon concentrations using an elemental analyser (FLASH EA 1112 Series CHN analyser, Thermo-Finnigan, Waltham, MA, USA). Leaf and root Si concentrations were analysed by pressing 2 g of sample powder into a 3 mm thick, 13 mm diameter pellet with a pressure of 13 bar. Silicon concentration (expressed as a percentage dry mass) was analysed from the pellets with an X-ray fluorescence spectrometer (Epsilon 3^x, PANalytical, EA Almelo, The Netherlands), as described in Reidinger *et al*. (2012). Total non-structural carbohydrate (TNC) concentration of the roots was determined using the method of Tissue & Wright (1995). The dried and milled plant material was extracted three times with a methanol: chloroform: water (12:5:3 v/v) solution to separate the soluble sugars from the pellet containing starch (Dickson 1979). The pellet was treated with 5 mL of perchloric acid (35% v/v) for 1 h to hydrolyse the starch (Sutton, Ting & Sutton

1981). The soluble sugars and the starch concentration were determined colorimetrically using the phenol-sulphuric acid method of Dubois *et al*. (1956).

5.3.6 Statistical analysis

R statistical interface (v3.0.1) was used for all statistical analyses.

Each week where photosynthetic rates were measured was analysed independently using two-way ANOVAs type = II, comparing 'Si' and 'CO_2' as treatments, while 'canegrub' was excluded because measurements were all taken prior to when canegrub treatment was applied. Sugarcane biomass, Si concentration (both leaves and roots), root C:N and root TNC responses were assessed using three-way analysis of variance (ANOVA)s type = II, from the R package 'car' (Fox & Weisberg 2011), comparing 'Si', 'CO_2' and 'canegrub' herbivory treatments as well as their interactions. Sugarcane belowground biomass and belowground Si concentrations did not meet the assumptions of ANOVA, therefore we applied a log transformation to normalise the distribution and stabilise the variance. Permutational multivariate analyses of variance (PERMANOVA) using the 'adonis' function within R package 'vegan' (Oksanen *et al.* 2015) were used to analyse leaf C:N response as the data did not meet the assumptions of ANOVA after transformations were applied.

Differences in the change of canegrub mass from the pot study in response to the silicon and CO_2 treatments, including the pots with only soil and larvae that accounted for any direct treatment effects on the canegrubs, were assessed using two-way ANOVA type = II from 'car' package. Those pots in which canegrubs had died were not considered in the analysis.

Canegrub feeding efficiency assays (RGR and RC) were analysed using PERMANOVA as they did not meet the assumptions of normality of ANOVA, even after transformations were applied. The 'canegrub' treatment was initially included in this analysis to account for any previous herbivory effects, but was dropped from the model due to non-significance.

All correlations reported were carried out using Pearson's product-moment correlation, where they met the assumptions of the test, or with Spearman rank-order correlation where they did not.

5.4 Results

5.4.1 Plant responses to eCO_2

Rates of sugarcane photosynthesis were variable throughout the growth period of the experiment (Fig. 5-1). The photosynthetic rates of plants under eCO_2 were significantly higher than those grown at aCO_2, apart from week 16. Elevated CO_2 significantly increased aboveground and belowground biomass (Table 5-1, Fig. 5-2a and 5-2b).

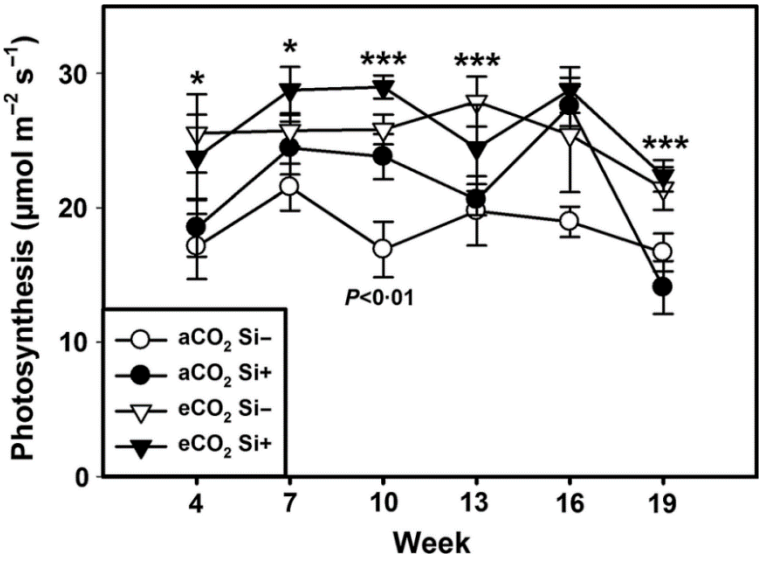

Figure 5-1. Rates of sugarcane photosynthesis (µmol m^{-2} s^{-1}) measured on various weeks throughout the experiment shown under different factorial treatment combinations. Values are means ± SE. *P*-values are given to indicate significance of the main effect of silicon treatment, while asterisks (*) indicate significance of main effect of CO_2 treatment. Degrees of significance are indicated as follows: *P < 0·05, ***P < 0·001. N = 14, except for aCO_2 under Si- treatment where N = 12.

Total Si concentration (expressed as % dry mass) was unaffected by eCO_2 in either leaves or roots (Table 5-1). Leaf C:N decreased under eCO_2 but this was only detectable under Si- due to an interaction between CO_2 and Si treatments (Table 5-1, Fig. 5-3a). Root C:N increased under eCO_2 by 12% (Fig. 5-3a), this was due to both an increase in C and a decrease in N concentration. Interestingly, the total non-structural carbohydrates (soluble sugars and starch) in the roots were 23% lower under eCO_2 compared with the controls (Table 5-1).

Table 5-1. Results of ANOVA model for the main effects and interactions of factors on aboveground (AB) and belowground (BG) sugarcane responses. Outputs from the PERMANOVA are indicated, where assumptions of ANOVA were not met. Significant impacts ($P \leq 0.05$) indicated in bold.

Response	Figure reference	Factors													
		CO_2		Si		Canegrub		CO_2 x Si		CO_2 x Canegrub		Si x Canegrub		CO_2 x Si x Canegrub	
		$F_{1,46}$	*P*	$F_{1,46}$	*P*	$F_{1,46}$	*P*	$F_{1,46}$	*P*	$F_{1,46}$	*P*	$F_{1,46}$	*P*	$F_{1,46}$	*P*
Total Biomass (g)	5-2a	**9.27**	**0.004**	**22.747**	**<0.001**	1.722	0.196	0.003	0.959	1.415	0.24	2.715	0.106	0.005	0.943
AG Biomass (g)	-	**7.837**	**0.007**	**24.212**	**<0.001**	0.231	0.633	0.151	0.699	0.953	0.334	3.079	0.086	0.022	0.883
BG Biomass* (g)	5-2b	**7.900**	**0.007**	**6.370**	**0.015**	**17.95**	**<0.001**	1.523	0.223	1.201	0.278	0.821	0.369	0.807	0.373
Leaf C:N†	5-3a	**5.637**	**0.025**	0.001	0.988	**9.673**	**0.002**	**6.182**	**0.018**	0.060	0.832	0.283	0.589	0.001	0.971
Root C:N	5-3a	**3.449**	**0.018**	1.267	0.266	0.001	0.993	0.368	0.546	2.506	0.12	1.511	0.225	0.019	0.889
Leaf Si (%DM)	5-3b	0.010	0.92	**12.879**	**<0.001**	**5.241**	**0.027**	0.65	0.424	0.419	0.52	2.224	0.143	1.127	0.294
Root Si* (%DM)	5-3b	0.001	0.996	**16.578**	**<0.001**	0.735	0.396	<0.001	0.993	0.1749	0.677	0.061	0.805	0.473	0.495
Root TNC (mg g^{-1})	-	**5.565**	**0.027**	1.150	0.309	**7.180**	**0.009**	0.274	0.614	2.631	0.109	0.406	0.481	1.739	0.207

* log transformation

† PERMANOVA

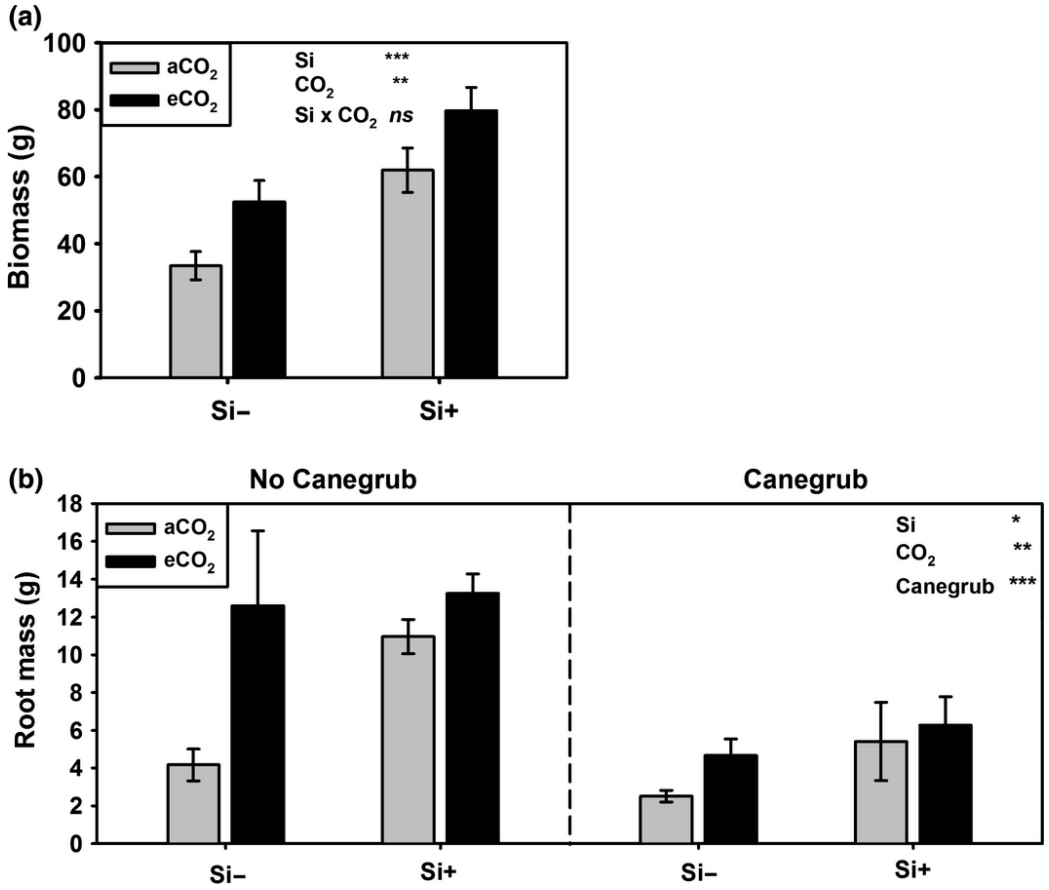

Figure 5-2. (a) Effects of silicon treatments on the total biomass (g) of sugarcane grown under aCO_2 and eCO_2. Effects of silicon treatments on the root mass **(b)** of sugarcane subjected to no-canegrub herbivory (left) and canegrub herbivory (right), also grown under aCO_2 and eCO_2. Levels of significance of main effects from factorial treatments Si and CO_2 on total biomass **(a)** are shown. Levels of significance from factorial treatments of Si, CO_2 and canegrub are shown for root mass **(b)**. Values are means ± SE. Degrees of significance are indicated as follows: ns = not significant, **P* < 0·05, ***P* < 0·01, ****P* < 0·001. *N* = 7, except for aCO_2 under Si- treatment where *N* = 6.

5.4.2 Plant responses to silicon

The mean rates of photosynthesis of sugarcane grown under Si+ were typically higher compared to Si- plants, but this effect was only detected as significant at week 10 (Fig. 5-1). The application of Si significantly increased both the aboveground and belowground biomass (Table 5-1, Fig. 5-2a and 5-2b). Total Si concentration increased in response to Si+ in both leaves and roots (Table 5-1, Fig. 5-3b), confirming the efficacy of the Si treatment. There was no difference in C:N between the Si- and Si+ plants (Fig 5-3a). Similarly, there was no effect of Si application on the total non-structural carbohydrates of the roots (Table 5-1; Appendix I Table S5-1).

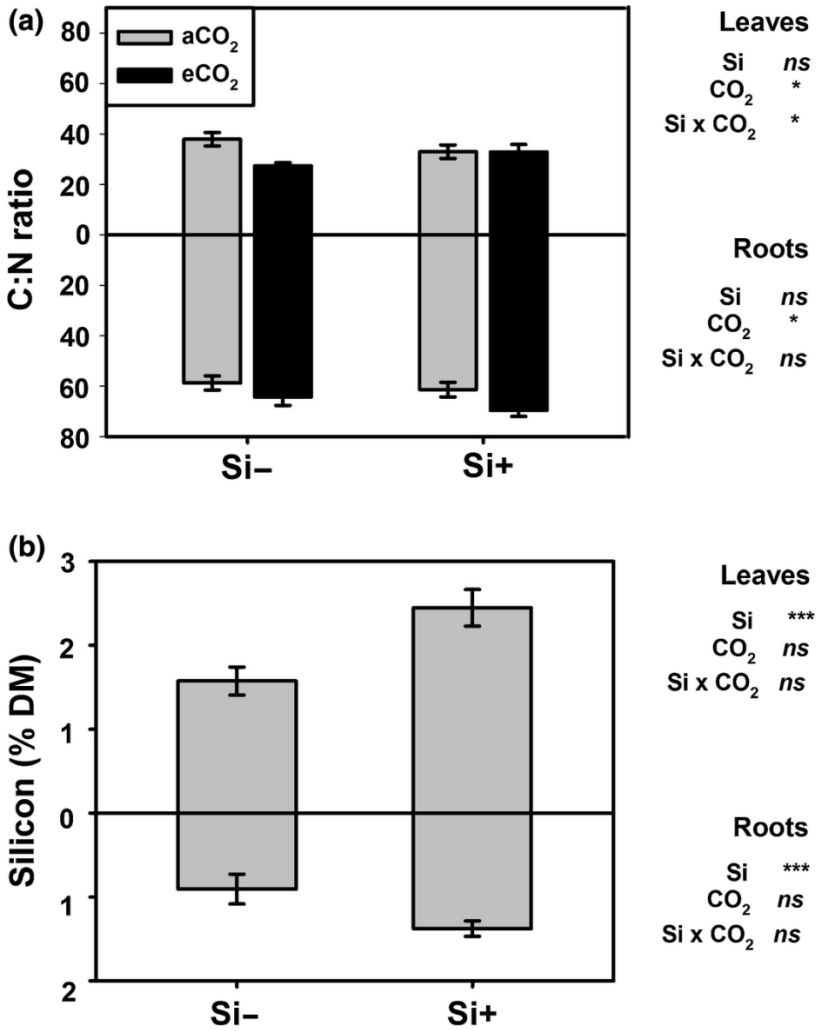

Figure 5-3. (a) Effects of silicon treatments on the carbon-to-nitrogen ratio of leaves and roots under aCO_2 and eCO_2. **(b)** Silicon concentration (% dry mass) of sugarcane grown under Si− and Si+ treatments. Levels of significance are shown for effects of silicon and CO_2 treatments. Values are means ± SE. Degrees of significance are indicated as follows: ns = not significant, $*P < 0{\cdot}05$, $***P < 0{\cdot}001$. $N = 7$, except for aCO_2 under Si- treatment where $N = 6$.

5.4.3 Impacts of root herbivory

Canegrub herbivory had no effect on the aboveground biomass of the sugarcane (Table 5-1) but significantly decreased belowground biomass (Table 5-1, Fig. 5-2b), decreasing root mass by almost 46%. Canegrub herbivory significantly lowered concentrations of leaf Si, about 16% less (Table 5-1; Table S5-1), mostly likely due to impaired Si uptake from root damage. Canegrub treatment had no impacts on root total Si concentrations (Table 5-1). Leaf C: N was higher under canegrub treatment (mean 36.24 ± 1.9), compared to the no-canegrub treatment (mean 29.56 ± 1.61) (Table 5-1), while root C: N was unaffected by canegrub treatment. However, the TNC of the roots was significantly lower under canegrub root herbivory (mean 429.4 ±

21.6 mg g^{-1}), compared to no canegrub treatment (mean 501.6 ± 19.9 mg g^{-1}) (Table 5-1).

5.4.4 Insect responses to eCO_2

The canegrubs from the pot study gained significantly more mass under eCO_2 (mean change in mass 0.75 ± 0.1g) compared with those feeding on plants under aCO_2 (mean change in mass 0.544 ± 0.09g). There was no significant difference in the change in mass between aCO_2 and eCO_2 (P = 0.939; mean change in mass 0.153 ± 0.049g and 0.148 ± 0.035g, respectively) from the pots which contained only soil, indicating no direct effects of eCO_2 on the canegrubs.

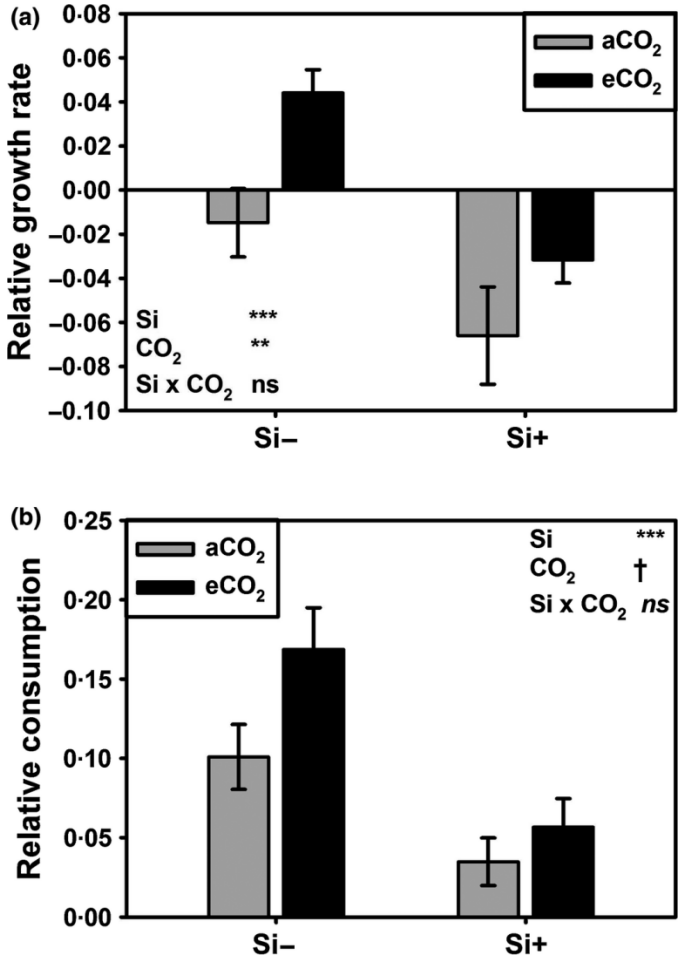

Figure 5-4. (a) Effects of silicon treatments on the relative growth rate [=mass gained (g)/time (days)] and **(b)** relative consumption of roots [=food ingested (mg change in dry mass)/initial body mass (mg fresh mass)] under aCO_2 and eCO_2. Levels of significance are shown for effects of silicon and CO_2 treatments. Values are means ± SE. Degrees of significance are indicated as follows: ns = not significant, $^{\dagger}P < 0{\cdot}1$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. N = 14, except for aCO_2 under Si- treatment where N = 12.

