The host specificity and efficacy of *Phenrica guerini* (Bechyné) (Coleoptera: Chrysomelidae), a candidate biological control agent of *Pereskia aculeata* Miller (Cactaceae) in Australia

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

MASTER OF SCIENCE

at

RHODES UNIVERSITY

By

Elizabeth Anne Dixon

ORCID ID

https://orcid.org/0000-0002-0207-4463

November 2021

Abstract

Pereskia aculeata Miller (Cactaceae) is a primitive creeping cactus that originated from South America and has become an invasive alien plant in South Africa and Australia. Due to its vine-like growth form mechanical and chemical control are not feasible options for its control, therefore biological control is the best option for controlling this plant. *Phenrica guerini* (Bechyné) (Chrysomelidae) is a leaf-feeding flea beetle that has been released as a biological control agent against *Pe. aculeata* in South Africa and has been shown to be damaging at some sites in the country. In this thesis, further studies into the use of *Ph. guerini* as a biological control agent against *Pe. aculeata* were conducted to determine whether the beetle should be released as an agent against the target weed in Australia.

The host range of a potential biological control agent is important to determine as only monophagous agents, or agents with suitably restricted host ranges, should be considered for release. Host specificity testing was conducted to determine if *Ph. guerini* was suitably specific for release in Australia. *Phenrica guerini* larvae only fed and partially developed on three out of 25 test plants, but failed to pupate on any plants besides *Pe. aculeata*. Adult beetles did not feed on any test plants and only oviposited on one test plant species. *Phenrica guerini* did not develop on any plant other than the target weed and is therefore suitably host specific for release in Australia.

The efficacy of a potential agent is also important to consider as the release of an ineffective agent can reduce the success of a biological control programme. A laboratory based pre-release efficacy assessment was done to determine the impact that *Ph. guerini* could have on *Pe. aculeata*. There was a significant difference between the control plants and those with larvae, with an average of 72 (S.E. ± 12.33) less leaves on plants with feeding and the main stem of the plant being 11.1 cm (S.E. ± 7.17) shorter. Although there were no significant differences observed in the number of additional stems, and weights of the different plant parts, this was not a long term study and many of these differences would not have been observed in a short period. Based on the impact observed on the leaves and the main stem, and various field studies that have been done to assess the impact of *Ph. guerini* on *Pe. aculeata* in the field in South Africa, *Ph. guerini* appears to be a potentially damaging agent.

As *Pe. aculeata* is still in its early stages of invasion in Australia the beetle has the potential to be damaging and reduce the invasion trajectory of the plant. This study showed that the beetle

is suitably host specific and potentially damaging enough to be recommended for release as a biological control agent against *Pe. aculeata* in Australia.

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Acknowledgments

A big thank you to my supervisor, Iain Paterson. Thank you for looking after my insects and experiments when I was in isolation or Zim sorting out my passport. Thank you for helping with collecting insects and plants for my experiments. And thank you for all of the advice and comments throughout the process.

Another massive thank you to my husband, Russell Dixon, for all the support and being my COVID-19, same-household field trip partner. Thank you for all your help collecting plants and insects, and for being some extra muscle when I was setting up and taking down large experiments for the impact assessment. Thank you for all the effort you have put in to help make this project possible!

Thank you to Pippa Muskett for all of the varied help. Thank you for looking after my insects and experiments when I was in isolation or in Zim sorting out passports! Thank you for helping me find cages and space to run things, and for your willingness to help in many ways!

Thank you to Guy Sutton for the help with the statistical analyses for the impact assessment chapter. Thank you for helping me better understand the stats and for being willing to look through my R code and data to ensure my stats were correct.

Thank you to other members of the Centre for Biological Control for their help and assistance with this project; Zeze Mnqeta for advice on rearing *Ph. guerini* from her MSc; Lenin Chari and Liam Yell for assisting Iain with collecting *Ph. guerini* on one of their field trips; Jeanne van der Merwe for all of her administrative help and making sure the project can go on!

A big thank you to my family and in-laws and my spiritual family at Grahamstown Baptist Church for all the support, love, prayers, and encouragement throughout my time in Grahamstown/ Makhanda.

Thank you to the New South Wales Department of Primary Industry (Australia) and to the South African Department of Forestry, Fisheries, and the Environment (DFFE) for funding.

And last but most certainly not least thank you to the LORD for all He has done and for carrying me through this project. All praise to Him! 1 Corinthians 10:31.

Chapter 1

General Introduction

Invasive alien plants pose a risk to economies and ecosystems worldwide (Vitousek *et al.* 1997; Rai & Singh 2020). Due to this threat, many countries invest significant funds to control these plants (van Wilgen *et al.* 2001). Biological control is a cost effective and environmentally sound method of controlling invasive alien plants (McFadyen 1998; Briese 2000). For biological control to be environmentally sound the agents used need to be suitably host specific and effective (McFadyen 1998). Host specificity testing is a fundamental part of biological control programmes as it determines the host range and potential risks of a biological control agent before release of the insect (McFadyen 1998; McEvoy 1996; van Klinken 2000).

In this study, host specificity testing and an impact assessment were conducted to determine whether *Phenrica guerini* (Bechyné) (Coleoptera: Chrysomelidae), a leaf-feeding flea-beetle, is suitably host specific and damaging for release as a biological control agent for the invasive alien weed, *Pereskia aculeata* Miller (Cactaceae), in Australia. In this chapter, a general introduction to the biological control of invasive alien plants, including the need for host specificity and pre-release impact assessments are given. Some background to *Pe. aculeata* is provided, focusing on the biological control used against it in South Africa and the need for its control in Australia.

1.1 Invasive alien plants

Plants have been moved across the globe for centuries and most alien plants are not harmful to their introduced habitats, however some of these plants become invasive in their introduced region and these can have significant negative impacts (Mack *et al.* 2000; Cappuccino & Carpenter 2005; Moran *et al.* 2005). Humans are the main agents of spread of these plants, with many introductions being deliberate (Vitousek *et al*. 1997; Mack *et al*. 2000). Invasive alien plants are considered to be as environmentally damaging as land clearing and disturbances, such as mown grasses, woodland clearing, and pine plantations, although the effect of the invasive species is often less obvious to observe (Samways *et al.* 1996). They can alter ecosystem properties and cause alterations in disturbance frequencies caused by fire, animals, and diseases (van Wilgen & Richardson 1985; Vitousek 1986; Vitousek *et al*. 1997; Mack *et al.* 2000; van Wilgen *et al.* 2001). The effects that they have on biodiversity and ecosystem-level processes have direct impacts on economies including losses in crops, fisheries, forestry, and grazing capacity (Mack *et al*. 2000; van Wilgen *et al*. 2001).

As these invasive alien plants cost countries large sums of money it is important to be able to predict which exotic plants, especially recently introduced exotic plants, are the most likely to become invasive (Cappuccino & Carpenter 2005), although invasions are relatively unpredictable (Williamson 1999). Almost all plant types are represented amongst the invasive species globally (Williamson & Brown 1986; Pyšek *et al*. 2017). Many hypotheses have been put forward to try and predict invasions, including six major hypotheses for invasion biology and what makes a good invader (Jeschke *et al*. 2012). These hypotheses include the "Biotic resistance hypothesis" and "Island susceptibility hypothesis" which focus on the diversity of the ecosystems being invaded, the "Tens rule" which focuses on the invader species, and the "Invasional meltdown hypothesis", "Novel weapons hypothesis", and "Enemy release hypothesis" which all focus on the interactions between both the invader and the ecosystem (Jeschke *et al*. 2012). According to Jeschke *et al*. (2012) the hypotheses with the most empirical support are those that focus on the invader-ecosystem interactions. It is likely that a combination of these hypotheses, rather than one, explains invasion success, and that the varying degree of importance of each hypothesis will change for each case of invasion.

The "Enemy release hypothesis" or "Natural enemy hypothesis" is the central principle to the success of the biological control of invasive alien plants (Maron & Vilà 2001). It is based on the assumption that, in its native range, a plant's spread and abundance is limited by natural enemies but when the plant is introduced into an area without its natural enemies its population will explode and the plant will become invasive (Jeschke *et al.* 2012). If this hypothesis is correct then it would imply that a plant should have reduced population sizes or growth rates in its native range as a result of native herbivory, and that the same plant in its introduced range should have insignificant herbivore damage and a larger population size and growth rate compared to the native range (Maron & Vilà 2001). In general, classical biological control has been successful (McFadyen 1998; Zachariades *et al*. 2017) and as it is based on the "Enemy release hypothesis" biological control can show a form of support for the hypothesis. Hinz & Schwarzlaender (2004) reviewed several studies that had compared herbivory of plants in their native and introduced ranges and concluded that, based on those studies, there was a correlation between release from natural enemies and invasion success, thus supporting the "Enemy release hypothesis". However, this is not always the case as there are many examples of invasive alien plant species that are abundant in their native ranges, including species that have abundant and seemingly damaging natural enemies (Maron & Vilà 2001). If a species is abundant in its native range despite herbivore pressure then it is unlikely that enemy release is

the only reason why it has become invasive in its introduced range (Maron & Vilà 2001). However, Müller-Schärer & Schaffner (2008) have argued that biological control is a suitable strategy even if the main cause of an invasion is not enemy release. This is due to the aim of classical biological control being to alter community-based imbalances in herbivore pressure in the introduced ranges rather than restoring herbivore pressure to the level found in the native range (Müller-Schärer & Schaffner 2008).

There are three main actions that are taken in the management of invasive alien plants, which include prevention, eradication, and impact reduction (Novoa *et al.* 2019). The first step is to try and prevent the introduction of potential invasive species (Novoa *et al*. 2019). If a species has already been introduced and is established or establishing but has not yet become invasive, then it may be possible to eradicate the species (van Wyk *et al.* 2012). This is done using "Early Detection and Rapid Response", where the first detection of an alien species should lead to a risk analysis to determine the likelihood of the species becoming invasive, and if there is sufficient risk, this should be followed by actions being taken to eradicate the species (Cappuccino & Carpenter 2005; van Wyk *et al.* 2012). Eradication can be the most effective and successful way of managing an invasion if it is done early (Simberloff 2003). Once a plant species has become too abundant to eradicate, it becomes necessary to control the plant by containing its spread and reducing the negative impacts the plant has on the economies and ecosystems of a country (Kaplan *et al*. 2017; Novoa *et al*. 2019). The control of widely established invasive plant species has been done using mechanical, chemical and biological control (Villamanga & Murphy 2010). In some cases, biological control is the most environmentally safe, sustainable and cost effective method for the control of invasive species (McFadyen 1998).

1.2 Biological control of invasive alien plants

Historically, there have been four types of weed biological control; classical, inundative (or augmentative), conservative, and broad spectrum (Briese 2000). The oldest, most common and effective method of biological control used in both Australia and South Africa for the control of invasive alien plants is classical biological control (Briese 2000; Moran *et al*. 2011). Classical biological control applies the "Natural enemy hypothesis" by using natural enemies of the invasive species from their country of origin against the plants in the invaded areas (McFadyen 1998; Briese 2000; Maron & Vilà 2001). The aim of biological control is to reduce the density, cover, and distribution of the weed population in the invaded areas through the

action of its natural enemies (Briese 2000; Denslow & d'Antonio 2005). The complete eradication of invasive species has never been the purpose of biological control and it should not be seen as the goal of this management practice (Briese 2000). Biological control aims to reduce the weed population to the point at which the weed no longer causes economic damage, or mechanical and chemical control become economically viable methods (Briese 2000; Hinz *et al*. 2020).