From the feeding assays, the majority of insects lost mass, but there was a significant increase in their change in mass overall from a mean change of -0.212 ± 0.048 g in the controls to -0.005 ± 0.049 g under eCO_2 (Table 5-1). A positive response to eCO_2 was also seen in the RGR (Fig. 5-4a) as well as the RC (Fig. 5-4b), although the latter was marginally significant (*P* = 0.05).

Table 5-2. Results of ANOVA model for the main effects and interactions of factors on canegrub responses from the 24 hour feeding efficiency assay and the three week pot study. Outputs from PERMANOVA are indicated, where assumptions of ANOVA were not met. Significant impacts (*P*≤0.05) indicated in bold.

		Factors					
Response	Figure reference	CO_2		Si		CO_2 x Si	
Feeding efficiency assay		$F_{1,50}$	*P*	$F_{1,50}$	*P*	$F_{1,50}$	*P*
Change in mass * (g)	5c	**17.929**	**<0.001**	**40.609**	**<0.001**	0.003	0.954
RGR †	4a	**8.779**	**0.004**	**18.232**	**0.001**	0.587	0.444
RC †	4b	**3.788**	**0.05**	**17.59**	**0.001**	0.952	0.341
Pot Study		$F_{1,22}$	*P*	$F_{1,22}$	*P*	$F_{1,22}$	*P*
Change in mass* (g)	5d	**4.102**	**0.055**	**8.333**	**0.008**	1.003	0.328

*log transformed
† PERMANOVA

5.4.5 Insect responses to silicon

From the pot study, the canegrubs gained significantly less mass overall under Si+ (mean 0.48 ± 0.08 g) compared to Si- (mean 0.82 ± 0.11 g) (Table 5-2). There was no significant difference in the change in larval mass between Si- and Si+ treatments (*P* = 0. 669; mean change in mass 0.136 ± 0.046g and 0.164 ± 0.037g, respectively) from the pots in the pot study which contained only soil, indicating no direct effects of silicon on canegrubs (i.e. they were entirely plant-mediated).

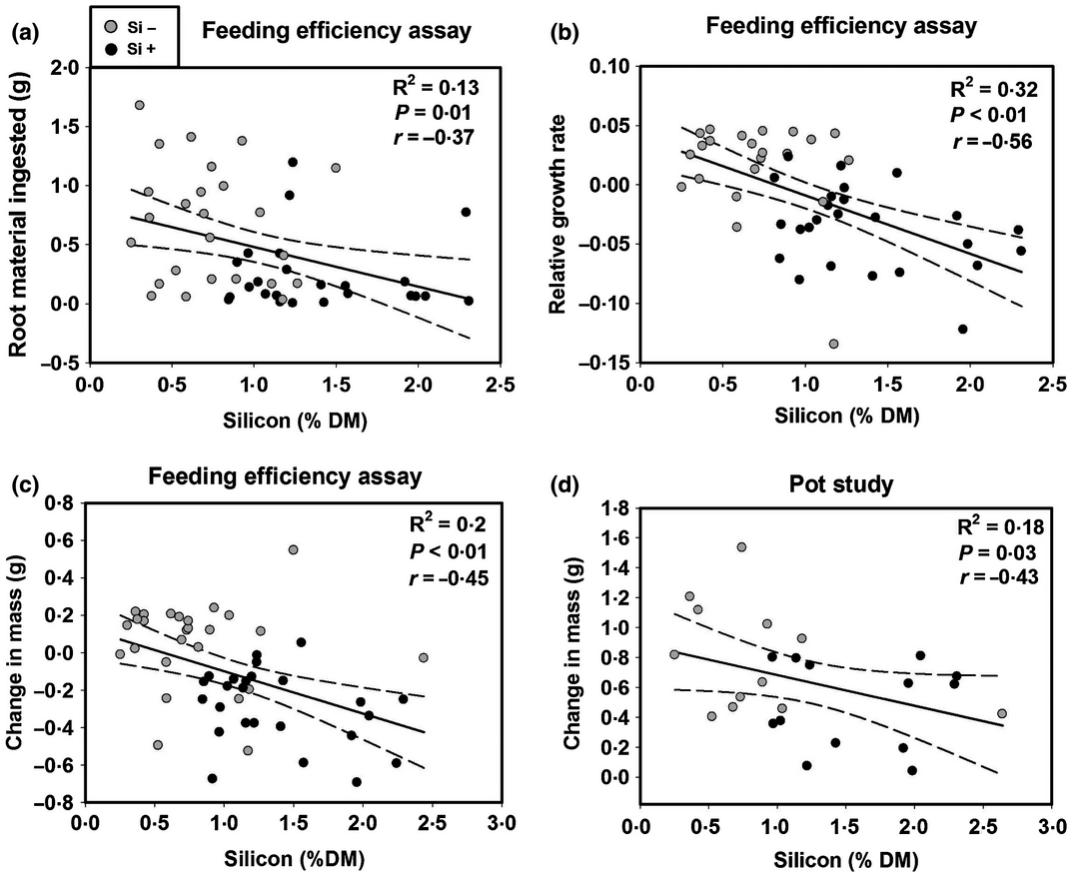

Figure 5-5. Correlations between the total root material ingested **(a)**, the canegrub relative growth rate **(b)**, and canegrub change in mass from the feeding assays **(c)** alongside the change in canegrub mass from the pot study **(d)** against total root silicon concentration (% dry mass). Solid lines represent linear regression through all the data points. Dashed lines represent 95% confidence intervals. Correlation coefficients (*r*), coefficient of determination (R^2) and *P*-values are shown. *N* = 14, except for aCO_2 under Si- treatment where *N* = 12.

From the feeding assays, the Si+ treatment had negative effects on the mass change of the canegrubs, which was reduced under Si+ (mean -0.278 ± 0.043 g) compared with Si- (mean 0.039 ± 0.043 g). Similarly, the Si+ significantly reduced the RGR of the canegrubs (Fig. 5-4a). Also, the RC was 65% lower overall under Si+ compared with Si- (Fig. 5-4b).

There was a negative correlation detected between the mass of root material ingested by the canegrubs and the root Si concentrations (Fig. 5-5a), with a similar pattern observed between the RGR and root Si concentrations (Fig. 5-5b). Canegrub change in mass from the feeding efficiency assays (Fig. 5-5c) and from the pot study (Fig. 5-5d) were both negatively correlated with root Si concentrations.

5.5 Discussion

We have demonstrated for the first time, to our knowledge, the impacts of eCO_2 and Si on sugarcane and how changes in plant chemistry then impact a belowground herbivore. Elevated CO_2 concentrations increased sugarcane growth as well as the performance of the root feeding canegrub. This increased performance under eCO_2 could signal an increase in damage to sugarcane crops in the future. However, we have also shown that the application of Si not only increases the growth of sugarcane but also significantly decreases the performance and damage of the canegrub, even under eCO_2.

Plants grown under eCO_2 had significantly higher rates of photosynthesis overall, while rates of photosynthesis of plants under Si+ tended to be higher than those under Si-. The mechanism behind increased photosynthesis under Si+ could be due to the role of Si in reducing oxidative damage during photosynthesis, thereby increasing the overall photosynthetic capacity (Shen *et al.* 2010). Increases in photosynthesis were reflected by significant increases in biomass of the sugarcane under both eCO_2 and Si+. It is important to highlight the form of soluble Si used ($NaSiO_3.9H_2O$) would have also supplied excess sodium, which is not considered an essential nutrient to plants but can reduce growth in high concentrations (Maathuis 2014). Therefore, plant growth responses observed here are possible underestimations of the impacts of plant available soil Si.

Despite being a C_4 plant, where photosynthesis is usually saturated even at ambient atmospheric CO_2 concentrations, such strong biomass responses by sugarcane to eCO_2 have been reported previously (De Souza *et al.* 2008). Several C_4 crops, including maize and sorghum, have shown little or no response to eCO_2, so it has been assumed that positive responses by a C_4 plant only occur when soil water availability is low (Seneweera, Ghannoum & Conroy 1998). Nevertheless, as was highlighted by Ghannoum *et al.* (2000), there are an increasing number of studies that show strong responses by C_4 plants to eCO_2 even under well-watered conditions. Notwithstanding this, despite the regular watering throughout our experiment, it is possible that transient drought was experienced, as was the case for the study by De Souza *et al*. (2008), due to pot size limitations, which could amplify eCO_2 responses.

In addition to these positive growth responses to eCO_2, we have demonstrated that the performance of a destructive belowground herbivore increases under eCO_2, while the relative consumption of root material also increased. This increased consumption is likely to be a compensatory feeding response to the increase in C: N ratio of the roots under eCO_2, as the canegrubs consume more material in attempts to satisfy their N requirements (Robinson, Ryan & Newman 2012). Compensatory feeding in response to higher root C: N has been reported for at least one other grass root feeding scarab (Johnson, Lopaticki & Hartley 2014).

Often, insects cannot fully compensate for decreased nutritional quality of the plants but there are several examples of compensatory and over-compensatory responses in terms of insect performance (Stiling & Cornelissen 2007). We found canegrubs performed better when feeding on roots grown under eCO_2. The mechanisms of this improved performance, at least from the pot study, may be due in part to the increase in root biomass under eCO_2, which showed a large significant increase, particularly under the Si- treatment. However, this cannot explain the positive responses from the feeding efficiency assays, as all canegrubs were given the same mass of roots to feed on. Interestingly we also found a negative response in the root TNC to eCO_2, which would mean less sugar available to the insects. The improved overall performance of the canegrubs under eCO_2 compared to aCO_2 may therefore be a response to an unmeasured plant root trait.

The TNC of the sugarcane roots were also significantly reduced in roots under canegrub herbivory, which may be due to reductions in allocation of C from the leaves to roots, as induced C reallocation in response to root herbivory has been documented (Robert *et al.* 2014). We also found that plants subjected to root herbivory had slightly higher leaf C: N. The decrease in leaf C: N in response to eCO_2 was unexpected, although there are prior examples where plant C: N has not responded to eCO_2 (McKenzie *et al.* 2016), as well as other instances where decreases in foliar C: N were reported (Ferrario-Méry *et al.* 1997). The decrease in C: N observed here in response to eCO_2 only occurred under the Si- treatment, which could indicate that under future CO_2 concentrations other foliar feeding insects may benefit from an increase in host plant quality, particularly in low Si soils.

While the impacts of eCO_2 on root herbivore performance and consumption here suggests a possible exacerbation of an already challenging pest problem, the application of Si to sugarcane dramatically reduced plant damage and decreased performance of the larvae. The effectiveness of the Si treatment in increasing the Si concentrations within sugarcane tissue was clear, and the resulting negative responses of decreased performance and root consumption from the canegrubs were even more evident. Reductions in insect herbivore performance have been reported in response to Si previously (Kvedaras & Keeping 2007; Massey & Hartley 2009), but has only once been reported in a root feeding insect (Frew *et al.* 2016c). The mechanisms of this response are likely to be similar to those reported for aboveground herbivores, where an increase in Si increases the overall roughness and toughness of plant tissue (Epstein 2009) which impede herbivore penetration and chewing. An increase in silica phytoliths also leads to a reduction in the digestibility and palatability of plant material (Massey & Hartley 2009), all of which are likely to contribute to the reductions in root consumption we observed here under Si+. Indeed, the correlations we found between Si concentrations of the root material and canegrub performance support our findings that the application of Si is effective in decreasing root herbivore performance and consumption.

As atmospheric CO_2 concentrations continue to rise, it is important to understand how changes in plant quality affect belowground herbivores and thereby impact both natural and managed ecosystem functioning. Understanding these changing interactions is also central to ensuring future food security to support the increasing global population under eCO_2. Although potentially less important in annual/semi-perennial plant systems, or systems subject to regular harvest, it is possible that organisms will acclimatise to changes in atmospheric CO_2 concentrations. Nonetheless, our results demonstrate the performance of a belowground root herbivore increases under eCO_2. This could suggest large impacts to agriculture, as the pest status of belowground herbivores has the potential to worsen in response to eCO_2. As current control strategies, such as prophylactic pesticide use, are expected to become more restricted, environmentally sustainable alternatives are continually being researched. Here we have shown the application of Si can

dramatically decrease the performance of an economically significant root feeding insect, while also decreasing root consumption. These effects are significant under current and future atmospheric CO_2 concentrations. As global temperature and rainfall patterns are also predicated to be significantly altered by the end of the century, it is pertinent for future research to investigate the impacts of these variables to better understand plant–herbivore interactions under climate change.

Our findings indicate that Si based defences should play a central role in climate change remediation regarding pest management. Bioavailable soil Si is often depleted in agricultural soils (Savant, Datnoff & Snyder 1997) and therefore characterisation of plant available soil Si would facilitate targeted application of Si fertilisers, which have already been commercially developed for use in agriculture (Guntzer, Keller & Meunier 2012). In the long term, breeding programmes should exploit the recent advances in the molecular understanding of Si uptake (Ma & Yamaji 2015) and the natural variation in plant Si concentrations (Soininen *et al.* 2013) to select for crop varieties with higher Si accumulation efficiency. This way, potential crop pest exacerbation by climate change can be remediated by exploiting a previously undervalued natural plant defence.

5.6 Acknowledgements

We thank teams at Sugar Research Australia Limited and the Hawkesbury Institute for the Environment. Funding was provided by Sugar Research Australia (project no.2014/104) and the Hawkesbury Institute for the Environment, Western Sydney University.

Chapter 6: Arbuscular mycorrhizal fungi promote silicon accumulation in plant roots with negative impacts on root herbivores

6.1 Abstract

Belowground herbivores and arbuscular mycorrhizal (AM) fungi share the soil environment and interact directly with plant roots. As root feeding insects extensively damage root systems, they reduce plant fitness, reduce root material available for colonisation, and can damage AM fungi directly. This suggests there should be strong selection pressure for AM fungi to support plant defences against root herbivores. While AM fungi are commonly observed to reduce the performance of root feeding insects, the mechanisms remain unclear. AM fungi are known to alter plant defences, impacting several defence mechanisms that can affect insect herbivores, yet one important plant defence has been neglected to be considered in these interactions. Plant silicon (Si) is an effective defence against root feeding insects, and AM fungi have been observed to increase Si in plants. This highlights the potential of role of Si in AM interactions with belowground insect herbivores.

We grew sugarcane (*Saccharum* spp. hybrid) in high and low Si soils, associated with native AM fungal communities, a commercial AM fungal community or with no AM fungi. Canegrub (*Dermolepida albohirtum*) performance was measured in a feeding assay.

Within low Si soil, both commercial and native AM communities reduced canegrub growth rates by 107% and 81%, respectively, while increasing root Si concentrations by 70% and 41%, respectively. Root AM colonisation strongly correlated with root Si concentrations in low Si soil. Conversely, within high Si soil, AM fungi had no impact on plant Si concentrations or canegrub growth rates. However, canegrub root consumption was reduced by AM fungi, which was a response independent of Si.

Our study suggests the negative impacts of AM fungi on root herbivores are associated with an increase in plant Si, when soil Si is limited. These results shed light on the mechanisms underpinning interactions between AM fungi and root feeding

insects and also highlight the complex and multifaceted nature of these relationships, requiring further research.

6.2 Introduction

Most terrestrial plants associate with arbuscular mycorrhizal (AM) fungi (Smith & Smith 2011). This symbiosis is frequently mutualistic and is generally based on the transfer of carbon from the host plant and soil nutrients such as phosphorus (P) and nitrogen (N) from the fungus (Smith & Read 2010). The degree to which this ancient relationship is mutualistic can be determined by plant and fungal community identities as well as environmental factors such as soil type and nutrient availability (Jones & Smith 2004).

The effects of AM fungi on foliar feeding insect herbivores are highly variable (Koricheva, Gange & Jones 2009) and the mechanisms remain unclear (Bennett, Alers-Garcia & Bever 2006). The majority of this research has focussed on aboveground insects, with relatively few studies investigating how AM fungi affect root herbivore performance (see Johnson & Rasmann 2015 and references therein). This is surprising as root herbivores not only impact plant fitness and communities but are of major importance to food webs and ecosystem functioning (Blossey & Hunt-Joshi 2003). Additionally, root feeding insects and AM fungi share the same soil environment and interact with the same part of the host plant, the roots. As such, there should be stronger evolutionary selection pressure for AM fungi to impact host plant suitability for belowground herbivores than they do aboveground (Johnson, Erb & Hartley 2016). This is because root herbivory decreases photosynthesis, reducing photoassimilates available for transfer to AM symbionts (Zvereva & Kozlov 2011). Additionally, root herbivores reduce root mass available for AM colonisation and can also potentially inflict damage to AM fungi directly (Johnson & Rasmann 2015). Indeed, of the handful of studies that have investigated the impacts of AM fungi on root feeding insects, all except one (Currie, Murray & Gange 2011), found that mycorrhizal colonisation of the host plant negatively impacted root herbivores, suggesting a significant role of AM fungi in plant defences. Yet the mechanisms behind these negative effects are still unclear (Gange 2001; Johnson & Rasmann 2015).

As the AM fungi–plant symbiosis is based on the transfer of nutrients, much of the literature emphasises the role of AM fungi in plant nutrition. Indeed, as AM fungi exert strong effects on plant nutritional status, this in turn can affect chemical defences against root herbivores. However AM fungi also initiate changes in plant defence pathways and chemicals (Jung *et al.* 2012) in ways that cannot be attributed to improved nutritional status alone (Liu *et al.* 2007). AM priming of plant defences is a major mechanism behind AM induced resistance to pathogens and herbivores (Pozo & Azcón-Aguilar 2007). This includes priming of the plant jasmonic acid pathway (Hause *et al.* 2002), which is responsible for the production of many chemical defences against chewing insects (Howe & Jander 2008). Overall, several plant mediated mechanisms have been implicated in AM fungi–insect interactions (Koricheva, Gange & Jones 2009; Jung *et al.* 2012). However, one plant defence that has been neglected to be considered as a potential mechanism through which AM fungi influence insect herbivores is silicon.

Silicon (Si) is the second most abundant element in the Earth's crust and is now recognised to have a significant role in several aspects of plant ecology and evolution (Cooke, DeGabriel & Hartley 2016). Silicon is taken up by plant roots from the soil in the form of silicic acid (H_4SiO_4) (Ma & Yamaji 2015) where it is deposited within plant tissue as SiO_2, commonly known as phytoliths or silica bodies. The efficacy of Si as a defence against a range of aboveground herbivores is well established (Keeping, Kvedaras & Bruton 2009; Massey & Hartley 2009; see examples in Reynolds, Keeping & Meyer 2009). These effects are mainly attributed to increases in physical toughness of plant tissue, increased mandibular wear, decreased nutritional value and decreased digestibility of plant tissue (Massey & Hartley 2009). We recently demonstrated, for the first time to our knowledge, the negative impacts of plant Si on a root feeding insect (Frew *et al.* 2016c). We also provided evidence supporting the theory of a potential trade-off between plant phenolics and Si found within Si accumulating plants (Schaller, Brackhage & Dudel 2012; Cooke & Leishman 2012). Indeed, it has been shown that AM fungi can increase Si uptake in plants (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996), although the exact mechanisms remain unclear. This highlights the potential role of Si within AM mediated defences

against root herbivory. Considering the importance of root herbivores and AM fungi to food webs and ecosystem functioning, it is important to gain a better understanding of how these organisms affect each other in order to gain a more comprehensive appreciation of their interactions and impacts.

As the effects of AM fungi on plant–insect interactions can vary depending on AM species and AM community composition (Bennett & Bever 2007), we investigated the potential role of Si using two different AM communities. We investigated the impacts of a commercially available AM community and the native soil AM community on plant growth, photosynthesis and chemistry alongside root herbivore performance within two different soil types, selected for their low and high Si concentrations. We carried out a pot experiment and feeding trials using sugarcane (*Saccharum* spp. hybrid L.) and greyback cane beetle larvae (*Dermolepida albohirtum* Waterhouse), colloquially known as canegrubs. The sugar industry of Australia loses up to AU$40 million a year as a result of damage from the canegrub, and sugarcane is a Si accumulating plant that forms associations with AM fungi. Considering these traits, alongside the economic significance of the canegrub, this presented a good model to test our hypotheses.

We hypothesised that AM fungi would promote plant growth regardless of soil type but that native AM communities would promote a larger growth response compared to the commercial AM communities. This is because commercially available AM fungi are possibly less likely than native AM fungi to be adapted to a specific local soil environment (Schechter & Bruns 2012; Johnson *et al.* 2016a), and therefore host plants could be expected to have stronger response to the native communities present within the field. We expected root herbivore performance to be lowest on plants associated with native AM fungal communities, but only within low Si soil, as effects of AM fungi on host plant growth and physiology are often limited in high nutrient soils (Treseder 2004). We therefore expected a similar response regarding Si, where plants in high Si soil are able to access and/or uptake sufficient Si without AM fungal facilitation. Therefore, we expected less of a difference in root herbivore performance between AM and non AM plants within high Si soil.

6.3 Materials and methods

6.3.1 Plant growth and AM treatments

We grew 60 sugarcane (*Saccharum* species hybrids L.) plants of Q138, a commonly grown cultivar within Australia, from single-eye cuttings. Plants were germinated in trays of gamma-irradiated potting mix (Richgro© All Purpose Potting Mix), receiving tap water *ad libitum* for three weeks in a shade house. All plants were then transferred to 10 litre pots with one of two different soils, a low Si soil (1,392mg/kg) or a high Si soil (2,221 mg/kg), most soils range from 1,000 to 3,000 mg/kg (See Appendix II Table S6-1 for soil nutrient analysis). Soils were sourced from two sugarcane fields in the Gordonvale region of north Queensland, Australia, fully described in Frew & Johnson (2017). Both soils were fully homogenised and gamma-irradiated, rather than autoclaved or heat sterilised, to minimise impacts of sterilisation on soil texture and nutrient availability. At this stage all plants received AM treatments of approx. 400 AM spores by pipetting onto seedling roots. The 'AM fungi' treatments comprising of one of the following:

- **Non AM** inoculum comprising of equal proportions of commercial AM and native AM inoculants (from both low Si and high Si soil equally) sterilised by autoclaving.
- **Commercial AM** inoculum Start-up Super© from Microbe Smart Pty. Ltd., Melrose Park DC, South Australia, listed to contain spores from four AM fungal species: *Glomus etunicatum*, *G. coronatum*, *G. intraradices* and *G. mosseae*. Spores were extracted from the inoculum using wet sieving and sucrose centrifugation extraction method (Daniels & Skipper 1982).
- **Native AM** inoculum comprising of AM spores extracted from sugarcane field soil from either the low Si soil or high Si soil, extracted using the same method (see above). Native AM inoculum was only applied to the respective native soil (i.e. low Si soil inoculant was the native AM treatment for plants grown in low Si soil).

To ensure all AM treatments received a similar number of spores, extraneous spore extraction solution (without spores) was removed to produce inoculants with a similar average spore density. All pots also received microbial filtrate (300 ml) to standardise the microbial community within each pot at the initiation of the

treatment. This filtrate was created by using the extraneous extraction solution (without spores) from the commercial AM fungal inoculant, low Si soil 'native' inoculant and high Si soil 'native' inoculant in equal proportions.

Pots were randomly distributed on benches within a shade house and received natural light throughout, which was approximately 640 mol^{-2} m^{-2} s^{-1} on a clear day. Temperature was logged every 30 mins throughout the experiment, mean day and night temperatures throughout the growth period were 25.8°C and 15.7°C, respectively. All pots received water *ad libitum.* Every two weeks all pots were randomly re-arranged within the shade house to reduce any spatial or edge effects.

Rates of photosynthesis were measured within the shade house approximately every three weeks with a Portable Photosynthesis System (LI-6400, Li-COR Inc., Lincoln, USA). Plants were grown for 26 weeks before being harvested. After three weeks, all plants were removed from the pots, along with the larvae. The leaves, stems and roots were separated, roots were thoroughly washed, and all plant material was placed in a 40°C oven for 72 hours, and then weighed. A subsample of fresh root material was retained from each plant to be used for a feeding assay.

To confirm colonisation of roots under the AM treatments and absence of colonisation of the roots under the 'non AM' treatment, a random sample of 1–2 g of fresh root from every plant was cleared with 10% KOH in a 90°C water bath for 10 mins and then stained with 5% ink-vinegar (Vierheilig *et al.* 1998). A random selection of the cleared and stained roots were mounted on glass slides with glycerine under a cover slip and scored for presence of AM fungi using the intersect method (McGonigle *et al.* 1990) for 50 intersects. When quantifying colonisation, only hyphae in which there was a visible connection to AM structures (arbuscules, vesicles, spores) were counted, to exclude other types of non-mycorrhizal hyphae. No colonisation was detected in the non AM plants.

6.3.2 Feeding assay

To investigate the impacts of AM fungi on the feeding behaviour and performance of the canegrub, feeding assays were carried out as in Frew *et al*. (2016c), adapted from Massey & Hartley (2009). Individual young third instar larvae, starved for 24

hours, were weighed before being placed in a Petri dish (14 cm diameter) with approximately 5 g of fresh sugarcane root material, taken from the harvested sugarcane plants. Larvae and root type were randomly allocated, kept at 26°C and were allowed to feed for 24 h, after which time they were starved for a further 12 h to ensure all frass was expelled, before being reweighed. Values of water content, derived from root samples from the same plants, were used when converting fresh mass of roots to dry mass, to account for any evaporative water loss during the experiment. Alongside canegrub mass gained/lost over the experimental period, two insect performance indices were calculated according to Slansky (1985):

- Relative growth rate calculates body mass growth relative to initial body mass, and was calculated from: mass gained (g) / initial body mass (g)/ time (days).

- Relative consumption estimates the mass of root material ingested over the 24 hour period relative to initial body mass and was calculated from: food ingested (mg change in dry root mass)/ mean body mass over experimental period (mg body mass).

6.3.3 Plant chemical analysis

All dry plant leaf and root samples were ball milled and a subsample of approximately 40 mg was analysed for N and carbon (C) concentrations using an elemental analyser (FLASH EA 1112 Series CHN analyser, Thermo-Finnigan, Waltham, MA, USA). Concentrations of Si and P were determined as described in (Hiltpold *et al.* 2017) by an X-ray fluorescence spectrometer (Epsilon 3x, PANalytical, EA Almelo, The Netherlands), based on the method of Reidinger *et al*. (2012). Total phenolic concentrations in the roots were determined as described in Salminen and Karonen (2011), in technical triplicates, using a Folin-Ciocalteu assay with gallic acid monohydrate (Sigma-Aldrich, St. Louis, MO, USA) as the quantification standard.

6.3.4 Statistical analysis

R statistical interface (v3.2.3) was used for all statistical analyses (R Core Team 2015).

Responses were analysed including ‘AM fungi’ and ‘soil type’ as explanatory factors (see Results section 6.4 and Table S6-2) in ANOVAs type = III, to give greater power

of analysis for interactions, from the R package 'car' (Fox & Weisberg 2011). This highlighted significant effects and interactions involving 'soil type' and therefore for ease of interpretation, responses within the low Si soil and the high Si soil were analysed independently.

Independent t-tests using the 't.test' function in R were used to analyse for any differences between commercial AM and native AM treatments on AM root colonisation. Sample sizes were unbalanced as some of the plants died before harvest.

Rates of photosynthesis within both soil types were analysed using a linear mixed effects model ('lmer' function) from the R package 'lme4' (Bates *et al.* 2015), with photosynthesis as the response variable and 'AM fungi' treatment as a fixed effect. To account for the effects of measurements taken over time and to account for non-independence of measurements taken on same individual plants, 'week' and 'plant number' were considered as random effects in the model.

Permutational multivariate analyses of variance (PERMANOVA) using the 'adonis' function within the R package 'vegan' (Oksanen *et al.* 2015) were used to analyse root C:N ratio response in low Si soil to 'AM fungi' as the data did not meet assumptions of normality even after transformations were applied. Spearman's rank correlation coefficient using the 'cor.test' function in R was used to analyse for correlations between root Si concentrations and AM root colonisation. All other plant responses (biomass, root total phenolics, root Si concentrations and root P concentrations) were analysed using ANOVAs type = II from the R package 'car', followed by Tukey's *post hoc* comparisons of means tests using the package 'agricolae' (Mendiburu 2015). Log transformations were applied to any data that did not meet the assumptions of normality (Tables S6-2 and S6-3).

From the feeding assays, differences in the larval relative consumption and the relative growth rate from both soil types were assessed using ANOVA type = II from the 'car' package, followed by Tukey's *post hoc* comparisons of means tests. The relative consumption data were not normally distributed and log transformations were applied to meet the assumptions of the model. Pearson's product-moment

correlation using 'cor.test' function in R was used to test correlations between the larval change in mass and root Si concentrations.

6.4 Results

6.4.1 AM colonisation

Arbuscular mycorrhizal root colonisation was significantly affected by AM treatments in both the low and high Si soils (low Si soil: $F_{2,26} = 58.38$, $P < 0.001$; high Si soil: $F_{2,23} = 59.87$, $P < 0.001$); the non AM roots showed no evidence of root colonisation (Fig. S6-1). There was no significant difference between the root colonisation under the commercial (27.4 ± 2.3%) and native (27.7 ± 2.8%) AM treatments ($t = 0.09$, $P = 0.92$) in the low Si soil. Similarly, there was no significant difference between the root colonisation under the commercial (17.1 ± 1.7%) and native (16.1 ± 1.3%) AM treatments ($t = 0.47$, $P = 0.65$) in the high Si soil.

6.4.2 Plant responses to AM fungi

The photosynthetic rates of plants were significantly increased by AM fungi throughout the experiment overall in both the low ($F_{2, 159} = 29.62$, $P < 0.001$; Fig. 6-1a) and high Si soil ($F_{2, 155} = 32.04$, $P < 0.001$; Fig. 6-1b) with no difference between commercial and native AM treatments in either soil type. These elevated photosynthetic rates were reflected in the plant growth as AM fungi significantly increased aboveground biomass, root biomass and total biomass of the plants in the low Si soil (Fig. 6-1c) and the high Si soil (Fig 6-1d; Table S6-2). In both soil types, there was no difference in biomass of plants between the commercial and native AM treatments (low Si soil: $t = 0.67$, $P = 0.51$; high Si soil: $t = 0.09$, $P = 0.92$).

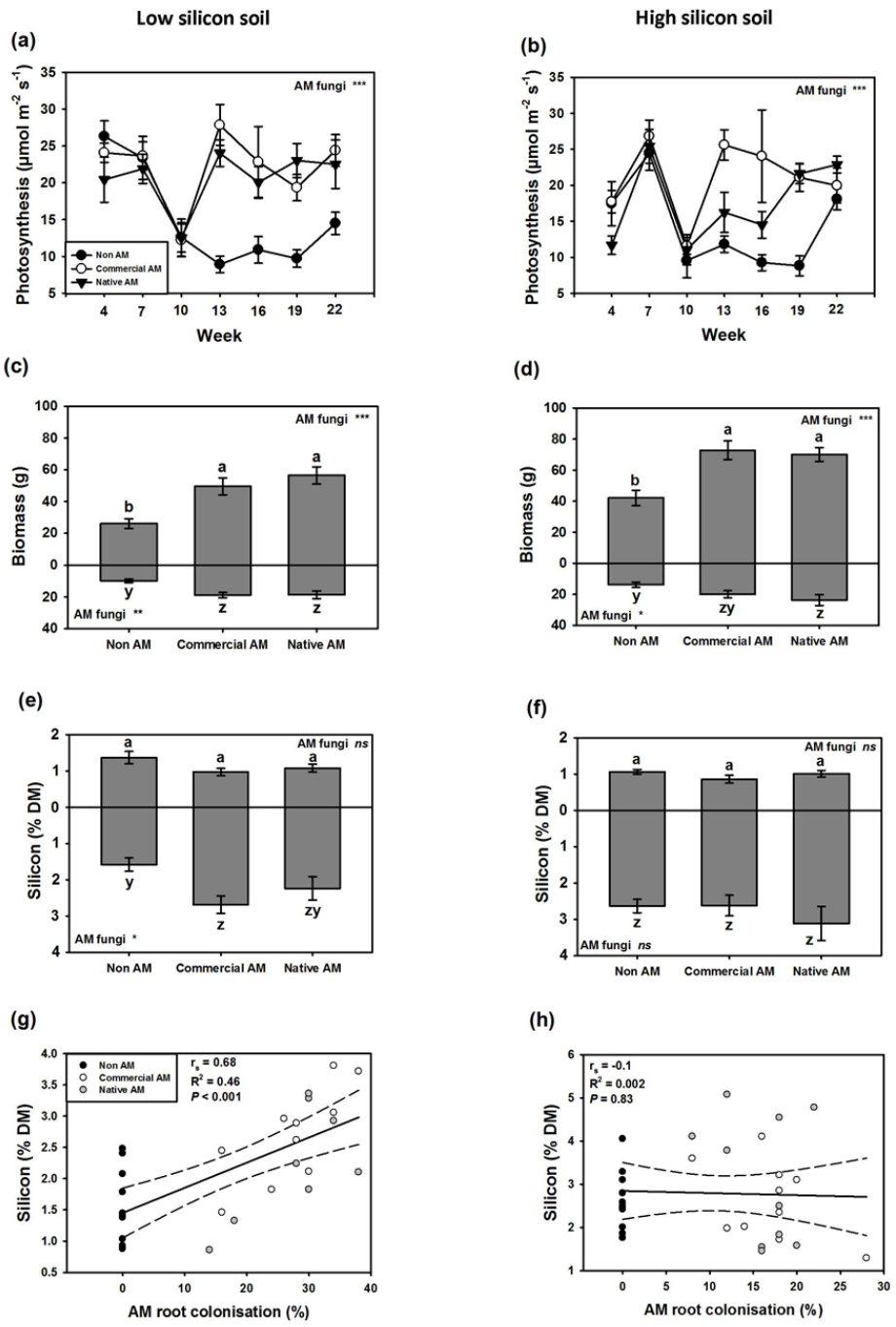

Figure 6-1. Rates of sugarcane (*Saccharum* spp. hybrid) photosynthesis (µmol m^{-2} s^{-1}) measured at different weeks throughout the experiment under different arbuscular mycorrhizal (AM) treatments (non AM, commercial AM and native AM) within low silicon (Si) **(a)** and high Si soil **(b)**. Aboveground and belowground biomass (g) of sugarcane in low Si **(c)** and high Si soil **(d)**, Si concentration (% dry mass) of sugarcane leaves and roots in low **(e)** and high Si soil **(f)**. Levels of significance are shown for effects of AM fungi treatments. Degrees of significance are indicated as follows: ns = not significant, . *P*<0.1 , * *P*<0.05, ** *P*<0.01, *** *P*<0.001. Where factor effects are significant, bars not sharing a common letter (a, b or y, z) differ significantly (Tukey; $P < 0.05$), note that these comparisons are made within the low and high silicon soils, not between them. Values are means ± SE. Correlations of AM root colonisation (%) and Si concentration (% dry mass) of roots all under different AM treatments within low Si **(g)** and high Si soil **(h)**. Solid line represents linear regression through all the data points, and dashed lines represent the associated 95% confidence intervals. Correlation coefficients (r), coefficient of determination (R^2) and *P*-values are shown. *N* = 10 for low Si soil, except for the non AM treatment where *N* = 9; *N* = 9 for high Si soil.

Silicon concentrations of plant leaves were not affected by AM fungi in either the low or high Si soil (low Si soil: $F_{2,26} = 2.17$, $P = 0.14$; high Si soil: $F_{2,24} = 1.56$, $P = 0.23$). In the low Si soil, the commercial and native AM treatments increased root Si by 70.2% and 41.7%, respectively, compared to the non AM plants (Fig. 6-1e; Table S6-3). There was no significant difference in root Si concentrations between the commercial and native AM plants (low Si soil: $t = 1.12$, $P = 0.28$). In the low Si soil there was a positive correlation between root colonisation and root Si concentration ($r_s = 0.68$, $R^2 = 0.46$, $P < 0.001$; Fig. 6-1g). Contrastingly, in the high Si soil AM fungi had no effect on the root Si concentrations (Fig. 6-1f; Table S6-3) and there was no correlation between root colonisation and root Si concentration ($r_s = -0.1$, $R^2 = 0.002$, $P = 0.83$; Fig. 6-1h). Root P concentrations were unaffected by AM fungi in either soil type. Similarly, the C:N ratio and total phenolic concentrations of the roots were unaffected by AM fungi in both soil types (Table S6-3).