Successful biological control depends on the damage the biological control agents can cause the plant, the ecology of the agent, and the ecology of the target weed (McFadyen 1998). As biological control is irreversible the choice of biological control agent is an important part of the success of biological control programmes (McFadyen 1998). There are many considerations to be taken when choosing a biological control agent. These include the synchronicity of the plant and its agent, the habitat, the ecoclimatic similarity between the native and introduced ranges, and how easily the agents can find and colonise new plants (Pemberton 1984; Briese 2004; Zalucki & van Klinken 2006). It is important to try and predict the distribution and abundance of potential agents in the introduced environments to help prioritise potential agents for testing and release (Zalucki & van Klinken 2006).

If successful, biological control results in the continual and permanent control of the weed (Harris 1988) and it can provide highly effective, selective, and long lasting control which is cost effective and mostly self-sustaining (McEvoy 1996; Briese 2000; van Wilgen *et al*. 2001; Hinz *et al*. 2020). Biological control has been an effective control strategy across many countries with many success stories (Schwarzländer *et al.* 2018). It was first practiced, as a mistake, in India when a cochineal insect was introduced for dye production on *Opuntia monacantha* (Wild.) Haw. (Cactaceae) (Winston *et al.* 2014; Day *et al*. 2020). Soon the *O. monacantha* populations were being decimated and the insect was purposefully redistributed for the purposes of controlling the plant (Day *et al*. 2020). There have now been over 500 biological control agents released globally against around 200 weed species (Winston *et al*. 2014). Although there are many countries that have released agents for the biological control of weed species the most active countries are The United States of America, Canada, South Africa, Australia, and New Zealand (Winston *et al*. 2014). The first biological control programmes in South Africa and Australia were started in 1913 and 1914, respectively, and were both against *O. monacantha*. Although biological control does not usually show immediate results there have been many long-term successes, and 65.7% of the releases of biological control agents globally have resulted in some level of control (Schwarzländer *et al.*

2018). However, not all successful cases of biological control result in the same degree of control of the invasive plant species (Briese 2000; Zalucki & van Klinken 2006).

Concerns that biological control will be harmful rather than helpful as it involves the release of another exotic organism into the invaded range have been raised in the past (Sheppard *et al*. 2003). Most of these concerns are due to a few examples of biological control agents that were released and have had significant negative impacts on non-target, rare species (Sheppard *et al*. 2003). However, a fundamental part of biological control is host specificity testing to ensure that the biological control agents are specific enough and will not cause non-target damage (van Klinken 2000; Hunt *et al*. 2008; Paynter *et al.* 2020a). In a review on non-target attack by Hinz *et al*. (2019) it was found that at least half of the unique non-target attack cases globally were predictable and for many of the unexplained cases of non-target attack the plant species that was attacked had not been tested before the release of the agent, making prediction of the attack impossible. This enforces the need for effective pre-release host specificity testing but also indicates that the examples of non-target attack do not raise a cause for concern over biological control as a practice, but rather the concern over how effective pre-release testing is. Although many more agents have been released since 1960 compared to before 1960, there has been a much lower proportion of non-target attack recorded, and this is attributed to better host specificity testing, including a more refined and well-chosen plant test list (Hinz *et al*. 2019).

Although it is crucial to determine the host specificity and therefore safety of an agent before release, it is also important to determine the impact of the agent on the target weed to predict whether it will be an effective control agent or not (Müller-Schärer *et al*. 2020). The release of ineffective agents adds to the costs and risk of biological control and so pre-release efficacy assessments should be done as part of the pre-release screening of a potential biological control agent (Sheppard 2003; Goolsby *et al.* 2004; McClay & Balciunas 2005). McClay & Balciunas (2005) have argued that the only reason to release an ineffective agent is due to an unavoidable inability to predict the agent's efficacy. It is therefore important to predict, as best as possible, an agent's efficacy pre-release as part of the determination of whether an agent is suitable for release or not. This is not easy to do, as even predictions based on impact studies in the agent's native range, or in quarantine, with a climatic based analysis cannot guarantee success because it is not possible to perfectly predict the many factors that will affect the agent in the introduced range (McFadyen 1998).

There are also many conflicts of interest that surround biological control. Some invasive species have some form of cultural or economic significance to the region that they have invaded and so there is conflict over the use of biological control for that plant (Cullen & Delfosse 1984; Zimmermann & Klein 2000). In Australia there was much conflict that resulted in legal battles over the biological control of the perennial shrub, Paterson's curse, *Echium plantagineum* L. (Boraginaceae), or Salvation Jane as it was named by those who were opposed to its biological control (Cullen & Delfosse 1984). The conflict was not due to possible non-target attack from the agent but due to apiarists who believed the plant had beneficial effects that were greater than the detrimental effects of the declared noxious weed (Cullen & Delfosse 1984). The biological control of *Opuntia ficus-indica* L. (Cactaceae) in South Africa has been met with similar conflicts of interest as there are farmers and game reserve managers who want the plant to be further controlled and removed, but there are also commercial growers of the plant who want the plant for the economic benefits that are brought from its fruit and as a fodder crop (Zimmermann & Klein 2000; Shackleton *et al*. 2011).

These conflicts of interest and concerns due to non-target attack of the agents require scientists and regulators to carefully weigh up and consider the risks and benefits of releasing insects for biological control (McEvoy 1996; Downey & Paterson 2016). The risk of the impacts from the target plant species on biodiversity needs to be considered against the potential non-target effect that may be associated with a biological control agent (Downey & Paterson 2016). This is why it is important to determine the host-specificity and potential impacts of any agent being considered for biological control before it is released into the invaded range (Harris 1988; Müller-Schärer *et al.* 2020).

1.3 Host specificity testing of potential biological control agents

Host specificity testing is an important aspect of biological control that is used to estimate a potential agent's host range and predict potential impacts that an agent could cause before it is released (McEvoy 1996; McFadyen 1998; van Klinken 2000; Briese 2004). It was first developed due to the potential threat that introduced phytophagous insects could have on economically valuable crops (Hunt *et al*. 2008). The techniques used for host specificity testing have been refined and standardized over the years and are now used to determine if any damage may be caused to both native flora in the agent's introduced range or to alien plants of value in the introduced range (Fowler *et al*. 2012). It is also meant to help avoid the non-target attack

that has been recorded in a few cases that have caused doubt over the safety of biological control (Hinz *et al*. 2019).

Host specificity testing is usually conducted using either choice, no-choice or field trials (van Klinken 2000). These tests are done in a laboratory or quarantine facility but can be done in the field in the native range of an agent or an area in which the agent has been introduced previously. The methods that are currently being used to determine the host range of potential agents are generally very accurate and have resulted in a good safety record for biological control (Hinz *et al.* 2019; Paynter *et al.* 2020a). If host specificity testing is done properly by using modern methods of plant choice selection and the biology and behaviour of the agent are both taken into account then it works well and can accurately predict the host range and safety of a potential agent. Further details on how this can be done, including the methods used and process of host specificity testing, are provided in Chapter 2.

1.4 Quantifying the efficacy of potential agents

Biological control agents must not only be suitably host specific to be safe for release, they should also be sufficiently damaging to control the target weed (Müller-Schärer *et al.* 2020). Pre-release efficacy assessments have not always been incorporated into pre-release biological control tests and there has been a reluctance to carry out the tests due to added costs and a lack of confidence in the data produced by pre-release efficacy testing (McClay & Balciunas 2005). The release of an ineffective agent should be avoided, as there is always an intrinsic risk with the release of a biological control agent, however small, and the risk is only worth taking if the agent is going to be damaging (Sheppard *et al*. 2003; McClay & Balciunas 2005). Therefore, pre-release efficacy testing should be done no matter how difficult, costly, or time consuming it may be to ensure that only effective agents are released (Sheppard 2003; McClay & Balciunas 2005; Morin *et al*. 2009).

Pre-release efficacy assessments can also be more cost effective than host specificity testing and in some cases may be used as a cheaper way of doing the initial screening of insects for biological control, followed by host specificity testing of the insects that are sufficiently damaging (McClay & Balciunas 2005). If the selection of agents is also largely based on the efficacy of the insect before release then this can minimise the number of agents that are necessary for successful control (Sheppard 2003). Pre-release efficacy studies can easily be done in the field in the agent's native range (Goolsby *et al*. 2004; Sutton *et al*. 2021) or under strict quarantine conditions (Balciunas & Smith 2006). Further details on the different methods

used and important considerations in pre-release efficacy assessments are provided in Chapter 3.

1.5 The biological control of Cactaceae

The Cactaceae are endemic to the Americas, so there are no native cacti in Australia or anywhere outside the Americas, with the possible exception of *Rhipsalis baccifera* (J. Müller) Stern which is considered indigenous in Africa and parts of Asia (Roland-Gosselin 1913; Barthlott & Taylor 1995; Paterson *et al.* 2011a). It is a family of plants that has invaded many parts of the world, including South Africa and Australia causing economic losses (Annecke & Moran 1978; Briese 2004). There are 57 cactus species listed as invasive worldwide, with the hotspots for cactus invasion being Australia, South Africa, and Spain (Winston *et al.* 2014; Novoa *et al.* 2019). Many cactus invasions at the small scale are controlled using physical control (Novoa *et al.* 2019). Herbicides are also used for the control of cacti but regulations make it difficult to use certain herbicides in some countries (Novoa *et al.* 2019). The most cost-effective and successful management tool for widespread cactus invasions has been biological control (Novoa *et al.* 2019). The lack of native cacti outside the Americas, increases the number of potential biological control agents available for invasive cacti because generalist cactophagous herbivores could be released and there is a lower risk of non-target attack on native plants (Moran & Zimmermann 1991a; Paterson *et al*. 2011a). Cactaceae are therefore particularly good targets for biological control (Paterson *et al.* 2011a).

The first successful biological control agent in South Africa was released in 1913 against *O. monacantha.* Since then there have been 18 agents released against 22 cactus weeds in South Africa, with many agents being successful on more than one cactus species (Zachariades 2018a; 2018b). Some of these cacti were not the original target weeds but agents have moved onto them and provided some form of control (Zachariades 2018a; 2018b). Of the 22 cactus weeds that are currently under biological control in South Africa, seven are under complete control, eight are under substantial control, one is under evaluation, the control level of four of the species has not been determined, one is under negligible to substantial control depending on the variety, and one is under negligible control (Zachariades 2018a; 2018b).

The biological control of cacti species has been relatively successful in comparison to the control of other invasive plant groups in South Africa (Paterson *et al*. 2021). Looking at just the plants that have agents established and some degree of control determined, the control of cacti has had a high proportion of successes (Zachariades 2018a; 2018b). Of the seventeen

cactus species that have established agents 41% of the cacti are under complete control and 47% are under substantial control with only 12% (2 species) of the 17 species having negligible control (Zachariades 2018a; 2018b). For the Fabaceae, another large group targeted for biological control in South Africa, only 6.7% (1 species) of the 15 species analysed are under complete control and 46.7% are under substantial control (Zachariades 2018a; 2018b). The other 46.7% of the 15 species of Fabaceae with established agents are under negligible control. For all 36 non-cacti species that have established agents in South Africa 44% are under negligible control, 36% are under substantial control and only 19% are under complete control. Biological control has also been extremely successful in controlling Cactaceae in Australia, with agents released against 21 cactus weed species and agents established and causing moderate to extensive damage on 14 of these target cactus weeds (Novoa *et al*. 2019). Australia is also home to one of the most famous examples of successful biological control due to the release of *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae) and *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae) against *Opuntia stricta* (Haw) Haw. (Cactaceae) which caused great reductions in the invasion of *O. stricta* and subsequent sharing of the agents with other countries because the control had been so successful in Australia (Raghu & Walton 2007; Hoffmann *et al*. 2020).