6.4.3 Insect responses to AM fungi

In the low Si soil, there was no significant main effect of AM fungi on the consumption of root material (Fig. 6-2a; Table S6-4). However there was a significant difference when comparing AM treatments (grouping commercial and native AM fungi) to the non AM treatment ($F_{2,26} = 4.88$, $P = 0.03$), where non AM plants saw significantly higher consumption by the canegrubs (0.14 ± 0.03 g g^{-1}) compared to the AM plants (0.06 ± 0.01 g g^{-1}). In the high Si soil, the consumption of root material was significantly impacted by AM fungi (Table S6-4), where larvae feeding on non AM roots had significantly higher relative consumption compared to those feeding on roots associated with the commercial AM fungi (Fig. 6-2b).

In the low Si soil, the relative growth rate of the larvae was negatively affected by AM fungi, where larvae feeding on non AM plants performed best (Fig. 6-2c, Table S6-4). In the high Si soil, there was no effect of AM fungi on the growth rate of larvae (Fig. 6-2d, Table S6-4). A negative correlation was found between the change in mass of the larvae from the feeding assay and the Si concentrations of the roots, in both the low ($r_p = -0.46$, $R^2 = 0.18$, $P = 0.01$; Fig. 3a) and high ($r_p = -0.38$, $R^2 = 0.11$, $P = 0.04$; Fig. 6-3b) Si soils.

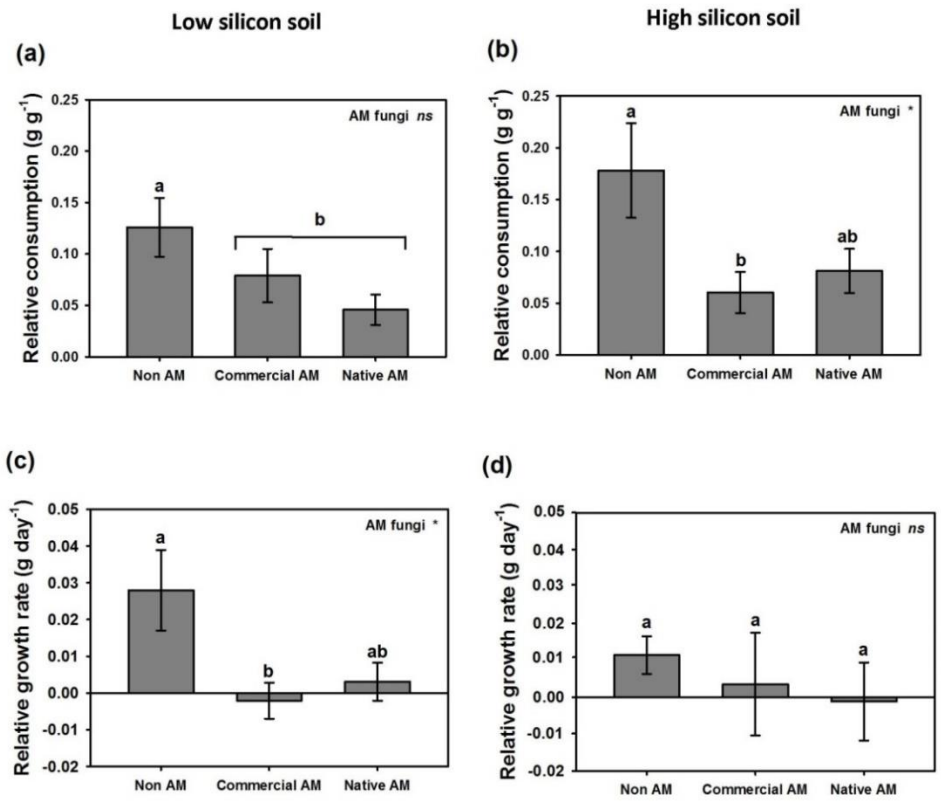

Figure 6-2. Relative consumption of roots [=food ingested (mg change in dry mass)/ mean body mass (mg fresh mass)] by canegrubs (*Dermolepida albohirtum*) feeding on roots grown under different arbuscular mycorrhizal (AM) fungal treatments (non AM, commercial AM and native AM) within a low silicon (Si) **(a)** and a high Si soil **(b)**. Relative growth rate [=mass gained (g)/initial body mass (g)/ time (days)] of canegrubs feeding on roots grown under different AM treatments within a low Si **(c)** and a high Si soil **(d)**. Values are means ± SE. Degrees of significance are indicated as follows: ns = not significant, * P<0.05. Where factor effects are significant, bars not sharing a common letter (a, b) differ significantly (Tukey; $P < 0.05$), note that these comparisons are made within the low and high silicon soil, not between them. N = 10 for low Si soil, except for the non AM treatment where N = 9; N = 9 for high Si soil.

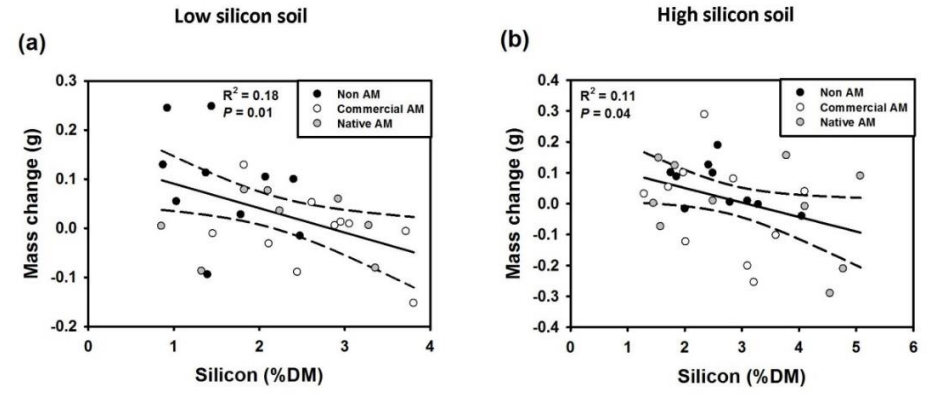

Figure 6-3. Correlations between the change in canegrub (*Dermolepida albohirtum*) mass (g) and root silicon (Si) concentrations (% dry mass) under different arbuscular mycorrhizal (AM) fungal treatments (non AM, commercial AM and native AM) within a low silicon (Si) soil **(a)** and a high Si soil **(b)**. Solid lines represent linear regression through all the data points. Dashed lines represent 95% confidence intervals. Coefficient of determination (R^2) and P-values are shown. N = 10 for low Si soil, except for the non AM treatment where N = 9; N = 9 for high Si soil.

6.5 Discussion

We have shown that AM fungi can negatively impact the performance of a root feeding insect and present evidence suggestive of a context dependent Si based mechanism. The degree to which AM fungi are mutualists is often dependent on soil nutrients such as N and P (Treseder 2004). Our study has shown this may also be the case for soil Si, where we propose AM fungi only provide a Si based defensive benefit to the host plant when soil Si concentrations are low. Within soils with higher concentrations of available Si, AM fungi can still impact root herbivore consumption, independent of Si, highlighting the multifaceted nature of interactions between AM fungi, their host plants and root feeding insects. Although other differences between the two soils may have impacted unmeasured plant traits or defence compounds, our evidence indicates Si based defences play an important role in the plant mediated interactions between AM fungi and root feeding insects.

Plants in both low Si and high Si soil showed increases in their rates of photosynthesis in response to AM fungi, with no difference between commercial and native communities. This effect only became clear during measurements at 13 weeks of growth (10 weeks after AM fungal inoculation), possibly as AM root colonisation and mycelial colonisation of the soil was established. Increases in photosynthesis in response to AM colonisation have been reported previously (Wright, Scholes & Read 1998; Wu & Xia 2006), and have been attributed to several mechanisms such as alterations in plant hormones (Drüge & Schonbeck 1993), increased transport of water and nutrition and an increased carbon sink for photoassimilates from the presence of AM fungi in the soil. Here, increases in photosynthesis were reflected by significant increases in biomass, again seen in both soil types, irrespective of AM community identity. These, almost parallel, plant responses to the native and commercial AM communities were surprising, as we expected commercial inocula to be less likely to contain species adapted for a particular local soil environment (Hartley & Gange 2009; Johnson *et al.* 2016a). However, as we did not confirm AM fungi species identity, it is possible that the AM inoculants shared some species.

Within the low Si soil, canegrub performance (growth rates and root consumption) was negatively impacted by AM fungi, irrespective of AM community identity. A nutritional explanation based on food quality is unlikely as there was no effect of AM fungi on the C:N ratio of the roots, which is an indicator of plant nutritional quality for insects, or on root P concentrations. Additionally, there was no impact of AM fungi on the root phenolic compounds. The reduction in canegrub performance is most likely a response to the higher concentrations of Si in the roots of the AM plants, particularly considering the strength of the relationship between AM root colonisation and root Si concentrations. Indeed AM fungi had previously been reported to increase Si concentrations in plants (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996), although the mechanisms are still unclear.

AM fungi had no impact on root Si concentrations within the high Si soil, and there was no relationship between AM root colonisation and root Si, which supports the hypothesis that AM fungi facilitate Si uptake when it is limiting in the soil. Indeed, there was no impact of AM fungi on canegrub growth rates. However, there was a significant impact of AM fungi, in the high Si soil, on canegrub root consumption which was not explainable by root Si concentrations. This suggests that insect herbivore responses to AM fungi is a complex interaction, likely to involve Si and several other mechanisms (Hartley & Gange 2009; Koricheva, Gange & Jones 2009). Indeed, it is likely that other unmeasured root phytochemicals were affected by AM fungi, which could explain this effect on canegrub root consumption in high Si soil. This highlights the need for more comprehensive identification and quantification of changes in plant metabolites in response to AM fungi to gain a more complete understanding of mechanisms underpinning these relationships. Nevertheless, within both soil types, there were negative correlations between canegrub mass change and root Si concentrations, which support the understanding that Si is an effective plant defence against root feeding insects.

Root feeding insects can potentially reduce the extent to which the AM fungi–plant symbiosis is beneficial. This suggests that by increasing Si in the host plant roots, AM fungi may have gained an evolutionary advantage by increasing plant fitness through reduced root herbivory. This way, those AM fungi that increased root Si, minimised

damage to both hyphae and plant roots, thereby maximising nutrient uptake and photosynthesis. This is an oversimplification, as highlighted by the Si-independent effects of AM fungi on canegrub root consumption in this study, as AM fungi impact root feeding insects in more ways than just altering the Si concentrations of their food. Even still, Si is likely to play a significant role in how AM fungi reduce root herbivore performance, and is therefore a possible driver of belowground insect responses to AM fungi, in some contexts.

There are several examples of AM fungi negatively impacting root feeding insects, but the mechanisms remain largely unknown. Silicon based defences have been shown to effectively reduce foliar feeding (Reynolds, Keeping & Meyer 2009) and root feeding (Frew *et al.* 2016c) insect performance, as well as play a key role in ecosystem interactions and plant evolution (Cooke, DeGabriel & Hartley 2016; Strömberg, Di Stilio & Song 2016). Our study implies that AM fungi can facilitate the uptake of Si and suggests a possible synergistic relationship between AM fungi and Si in the defence of plants against root feeding insects in some contexts (e.g., where soil Si concentrations are relatively low). However, AM fungi also reduced consumption of roots grown in soil where Si was abundant, in a way that was independent of Si uptake, thus the mechanisms underlying this relationship are still unclear and require further research.

6.6 Acknowledgements

We thank the teams at Sugar Research Australia Limited and the Hawkesbury Institute for the Environment for their support, particularly Andrew Gherlenda, Lisa Derby and Allen Eaton. Funding was provided by Sugar Research Australia (project no. 2014/104).

Chapter 7: Arbuscular mycorrhizal fungi stimulate immune function whereas root silicon diminishes growth in a soil dwelling herbivore

7.1 Abstract

In order to survive insects have evolved immune defences to protect themselves from pathogens and natural enemies. These immune defences, alongside insect growth, are known to be impacted by host plant quality. Arbuscular mycorrhizal (AM) fungi alter host plant nutritional quality, and can increase plant silicon (Si) concentrations. As plant Si defences also alter host plant quality, this highlights the potential for AM fungi and Si to impact root herbivore growth and immune function. The majority of studies have examined the effects of AM fungi on aboveground insect herbivores, and none have investigated the impacts on insect immune function. This aim of this study was to test the effects of AM fungi and Si on plant growth alongside the growth and immune function of a root feeding insect.

This was tested using two sugarcane varieties (*Saccharum* species hybrids), each of which were grown with (AM) or without (non AM) AM fungi, half of which received Si supplementation (Si+) while the other half did not (Si-). Canegrubs (*Dermolepida albohirtum*) fed on half the plants and their immune function was assessed through measurement of phenoloxidase activity in a pathogen bioassay. A 24-hour feeding trial was also carried out to assess insect growth and root consumption.

Si increased plant biomass by 15%, while AM fungi promoted plant growth only when no Si was applied. Si decreased insect growth rates and root consumption, the latter by 71%. Insect growth rate and root consumption were reduced by AM fungi, but only when no Si was applied and only in one plant variety. Insect immune function was increased by 62% under the AM treatment, which was unrelated to host plant nutritional quality, while immune function was negatively correlated with insect mass.

This study demonstrates that the negative impacts of AM fungi on root feeding insects can depend on Si availability and plant variety. The results also suggest AM

fungi can prime insect immune function, independent of host plant quality or Si concentrations, highlighting the complexity of interactions between AM fungi and soil dwelling insects.

7.2 Introduction

The performance of insect herbivores is dependent on the quality and defensive traits of their host plants (Awmack & Leather 2002). In order to survive, insects have evolved mechanisms to defend themselves against pathogens and natural enemies, namely their immune defences (Schmid-Hempel 2005). The insect immune system comprises several levels of defence, but the primary defence mechanisms occur through encapsulation and melanisation (Smilanich, Dyer & Gentry 2009). Phenoloxidase (PO) is a key enzyme to the production of melanin (Cerenius & Söderhäll 2004), which is used in the melanisation and encapsulation processes to externalise an invading body. As such, the measurement of PO activity is often used as an indicator of the immune system activity of an insect (Cotter & Wilson 2002; Cotter *et al.* 2004; Triggs & Knell 2012). Host plant quality impacts insect growth, but also insect immunity, where reductions in plant quality can reduce insect immune function (Lee, Simpson & Wilson 2008; Gherlenda *et al.* 2016).

The nutritional quality of plants is dependent on soil fertility but also interactions with co-evolved microbial symbioses. For example, arbuscular mycorrhizal (AM) fungi associate with the majority of land plants, a symbiosis that is based on the transfer of soil nutrients such as phosphorus (P) and nitrogen (N) in exchange for plant photoassimilates. Therefore, it is not surprising that AM fungi can impact the performance of herbivorous insects, as plant concentrations of N as well as P are known to affect herbivore performance (Elser *et al.* 2000). Yet no studies, to our knowledge, have investigated the impacts of AM fungi on insect immunity. The majority of research has focussed on the effects of AM fungi on aboveground insects with only a handful of studies investigating the response of root feeding insects (Johnson & Rasmann 2015). This is surprising as belowground herbivores and AM fungi share the same soil environment and the same organ of the host plant. Most studies have found AM fungi negatively impact root feeding insects (Johnson &

Rasmann 2015). Although the mechanisms remain unclear, Si uptake has been implicated in some of these cases.

In addition to increasing P and N uptake, AM fungi can increase silicon (Si) uptake in plants (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996; Garg & Bhandari 2016) and have recently been observed to increase plant Si in soils with low Si concentrations, with negative impacts on root herbivore performance (chapter 6). The efficacy of plant Si as a defence against herbivorous insects is well documented (Massey & Hartley 2009; Reynolds, Keeping & Meyer 2009) and has also been shown to be effective against root feeding insects (Frew *et al.* 2016a; c). These impacts on insect herbivores are largely attributed to increased plant toughness, reduced palatability and digestibility by mechanical protection of the parenchyma cells, where insects retrieve much of their starch and protein. Therefore, in reducing the palatability and digestibility, Si reduces plant quality as a food source for insects. This highlights the potential for Si to impact soil insect growth, root consumption as well as their immune function (Lee, Simpson & Wilson 2008). Indeed, the functional significance of plant Si has only recently been acknowledged (Cooke, DeGabriel & Hartley 2016) where it has been suggested that insect herbivory constituted evolutionary selection pressure for Si accumulation in plants (Strömberg, Di Stilio & Song 2016). The effects of Si and AM fungi on host plant quality highlights their potential to impact insect growth and immune function.

We studied the roles of AM fungi and Si on the growth and immunity of a root feeding insect using sugarcane (*Saccharum* species hybrids L.), a known Si accumulator that is also mycorrhizal, and the larvae of the greyback cane beetle (*Dermolepida albohirtum* Waterhouse), colloquially known as canegrubs. There can be wide variation between plant varieties in terms of their Si uptake efficiency (Soininen *et al.* 2013) as well as their responsiveness to AM fungi (Sawers, Gutjahr & Paszkowski 2008). We, therefore, examined the effects of Si and AM fungi on two varieties of sugarcane with distinct breeding lineages, one perceived to be more resilient to biotic stresses (Q240) than the other (Q200). We also examined the impacts on canegrub growth, consumption and the effects of the treatments on the immune function of the canegrubs in a bioassay.

We predicted the following: (i) AM fungi and Si increase plant growth but decrease insect growth via increases in root Si concentrations; (ii) the effects of AM fungi on plant growth alongside the effects on insect growth and immunity are strongest when no Si treatment is applied as high Si availability is likely to mask AM effects; (iii) as both AM fungi and Si will reduce the quality of the plant as a food source due to increases in Si, immune activity of the insect will also be reduced. However, as AM fungi can increase P and N uptake, this could increase host plant quality and thereby increase insect immune function.

7.3 Materials and methods

7.3.1 Experimental set-up

A factorial experiment with three factors including AM fungi, Si and root herbivory (RH) in a fully crossed design was carried out using 80 sugarcane (*Saccharum* species hybrids: Poaceae) plants of variety Q200 and 80 of variety Q240 grown from single-eye cuttings. Plants were germinated in trays of gamma irradiated potting mix (Richgro© All Purpose Potting Mix), receiving tap water *ad libitum* for two weeks in a shade house. All plants were then transferred to 3.7 L pots with gamma irradiated soil originally sourced from a sugarcane field in Queensland, Australia, fully described in Frew & Johnson (2017) as 'soil A'.

Half of the plants were inoculated with approx. 400 AM fungal spores from a commercial inoculum, Start-up Super© (Microbe Smart Pty. Ltd., Melrose Park DC, South Australia) comprising spores from four species identified as *Glomus etunicatum*, *G. coronatum*, *G. intraradices* and *G. mosseae*. Spores were extracted from the inoculum using wet sieving and a sucrose centrifugation extraction method (Daniels & Skipper 1982). All pots also received microbial filtrate (300 mL) to standardise the microbial community within each pot at the initiation of the treatment. This filtrate was created by using the extraneous extraction solution (without spores) from the AM fungal inoculant. Half of the plants received 200 mL of 500 mgL^{-1} soluble Si in the form of $NaSiO_3.9H_2O$ (Cid *et al.* 1990) every two days (Si+), while the other half received only water (Si-). $NaSiO_3.9H_2O$ is a highly efficient Si

fertiliser in other grass species (Mecfel *et al.* 2007), and has been used in several previous studies (Reynolds, Keeping & Meyer 2009).

Pots were randomly distributed within the shade house and received natural light throughout, which was approximately 640 mol^{-2} m^{-2} s^{-1} on a clear day. Air temperature was logged throughout the experiment; mean day and night temperatures throughout the growth period were 26.9°C and 14.3°C, respectively. Every two weeks all pots were randomly re-arranged within the shade house to reduce any spatial or edge effects. Throughout the experiment all plants received tap water as required. Plants were grown under their respective treatments for 23 weeks before being harvested.

Three weeks prior to harvesting the plants, a third instar canegrub (*D. albohirtum*) was weighed, and placed in the soil of each plant that was designated to be subject to root herbivore treatment (RH+), while the other half received no canegrub (RH-). To account for any direct impacts of the treatments on the larvae, 16 pots with no plants, only soil, were placed into the shadehouse and individual canegrubs were also placed into these pots. These pots were also treated with and without Si treatments. After three weeks, all plants were removed from the pots, along with the larvae which formed the RH treated, which were weighed as a measure of performance and then used for the assessment of immune function. The leaves, stems and roots were separated, roots were thoroughly washed, and all plant material were placed in a 40°C oven for 72 hours, and then weighed. One subsample of fresh root material was retained from each plant to be used for feeding efficiency assays (see *Insect feeding assays* section 7.3.2).

To confirm colonisation of roots under the AM treatment and absence of colonisation of uninoculated controls, a random sample of approximately 1–2 g of fresh root from a subsample of five plants from each treatment combination was cleared with 10% KOH in a 90°C water bath for 10 mins and then stained with 5% ink-vinegar (Vierheilig *et al.* 1998). A random selection of the cleared and stained roots were mounted on glass slides with glycerine under a cover slip and scored for presence of AM fungi using the intersect method (McGonigle *et al.* 1990) for 100

intersects. When quantifying colonisation, only hyphae in which there was a visible connection to an AM fungal structure (arbuscule, vesicle or spore) were counted to exclude other types of non-mycorrhizal hyphae.

7.3.2 Insect immune function assay

To assess immune function, all larvae recovered from the pots (RH+ treatment) were washed and surface sterilised with a cotton swab dipped in 70% ethanol. Small pots (50mL) of sterilised sand were inoculated with 50 entomopathogenic nematodes (EPN) (*Heterorhabditis zealandica* Poinar), natural pathogens of soil dwelling insects, before placing one larva into each pot to allow larvae to be infected and for an immune response to develop. This rate of EPN application was confirmed from test infectivity trials to determine the rate that produced high canegrub infection success with minimal early mortality (data not shown). All larvae were left for 72 hours to allow EPN infection to occur and an immune response to develop. Canegrub larvae were then weighed and surface sterilised with a cotton swab dipped in 70% ethanol prior to puncturing with a sterile 26G Hamilton syringe through which haemolymph was collected using a 10 µL capillary tube. A buffered haemolymph solution was made by adding 5 µL of haemolymph to 200 µL of ice-cold phosphate buffered saline solution (PBS, pH 6.4) and was frozen at -80 °C until analysis (Catalán *et al.* 2012). Phenoloxidase activity can be an indicator of insect encapsulation response through the melanisation pathway in response to foreign invaders (Cotter *et al.* 2004). Both PO activity and haemolymph protein concentration were measured to assess PO activity per mg of protein as per Cotter & Wilson (2002). PO activity was assessed by adding 100 µL of buffered haemolymph solution to 100 µL of 20 mM L-DOPA (Sigma–Aldrich, D9628) in a 96 well plate. Absorbance was measured at 492 nm at 25°C at 1 min intervals for 30 min. It had been confirmed in test trials with a time series that the reaction reached linear phase during this time period (data not shown). The PO activity was then expressed as the change in optical density divided by the amount of haemolymph in the buffered solution (Rantala, Vainikka & Kortet 2003). Solubilised protein concentration of the haemolymph was assessed using the Bio-Rad protein assay kit (Bio-Rad, Hercules, California, USA) with bovine serum albumin as the standard, resulting in the calibration of PO activity per mg of protein. A subset

of samples were also run with phenylthiocarbamide (PTC; final concentration 2 µM), a known inhibitor of PO (Thomas *et al.* 1989) as per Gherlenda *et al*. (2016), to confirm no other proteins were present within canegrub haemolymph which could influence the readings of PO activity. Inter-plate variation of readings was controlled for by splitting treatment in equal presentation across plates.

7.3.3 Insect feeding assay

To investigate the impacts of AM fungi and Si on the feeding behaviour and performance of the canegrubs, feeding assays were based on the methods described in Frew *et al*. (2016a) and Massey & Hartley (2009). Individual third instar larvae, which were previously fed exclusively on carrot, were starved for 24 hours and weighed. Each larva was then randomly selected and placed in a Petri dish (14 cm diameter) with approximately 5 g of fresh sugarcane root material, taken from the harvested sugarcane plants. Larvae and root type were randomly allocated, kept at 26°C and were allowed to feed for 24 hours, after which time they were starved for a further 12 hours to ensure all frass was expelled, before being reweighed. Values of water content, derived from root samples from the same plants, were used when converting fresh mass of roots to dry mass, to account for any evaporative water loss during the experiment. Two insect performance indices were calculated according to Slansky (1985):

- Relative growth rate calculates body mass growth relative to initial body mass, and was calculated from: mass gained (g) / initial body mass (g)/ time (days).

- Relative consumption estimates the mass of root material ingested over the 24 hour period relative to initial body mass and was calculated from: food ingested (mg change in dry mass)/ mean body mass over experimental period (mg fresh mass).

7.3.4 Plant chemical analysis

All dry plant root samples were ball milled and a subsample of approximately 40 mg was analysed for nitrogen (N) and carbon (C) concentrations using an elemental analyser (FLASH EA 1112 Series CHN analyser, Thermo-Finnigan, Waltham, MA USA). Concentrations of silicon (Si) and phosphorus (P) were determined as described in

Hiltpold *et al*. (2017) by an X-ray fluorescence spectrometer (Epsilon 3x, PANalytical, EA Almelo, The Netherlands), based on the method of Reidinger *et al*. (2012).

7.3.5 Statistical analysis

R statistical interface (v3.2.3) was used for all statistical analyses (R Core Team 2015).

Sugarcane biomass and root C:N ratio alongside root concentrations of P were all analysed separately by analysis of variance (ANOVA) calculating the marginal sums of squares using the 'Anova' function from the R package 'car' (Fox & Weisberg 2011), testing the effects of 'variety', 'Si', 'AM fungi', 'RH' and their interactions. Sugarcane root mass and concentrations of Si were analysed separately using permutational multivariate analyses of variance (PERMANOVA) using the 'adonis' function within the R package 'vegan' (Oksanen *et al.* 2015) as the data did not meet the assumptions of ANOVA even after transformations were applied.

Differences in the change in mass in the insects used as the root herbivore (RH+) treatment were assessed using two-way ANOVA using the 'Anova' function from the 'car' package. Those pots in which canegrubs had died (four larvae) were not considered in the analysis. From the feeding trials, insect relative growth rate, relative consumption and PO activity were also analysed using the 'Anova' function from the 'car' package, where consumption and PO activity were log transformed to normalise the distribution and stabilise the variance. The RH treatment was initially included as a factor here to test for any effects of previous herbivory (in the plants under RH+ treatment) on the insect responses from the feeding trial, but was dropped from the model due to non-significance. Correlation between insect mass and PO activity was analysed using Pearson's product moment correlation test using the 'cor.test' function.

7.4 Results

7.4.1 Plant varietal differences

Biomass of the two varieties were significantly different from each other (Table 7-1), where Q240 had 5.4% more biomass overall compared to Q200. This was largely driven by root mass as Q240 had 29.5% greater root mass than Q200 (Table 7-1, Fig.

7-1a). Despite that Si uptake can vary between plant varieties, here there was no difference in root Si concentrations between these varieties (Table 7-1, Fig. 7-1b). However, there were differences between the varieties in root C:N ratio (Table 7-1), where Q240 had a greater C: N ratio compared with Q200 (Fig. 7-2).

7.4.2 Plant responses to AM fungi

Mycorrhizal colonisation of roots under the AM treatment (mean 20.08 ± 1.73%) was significantly greater than uninoculated controls ($P < 0.001$; mean 1.23 ± 0.18%). Although there were low levels of colonisation detected in non AM plants, these did not exceed 3% (Fig. S7-1). There was no difference in root colonisation between the varieties ($P = 0.725$; data not shown).

In accordance with our hypothesis, plant biomass was higher in AM plants compared to non AM plants, only when no Si was applied, therefore an interaction between Si and AM fungi was detected (Table 7-1, Fig. 7-1a). Also as hypothesised, root Si concentrations were significantly increased by AM fungi only when no Si was applied (Fig. 7-1b), therefore a significant interaction between AM fungi and Si was detected (Table 7-1).

AM fungi had no observed impact on root C:N ratio (Fig. 7-2) or P concentrations (Table 7-1).

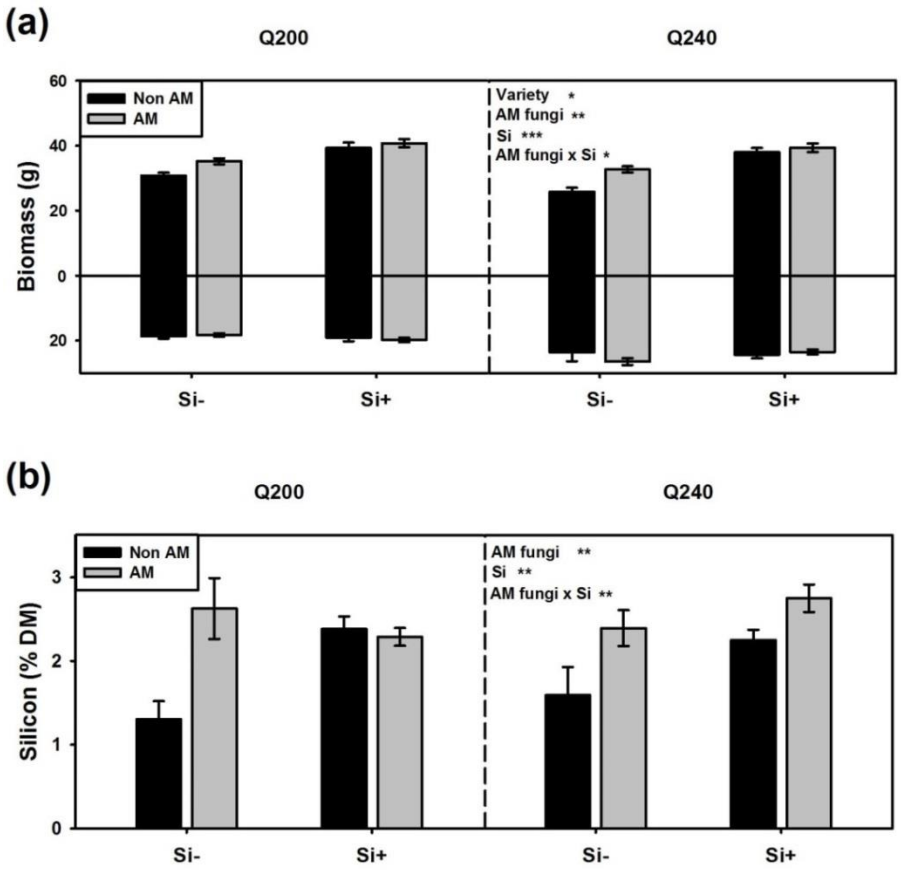

Figure 7-1. (a) Effects of Si treatments on the aboveground and belowground biomass (g) of two sugarcane (*Saccharum* species hybrids) varieties grown with (AM) and without (non AM) arbuscular mycorrhizal (AM) fungi. **(b)** Effects of silicon (Si) treatments on sugarcane root Si concentrations (% dry mass) grown with (AM) and without (non AM) arbuscular mycorrhizal fungi. Significant factors and interactions are shown, degrees of significance are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values are means ± SE, $N = 10$.

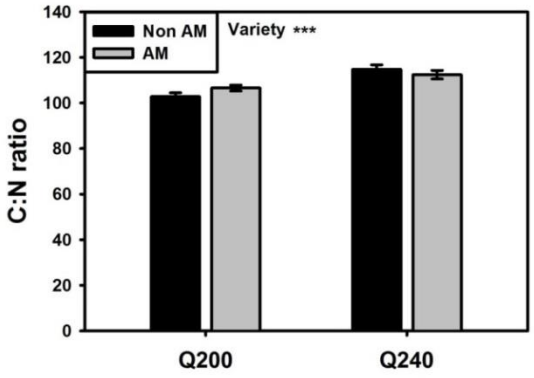

Figure 7-2. Differences in C:N ratio between sugarcane (*Saccharum* species hybrids) roots of varieties Q200 and Q240, grown with or without AM fungi. Significant factors are shown, degrees of significance are indicated as follows: *** $P < 0.001$. Values are means ± SE, $N = 10$.

Table 7-1. Results of ANOVA model for the main effects and interactions of factors on sugarcane responses. Log transformed data and outputs from the PERMANOVA are indicated, where assumptions of ANOVA were not met. Significant impacts ($P < 0.05$) indicated in bold, $N = 10$.

	Response									
Factor	Biomass		Root mass †		Root C:N		Root P		Root Si †	
Figure reference	7-1a		7-1a		7-2		-		7-1b	
	$F_{1,152}$	*P*	$F_{1,152}$	*P*	$F_{1,152}$	*P*	$F_{1,152}$	*P*	$F_{1,152}$	*P*
Variety	**5.869**	**0.017**	**32.917**	**<0.001**	**27.789**	**<0.001**	**47.45**	**<0.001**	0.002	0.557
AM fungi	**11.127**	**0.001**	2.174	0.143	0.19	0.664	0.542	0.46	**0.083**	**0.001**
Si	**43.35**	**<0.001**	0.423	0.516	3.431	0.07	0.963	0.313	**0.039**	**0.007**
RH	0.026	0.871	0.65	0.421	0.609	0.436	1.725	0.192	0.003	0.482
Variety x AM fungi	0.679	0.411	1.06	0.304	3.187	0.076	1.495	0.222	0.001	0.912
Variety x Si	0.022	0.881	0.135	0.714	0.001	0.987	0.107	0.741	0.001	0.657
AM fungi x Si	**4.89**	**0.029**	1.259	0.264	1.156	0.284	0.192	0.655	**0.038**	**0.008**
Variety x RH	0.453	0.502	0.111	0.739	0.409	0.523	2.473	0.116	0.001	0.659
AM fungi x RH	1.892	0.171	0.001	0.979	1.606	0.207	1.091	0.294	0.013	0.132
Si x RH	0.051	0.822	0.193	0.662	0.066	0.798	1.214	0.272	0.001	0.632
Variety x AM fungi x Si	2.283	0.133	4.083	0.05	2.187	0.141	0.013	0.913	0.016	0.092
Variety x AM fungi x RH	0.523	0.471	0.002	0.959	0.386	0.535	0.552	0.454	0.001	0.650
Variety x Si x RH	0.012	0.912	0.073	0.788	1.107	0.294	0.011	0.915	0.001	0.794
AM fungi x Si x RH	0.129	0.72	0.011	0.915	1.559	0.214	4.313	0.051	0.000	0.983
Variety x AM fungi x Si x RH	0.002	0.968	0.052	0.821	2.948	0.089	1.106	0.292	0.008	0.224

* log transformed
† permanova

7.4.3 Plant responses to silicon

As hypothesised, plant biomass increased in response to Si, in both varieties (Table 7-1, Fig. 7-1a) and Si treatment increased root Si concentrations by 22.2% (Table 7-1, Fig. 7-1b).

7.4.4 Plant responses to RH

Biomass and root mass were not impacted by RH treatments (Table 7-1). RH also had no effect on any other plant trait measured (Table 7-1).

7.4.5 Insect response between varieties

The insects used as the RH treatment showed no difference in their mass change between Q200 (mean -0.237 ± 0.095g) and Q240 (mean -0.259 ± 0.054g; Table 7-2). From the feeding trial, insect growth rate was 57.9 % lower on Q240 compared to Q200 (Fig. 7-3a, Table 7-2). Conversely, root consumption was 10.6% higher in Q240 compared to Q200 (Fig. 7-3b, Table 7-2). Insect immune function was also higher when feeding on Q240, by around 76%, compared with Q200 (Fig. 7-4, Table 7-2).

Table 7-2. Results of ANOVA model for the main effects and interactions of factors on insect responses from the larvae used as the root herbivore treatment (RH) and from the feeding assay. Log transformed data are indicated. Significant impacts ($P < 0.05$) are indicated in bold, $N = 20$.

	Response							
Factor	Mass change (RH insects)		RGR		RC*		PO activity*	
Figure reference	-		7-3a		7-3b		7-4	
	$F_{1,75}$	*P*	$F_{1,152}$	*P*	$F_{1,152}$	*P*	$F_{1,75}$	*P*
Variety	0.241	0.625	**14.61**	**<0.001**	**4.001**	**0.047**	3.094	0.08
AM fungi	**6.164**	**0.016**	**6.543**	**0.012**	**5.289**	**0.023**	**6.206**	**0.016**
Si	**14.44**	**<0.001**	**81.12**	**<0.001**	**11.68**	**<0.001**	0.512	0.478
Variety x AM fungi	3.478	0.071	3.419	0.07	**5.726**	**0.018**	0.428	0.516
Variety x Si	0.398	0.53	3.927	0.057	2.761	0.098	0.051	0.885
AM fungi x Si	0.048	0.828	3.446	0.065	0.642	0.424	2.235	0.141
Variety x AM fungi x Si	0.001	0.971	**10.96**	**0.001**	0.358	0.55	3.614	0.063

*log transformed

7.4.6 Insect response to AM fungi

The insects used as the RH treatment exhibited a greater reduction in mass under the AM treatment (mean change in mass -0.357 ± 0.085g) compared to the non AM treatment (mean change in mass -0.133 ± 0.058g; Table 7-2).