There has been much work shared between South Africa and Australia in the history of cactus control (Annecke & Moran 1978; Moran & Zimmermann 1991a; Paterson *et al.* 2019a). Australia had great success against *Opuntia* species in the 1930s and South Africa used the knowledge gained form Australian entomologists to start biological control programmes against *Opuntia* species in South Africa (Annecke & Moran 1978; Moran & Zimmermann 1991a). Although the moth, *C. cactorum* has become a famous example of cactus control the cochineal insects (*Dactylopius* spp.) and the mealybug *Hypogeococcus* sp. have contributed the most to the successful biological control of Cactaceae globally (Moran & Zimmermann 1984; Moran & Zimmermann 1991b; Paterson *et al.* 2019a; Hoffmann *et al*. 2020; Paterson *et al*. 2021). *Pereskia aculeata* is a very different type of cactus and is not a suitable host for any cochineal insects or *Hypogeococcus* sp., but based on the significant success with cactus biological control in the past, it should be an excellent target for biological control with a high chance of success.

1.6 *Pereskia aculeata*

Pereskia aculeata (Fig 1.1), commonly known as leaf cactus or Barbados gooseberry, is a primitive creeping cactus originating from Central and South America which has become an invasive species in Australia and South Africa (Leuenberger 1986; Moran & Zimmermann 1991a; Klein 1999; Paterson *et al*. 2011a). The genus *Pereskia* is different from other cacti in that the species have well developed leaves with woody stems and branches, and do not have the typical succulent modified stems of the family (Leuenberger 1986; Moran & Zimmermann 1991a) (Fig 1.1b). *Pereskia aculeata* has paired hooked thorns on the younger green shoots and clusters of long needle-like spines on older woody stems (Leuenberger 1986) (Fig 1.1b). It has creamy to yellow coloured flowers (Fig 1.1c) that become yellow fruit with spines when developing and once ripe the fruit becomes orange and the spines are lost (Leuenberger 1986). The leaves are eaten in Brazil (Leuenberger 1986) but have not become a major food source in their introduced ranges.

The native range of *Pe. aculeata* is disjunct and separated into two main areas (Leuenberger 1986). Its northern population is found in Central America, northern Venezuala and the Caribbean whereas its southern population is found in southern and south-eastern Brazil, southern Paraguay and northern Argentina (Leuenberger 1986). There are naturalised populations of the cactus in Mexico, Central America, and Florida (USA) (Leuenberger 1986). In its native range *Pe. aculeata* has a variety of morphotypes and it appears that there is no distinct original morphotype in South Africa (Paterson *et al.* 2009). Therefore, genetic studies were done on the plants from both the invaded range in South Africa and its native range in the Americas to determine the origin of the plant (Paterson *et al.* 2009). The results from this study indicated that the *Pe. aculeata* in South Africa likely originated from the Rio de Janeiro Province in Brazil (Paterson *et al.* 2009). However, the results also suggest that the invasive leaf cactus in South Africa originated from a garden variety of the plant, so after the original plant was collected in Brazil the lineage was cultivated for many years as a garden variety in Europe prior to introduction into South Africa (Paterson *et al*. 2009). The first record of the plant in South Africa was at a botanical garden in Cape Town in 1858, and it was recorded as problematic in the country for the first time in the 1970s (Moran & Zimmermann 1991a; Paterson *et al.* 2009).

Pereskia aculeata has the ability to invade both disturbed and pristine sites due to its ability to propagate asexually as well as having fruits that are eaten and transported by birds (Paterson

et al. 2011b; Northern Territory Government 2012). *Pereskia aculeata* has the ability to grow up into forest canopies (Fig 1.1d), blocking out the sunlight for the trees it is on, and even causing large trees to collapse under its weight causing large gaps in forests (Moran & Zimmermann 1991a; Klein 1999; Paterson *et al*. 2009; 2011a; 2011b). This is especially a threat to the South African forest biome which has many rare vegetation types with high levels of endemism (Paterson *et al.* 2011b). Infestations of the plant negatively impact biodiversity, causing significant decreases in indigenous plant species richness and diversity (Paterson *et al.* 2011b). Control measures would only protect biodiversity if the weed densities were kept below 50 % cover (Paterson *et al*. 2011b).

Figure 1.1: *Pereskia aculeata* Miller (Cactaceae) (a) infestation along a road, with the plant developing fruit; (b) leaves and typical groups of spines along a well-developed woody stem; (c) flower; (d) infestation growing up trees and a slope, covering indigenous plants.

Most invasive plant species appear to be dominant in the systems that they are found in, however, this does not mean that they are 'drivers' of biodiversity loss as they may sometimes be 'passengers' of disturbances that facilitate their invasion and their impact on biodiversity loss (MacDougall & Turkington 2005). Due to the leaf cactus's ability to invade pristine sites without known disturbances, Paterson *et al*. (2014b) suggest that it is a 'driver' of biodiversity

loss and not a 'passenger' of disturbance. This means that the plant will outcompete native plants and cause significant losses in biodiversity without any other disturbances present (MacDougall & Turkington 2005).

Although *Pe. aculeata* has been recorded in South Africa since 1858 it was only declared as a national weed in 1979 (Conservation of Agricultural Resources Act, 1983 (CARA 1983); Moran & Zimmermann 1991a). It is also on the Australian government's "Alert List for Environmental Weeds" (Anonymous 2003; Northern Territory Government 2012). Chemical control is not a possibility for the leaf cactus as its vines intertwine with native plants and the native flora will also be impacted by any chemicals used (Moran & Zimmermann 1991a; Klein 1999; Paterson *et al.* 2009; 2011b). Mechanical control is also ineffective due to the plant's ability to regenerate from a small stem that may have been missed during clearing which will root and regrow, making biological control the only viable option of control (Moran & Zimmermann 1991a; Klein 1999; Paterson *et al*. 2009; 2011b).

1.7 The biological control of *Pereskia aculeata* in South Africa

Phenrica guerini is a leaf feeding flea beetle in which both the larval and adult stage feed and defoliate the plant (Klein 1999). In 1991 it was released as the first biological control agent against *Pe. aculeata* in South Africa (Klein 1999; Mnqeta & Paterson 2019). Another agent, *Epipagis cambogialis* (Guenée) (Lepidotera: Pyralidae) was also brought to South Africa for testing in the early 1990s but it developed on non-target species including species outside of the family Cactaceae, resulting in the rejection of the moth as a potential agent for the leaf cactus (Klein 1999). Another moth, *Maracayia chlorisalis* (Walker) (Lepidotera: Crambidae) was considered but due to unreliable initial host specificity test results and difficulty with rearing the moth it was not released (Klein 1999). After *Ph. guerini* established in South Africa the search for other potential agents continued, with renewed surveys in South and Central America (Paterson *et al.* 2014a).

These surveys resulted in a few promising potential biological control agents. One of the most promising potential new agents was the weevil, *Pereskiophaga brasiliensis* Anderson (Coleoptera: Curculionidae), however, it was rejected as an agent as it was not suitably host specific for release, feeding on plants outside of the family Cactaceae (Paterson *et al*. 2019b). A second agent was released in 2014 against the leaf cactus in South Africa (Paterson *et al.* 2019b). This agent was the bug, *Catorhintha schaffneri* Brailovsky & Garcia (Hemiptera: Coreidae). The bug is a sap-sucker that causes the wilting and death of growing shoots of the

plant (Paterson *et al.* 2014a; 2014b; Mnqeta & Paterson 2019). It has established successfully at some sites in South Africa (Mnqeta & Paterson 2019; Muskett *et al.* 2020; King *et al*. 2021). There are therefore two biological control agents established in South Africa, the flea beetle, *Ph. guerini*, which is widely established and has been present in the country for over 25 years, and the recently released stem-wilting bug, *C. schaffneri*, that is only present a few sites in the country (Muskett *et al*. 2020; King *et al*. 2021).

A post-release evaluation of *Ph. guerini* has been done, including a more detailed evaluation at one of the sites where the beetle has established in South Africa, and the beetle was shown to be damaging to individual plants, and although it has not completely controlled *Pe. aculeata* in South Africa, it should be considered an effective agent as it has shown potential for reducing the density of the cactus (Mnqeta 2017). The same study also suggested that the damage level recorded for *Ph. guerini* should be changed from trivial to moderate for some of the sites where it is found, although due to a lack of pre-release evaluations it is not clear what the true extent of damage caused by the beetle is (Mnqeta 2017). Although there are two agents currently released against *Pe. aculeata* in South Africa, the level of control so far can be attributed to *Ph. guerini* as the stem-wilting bug has not been released for long enough. Currently the level of control of *Pe. aculeata* in South Africa, due to the flea beetle, is described as the target weed being suppressed or, in some cases, stabilised due to biological control so that other methods or further agents are needed for full control but the invasion has been reduced compared to if *Ph. guerini* was not present (Moran *et al*. 2021).

1.8 The need for biological control of *Pereskia aculeata* in Australia

Pereskia aculeata is on the "Australian National Weeds Alert List" (Poon *et al*. 2007). It is considered a weed in Australia and was marked for eradication as it has a weed history outside of Australia and poses a threat to Australian ecosystems (Csurhes & Edwards 1998; Anonymous 2003; Northern Territory Government 2012). However, eradication of the cactus from the country is impossible and so the intention is to reduce the negative impacts of the weed through control (Csurhes & Edwards 1998). As it is not possible to control the weed mechanically or chemically, biological control is considered the best option to reduce the negative impacts of the weed in Australia.

The leaf cactus was originally traded and cultivated in Australia as a garden ornamental and hedge plant due to its attractive flowers, edible fruit, and unusual growth form for a cactus (Northern Territory Government 2012). It is thought to have first been introduced to Australia

in the 1920s and was first recorded as naturalised in a localised population in New South Wales in 1989 (Anonymous 2003). The cactus poses a risk to the eucalypt communities and riparian vegetation in Australia and its likelihood of being a threat is increased due to the cactus' weed status in South Africa and the similarity between the climatic and environmental conditions of South Africa and Australia (Csurhes & Edwards 1998; Anonymous 2003). The cactus is currently known to be found in the coastal sub-tropical eucalypt communities of Queensland, the riverside vegetation in the suburb of Sherwood (Brisbane), and on the north coast in New South Wales, with an old record of the cactus at Bar Island on the central coast of New South Wales, which was allegedly removed by a bush regeneration team (Anonymous 2003; Northern Territory Government 2012).

Climate matching has been an important part of determining which areas are most at risk of invasions from other countries due to similarity in climate (Curnutt 2000). This same principal can be used in determining which countries can use similar biological control agents for shared weeds. There are many shared invasive species between climatically similar countries across the world and there has been much sharing of information between the countries in the establishment of biological control against these shared plants (Annecke & Moran 1978; Moran & Zimmermann 1991a; Paterson *et al.* 2019a). In general it has been found that if there has been successful biological control of an invasive species in one country the chances of success using the same agents in a climatically similar country are greatly increased (McFadyen 1998). Australia and South Africa have similar climates that can allow for shared biological control opportunities (Tatem & Hay 2007; Bennett 2011). This can reduce the costs of biological control programmes as initial surveys and some of the testing may have already been done, although it is important to determine the host specificity of an agent against potential non-target species in each introduced region individually, because the list of test plant species will differ (McFadyen 1998). Biological control programmes that rely on previous work conducted in other countries are often referred to as piggyback projects.

Piggyback biological control projects have been successful in many places across the world. In many low-income and middle-income countries most of their biological control projects have been as a result of projects against the same weed in a different country and the release of the agents has been relatively successful (Day *et al.* 2020). The first of many deliberate introductions of an agent against a weed in Latin America was a piggyback project from the USA and the release of the agent in Latin America was successful (Barreto 2008). As South Africa already has two biological control agents established for the biological control of *Pe.*

aculeata, one or both of these agents could potentially be used in Australia against the leaf cactus without Australian entomologists needing to do initial surveying for potential agents. An application for release of the bug, *C. schaffneri*, in Australia as a control agent against the leaf cactus has been submitted but is still pending approval (CSIRO 2021).

1.9 Thesis aims and rationale

This study aimed to determine whether *Ph. guerini* is a suitable agent for the biological control of *Pe. aculeata* in Australia. Host specificity no-choice testing of both the larvae and adults of *Ph. guerini* was conducted to determine the host range of the insect and to determine if the agent is safe for release in Australia. An impact assessment was also conducted to determine how much damage the beetle could cause to the leaf cactus and was interpreted in light of what is known about the impact of *Ph. guerini* in the field in South Africa. This study is the first step towards a potential application for release of the beetle in Australia.

Chapter 2

Host Specificity Testing of *Phenrica guerini*

2.1 Introduction

One of the primary criteria used by scientists and regulators when deciding whether or not to release a candidate biological control agent is its host specificity (McEvoy 1996; McFadyen 1998; Briese 2004). Most species of phytophagous insects have specialised in their diet to some extent, and therefore feed on only a small proportion of the plant species within their habitat (Jaenike 1990; Fry 1996). Phytophagous insects generally feed on only one or a few plant genera or on a single plant family or subfamily, with only a small proportion of phytophagous species feeding on plants in more than three different plant families (Bernays & Graham 1988; Ødegaard *et al*. 2005).

Since most phytophagous insects are specialised to some degree they can be useful in biological control, as specialist agents should only attack their target weeds. However, as there are still many phytophagous insects that are generalists or are not specialised to the degree needed for the biological control of a particular weed in an invaded area, it is important to demonstrate a candidate agent's host specificity prior to its release.

2.1.1 Importance of host specificity testing in biological control

The need to demonstrate the host specificity of a candidate biological control agent was first recognised in the 1950s as a result of the threat posed to valuable crop species due to the release of biological control agents for invasive alien plants (McEvoy 1996; Hunt *et al.* 2008). This led to the development of various host specificity testing procedures that have been refined over time to what they are today, so that the risk to both economically important plants and native flora is addressed (Pemberton 2000). There have been cases of significant non-target attack due to biological control agents that have caused negative impacts on rare species (Sheppard *et al*. 2003). However, most well-known examples of non-target impacts from biological control were in fact predicted prior to release of the agent based on evidence from host specificity testing (Julien & Griffiths 1998; Delfosse 2005; Downey & Paterson 2016; Hinz *et al*. 2019), and there is strong evidence that host range testing accurately predicts host range and therefore the likelihood of non-target impacts after release (Paynter *et al*. 2015). Despite this evidence, the perception of risks due to non-target attack by biological control agents has caused decreases in support and funding of biological control and the number of agents released in places like the USA (Schwarzländer *et al*. 2018), and has led to hesitancy

with the uptake of biological control of weeds in Europe (Barratt *et al*. 2018). However, due to the current methods used in host specificity testing there has been a lower proportion of non-target attack despite an increase in the number of agents released over the past few decades (Hinz *et al*. 2019; Paynter *et al*. 2020a). Host specificity testing along with other pre-release studies have resulted in biological control having a superior safety record compared with other weed management methods (Blossey *et al*. 2018).

Biological control is a long lasting and cost effective management approach against invasive alien plants (Briese 2000; Hinz *et al*. 2020). However, as classical biological control involves the release of alien insects into novel environments, it is irreversible, and so all possible consequences should be considered and addressed before the release of an agent (McFadyen 1998). There is legislation around the use of biological control and a fundamental part of the decisions made around the release of an insect is its host range (McFadyen 1998; Briese 2004). In most countries in which biological control is practiced, legislation includes many steps that need to be taken before an agent is approved and these steps are mostly precautionary and aim to ensure that any agents released are safe (Palmer *et al.* 2010 (Australia); Klein *et al*. 2011 (South Africa); Hinz *et al*. 2014 (United States of America)).

Host specificity testing aims to determine the fundamental, and where possible realised, host range of an agent and is used to predict any potential risks associated with utilising the agent for biological control (McFadyen 1998). The fundamental host range of the agent consists of all of the possible host plants on which the agent can develop and complete its life cycle, whilst the realised host range contains only the hosts on which the agent will occur and develop on after release (Schaffner 2001). The realised host range is usually much more restricted than the fundamental host range of an agent, with field host ranges almost always being narrower than those determined in experimental host range testing (Blossey *et al.* 2018; Schaffner *et al.* 2018). This is because experimental host range testing often involves no-choice testing, which can result in an agent feeding on a plant species because that plant is the only option available to them when they would not feed on it under field conditions (Paynter *et al.* 2015). The use of host specificity testing has resulted in biological control having a good safety record with minimal non-target attack and this is attributed to the accuracy with which current methods determine candidate agent's host ranges (Blossey *et al*. 2018; Hinz *et al*. 2019; Paynter *et al*. 2020a).

2.1.2 What is host specificity testing and how does it work?

Phytophagous insects need to find suitable hosts for feeding and oviposition to ensure their survival (Anderson *et al*. 2013; Nishida 2014). There are two types of behaviour that are important in this process; pre-alighting behaviour, which is exhibited in the finding or locating of a host, and the post-alighting behaviour, which is exhibited in the acceptance of a host (Cunningham & West 2001). The first and most essential step in an insect-plant interaction is locating an appropriate host (Reeves 2011). This is done by insects in a number of different ways, including the use of visual, olfactory, gustatory, and tactile cues (Bruce & Pickett 2011; Reeves 2011; Nishida 2014). Although phytophagous insects do not all select their hosts in the same way, it is possible to determine the host selection behaviour and to demonstrate that the insects do not use or choose unsuitable hosts, either through pre-alighting or post-alighting behaviours (Cunningham & West 2001; Bruce & Pickett 2011; Nishida 2014). It is therefore possible to use host selection behaviour in the selection of possible host plants and for host specificity testing of a candidate biological control agent.

Host specificity tests have become largely standardized and have provided increasingly safe weed biological control agents (Blossey *et al*. 2018). It involves exposing the potential agent to a series of different trials where their survival and preference on non-target plant species is determined. The bulk of this work is usually conducted under quarantine conditions, as the agent is not known to be safe for release until the host specificity testing is completed. Biological control practitioners need to design and run experiments that are likely to accurately predict any potential non-target attack whilst minimising the chances for false positive results (where a test plant is attacked in testing but would not be attacked in the field) and false negative results (where a test plant does not appear to be in the insect's host range but does experience attack in the field) (Schaffner *et al*. 2018). An understanding of what drives host selection in herbivorous insects, and of insect behaviour more generally, is essential when designing and interpreting the results of host specificity testing in order to avoid these false results.

Biological control researchers need to identify the stages of the insect's life history that need to be host specific, usually with regards to damage and development, and ensure that these stages are adequately tested (van Klinken 2000). Tests of feeding preference are usually performed on any mobile stages of the insect (McFadyen 1998) as they are considered the "deciding" stage (Marohasy 1998). In many phytophagous insects the immature stages have a

limited ability to change their location and so their host plants are largely determined by where their mobile parents oviposit (Mayhew 1997). Although this does not guarantee whether or not the immature stages will survive on the plant they hatch on, it is important to consider (Mayhew 1997). If an adult is likely to lay eggs on a plant but the larvae cannot develop on the plant then this would cause false positive results in the laboratory if only oviposition were considered, and an agent may be rejected for use although it is theoretically safe because the larvae would not be able to develop on the non-target plant. It is also possible that larvae may develop or cause a degree of damage on a non-target plant but the parent will not lay eggs on that plant and so larvae testing would show that the insect is not suitably host specific. This would result in a false negative if only larval development were tested, as the adults will not lay eggs on the plant and so the larvae will never get the chance to feed on the plant in the field. Even if the larvae cannot develop on a plant, Cullen (1989) suggested that adult feeding is still important due to secondary wandering by adults that could be a risk if the population size becomes too large. It is also important to use the results of these tests to predict the chances of spill-over, where an agent will be found feeding on a non-target plant that is in close proximity with the target plant in the field when the agent's population numbers are large (Sutton *et al.* 2017; Paynter *et al*. 2020a). Although spill-over does not have many long-term ecological consequences or cause much damage on non-target plants, it is important to predict and ensure that when spill-over happens it does not result in public distrust in how accurate host range predictions are (Paynter *et al*. 2008; Sutton *et al.* 2017; Paynter *et al*. 2020a).

Insect learning can also be an important part of host selection, (Cunningham & West 2001; Dukas 2013; Tapia *et al*. 2015) and can influence host specificity testing. There is the possibility for an insect to learn to feed only on the plant that it hatches on, and reject other suitable hosts due to its prior feeding and learning (Heard 2000). This can be an issue in host specificity testing if the candidate agent learns to feed on the target plant and therefore does not feed on a plant that would be a suitable host if they had not fed on another plant previously. To deal with this issue, it is best to ensure that the insects do not have a chance to start feeding on and subsequently learn to feed on or develop a preference to any plant that is not the test plant in question. This can be done by placing hatching larvae directly onto test plants before any feeding begins, or transferring eggs onto test plants depending on what is most practical for the agent in question.

Host specificity testing is usually done in quarantine in the country in which it is being considered for release. These tests will therefore be conducted in conditions that may not be

representative of the field and will involve the insects being placed in cages or some other sort of barriers. Cages or physical barriers can disrupt agent behaviour (Cullen 1989; Heard 2000). There are insects that can be tested reliably in small cages but others will be influenced by the cages and so all results should be interpreted in light of an insect's behaviour (Heard 2000). If an insect uses olfactory cues or other stimulants from the plant to select its host then it is important to take into account the potential for the target plant to give off cues that make a non-target plant in close proximity to the target weed attractive to the agent (Paynter *et al.* 2015; Sutton *et al*. 2017). The experimental design for host specificity needs to address these concerns to ensure reliable host range data. Despite some of these challenges, quantitative laboratory-based host specificity testing is sufficient to predict the likelihood of non-target attack under field conditions (Paynter *et al*. 2015). The three main experimental methods used in host specificity testing are "no-choice tests", "choice tests", and "field based testing", which will all be discussed below.