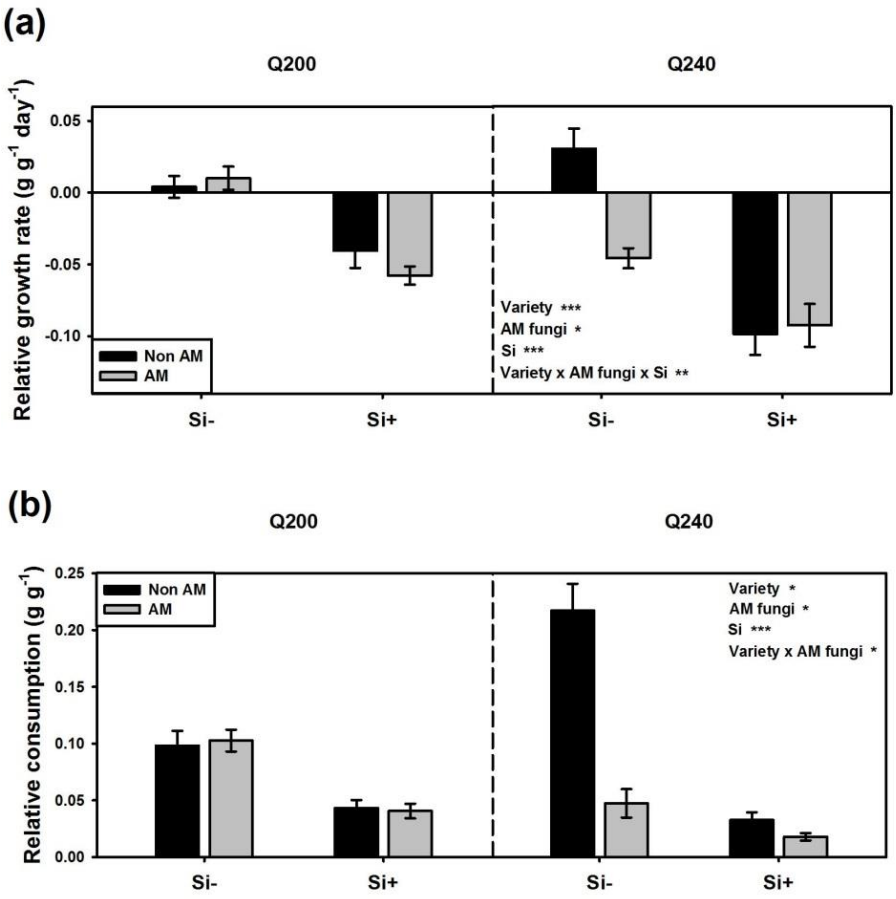

Figure 7-3. (a) Effects of silicon (Si) treatments on canegrub (*Dermolepida albohirtum*) relative growth rate (g g^{-1} day^{-1}) and **(b)** relative consumption (g g^{-1}) of sugarcane (*Saccharum* species hybrids) roots of two varieties (Q200 and Q240) grown with (AM) and without (non AM) arbuscular mycorrhizal (AM) fungi. Significant factors and interactions are shown, degrees of significance are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values are means ± SE, $N = 20$.

From the feeding trial, as hypothesised, insect growth was reduced by AM fungi, but this was only observed in Si- plants and only in Q240, hence there was a significant interaction between variety, AM fungi and Si treatments (Fig. 7-3a, Table 7-2). Relative consumption was lower on AM plants, which was also only observed within Q240, therefore an interaction between variety and AM fungi was detected (Fig. 7-3b, Table 7-2).

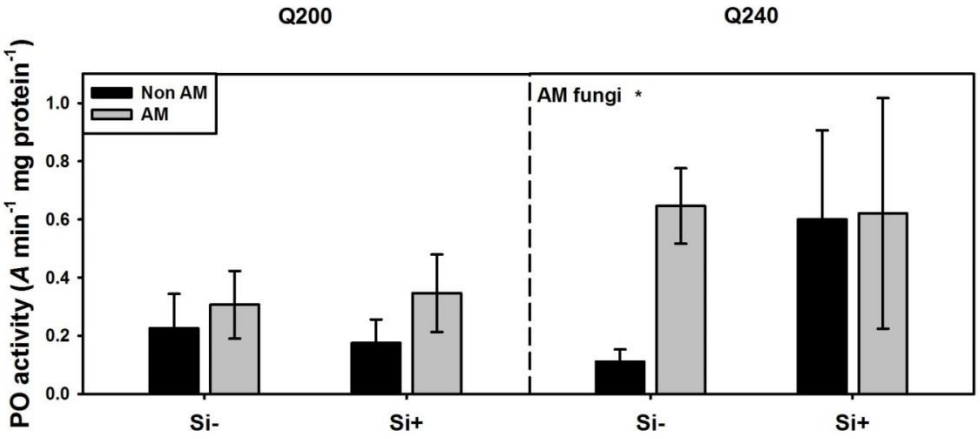

Figure 7-4. Canegrub (*Dermolepida albohirtum*) PO activity (*A* min^{-1} mg $protein^{-1}$) measured after feeding on two sugarcane (*Saccharum* species hybrids) varieties under different silicon (Si) treatments, with (AM) and without (non AM) arbuscular mycorrhizal fungi. Significant factors are shown, degrees of significance are indicated as follows: * $P < 0.05$. Values are means ± SE, $N = 20$.

AM fungi increased insect immune function by around 62.1% compared to the non AM treatment (Fig. 7-4, Table 7-2). This effect was largest within Si- treated plants of Q240; hence there was a marginally significant three-way interaction between variety, AM fungi and Si treatments. There was also a strong negative correlation ($r_p = -0.51$, $R^2 = 0.25$, $P < 0.001$) between insect mass from the immune function assay and PO activity (Fig. 7-5).

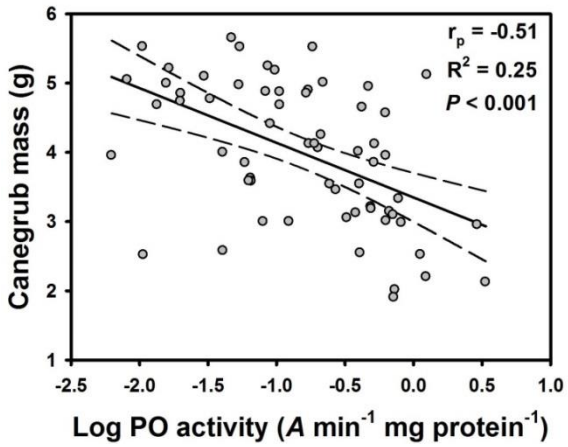

Figure 7-5. Correlation between canegrub mass (g) and log transformed PO activity (*A* min^{-1} mg $protein^{-1}$). Solid line represents linear regression through all the data points. Dashed lines represent 95% confidence intervals. Pearson's product moment correlation coefficient (r_p), coefficient of determination (R^2) and *P*-value are shown.

7.4.7 Insect response to silicon

The insects used as the RH treatment were negatively affected by the Si+ treatment (mean change in mass -0.427 ± 0.079g), while performing better on plants under Si- (mean change in mass 0.071 ± 0.057g; Table 7-2). The insects in pots with only soil showed no difference in their change in mass between Si- and Si+ (P = 0.645, mean change in mass 0.112 ± 0.046g and 0.14 ± 0.037g, respectively), suggesting there were no direct effects of Si on insect growth.

From the feeding trial, as hypothesised, insect growth was significantly lower on plants under Si+ compared to Si- (Fig. 7-3a, Table 7-2), and root consumption was reduced by around 71.2% on Si+ treated plants compared to Si- (Fig. 7-3b, Table 7-2). Unexpectedly, there was no significant effect of Si treatment on canegrub immune function (Fig. 7-4, Table 7-2).

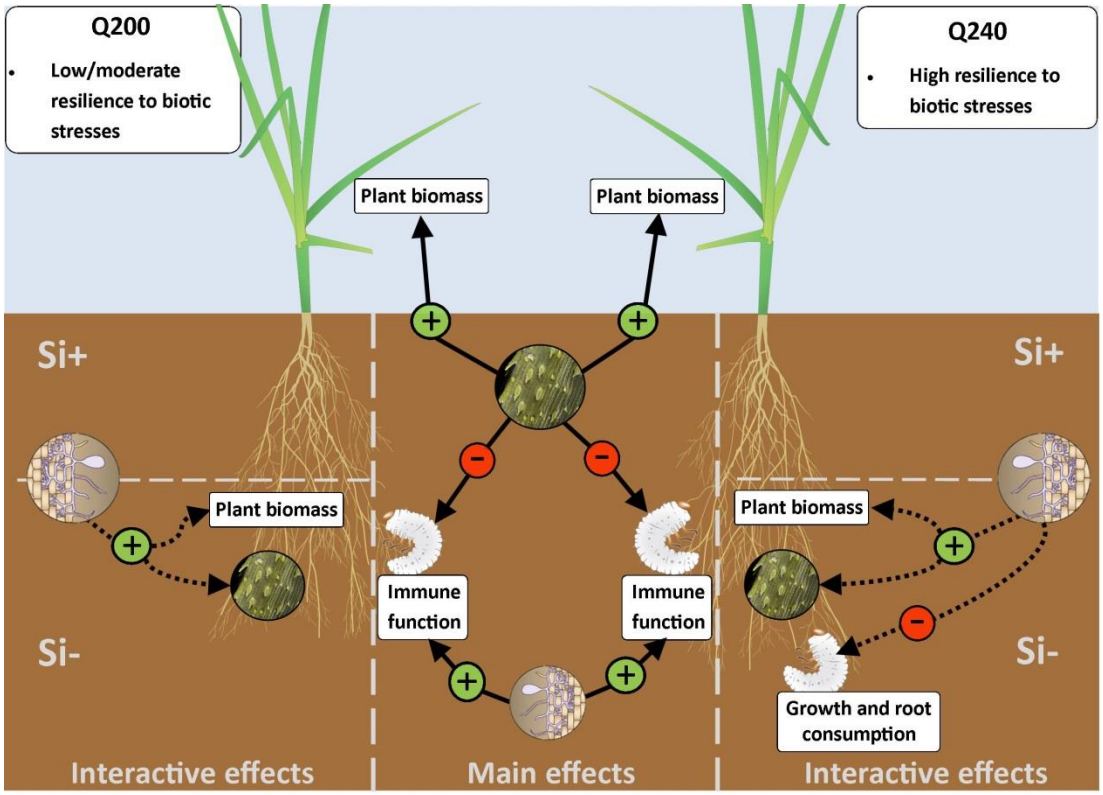

Figure 7-6. Impacts of arbuscular mycorrhizal (AM) fungi and silicon (Si) treatments on sugarcane (*Saccharum* species hybrids) varieties Q200 and Q240, and canegrub (*Dermolepida albohirtum*) performance and immune function. Main factor effects indicated by solid lines, shown in centre of diagram; interactive effects indicated by dashed lines (e.g. positive effects of AM fungi under Si- treatment only), shown on the far left (for Q200) and far right (for Q240).

7.5 Discussion

We have demonstrated for the first time, to our knowledge, the differential impacts of AM fungi and Si within different plant varieties on the growth and immune function of a root feeding insect. Si effectively reduced insect performance while the negative impacts of AM fungi were dependent on Si and also on plant variety. AM fungi promoted insect immune function independent of any measured plant trait suggesting a possible direct priming of insect immunity, while also highlighting a growth-immunity trade off, both of which could play an important role in the responses of soil dwelling insects to AM fungi.

Plant growth was increased by Si, an effect that is well documented across plant species (Ma 2004; Cooke & Leishman 2016), while the positive effects of AM fungi were observed only when no Si was applied. Therefore, AM fungi and Si were not found here to have additive effects on plant growth, possibly as the Si treatment promoted plant growth to capacity. The RH treatment did not impact biomass or root mass of the plants possibly because only one canegrub was applied, as previous work has shown the addition of three canegrubs to pots of the same volume significantly decreases root mass (Frew *et al.* 2016a).

When Si was readily available, it is likely the plants were able to access the higher concentrations of Si without facilitated uptake by AM fungi, a response that has been observed previously (chapter 6). When no Si treatment was applied AM fungi increased root Si concentrations, which could be direct Si uptake from the soil by AM fungal hyphae or due to higher rates of photosynthesis induced by AM colonisation (Wu & Xia 2006). As Si uptake in plants is known to involve several aquaporin transporters involved in the uptake of water (Ma & Yamaji 2015), an increase in photosynthesis, and therefore increased transpiration and water uptake, could cause an increase in Si uptake via the lateral roots. Although the exact mechanisms behind increases in Si concentrations in plants colonised by AM fungi remain unclear, there are several examples of enhanced Si acquisition due to mycorrhizal colonisation (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996; Garg & Bhandari 2016).

Insect performance was effectively reduced by Si on both plant varieties. Reduced performance of insect herbivores in response to Si has been well documented (Reynolds, Keeping & Meyer 2009), and has also been observed in root feeding insects (Frew *et al.* 2016a; c). This reduced performance is likely to be due to increases in toughness of plant tissue, which is an effective defence trait against root feeding insects (Johnson *et al.* 2010b), alongside reductions in overall plant quality as a food source, as high Si concentrations reduce the digestibility of plant material (Massey & Hartley 2009).

In contrast to the effects of Si, the impacts of AM fungi on insect growth and consumption were dependent on plant variety, as well as plant Si availability. The negative impacts of AM fungi on insect performance were evident in Q240, under Si-. This response is unlikely to be linked to changes in plant nutrition from AM colonisation as we did not detect an effect of AM fungi on plant C:N or P concentrations, and previous studies have highlighted there is little support for a nutritional mechanism underpinning the impacts of AM fungi on root feeding insects (Gange 2001). Therefore the reduction in canegrub growth rates here are likely to be a response to the higher concentrations of root Si from enhanced Si uptake by AM colonisation. Insect root consumption was also reduced by AM fungi within Q240, independent of Si treatment, although the effect was larger under Si- compared to Si+ treated plants. This is likely to be due to an increase in root Si concentrations from AM colonisation, although this does highlight that AM fungi impact insect herbivores through multiple mechanisms, as mycorrhizae are known to alter plant chemistry in a number of ways (Pozo & Azcón-Aguilar 2007; Koricheva, Gange & Jones 2009; Johnson & Rasmann 2015). Indeed, although the two sugarcane varieties here differ in their observed resilience to biotic stresses, it is important to consider these are both products of substantial breeding programs. As such, it is possible that other environmental factors or uncharacterised varietal traits, which were not accounted for here, may have contributed to the effects observed in this study.

Arbuscular mycorrhizal fungi increased the insect immune function regardless of plant variety or Si availability. Insect PO activity can be an indicator of insect non-specific immune response to foreign invaders including entomopathogenic

nematodes, bacteria and fungi. Arbuscular mycorrhizal fungi are known to increase nutrient acquisition by their host plants, thereby can potentially increase plant nutritional quality which could promote insect immune function. Here however, the increase in PO activity in response to AM fungi is unlikely to be a response to changes in host plant nutritional quality as AM fungi had no observed impact on the concentrations of P (a principal nutrient in the AM fungi–plant symbiosis) or any root elements other than Si. Additionally, AM fungi had no impact on the C:N ratio of the root material, which can be an indicator of plant quality for insect herbivores.

An alternative hypothesis is that the insect immune system may have been primed directly by contact with AM fungi in the soil. The insect immune system recognises non-self by cell surface molecules such as β-1, 3-glucans, lipopolysaccharides and peptidoglycans, collectively known as pathogen-associated molecular patterns, or PAMPs (Theopold *et al.* 1999; Cerenius & Söderhäll 2004). Exposure to PAMPs in soil entomopathogenic fungi have been shown to prime insect immune function, increasing the immune response to subsequent infections (Moret & Siva-Jothy 2003; Krams *et al.* 2013). Arbuscular mycorrhizal fungi are known to possess similar groups of PAMPs as entomopathogenic fungi, such as β-1, 3-glucans (Lemoine, Gollotte & Gianinazzi-Pearson 1995). Therefore it is possible AM fungi are able to elicit insect PO activation, particularly within a soil dwelling insect, thereby priming canegrub immune function, and, in this case, resulting in higher PO activity in response to a subsequent immune challenge. The interactions between AM fungi and root feeding insects are likely to be complex and research to date has focussed on plant-mediated interactions between AM fungi and root feeding insects. Our results highlight the need for future research to consider possible direct interactions between AM fungi and soil dwelling insects if the ideas presented here are to progress further than speculation.

Additionally, the negative correlation between PO activity and canegrub mass is also noteworthy. The different physiological costs of insect immune defence, including trade-offs between immunity and growth, have been well documented (Moret & Schmid-Hempel 2000; Siva-Jothy, Moret & Rolff 2005; Bascuñán-García, Lara & Córdoba-Aguilar 2010; Triggs & Knell 2012; Ardia *et al.* 2012), although remain

understudied in belowground insects. In this case, canegrub investment into immune response has a potential growth cost, where immune function as measured by PO activity, can result in growth reduction. This trade-off may also play a role in the previously observed reductions in growth from belowground insects to AM fungi (Gange 2001), where AM fungi may be stimulating an immune response to AM fungi and therefore energy investment into growth is reduced. Further investigations into the growth and immune function responses of root feeding insects to AM fungi, including possible direct interactions, are necessary to better understand these observations.

The performance of root feeding insects is strongly influenced by their host plants, the majority of which associate with AM fungi. These fungi reside both in the soil and within plant tissue and are known to alter plant quality and defences, including Si concentrations in certain contexts. Our findings not only highlight the efficacy of Si based plant defences against root feeding insects but also suggest AM fungi impact root feeding insects via Si uptake, depending on Si availability to the plant. Root feeding insects grow and develop within the soil environment, and as such are in direct contact with soil microbial communities, including AM fungi. The close proximity of these organisms suggests they could impact each other in more ways than via their shared host plant. This study found insect immune function was increased by AM fungi in a way that was independent of any measured plant trait. Further work is required to better understand the mechanisms behind these responses and future work should look to consider not only plant mediated effects, but also direct interactions between these soil dwelling organisms.

7.6 Acknowledgements

We thank the teams at Sugar Research Australia Limited and the Hawkesbury Institute for the Environment, Western Sydney University. Funding was provided by Sugar Research Australia (project no.2014/104).

Chapter 8: General discussion

Table 8-1. Glossary of terms.

Term	Explanation
AM fungi	Arbuscular mycorrhizal fungi. Obligate symbiotic fungi that colonise the roots of most land plants, transferring soil nutrients such as phosphorus (P) to the plant in exchange for sugars.
Canegrub	Larvae of scarab beetles, *Dermolepida albohirtum* (Waterhouse), native to Australia which feed extensively on grass roots, known for their destructive feeding of sugarcane roots.
CO_2	Carbon dioxide. Elevated atmospheric CO_2 concentrations (eCO_2) are predicted to have significant impacts on the global environment, including plant–insect interactions.
EPN	Entomopathogenic nematodes. Nematodes (thread worms) which kill insects via the bacteria they harbour inside them.
PO	Phenoloxidase. An enzyme central to the initiation of insect immune response to foreign bodies that is used to assess insect immunity.
Si	Silicon. The second most abundant element in the Earth's crust and an important defence in plants against herbivores.