2.1.2.1 No-choice tests

No-choice starvation tests consist of placing the biological control agent with one test plant species under standardized conditions (Sutton *et al*. 2017). They often involve putting the immature stages of the insect onto a test plant or control plant and waiting for the insect to either feed and develop, or starve and die (Paynter *et al.* 2015). They ensure that there is no alternative, more acceptable host that could confound the insect's response (van Klinken 2000). A control, consisting of the target plant with the same number of insects placed on it, is always set up in the same place and at the same time as any test plants. If the insects do not feed or oviposit as expected, or if they die unexpectedly on the control plants then this is used as an indication that there were variables other than the test plant that could have caused the lack of feeding and subsequent deaths, indicating that those tests were invalid. No-choice starvation tests are almost infallible at predicting the fundamental host range of an insect (Fowler *et al*. 2012).

No-choice tests are less likely to result in a false negative result (Marohasy 1998), but they can result in the costly rejection of potentially useful biological control agents (Schaffner *et al*. 2018). This is due to no-choice testing potentially producing false positive results where the non-target attack is over-stated because the insects may feed on a species that they would not attack under field conditions, resulting in the rejection of the insect which may have been a safe biological control agent (Marohasy 1998; Fowler *et al.* 2012; Paynter *et al.* 2015; Downey

& Paterson 2016; Sutton *et al*. 2017; Schaffner *et al*. 2018). It is also likely that no-choice oviposition tests can produce false positives as the behaviour of the females may change in restrictive environments and they may 'dump' their eggs in the absence of their host (Marohasy 1998; Paynter *et al*. 2015). No-choice tests must always be done in cases where there is likely to be a no-choice situation in the field (for example, as a result of asynchrony between the agent and target plant due to season) as this can determine whether an agent needs to be rejected even if it prefers the target weed under choice conditions (Hinz *et al*. 2019).

Although no-choice testing always involves placing the candidate agent on a single test plant species, isolated from any other choices, it may be done in parallel or in sequence (Heard 2000). The sequential no-choice tests is not a commonly used design but can be useful in some situations. It combines multiple no-choice tests into one experiment by putting the agent on a series of test plant species, alternating between the target and test species (Sutton *et al*. 2017). This design may reduce the probability of obtaining false results, and it incorporates aspects of the insect's host selection (Sutton *et al.* 2017). However, Heard (2000) warned that this design may make the control less valid if it is not done at the same time as the other tests and if the insects are in a different behavioural physiological state this may influence the results. Sutton *et al.* (2017) set up a sequential control no-choice test to ensure that their control was valid. This involved placing the agents on either a control or test plant in sequence, as with other sequential no-choice tests, but also including a separate control plant at each step in the sequence for comparison (Sutton *et al.* 2017). The parallel method, which is more commonly used, involves running test and control plants at the same time but in isolation from one another with no exchange of agents between the plants (Heard 2000).

2.1.2.2 Choice tests

Choice tests were designed to provide conditions more representative of the field than no-choice tests (Marohasy 1998; Paynter *et al*. 2015). Choice tests involve placing the agents in a cage or room with more than one test plant, with multiple experimental design options available (Marohasy 1998). In paired-choice tests an agent is usually placed into a cage with both the target plant and a test species and the damage done to both plants in the set time is compared (Sutton *et al*. 2017). No-choice tests demonstrate the fundamental host range of the agent while paired-choice tests give a better indication of the realised host range of the agent. Therefore, if an agent is completely host specific and only feeds on the target weed under no-choice tests,

paired-choice tests are unnecessary as the agent will not feed on the non-target plants when given a choice (Paterson *et al*. 2014b).

In multi-choice tests the agent is placed into a confined area with the target plant and more than one other test plant and any damage or development on the plants is recorded, indicating the preference of the insects (Marohasy 1998; Sutton *et al*. 2017). This is often done with the target weed and any non-target species that were attacked or supported development under no-choice conditions to better determine the realised host range of the agents (Stutz *et al*. 2020). Both the paired-choice and multi-choice designs can cause false negative results as the agent may not respond to a lower-ranked plant species if it has access to the target plant (Paynter *et al*. 2015). To overcome this potential problem, the choice-minus-target test design was developed (Heard 2000). This involves presenting the candidate agent with two or more non-target test species, without the target species available, to determine use and choice of non-target species if the target is absent (Heard 2000; Paynter *et al*. 2015).

The choice design has some potential problems, many of which are solved through using a combination of no-choice and choice tests in predicting a potential agent's host range. As mentioned, false negative results may arise from choice testing, as an insect may not feed on the non-target plant when also offered the target plant although it would feed on the non-target plant if it had no choice, which may result in the release of an agent that then goes on to cause significant non-target damage (Marohasy 1998; Paynter *et al*. 2015). There is also the possibility of neighbouring plants having an influence on the test results within choice tests. If an agent selects its target using olfactory cues then plant volatiles produced by the target plant may cause the agent to use a non-target plant due to the associated chemistry of the physically close target plant (Paynter *et al*. 2015; Sutton *et al*. 2017; Schaffner *et al*. 2018). Alternatively, if 'repellent' plants are used in close proximity to a plant that could experience non-target attack then the agent may avoid the potential host as it avoids the 'repellent' plant (Schaffner *et al*. 2018). These are due to the plants and agents being confined in close proximity which results in the mixing of secondary metabolites which could confuse the agent if it uses secondary metabolites as part of its host selection. When using choice tests it is important to consider how the agent selects its host to ensure a correct understanding and interpretation of the results (Sutton *et al*. 2017). The analyses of multiple-choice experiments may also be difficult as the data obtained are not independent (Schaffner *et al*. 2018).

2.1.2.3 Field based host specificity testing

Open field host specificity testing aims to predict what a candidate agent will do when it encounters its target species among possible host plants in a novel environment, and it has recently been used to clarify results obtained in confinement (Schaffner *et al*. 2018). It is considered an ideal testing option as it consists of a scenario more representative of the field without any artificial barriers to insect movement resulting in more natural behaviour and it can therefore produce more reliable results than other forms of host-range testing (Clement & Cristofaro 1995; Fowler *et al.* 2012; Paynter *et al*. 2015; Sutton *et al*. 2021). If a candidate agent is not proven to be host specific during testing in quarantine or a laboratory, open field testing can help predict the realised host range and therefore the likelihood of non-target attack when the agent is faced with conditions that are more similar to those that will be faced after release (Schaffner *et al.* 2018; Stutz *et al.* 2020).

Open field tests occur either in the insect's country of origin or in a country where it has been previously released (Clement & Cristofaro 1995; Fowler *et al.* 2012; Schaffner *et al*. 2018; Sutton *et al.* 2021). These tests could either be done through surveying sympatrically growing close relatives with natural populations of the potential biological control agent (Sutton *et al.* 2021) or through manipulative experiments (Schaffner *et al*. 2018). The open field tests are mostly multiple choice tests with the insect being exposed to naturally occurring, transplanted, or potted test and target plants in various arrangements (Clement & Cristofaro 1995; Schaffner *et al*. 2018). Open field tests face similar challenges to choice tests as the proximity of plants to one another may influence agent behaviour and there may be difficulties with the way the results are analysed and interpreted (Paynter *et al*. 2015; Schaffner *et al*. 2018). To overcome the potential issues of false negative results obtained due to the presence of the target plant, Heard (2000) proposed that a two phase design should be used in open field tests which involves a choice design followed by the choice-minus-control design. The cues the insect uses for host selection are therefore also important to consider in open field testing (Sutton *et al*. 2017).

However, there are also many more logistical difficulties that open field testing faces. Since the testing is usually done in the native range of the insect, some of the key test plants may not occur within the insect's country of origin, and it is difficult to import plants to be used outside of quarantine for testing due to the danger of the plant naturalising and becoming an invasive species itself (Clement & Cristofaro 1995; Fowler *et al.* 2012; Paynter *et al*. 2015; Schaffner

et al. 2018). It is also necessary to have a certain density of insects for the tests to be reliable, however, in the insect's native range they may be rare and in some cases, additional insects have to be introduced (Clement & Cristofaro 1995). This can be an issue, however, when the insects that are collected and released at the test site disperse immediately on release (Schaffner *et al*. 2018).

Despite these difficulties, if it is possible to do, field based host specificity testing is a helpful indicator of the more natural host-range of a candidate agent and may be used to make decisions about an agent that feeds on non-target species in other tests (Fowler *et al.* 2012; Paynter *et al*. 2015; Schaffner *et al*. 2018). However, it is not always necessary to do open field testing to make the final decisions as laboratory based tests are sufficient to demonstrate the fundamental host range of an agent if the experimental design and test plant list used are suitable (Paynter *et al*. 2015).

2.1.2.4 Test plant selection

If test plant species were appropriately chosen then more than 90% of unpredicted non-target attack recorded could have been avoided (Hinz *et al*. 2019). It is impossible to test all plants that the insect may encounter for non-target attack and so the key to accurately predicting the host specificity of a candidate agent is to carefully choose a sub-set of plant species that could accurately demonstrate its host range (Briese 2006).

The selection of test plants has changed in the history of host specificity testing, starting with the use of entomologists' expertise, and then testing of valuable crop plants, to the additive and subtractive procedures that are used in some form today (McEvoy 1996). Plants that are in the same genus or are more closely related to the target weed are more likely to face non-target attack than more distantly related species (Pemberton 2000; Hinz *et al*. 2019) and this has been the basis for plant test list choices recently. Wapshere's centrifugal phylogenetic method (Wapshere 1974), which is based on the taxonomic relationships of test plant species to the target weed, was the main method used to choose plant test lists until a method using degrees of phylogenetic separation was proposed by Briese (2003). Wapshere's centrifugal phylogenetic method involved the sequential testing of plant taxa most closely related to the target weed, based on their taxonomic relationship and proximity to the target weed, until the host range was circumscribed (Briese & Walker 2002; 2008). It also involved the use of "safeguard species", such as the testing of certain cultivated plants, even if those species should not have been at risk due to phylogenetic relatedness (Briese & Walker 2002; Briese 2003).

Although this method was used for a long time, Briese & Walker (2002) argued that test plant lists should be chosen solely on their phylogenetic relatedness to the target weed and that the use of distantly related "safeguard species" of economic or conservation importance should be discontinued. They also emphasized the need for use of phylogenetic degrees of relatedness being the criteria used to choose the plants as this meant that relationship was the focus, rather than categories of taxa (Briese & Walker 2008).

The method proposed by Briese (2003) has been adopted by certain regulatory bodies (Briese & Walker 2008) and therefore, the plant test lists that are chosen for modern host specificity testing are largely based on degrees of phylogenetic relatedness. There will sometimes be the need to test species that do not fall into this category but are considered more as "safeguard species" if the decision makers require, but scientific evidence indicates that this is not actually necessary (Briese 2003; 2006). It can also be difficult to use this method as there are some plant taxa that still have controversial and changing evolutionary relationships, and not all plant groups have been the subject of phylogenetic studies (Hinz *et al*. 2008). Although some researchers use the principals of Wapshere's centrifugal phylogenetic approach adapted to the proposals by Briese (2003) (Stutz *et al*. 2020), the degrees of phylogenetic rather than taxonomic relatedness is now commonly used and has resulted in host specificity testing that produces safe agents without the need for "safeguard species" (Briese & Walker 2008; Hinz *et al*. 2008; 2019; 2020). If a plant list is too large from using these criteria it is possible to filter it by also taking into account the biogeography and ecology of the target species compared with the other test plants (Briese 2003).

2.1.3 Does host specificity testing work?

Modern host specificity testing has resulted in biological control having a good safety record (Blossey *et al.* 2018; Hinz *et al.* 2019; 2020). Although there has been some debate over the ability of biological control agents to evolve and increase their host ranges, it is generally accepted that biological control agents have conservative host ranges and an evolutionary increase in their host range is highly unlikely (Briese 2000; Blossey *et al*. 2018; Müller-Schärer *et al*. 2020; Paynter *et al*. 2020a).

However, there are some adaptive changes that biological control practitioners need to consider when determining the host range of a candidate agent. If a non-target species has experienced attack in host testing but is out of the climatic range or ecological host range of the candidate agent then the insect may be approved for release (Pemberton 2000). Due to climate change,

however, this is a potential problem if an agent's thermal tolerance changes or an area's climate changes resulting in the agent moving into areas that had been deemed safe from agent attack with the potential for significant non-target attack occurring (Müller-Schärer *et al*. 2020; Paynter *et al*. 2020a).

There are a few reports of evolutionary changes within an agent's host range, such as the expansion of the leaf beetle, *Ophraella communa* LeSage (Coleoptera; Chrysomelidae) to feed on *Ambrosia trifida* L. (Asteraceae) in its introduced range whereas it could not feed on *A. trifida* in its original distribution (Fukano *et al.* 2016). Paynter *et al.* (2020b) also reported on the expansion of host use of a butterfly used as a biological control agent in Rarotonga in the Cook Islands. The larvae of *Heliconius erato cyrbia* Godart (Lepidoptera; Nymphalidae) were found feeding on seedlings of *Passiflora edulis* Sims (Passifloraceae), which was not predicted in host range testing (Paynter *et al.* 2020b). However, this was not due to a major host range expansion but rather due to the plant's heteroblasty resulting in the seedlings having a more suitable chemistry for larval survival than the older *P. edulis* plants (Paynter *et al.* 2020b). Despite records of perceived host range expansion such as these, there have been no recorded shifts in host use patterns and host range of biological control agents (Pemberton 2000; Blossey *et al*. 2018; Müller-Schärer *et al*. 2020). This means that there may be observed changes in host use in the field, but all changes should be to plants that were shown, or should have been shown, to be in the fundamental host range of the insect from host specificity testing.

There are some agents that are released although minor non-target attack is predicted to occur. Non-target attack in the field is most likely to occur when a plant is part of the insect's fundamental host range but is not a part of the realised host range and is therefore approved and released, however, due to either dispersal or large reductions in target plant populations the target plant becomes unavailable and the agent is under pressure to make use of an alternative host (Browne & Withers 2002; Paynter *et al*. 2020b). If an agent is feeding on a non-target species there may be some selection pressures that result in increased levels of non-target attack as the agent may adapt and become more efficient at feeding on the non-target species (Blossey *et al*. 2018; Paynter *et al*. 2020a). This is demonstrated by larvae surviving on non-target plants and having offspring that have an improved fitness on the non-target host showing a better use of their host range (Fowler *et al*. 2012). The potential for even very low survival of potential agents on non-target plants must therefore be considered in the context of the potential for the agent to adapt in this way before an agent is released.

Insect learning, as suggested by Heard (2000), can also be a problem in the field if non-target attack occurs and certain individuals and their offspring learn to feed on non-target species over target species. However, insect learning has been shown to be more prominent in the field in polyphagous or generalist insects rather than monophagous or specialist insects (Anderson *et al*. 2013; Tapia *et al*. 2015) and so learning by the agent once in the field should not be too much of a concern for biological control practitioners. This will be especially true for insects that are demonstrated to be strictly monophagous in host specificity testing.

This chapter aimed to test the host specificity of *Ph. guerini* as a potential biological control agent for *Pe. aculeata* in Australia. Host specificity testing was done in the late 1980s to early 1990s to establish whether the flea beetle was safe for release in South Africa (de Beer, unpubl.). It was deemed safe for release by the South African authorities at the time, and has since established as a biological control agent against *Pe. aculeata* in South Africa (Mnqeta & Paterson 2019). However, the methods for test plant selection have changed since then, and regulatory bodies are far more conservative, so much more evidence of the safety of agents is required before releases can be made. As this study is being done for Australia it is also important to include a test plant list that is relevant to Australia. This study used the updated methods and most modern phylogenies to test the host specificity of *Ph. guerini* to an acceptable standard for the relevant authorities in Australia.

2.2 Materials and Methods

2.2.1 Insect culture

Phenrica guerini adults, larvae, and eggs were collected from sites in Port Alfred, in the Eastern Cape Province of South Africa (-33° 35′ 48′′, 26° 53′ 18′′). These were then transported to Rhodes University, Makhanda, where they were reared in the Department of Zoology and Entomology. The culture was kept in a controlled environment room at 25°C with a 12 hour day length.

The adults were placed in an insect cage with a potted *Pe. aculeata* plant. The larvae were kept in a separate insect cage with a potted *Pe. aculeata* plant alongside the adults' cage. Both cages were sprayed frequently with a fine mist of water, up to three times a day. Each day the plant in the adults' cage was checked for new eggs. Any leaves with new eggs were removed and placed in a petri dish, sprayed with a fine mist of water and kept in a ziploc bag or sealed plastic container. All hatched egg batches were moved from the petri dish into the larval rearing cage. Larval feeding stops in the fifth instar and so the larval cage was checked each day for any late

fifth instar larvae. These larvae were transferred into plastic tubs with moist potting soil and a few leaves of *Pe. aculeata* to ensure that if they were still feeding they would have food. The larvae were allowed to burrow into the potting soil to pupate. The soil in the pupal containers was kept moist by adding water to the tray in which the pupal containers were placed, allowing water to seep into the pupation medium through holes in the bottom of the pupation chamber. Any newly eclosed adults from the larval cage or pupal tubs were moved into the adults' cage.

The *Pe. aculeata* plants were replaced when there was insufficient food or appropriate oviposition sites (i.e. the leaves). The insects were provided with extra branches of *Pe. aculeata* from plants that grow outside the Department of Zoology and Entomology placed in the florist's foam in the cage when necessary. The insects reared in this way were used for all host specificity testing.

2.2.2 Host specificity testing

2.2.2.1 Life cycle of Phenrica guerini *on* Pereskia aculeata

Twenty larvae were monitored daily to determine the life history of the beetle, *Ph. guerini*. The average number of days taken for eggs to hatch, the duration of each instar and pupal length were determined. The number of days it took for adults to first oviposit and the average number of eggs per batch laid by an adult were also determined. Adult *Ph. guerini* (Fig 2.1a) lay batches of upright, pink-red eggs, usually on the underside of leaves (Fig 2.1b). These batches range in number from about 20-35 eggs per batch with an average of 27 eggs per batch (S.E. ± 0.78) (n=26). The egg batches hatch after an average of 6.1 days (S.E. ± 0.14) (n=26) (Fig 2.1c). The first instar larvae are a dark pink colour which gradually change to grey-brown in the first hour or so and last for one to two days (2 S.E. ± 0.11) (n=20) (Fig 2.1c). The second and third instar larvae are both yellow, with the second instar larvae being a darker yellow colour and the third instar larvae a brighter yellow (Fig 2.1d). It is not always easy to tell the difference between the second and third instar larvae although they do appear to have a period of not moving and feeding which is possibly their moulting phase from second to third instars which occurs about 7.0 days (S.E. ± 0.26) (n=20) after hatching. The larvae become fourth instars about 11.0 days (S.E. ± 0.32) (n=20) after hatching and they are dark brown to black. About 18.0 days (S.E. ± 0.71) (n=20) after hatching they become light brown fifth instar larvae (Fig 2.1d). A few days before pupating the fifth instar larvae stop feeding and slowly make their way down the plant on which they were feeding. Around 28.5 days (S.E. ± 0.73) (n=20) after hatching, the fifth instar larvae burrow into the top soil and pupate. The pupae are bright yellow, although not

usually seen as they are buried (Fig 2.1e). After 17.5 days (S.E. ± 0.41) (n=20) in the soil they eclose as adults (Fig 2.1f). The adults then take another 6.1 days (S.E. ± 0.40) (n=8) to oviposit their first batches of eggs. Adults live for over 40 days (see Table 2.3). Both the adults and larvae feed on the leaves of *Pe. aculeata* (Fig 2.1g).

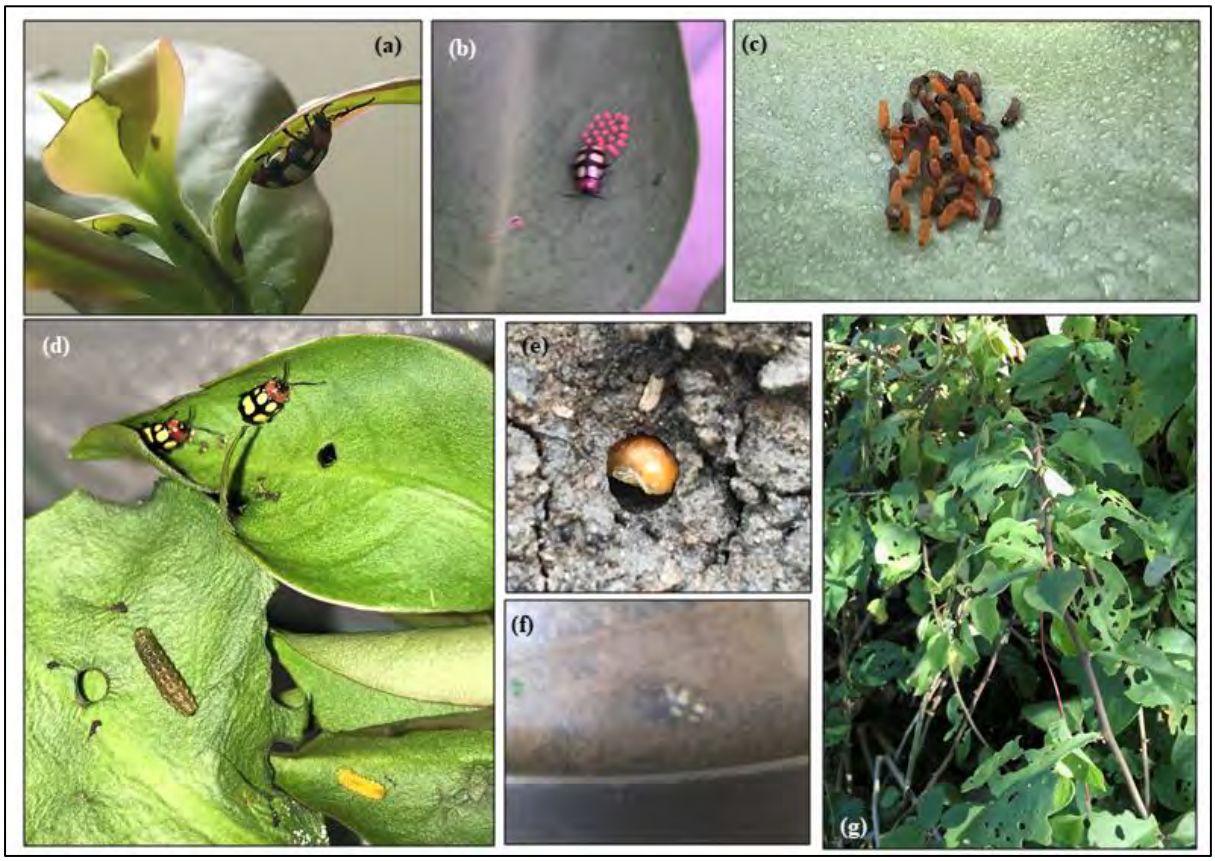

Figure 2.1: Life history of and feeding damaged caused by *Phenrica guerini* (Bechyné) (Coleoptera: Chrysomelidae), showing (a) an adult; (b) an adult female laying eggs; (c) recently hatched first instar larvae; (d) two adults, one fifth instar larva (brown in colour), and one late third instar larva (bright yellow in colour); (e) a pupa in the soil; (f) an almost fully developed adult in the soil a few days before eclosing; (g) evidence of typical feeding by *Ph. guerini*.