The work reported in this thesis examined the impacts of different abiotic and biotic factors within the soil environment on root feeding scarab larvae. This was initially investigated by assessing the impacts of irrigation and fertilisation on scarab larval communities within a eucalypt forest plantation (chapter 3). Subsequent investigations focussed on a single scarab species, the canegrub (*Dermolepida albohirtum*) and sugarcane (*Saccharum* species hybrids). The effects of plant silicon (Si) on canegrub performance was assessed alongside the impacts of root phenolic compounds (chapter 4) and elevated atmospheric carbon dioxide concentrations (eCO_2) (chapter 5) on canegrub performance and root consumption. The impacts of different arbuscular mycorrhizal (AM) fungal communities on canegrub performance under two soil types was investigated with a view to highlighting the potential role of Si in AM fungi–root feeding insect interactions (chapter 6). This was then tested directly by looking at the effects of AM fungi and Si supplementation on canegrub performance and immune

function on two different sugarcane varieties (chapter 7). The key findings of each research chapter are summarised in Figure 8-1.

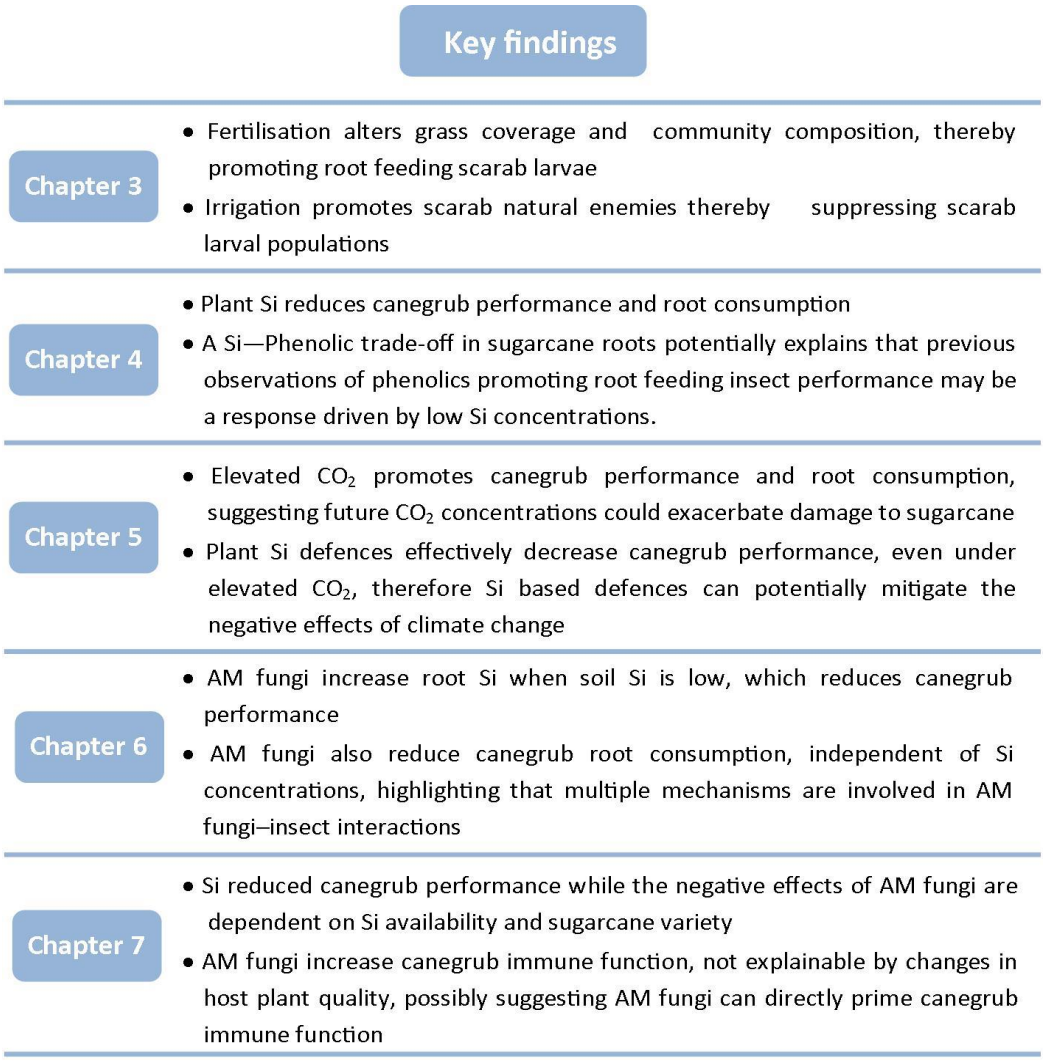

Figure 8-1. Key findings of research chapters (chapters 3 to 7).

8.1 Key Findings

8.1.1 Silicon defences against root feeding insects

Si is the second most abundant element in the Earth's crust and almost all plants take up bioavailable Si from the soil as silicic acid (H_4SiO_4) via the lateral roots. Si can constitute up to more than 10% dry weight of plant tissue (Epstein 1999), yet the functional significance of plant Si is only now gaining widespread recognition (Cooke, DeGabriel & Hartley 2016). Plant Si is known to enhance tolerance to several abiotic and biotic stresses including water stress, heat stress, heavy metal toxicity, and pathogen and herbivore attack. The efficacy of Si in reducing

performance of many aboveground insect herbivores is now well established (Reynolds, Keeping & Meyer 2009), and has also been shown to be an effective defence against insect pests of sugarcane (Kvedaras & Keeping 2007).

Several studies have previously observed that many Si accumulating plants also have low concentrations of carbon (C) based defence compounds, specifically phenolics (Cooke & Leishman 2012) suggesting some plants substitute C for Si in structure and defence (Schaller, Brackhage & Dudel 2012). This trade-off is hypothesised to have evolved around the Oligocene and Miocene when atmospheric carbon dioxide (CO_2) was low and therefore the ability to uptake Si for structural support would have been an advantage (Craine 2009). At this time there was the radiation of the grasses, which are typically high Si accumulators. Around the same time there was the appearance of larger mammalian herbivores with high-crowned (hypsodont) teeth that were suited to feeding on a Si rich diet (Strömberg 2011). This precipitated the hypothesis of a co-evolutionary 'arms-race' between herbivores and Si rich grasses, where grasses increased Si accumulation over time in response to herbivore pressure (McNaughton & Tarrants 1983). However a recent phylogenetic analysis has proposed that there is no correlation between mammalian grass eaters, the radiation of the grasses and Si accumulation (Strömberg, Di Stilio & Song 2016). Rather it was suggested that insect herbivory during the Cretaceous may have constituted selective pressure for Si accumulation in grasses.

Defences can be expensive and due to the cost of defence, plants must divert resources from other needs, such as growth and reproduction (Stamp 2003). If Si accumulation became evolutionarily advantageous due to limited C availability, then it would seem sensible, according to the optimal defence hypothesis, to exploit a resource that is more readily available and can serve a similar purpose i.e. Si. This would allow the limited C to be utilised for other functions. Indeed, there have been several examples where phenolics seemingly promoted performance of belowground insects, which is contrary to predictions (Johnson & Nielsen 2012). The work in this thesis has provided evidence that Si accumulating plants exhibit a 'trade-off' between phenolic compounds and Si, which suggested

that positive responses from root feeding insects to phenolics, may in fact be a response driven by low concentrations of Si in the roots (chapter 4).

This thesis has provided several examples where Si in the roots effectively reduced root herbivore performance (chapters 4 to 7), something which had only previously been demonstrated in aboveground herbivores and in one mammalian root herbivore (Wieczorek *et al.* 2015). The mechanisms behind these responses are likely to include increased plant toughness via deposition of silica (SiO_2) phytoliths that wear down insect mandibles (Massey & Hartley 2009) and reduce the palatability and digestibility of plant tissue through mechanical protection of the chlorenchyma cells, which is where insects retrieve much of their starch and protein (Hunt *et al.* 2008). Additional changes in plant chemistry may also play a role, for example Si has been shown to increase plant peroxidase and polyphenoloxidase concentrations (Gomes *et al.* 2005). These enzymes are involved in processes causing plant lignification and reductions in protein digestibility. These mechanisms are also likely to have played a role in the reduction in canegrub performance in response to Si reported within the work of this thesis (chapters 4 to 7).

8.1.2 Potential of silicon to mitigate effects of climate change

It has been suggested that increasing populations, together with increasing demand for food, water and energy, combine with climate change and threaten to create a 'perfect storm' scenario of global events (Beddington 2009). To meet the increasing demand for food and crop losses from insect herbivore pests, the usage of insecticides has increased sevenfold in the past 40 years (Tilman *et al.* 2001). These insecticides are costly and often environmentally damaging; therefore novel, economically and ecologically sustainable strategies of pest management are needed. This need is even more pressing when the possible implications of climate change are also taken into account. The rising atmospheric concentrations of CO_2 will impact directly on plants and the many insect herbivores that feed upon them. Elevated atmospheric concentrations of CO_2 (eCO_2) have been shown to increase the susceptibility of some plants to insect herbivore attack. This can be through breakdown of defences, for example eCO_2

is known to downregulate the jasmonic acid pathway which is key to the production of many plant chemical defences against chewing herbivores (Ode, Johnson & Moore 2014). Also, under eCO_2, many plants increase their CO_2 assimilation rates, producing more carbohydrates for growth (Conroy & Hocking 1993), which has the effect of diluting the concentrations of N in plant tissue, increasing the C:N ratio (Cotrufo, Ineson & Scott 1998). As N is often a limiting factor to insect nutrition (Mattson 1980), this reduces the plant quality as a food source for insects who then must increase their consumption in attempts to meet their dietary needs (Stiling & Cornelissen 2007).

Chapter 5 is the first study to investigate the impacts of eCO_2 and Si on the performance of a root feeding insect. Elevated CO_2 caused an increase, not only in consumption, but also in growth rates of a destructive root feeding insect, the canegrub. The increase in consumption was likely a compensatory feeding response to the reduction in the nutritional quality of the roots, as the canegrubs consumed more root material to satisfy their N requirements. Indeed, compensatory feeding in response to eCO_2 has been observed previously in scarab larvae (Johnson, Lopaticki & Hartley 2014). In this instance, canegrub root consumption increased under eCO_2 by around 57% which, directly translated, could indicate an increase of AU$21.6 million a year in crop losses to the Australian sugar industry (Allsopp 2010). Typically, insect growth rates are reduced under eCO_2 (Robinson, Ryan & Newman 2012), whereas here, canegrub relative growth rates increased by around 116%, a response that was not explainable by any measured plant trait. Therefore, considering eCO_2 is also known to break down plant chemical defence pathways (Ode, Johnson & Moore 2014), other, unmeasured plant responses to eCO_2 may have also played a role in promoting canegrub performance in this case.

This study found the application of Si to the soil promoted plant growth and increased root Si concentrations, which dramatically decreased canegrub consumption and growth rates, essentially mitigating the effects of eCO_2 on canegrub performance (chapter 5). These findings highlight the possible exacerbation of root feeding insect pests by climate change as eCO_2 alters plant

chemistry and increases plant susceptibility to insect herbivore damage. However, the results of chapter 5 suggest the impacts of eCO_2 can potentially be mitigated by exploiting plant Si based defences.

8.1.3 Impacts of AM fungi on the canegrub

AM fungi are known to induce many physiological and biochemical changes in the host plant which alter plant quality as a host for insect herbivores. The responses of aboveground insect herbivores to AM fungi are highly variable and context dependent (Koricheva, Gange & Jones 2009). There are very few studies that have assessed the impacts of AM fungi on the performance of root feeding insects. Those that have, tend to find AM fungi negatively affect soil insects (Johnson & Rasmann 2015), yet the mechanisms behind these responses remain unknown. Possible mechanisms include improved plant nutrition, improved compensatory plant growth after damage and activation of plant defence mechanisms. Concerning the latter, AM priming of plant defences is said to be key mechanism in mycorrhizal induced plant resistance to insect herbivores. For example, AM fungi are known to upregulate the jasmonic acid pathway (Jung *et al.* 2012), which is central to the production of many plant chemical defences against chewing herbivores.

The AM fungi–plant symbiosis is largely based on the bi-directional transfer of nutrients, and AM fungi had been previously observed to increase concentrations of plant Si (Kothari, Marschner & Römheld 1990; Clark & Zeto 1996; Garg & Bhandari 2016). The mechanisms behind these increases are unknown, but could be due to direct Si uptake from the soil by AM fungal hyphae or due to higher rates of photosynthesis induced by AM fungi (Wu & Xia 2006; Frew 2017). As Si uptake in plants is known to involve several aquaporin transporters involved in the uptake of water (Ma & Yamaji 2015), an increase in photosynthesis, and therefore increased transpiration and water uptake, could cause an increase in Si uptake via the lateral roots. Silicon in plants reduces performance of aboveground insect herbivores, and has been shown, as part of this thesis, to effectively reduce performance of a root feeding insect. It is possible, therefore,

that Si plays some mechanistic role in the relationship between AM fungi, the host plant, and root feeding insects.

The effects of AM fungi on root feeding insects can be dependent on soil Si availability, as demonstrated in chapters 6 and 7. Arbuscular mycorrhizal fungi were observed to increase sugarcane root Si concentrations, but only when available soil Si was low, either naturally or because no Si fertiliser was applied. Where soil Si availability was low, root Si concentrations correlated positively with AM root colonisation, while Si correlated negatively with canegrub performance (chapter 6). Within soil with high Si availability, there was no relationship between AM colonisation and Si concentrations, and there was no difference in root Si concentrations between the different AM treatments. Canegrub performance, however, still correlated negatively with root Si concentrations (chapter 6). These results suggest AM fungi can facilitate Si uptake in plants when Si is limiting, something that had already been observed for phosphorus (P) and N (Treseder 2004). This increase in Si uptake can negatively affect root feeding insects, as previously discussed, by reducing plant quality via increased toughness, decreased palatability and digestibility. Therefore Si may play an important mechanistic role in the effects of AM fungi on root feeding insects.

Arbuscular mycorrhizal fungi are known to alter host plant quality for insect herbivores, and host plant quality is known to affect insect immunity which suggests that AM fungi might alter insect immune function. The work reported in chapter 7 found that AM fungi increased the immune function of the canegrub, a response that was not explainable by any measured plant trait. Arbuscular mycorrhizal fungi have similar cell surface molecules, known as pathogen associated molecular patterns (PAMPs), as soil entomopathogenic fungi. These PAMPs found on entomopathogens initiate the insect immune response. Therefore soil dwelling insects, having evolved within the same soil environment as both entomopathogenic fungi and AM fungi, may mount an immune reaction in response to direct contact with AM fungi. Although this remains speculative, it

highlights the need to investigate possible direct interactions between root feeding insects and AM fungi.

8.1.4 Varietal responses to silicon and AM fungi

Since the cultivation of sugarcane, selective breeding for desirable traits, such as resistance and tolerance to insect herbivory, has produced numerous varieties (Allsopp & Cox 2002). Sugarcane is a known Si accumulator that forms symbiosis with AM fungi; however, both Si uptake efficiency and AM responsiveness are rarely taken into account. The work of this thesis has involved three different varieties of sugarcane, Q200, Q138 and Q240. Both cultivars Q138 and Q240 are observed to exhibit levels of tolerance to root herbivory, while Q200 is relatively more susceptible to root herbivore damage (Sugar Research Australia Ltd 2015). Prior to this work, however, there was no available information on if, and how, these varieties differed in their Si concentrations and their responsiveness to AM fungi.

Differences in responsiveness to AM colonisation between crop varieties is well reported (Sawers, Gutjahr & Paszkowski 2008). The results of this thesis found that Si fertilisation benefits sugarcane growth and decreases canegrub performance across all varieties (chapters 4 to 7). Contrastingly, despite the fact that roots of all varieties were successfully colonised by AM fungi, not all varieties exhibited the same responsiveness. For example, AM fungi successfully colonised Q200, and increased root Si concentrations, yet there was no impact on canegrub performance (chapter 7). This observed difference in canegrub responsiveness to AM fungi between the two varieties is possibly due to differences in the chemical profiles of the varieties in response to AM colonisation. Then again, as the effects of AM fungi on plant growth and insect performance can depend on plant species identity and AM species identity (Bennett & Bever 2007; Gehring & Bennett 2009), it is possible that association with a different AM community could have yielded a different response.

8.2 Implications and potential applications

The work of this thesis has highlighted the impacts of different soil factors on root feeding scarab larvae. Firstly, this work has found that fertilisation, a common agricultural management practice, can increase the populations of scarab larvae belowground. This has implications for a variety of production systems, as fertilisation using N, P, K fertiliser is a common practice on pastures, crops and tree plantations. Contrastingly, irrigation had no impact on scarab larvae, but increased natural enemy (EPNs) abundance. Therefore, timing the application of irrigation with the scarab lifecycle to negatively impact larvae when they are at their most vulnerable to stress (see 'Applied perspectives' section in chapter 2), while also promoting EPN abundance, could potentially suppress scarab larval populations and minimise pest exacerbation.

The ecological significance of Si is now becoming apparent, while the potential to exploit this previously undervalued plant trait is also now being recognised. This thesis has demonstrated that Si effectively reduces the performance of a root feeding insect across different soil types and host plant varieties. This highlights the potential benefits of using Si in crop protection against root feeding insect pests. Despite that Si is the second most abundant element in the Earth's crust, the majority of this is normally not available to plants. Plants only take up Si in the form of silicic acid (H_4SiO_4), the availability of which is dependent on the solubility of the silicates found in the soil (Liang *et al.* 2015). Plant available Si is already commonly added to crops in the USA and across Asia, and commercial Si fertilisers are currently available in several countries, including Australia (Guntzer, Keller & Meunier 2012). Agricultural soils can become Si depleted (Savant, Datnoff & Snyder 1997), therefore characterisation of soil Si availability would allow targeted application of Si fertilisers which can increase crop yield and decrease root damage from belowground insect herbivores. From a long term perspective, crop breeders should take advantage of the recent advances in the understanding of the molecular mechanisms underpinning plant Si uptake (Ma & Yamaji 2015), as well as the natural variation of Si uptake between crop cultivars (Hodson *et al.* 2005; Soininen *et al.* 2013), to select for varieties with high Si

uptake efficiency. This way crops can maximise their Si uptake from the soil, while Si fertilisers can be applied to Si depleted soils.