2.2.2.2 Test plant selection

The test plants used for the host specificity testing were chosen using the degrees of phylogenetic separation method proposed by Briese (2003), as a revision of Wapshere's 1974 centrifugal phylogenetic method. The plants were also chosen as representatives of what is present or of importance in Australia as that is the target region for potential release. The monophyletic suborder, Portulacineae (or Cactineae), within the order Caryophyllales contains the Cactaceae and closely related families, Portulacaceae, Basellaceae, Didieriaceae,

Halophytaceae, Talinaceae, Montiaceae, and Anacampserotaceae (Nyffeler & Eggli 2010; Ocampo & Columbus 2010). This suborder is distributed mainly in the Americas, Africa, and Australia, and is represented more in the southern hemisphere (Ocampo & Columbus 2010). The Portulacaceae, Montiaceae, and Anacampserotaceae are represented throughout most of the world, including South America and Australia (Nyffeler & Eggli 2010). The Talinaceae are indigenous to the Americas and Africa but not Australia, although *Talinum triangulare* (Jacq.) Wild. and *Talinum paniculatum* (Jacq.) Gaertn. are pantropical weeds (Nyffeler & Eggli 2010). The Basellaceae are native to the Americas although there is one species found in Africa, as well as a cultivated species in Africa, and three endemic species in Madagascar but no native species in Australia (Eriksson 2007). The Didiereaceae are endemic to Madagascar and only found as garden plants elsewhere in the world (Applequist & Wallace 2000). The Halophytaceae are only found in Argentina, and the Montiaceae are found in the Americas, northern Asia and Europe, as well as Australia and New Zealand (Nyffeler & Eggli 2010). The family Cactaceae contains over 1450 species, with the greatest species richness primarily in Mexico and secondary centres of species richness found in the southwestern Andean region and in eastern Brazil (Hernández-Hernández *et al*. 2011). There is only one species of Cactaceae, *R. baccifera*, that is thought to be native outside of North and South America, and it is found in Africa, Sri Lanka, and various islands of the Indian Ocean (Nyffeler & Eggli 2010). There are no native Cactaceae in Australia, but some are considered useful and are cultivated on a small scale (Nyffeler & Eggli 2010).

The list of test plant species used in this study is provided in Table 2.1. A total of 25 plant species from four orders and 11 families were tested. Two other species of *Pereskia*, *Pereskia grandifolia* Haw. and *Pereskia quisqueyana* AH Liogier were tested as they are present in Australia and are within the same genus as *Pe. aculeata* and so would be the most likely to experience attack from *Ph. guerini*. *Pereskia grandifolia* is the closest relative to *Pe. aculeata* and *Pe. quisqueyana* is in a more distantly related clade within the genus (Buttersworth & Wallace 2003; Ocampo & Columbus 2010). *Rhipsalis baccifera* was tested as it is the only possible native old world cactus species (Nyffeler & Eggli 2010) with some authors placing it more closely related to *Pereskia* than other Cactaceae species (Cuenoud *et al*. 2002; Ocampo & Columbus 2010), although others have placed it in a group that is further removed than any of the other Cactaceae in this test plant list (Hernández-Hernández *et al*. 2011). *Rhipsalis cereuscula* Haw. (Cactaceae) is an introduced, ornamental species from the same genus and was tested as it was readily available. Both *Opuntia aurantiaca* Lindley (Cactaceae) and *O.*

ficus-indica, which are part of the Opuntioideae group within the Cactaceae, the closest clade to the *Pereskia* group (Hernández-Hernández *et al*. 2011), were tested. They are both important invasive cacti in Australia (Auld *et al*. 1982-83; Hosking *et al*. 1988), although *O. ficus-indica* can also be a valued crop species (Zimmermann & Moran 1991). *Hylocereus undatus* (Haw.) Britton and Rose (Cactaceae) was tested as it is an ornamental and highly cultivated cactus species worldwide, including in Australia where it has been introduced and cultivated (Patwary *et al*. 2013). It is in a different clade within the Cactaceae to *Pe. aculeata* and the *Opuntia* species and is therefore more distantly related to the target weed (Hernández-Hernández *et al*. 2011).

The Portulacaceae are the family most closely related to the Cactaceae although the family is paraphyletic (Cuenoud *et al*. 2002) and was therefore split into four monophyletic family groups, namely, Portulacacae, Talinaceae, Montiaceae, and Anacampserotaceae (Nyffeler & Eggli 2010; Ocampo & Columbus 2010). Ocampo & Columbus (2010) place the Portulacaceae as the closest relative of the Cactaceae, and therefore various species from this group were tested. *Portulacaria afra* (L.) Jacq. (Portulacaceae) is an introduced, ornamental species in Australia that was tested. *Portulaca oleracea* L. (Portulacaceae) is native to Australia (Masoodi *et al*. 2011), and *Portulaca grandiflora* Hook. is an introduced ornamental species in Australia. Nyffeler & Eggli (2010) place Anacampserotaceae as more closely related to the Cactaceae than Portulacaceae and therefore a relative of this closely related group was required. *Anacampseros telephiastrum* DC. (Anacampserotaceae), an introduced species in Australia, was tested.

The next most closely related clade to the Cactaceae is the Talinaceae (Nyffeler & Eggli 2010; Ocampo & Columbus 2010). *Talinum paniculatum* is a pantropical weed (Nyffeler & Eggli 2010) and *Talinum caffrum* (Thunb.) Eckl. & Zehy. (Talinaceae) is an ornamental species in Australia from the Talinaceae that were tested. Cuenoud *et al*. (2002) and Ocampo & Columbus (2010) place the Basellaceae as the next clade along in the Carryophyllaceae order. Three species of Basellaceae were tested, including *Anredera cordifolia* (Ten.) Steenis (Basellaceae), which is an introduced and now invasive species in Australia, and is native to South America (Vivian-Smith *et al*. 2007). The other two species were *Basella rubra* L., and *Basella alba* L. (Basellaceae) which are both introduced species in Australia which are occasionally cultivated as a home-grown vegetable, and were thus tested as both representatives of this clade and their horticultural and agricultural value. The next most closely related family is the Didiereaceae (Ocampo & Columbus 2010) which includes *Alluaudia procera* (Drake) Drake, a species

endemic to Madagascar which has been introduced in Australia and elsewhere in the world as a garden plant, and was therefore tested as a representative of the family so that the host range could be accurately circumscribed.

The Mesembryanthemaceae and Caryophyllaceae are both families within the Caryophyllales order but are not a part of the Cactinae suborder. *Carpobrotus edulis* (L.) Brown. (Mesembryanthemaceae) is an introduced species in Australia and was tested as a representative of the native Australian *Carpobrotus* species. *Faucaria felina* (L.) Schwantes (Mesembryanthemaceae), *Glottiphyllym longum* (Haw.) N.E.Br. (Mesembryanthemaceae), and *Delosperma lydenbergense* L.Bolus (Mesembryanthemaceae) are all introduced Caryophyllales in Australia. *Silene primuliflora* Eckl. & Zeyh. (Caryophyllaceae) was used as a phylogenetic proxy and a representative of the naturalised *Silene* species found in Australia and from the Carryophyllales order.

The Crassulaceae (Saxifragales), Aspholdolaceae (Asparagales), and Euphorbiaceae (Malpighiales) are not Carryophyllales but all belong to the next most closely related orders to the Caryophyllales. *Crassula ovata* (Mill.) Druce. (Crassulaceae), *Aloe arborescence* Mill. (Aspholdolaceae), and *Euphorbia tirucalli* L. (Euphorbiaceae) have all been introduced in Australia and were tested as representatives of these orders.

Table 2.1: Plant selection list with the family and order that each plant in the test plant list belongs to and its status in Australia. All plants were used in larva no-choice testing and those with an asterisk (*) were also used in no-choice adult oviposition testing.

Plant Species	**Order**	**Family**	**Status in Australia**
Pereskia aculeata Miller *	Carryophyllales	Cactaceae	Target weed
Pereskia grandifolia Haw. *	Carryophyllales	Cactaceae	Naturalised
Pereskia quisqueyana AH Liogier *	Carryophyllales	Cactaceae	Introduced
Opuntia aurantiaca Lindley	Carryophyllales	Cactaceae	Invasive
Opuntia ficus-indica L. Mill.	Carryophyllales	Cactaceae	Invasive
Hylocereus undatus (Haw.) Britton and Rose	Carryophyllales	Cactaceae	Introduced ornamental/horticultural
Rhipsalis baccifera (J. Müller) Stern	Carryophyllales	Cactaceae	Introduced ornamental
Rhipsalis cereuscula Haw.	Carryophyllales	Cactaceae	Introduced ornamental
Portulaca oleracea L. *	Carryophyllales	Portulacaceae	Native
Portulaca grandiflora Hook.	Carryophyllales	Portulacaceae	Introduced ornamental
Portulacaria afra (L.) Jacq.	Carryophyllales	Portulacaceae	Introduced ornamental
Anacampseros telephiastrum DC.	Carryophyllales	Anacampserotaceae	Introduced ornamental
Talinum caffrum (Thunb.) Eckl. & Zeyh. *	Carryophyllales	Talinaceae	Introduced ornamental
Talinum paniculatum (Jacq.) Gaertn.	Carryophyllales	Talinaceae	Pantropical weed/ ornamental
Anredera cordifolia (Ten.) Steenis *	Carryophyllales	Basellaceae	Invasive
Basella alba L.	Carryophyllales	Basellaceae	Naturalised
Basella rubra L.	Carryophyllales	Basellaceae	Introduced
Alluaudia procera (Drake) Drake *	Carryophyllales	Didiereaceae	Introduced ornamental

Plant Species	Order	Family	Status in Australia
Carpobrotus edulis (L.) Brown.	Carryophyllales	Mesembryanthemaceae	Unknown
Delosperma lydenbergense L.Bolus	Carryophyllales	Mesembryanthemaceae	Unknown
Faucaria felina (L.) Schwantes	Carryophyllales	Mesembryanthemaceae	Introduced ornamental
Glottiphylum longum (Haw.) N.E.Br.	Carryophyllales	Mesembryanthemaceae	Unknown
Silene primuliflora Eckl. & Zeyh.	Carryophyllales	Carryophyllaceae	Unknown
Crassula ovata (Mill.) Druce.	Saxifragales	Crassulaceae	Introduced ornamental
Aloe arborescens Mill.	Asparagales	Asphodelaceae	Introduced ornamental
Euphorbia tirucalli L.	Malpighiales	Euphorbiaceae	Introduced ornamental

2.2.2.3 Sourcing test plants

Test plants were sourced either from local nurseries, herbal shops, or from areas in and around Makhanda. If plants were bought from nurseries they were sprayed or watered frequently, and given at least three weeks before testing to ensure that any pesticides used by the nurseries had been washed off. Plants that were grown from seed were grown until they were big enough to support the larvae, should the larvae survive, before being used. The plants were kept in a greenhouse outside the Department of Zoology and Entomology and were watered as often as needed.