The work of this thesis has also shown that root Si concentrations increase in response to AM colonisation when soil Si availability is low. The work of chapter 6 found no observable difference between the effects of commercially available AM fungi and the native AM fungi present in field soil on the host plant or on insect herbivore performance. Therefore, the implementation of agricultural practices that encourage mycorrhizal communities could provide additional benefit to crops, (see examples within Bowles *et al*. (2016)). The responsiveness of different crop varieties to AM fungi can differ, as found in chapter 7. Assessing the responsiveness of new crop varieties to AM fungi would facilitate targeted implementation of management practices that encourage native AM fungi on crops that are most likely to respond.

8.3 Constraints, caveats and future work

The majority of the work of this thesis was based on pot studies (chapters 4 to 7), where treatment effects have been examined on individual plants in their individual soil environments. In response to treatments, plants grown in pots may have different responses compared to plants grown in the field where neighbouring plants are likely to interact, either directly or indirectly through chemical exudation or soil microbial interactions. For example, AM fungi are known to form extensive common mycorrhizal networks (CMNs) belowground. It is hypothesised that these CMNs facilitate plant–plant communication that allows systemic defence signalling across plant populations (Barto *et al.* 2012). Therefore plant responses in the field could differ to those observed in pots, and as such, the responses of root feeding insects may also vary. The logical next step is to conduct field based experiments to investigate if and how the impacts of different soil factors (i.e. Si and AM fungi) on root feeding insects observed within this work might differ out in the field.

The experiments reported in chapters 4 and 5 were carried out in a glasshouse environment which presents particular constraints. In particular, the work of

chapter 5, where different CO_2 treatments were applied by placing plants in different chambers, is faced with the problem of pseudoreplication. This is a common challenge for climate change research (Newman *et al.* 2011) as when environmental factors are applied to a controlled glasshouse chamber, the unit of replication is the chamber itself. The individual plants are not independently subjected to treatments. It is therefore important to consider this when making biological conclusions based on experiments which use glasshouse facilities such as those used in the work reported in this thesis, as these studies lack true independence between replicates. Avoiding pseudoreplication in these cases is normally difficult as this would require multiple chambers and/or glasshouses to act as true replicates, and these extensive facilities are often not an option for researchers. Alternatively, repeating an experiment several times can help mitigate the problem, but this again is costly, time consuming, and often not available. Efforts were made to minimise the effects of pseudoreplication in the glasshouse studies of this thesis by 'chamber swapping' regularly throughout the experimental period. This involved moving the individual plants within, and between glasshouse chambers, with appropriate changes in the chamber environmental conditions. This method was shown to be effective at minimising the effects of pseudoreplication by Johnson *et al*. (2016b), where all three methods (i.e. using multiple chambers, performing multiple runs and 'chamber swapping') were compared and produced similar results.

Global climate change involves multiple environmental factors including atmospheric concentrations of CO_2, rainfall and temperature. The work reported in chapter 5 of this thesis investigated the effects of elevated concentrations of atmospheric CO_2 on root feeding insect performance. As multiple parameters are predicted to be altered under climate change, it is important to consider that these factors could potentially interact and differentially impact plant–insect relationships. The work in chapter 5 focussed on one climate change parameter and demonstrated strong responses from the canegrub to eCO_2 and Si. This work should be taken further to investigate how predicted future rainfall patterns and/or temperatures might impact the role of Si defences against root feeding

insects. For example, higher temperatures can increase plant transpiration (Jarvis & McNaughton 1986), and as plants take up Si through aquaporin transporters (Ma & Yamaji 2015) which are involved with water uptake, it is likely that elevated temperatures could result in increased Si uptake. Investigating the different factors involved in climate change and their interactions will provide a more complete understanding of how climate change might alter plant–insect interactions which can then facilitate the development of novel strategies to mitigate the negative effects of climate change.

The work reported in this thesis has investigated the impacts of soil factors such as Si and AM fungi on plants and their root herbivores, specifically focussing on sugarcane and the canegrub. This has involved measuring specific plant nutrients (e.g. C, N and Si) or metabolites (phenolics, soluble sugars) of interest that could potentially affect plant–herbivore interactions. However, there can often be responses observed that are not fully explainable by the parameters measured. For example, chapter 5 observed an increase in canegrub consumption, which was potentially due to the increase in the C:N ratio of the root tissue, yet canegrub performance also increased, which was not explainable by any measured plant parameter. Similarly, the work of chapters 6 and 7 saw changes in canegrub performance in response to AM fungi that were explainable by changes in Si concentrations, to some degree. Yet in both chapters, canegrubs showed decreases in either root consumption (chapter 6) or growth rates (chapter 7) that were not explained by Si. This highlights the need for a more comprehensive tool of identifying and quantifying changes in plant metabolites in response to experimental treatments. Metabolomics enables untargeted, simultaneous analysis of primary and secondary plant compounds, and is a highly valuable technique for ecologists to understand metabolic changes in plants and the impact on insect herbivores (Bezemer & van Dam 2005). Although requiring expensive high-throughput equipment, metabolomics could be a particularly useful tool in future work assessing plant responses to AM fungi, especially considering AM fungi alter multiple features of plant physiology and biochemistry (Koricheva, Gange & Jones 2009; Smith & Read 2010). As mentioned previously,

numerous soil organisms and processes are involved in the interactions between root feeding insects and their host plants. As such, future work should also consider the use of metagenomics, using environmental samples in field studies, to facilitate a comprehensive insight into the communities of biotic 'players' involved in these interactions, in a more holistic and real-world approach.

The work of chapter 7 found that AM fungi increased the immune function of the canegrub, a response that was not explainable by any measured plant trait. This is a further example where metabolomics could be useful to uncover the mechanistic basis of an insect response to AM fungi. Here, the next steps could involve insect feeding trials using intact plants in pots, either with or without AM fungi. Analysis of the plant roots using metabolomics could potentially reveal changes in root compounds in response to AM fungal colonisation that might impact on insect immune function and overall performance. To test the hypothesis that AM fungi may directly promote insect immune function, insect feeding trials using plant roots colonised by AM fungi, plant roots without AM fungi, just AM hyphae and spores, and no roots or AM fungi (no food) could be carried out. Insect immune function could then be assessed to determine if and how AM fungi might be directly impacting root feeding insect immunity.

8.4 Conclusions

The work reported in this thesis has addressed the impacts of different soil abiotic (moisture, N, P, K and Si) and biotic (AM fungi) factors on root feeding scarab larvae, with a focus on sugarcane and the canegrub. This work found that the addition of N, P, K fertiliser can increase scarab larval populations via changes in host plant communities, while irrigation increases the abundance of scarab natural enemies, which can suppress larval populations. This work also demonstrated, for the first time, the negative impacts of root Si on the performance of a root feeding insect while highlighting a trade-off between Si and phenolic compounds in the roots. Predicted future concentrations of atmospheric CO_2 were shown to increase the performance and root consumption of a root feeding insect; however increasing root Si concentrations mitigated these effects, highlighting the potential of Si to be used in future pest

management strategies. The negative impacts of AM fungi on a root feeding insect were potentially explained by AM facilitated increases in host plant Si uptake. However this work also highlighted that the interactions between root feeding insects and AM fungi are complex and involve multiple mechanisms, possibly including direct interactions.

This research expands our current knowledge of how factors within the soil environment, in particular Si and AM fungi, can impact root feeding insects. Despite advances in the recognition of the ecological significance of root feeding insects, our understanding of belowground insect herbivory still lags behind that of foliar feeding insects. The findings of this thesis contribute to a better understanding of the interactions between root feeding insects and their host plants. These findings not only highlight new areas for developing novel insect management strategies but have shed light on the fundamental biology and ecology of these hidden herbivores.

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Appendices

9.1 Appendix I – Chapter 5 supplementary material

Table S5-1. Mean (±SE) aboveground (AB) and belowground (BG) biomass, C:N, silicon concentration and root total non-structural carbohydrates of sugarcane grown at ambient (aCO_2) or elevated (eCO_2) [CO_2], control (Si-) or added (Si+) silicon treatment and without (CG-) or with (CG+) canegrub herbivory.

	AG biomass (g)	BG biomass (g)	Leaf C:N (%DM)	Root C:N (%DM)	Leaf silicon (%DM)	Root silicon (%DM)	Root TNC (mg g^{-1})
aCO_2 : Si- : CG-	26.07±5.74	4.17±0.84	35.16±3.42	55.06±3.0	1.94±0.35	0.71±0.09	528.9±33.5
aCO_2 : Si- : CG+	36.28±2.49	2.51±0.31	45.6±3.8	64.26±5.54	1.02±0.18	1.5±0.94	384.7±16.4
aCO_2 : Si+ : CG-	59.08±5.52	10.96±0.9	26.94±1.46	61.39±3.63	2.23±0.39	1.32±0.23	585.6±7.7
aCO_2 : Si+ : CG+	52.45±8.16	5.41±2.08	36.79±3.75	61.85±4.12	2.73±0.46	1.49±0.19	480.6±40.9
eCO_2 : Si- : CG-	43.39±7.93	12.6±3.96	24.13±0.96	62.5±6.92	1.97±0.38	0.89±0.22	405.4±23.3
eCO_2 : Si- : CG+	44.81±7.23	4.67±0.87	30.14±1.58	60.81±2.23	1.19±0.17	0.77±0.12	426.7±58.3
eCO_2 : Si+ : CG-	82.19±4.48	13.25±1.03	27.53±1.0	71.72±3.41	2.67±0.41	1.19±0.11	500.5±38.7
eCO_2 : Si+ : CG+	63.67±8.57	6.28±1.5	36.27±4.45	63.05±4.57	2.0±0.38	1.5±0.18	408.3±37.1

9.2 Appendix II – Chapter 6 supplementary material

Table S6-1. Nutrient analysis results of 'Low Si' and 'High Si' soils, both fully homogenised prior to analysis. Analysis carried out by Environmental Analysis Laboratory, Southern Cross University, Lismore, Australia. LECO IR analyser and total acid extractable techniques give an indicator of a store of nutrients while $CaCl_2$ extractable indicates nutrient availability for plant growth.

Method	Nutrient		Units	Low silicon soil	High silicon soil
LECO IR	Carbon	C	%	2.20	1.08
Analyser	Nitrogen	N	%	0.11	0.09
	C:N ratio			20	11.5
Total Acid Extractable	Calcium	Ca	mg/kg	348	1,167
	Magnesium	Mg		401	752
	Potassium	K		983	1,653
	Sulphur	S		120	192
	Phosphorus	P		363	266
	Silicon	Si		1,392	2,221
	Aluminium	Al		9,880	13,854
$CaCl_2$ Extractable	Silicon	Si	mg/kg	23	41
	Boron	B		0.17	0.35

Table S6-2. Summary of outputs from ANOVA results of plant (*Saccharum* spp. hybrid) photosynthesis (µmol m^{-2} s^{-1}), biomass (g) and root silicon (Si) concentrations (% dry mass) responses to arbuscular mycorrhizal (AM) fungi and soil type (soil) treatments and any interactions. Significant effects ($P \leq 0.05$) are highlighted in bold.

	AM fungi		Soil		AM fungi x Soil	
	$F_{2,314}$	*P*	$F_{2,314}$	*P*	$F_{2,314}$	*P*
Photosynthesis	**56.44**	**<0.001**	**3.72**	**0.049**	1.25	0.53
	$F_{3,52}$	*P*	$F_{3,52}$	*P*	$F_{3,52}$	*P*
Biomass	**14.12**	**<0.001**	**11.36**	**0.001**	0.11	0.74
Root Si*	1.51	0.22	**3.71**	**0.05**	**5.07**	**0.02**

*log transformation

Table S6-3. Summary of outputs from ANOVA results of plant (*Saccharum* spp. hybrid) responses to arbuscular mycorrhizal (AM) fungal treatments in low and high silicon soil, mean values ± SE in response to treatments are shown, significant effects ($P \leq 0.05$) highlighted in bold.

	Low silicon soil						High silicon soil				
				AM fungi						AM fungi	
	Non AM	Commercial AM	Native AM	$F_{2,26}$	*P*	Non AM	Commercial AM	Native AM	$F_{2,24}$	*P*
Biomass (g)	35.96 ± 4.15	68.41 ± 6.67	75.08 ± 7.45	**12.37**	**0.001**	55.98 ± 6.53	92.77 ± 7.99	93.69 ± 5.69	**9.98**	**<0.001**
Aboveground biomass (g)	26.08 ± 3.04	49.51 ± 5.34	56.41 ± 5.37	**11.96**	**0.001**	42.09 ± 4.96	72.79 ± 6.03	69.97 ± 4.51	**10.62**	**<0.001**
Root biomass (g)	9.88 ± 1.21	18.91 ± 1.67	18.67 ± 2.38	**8.33**	**0.002**	13.89 ± 1.75	19.97 ± 2.26	23.72 ± 3.5	**3.62**	**0.04**
Root C:N ratio †	69.86 ± 6.71	60.86 ± 6.71	71.6 ± 9.8	0.54	0.58	56.91 ± 4.95	51.83 ± 7.93	52.53 ± 7.52	0.14	0.87
Root phenolics (mg g^{-1} GAE)	10.73 ± 1.56	11.92 ± 1.23	14.5 ±1.67	1.48	0.25	11.77 ± 1.37	12.39 ± 1.7	10.72 ±1.23	0.3	0.74
Root Si (%) *	1.58 ± 0.19	2.69 ± 0.24	2.24 ± 0.32	**4.76**	**0.019**	* 2.64 ± 0.22	2.62 ± 0.28	3.12 ± 0.47	0.22	0.79
Root P (ppm)	947.7 ± 95.73	919.67 ± 64.96	771.65 ± 43.99	1.56	0.23	481.94 ± 22.5	503.26 ± 37.46	552.08 ± 56.84	0.95	0.39

*log transformation; † permanova

Table S6-4. Summary of outputs from ANOVA results of insect (*Dermolepida albohirtum*) responses including relative consumption and relative growth rate to arbuscular mycorrhizal (AM) fungal treatments in low and high silicon soil, mean values ± SE in response to treatments are shown, significant effects ($P \leq 0.05$) highlighted in bold.

	Low silicon soil						High silicon soil				
				AM fungi						AM fungi	
	Non AM	Commercial AM	Native AM	$F_{2,26}$	*P*		Non AM	Commercial AM	Native AM	$F_{2,24}$	*P*
Relative consumption ($g\ g^{-1}$) *	0.138 ± 0.026	0.079 ± 0.026	0.045 ± 0.014	2.43	0.11	*	0.18 ± 0.045	0.061 ± 0.019	0.081 ± 0.021	**3.49**	**0.04**
Relative growth rate ($g\ day^{-1}$)	0.019 ± 0.007	-0.002 ± 0.004	0.003 ± 0.005	**3.58**	**0.04**		0.011 ± 0.005	-0.003 ± 0.011	-0.001 ± 0.011	0.73	0.49

*log transformation

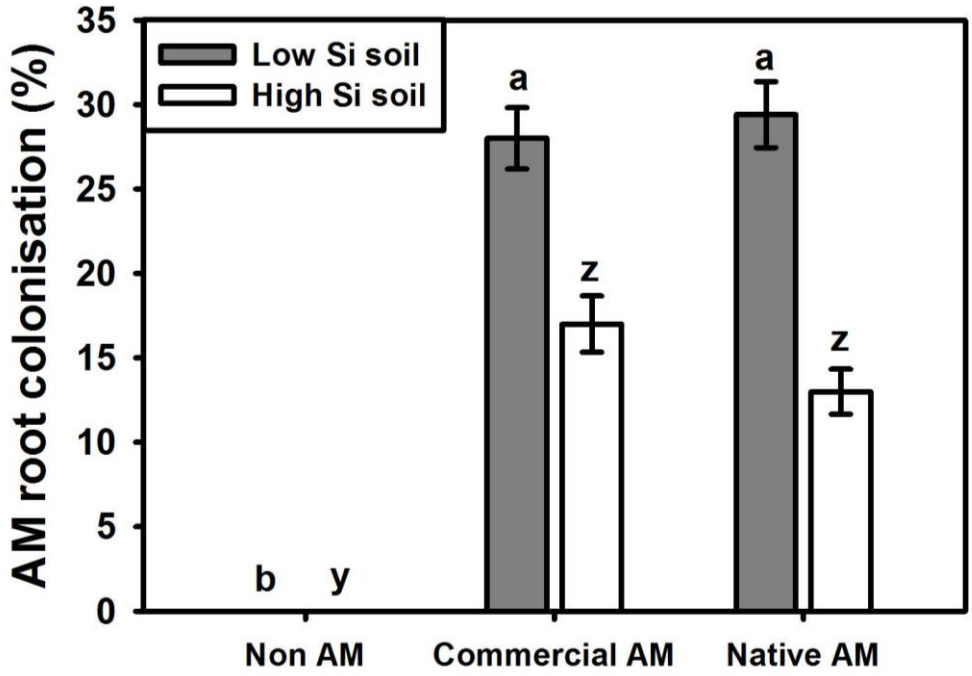

Figure S6-1. Arbuscular mycorrhizal (AM) colonisation (%) of sugarcane roots by grown under different AM treatments within a low Si and a high Si soil. Values are means ± SE. Where factor effects are significant, bars not sharing a common letter (a, b or y, z) differ significantly (Tukey; $P < 0.05$).

9.3 Appendix III – Chapter 7 supplementary material

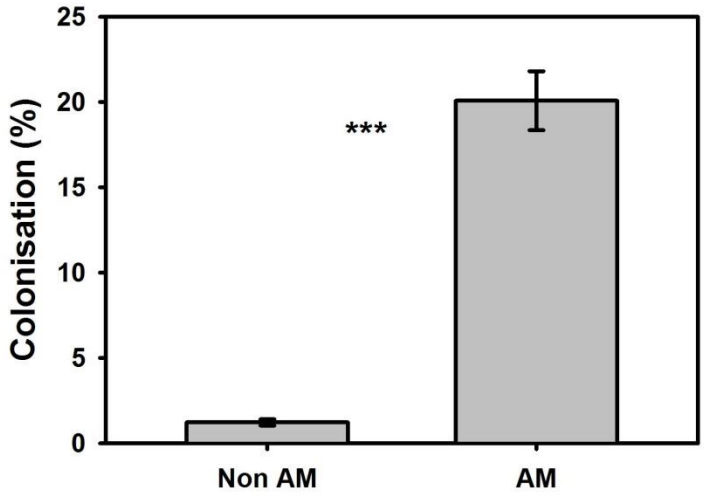

Figure S7-1. Colonisation (%) of sugarcane (*Saccharum* species hybrids) roots grown with (AM) or without (non AM) arbuscular mycorrhizal inoculation. Values are means ± SE. Degrees of significance are indicated as follows: *** $P < 0.001$.