2.2.2.4 Larval no-choice testing

Survival of larvae of *Ph. guerini* on different plants was evaluated with a no-choice design. An individual test plant (one of the species from the Table 2.1), or a control plant, was placed into an insect cage, of either 13.5 in x 24 in or 23 in x 35 in, in a laboratory at the Department of Zoology and Entomology or Waainek Research Facility at Rhodes University. All test plants from Table 2.1 were tested. Ten newly hatched *Ph. guerini* larvae were moved with a fine paintbrush onto the plants in the insect cages. The larvae had to be newly hatched to ensure no feeding on the *Pe. aculeata* leaf had occurred resulting in learning to eat only that plant (Heard 2000). Ten larvae were used as the mortality rate of moving larvae with a paintbrush was sometimes too high for a smaller number to be used and to still obtain statistically relevant results.

A control plant of the same size as the test plant in the same size cage, with the same number of larvae in the same location as the test plants was always run at the same time. The same control was used for new test plants up to five days after the control was started. Beyond five days, a new control was started to ensure similar larval stages were facing the same conditions on the control and test plant. A sample size of ten plants was used for the other *Pereskia* species and the plant species that experienced larvae feeding, all other test plant species had a sample size of five plants (Table 2.2).

Every day the number of surviving larvae, the instar of the surviving larvae, and the damage on the plants were recorded by visually estimating the damage caused and ranking it on a scale from zero to 4 (0 = no damage; 1 = evidence of exploratory feeding; 2 = minimal feeding; 3 = minimal feeding damage; 4 = extensive feeding damage). Any larvae that survived to the fifth instar were taken out of the insect cages and placed in pupation tubs with some food of the plant that they had been feeding on to record their development to adulthood.

2.2.2.5 Adult no-choice oviposition testing

To evaluate the selection of plants for oviposition two pairs of adult *Ph. guerini* were placed in an insect cage with either a control plant or a test plant. The test plants used were *Pereskia grandifolia*, *Pe. quisqueyana*, *Alluaudia procera*, *Talinum caffrum*, *Anredera cordifolia*, and *Portulaca oleraceae* as this was a fair representation of the plants more closely related to *Pe. aculeata* and included the three plants on which larvae fed and survived to and beyond the second instar (see results section below). A sample size of 5 plants was used for every plant species. Every day the damage to the plant and survival of the adults were recorded. The number of egg batches oviposited on the plant, the number of fertile egg batches oviposited on the plant, and the number of egg batches oviposited on the cage or pot were also recorded. The number of eggs per batch were counted.

2.2.3 Statistical analysis

As the data were not normally distributed a Kruskal-Wallis test (package = stats; function = kruskal.test) was run in RStudio (R Core Team 2021) for all the data and then for just the species that had feeding damage or eggs laid. A Pairwise Wilcoxon Rank Sum Test was run to check where the significant differences were between the plant species that had feeding damage or eggs laid (package = stats; function = pairwise.wilcox.test; p.adjust.method = "BH"). This was also done for the days to death of the adults.

2.3 Results

Phenrica guerini only successfully completed their life cycle on the target plant, *Pe. aculeata*. The beetles were unable to survive to pupation on any of the test plant species. The adult beetles laid significantly more eggs on the target plant compared to the test plant species. Significantly more damage was caused, by both the larvae and adult beetles, on the target plant compared to the test plant species.

2.3.1 Larval no-choice testing

Phenrica guerini larvae survived significantly longer on and caused more damage to the target plant than any of the test plants. There was a survival of 45% of larvae to the fifth instar on *Pereskia aculeata* with most of these larvae successfully pupating and eclosing (Table 2.2). Out of 25 test plants, the larvae only successfully fed and survived to or beyond the second instar on three plant species, *Portulaca oleraceae* (Portulacaceae), *Talinum caffrum* (Talinaceae), and *Anredera cordifolia* (Basellaceae) (Table 2.2). *Portulaca oleraceae* had the

highest survival rate for the larvae on test plants, with 17% surviving to the fifth instar, whilst only 1% of the larvae survived to the fourth instar on *Talinum caffrum* and 6% of the larvae survived to the third instar on *Anredera cordifolia*. On *Pe. aculeata* 72% of the larvae survived to the second instar compared with 39% on *Po. oleraceae*, 13% on *T. caffrum*, and 6% on *A. cordifolia*. An average of 64.7% survived to the third instar on *Pe. aculeata* compared with 32% on *Po. oleraceae*, 3% on *T. caffrum*, and 6% on *A. cordifolia*. No larvae survived to the fourth instar on *A. cordifolia* but 55.3% survived on *Pe. aculeata*, 22% on *Po. oleraceae* and 1% on *T. caffrum*. The percentage survival of larvae to the fifth instar on *Po. oleraceae* was significantly lower than the survival of larvae on *Pe. aculeata* ($\chi^2 = 26.515$, d.f. = 3, $p < 0.0001$). No larvae successfully pupated on any of the test plant species, with the exception of the control *Pe. aculeata*. The level of damage was significantly higher on *Pe. aculeata* than any of the other test plants on which feeding occurred ($\chi^2 = 32.592$, d.f. = 3, $p < 0.0001$). Of the three test plant species on which the larvae caused some damage, the most damage was recorded on *Po. oleraceae*, followed by *A. cordifolia* and minimal damage on *T. caffrum* (Table 2.2).

Table 2.2: *Phenrica guerini* larval no-choice test conducted on various test plant species showing the mean survival rate (± SE) and damage caused (± SE). The damage level is on a scale from 0 (no damage) to 4 (extensive damage).

Plant species	Family	Sample size (n=)	% Survival to second instar	% Survival to third instar	% Survival to fourth instar	% Survival to fifth instar	% Successfully pupated	% Successfully eclosed	Damage level caused
***Pereskia aculeata* (target weed)**	**Cactaceae**	**15**	**72±4.90**	**64.7±5.59**	**55.3±6.89**	**45.3±6.16**	**44±6**	**42.7±6.05**	**3.5±0.13**
Pereskia grandifolia	Cactaceae	10	0	0	0	0	0	0	0
Pereskia quisqueyana	Cactaceae	10	0	0	0	0	0	0	0
Opuntia aurantiaca	Cactaceae	5	0	0	0	0	0	0	0
Opuntia ficus-indica	Cactaceae	5	0	0	0	0	0	0	0
Hylocereus undatus	Cactaceae	5	0	0	0	0	0	0	0
Rhipsalis baciferra	Cactaceae	5	0	0	0	0	0	0	0
Rhipsalis cereuscula	Cactaceae	5	0	0	0	0	0	0	0
Portulaca oleracea	Portulacaceae	10	39±8.23	32±8	22±9.04	17±8.83	0	0	2±0.30
Portulaca grandiflora	Portulacaceae	5	0	0	0	0	0	0	0
Portulacaria afra	Portulacaceae	5	0	0	0	0	0	0	0
Anacampseros telephiastrum	Anacampserotaceae	5	0	0	0	0	0	0	0
Talinum caffrum	Talinaceae	10	13±6.16	3±1.53	1±1	0	0	0	0.1±0.1
Talinum paniculatum	Talinaceae	3	0	0	0	0	0	0	0
Anredera cordifolia	Basellaceae	5	6±2.45	6±2.45	0	0	0	0	1±0.24
Basella alba	Basellaceae	5	0	0	0	0	0	0	0
Basella rubra	Basellaceae	5	0	0	0	0	0	0	0
Alluaudia procera	Didiereaceae	5	0	0	0	0	0	0	0
Carpobrotus edulis	Mesembryanthemaceae	5	0	0	0	0	0	0	0

Plant species	Family	Sample size (n=)	% Survival to second instar	% Survival to third instar	% Survival to fourth instar	% Survival to fifth instar	% Successfully pupated	% Successfully eclosed	Damage level caused
Delosperma lydenbergense	Mesembryanthemaceae	5	0	0	0	0	0	0	0
Faucaria felina	Mesembryanthemaceae	5	0	0	0	0	0	0	0
Glottiphylum longum	Mesembryanthemaceae	5	0	0	0	0	0	0	0
Silene primuliflora	Carryophyllaceae	5	0	0	0	0	0	0	0
Crassula ovata	Crassulaceae	5	0	0	0	0	0	0	0
Aloe arborescens	Aspholdolaceae	5	0	0	0	0	0	0	0
Euphorbia tirucalli	Euphorbiaceae	5	0	0	0	0	0	0	0

2.3.2 Adult no-choice oviposition testing

Adult *Ph. guerini* survived for significantly longer, laid significantly more eggs on, and only caused damage on the target weed. Adult *Ph. guerini* did not show signs of feeding on any test plant species, but fed and caused damage on the target weed *Pe. aculeata* (Table 2.3). An average of 0.2 batches of fertile eggs were oviposited on one test plant species, *T. caffrum*, and an average of 0.2 batches of fertile eggs were oviposited on the cage/pot in the presence of *T. caffrum* and *Po. oleraceae*. There were no batches of eggs oviposited on any other test plant species. There was an average of 5.4 fertile batches and 0.67 infertile batches of eggs oviposited on the target weed and an average of 0.17 fertile batches of eggs oviposited on the cage/pot in the presence of the target weed. There were significantly more eggs laid by *Ph. guerini* on *Pe. aculeata* than on any of the test plants ($\chi^2 = 29.805$, d.f. = 6, $p < 0.0001$) with an average of 158.8 eggs laid on *Pe. aculeata* compared to an average of 5.2 eggs laid on *T. caffrum* which was the only test plant that the adults laid eggs on. Adult *Ph. guerini* survived an average of at least 27 days longer on *Pe. aculeata* than on the other test plant species ($\chi^2 = 69.806$, d.f. = 6, $p < 0.0001$).

Table 2.3: The average (± S.E.) number of eggs laid and damage caused by adult *Phenrica guerini* on various test plants during no-choice oviposition testing.

Plant species	Family name	Sample Size (n=)	Average number days to death	Damage	Average number fertile batches eggs on plant	Average number batches on cage or pot	Average number infertile egg batches on plant	Average number fertile eggs laid on plant
Pereskia aculeata	**Cactaceae**	**5**	**>40.65±0.55**	**YES**	**5.4±0.75**	**0.17±0.17**	**0.67±0.67**	**158.8±21.44**
Pereskia grandifolia	Cactaceae	5	11.4±0.81	NO	-	-	-	-
Pereskia quisqueyana	Cactaceae	5	6.85±1.06	NO	-	-	-	-
Portulaca oleracea	Portulacaceae	5	11.05±0.97	NO	-	0.2±0.2	-	-
Talinum caffrum	Talinaceae	5	9.6±0.44	NO	0.2±0.2	0.2±0.2	-	5.2±5.2
Anredera cordifolia	Basellaceae	5	9.05±0.81	NO	-	-	-	-
Alluaudia procera	Didiereaceae	5	13.65±1.30	NO	-	-	-	-

2.4 Discussion

The only plant species on which *Ph. guerini* successfully completed its life cycle was the target weed, *Pe. aculeata*. Although there was some feeding and damage caused by *Ph. guerini* larvae on three other plant species, this feeding is not a concern for the host specificity of the insect. The oviposition testing showed no feeding of adults and minimal oviposition on the test plants. *Phenrica guerini* is suitably host specific to recommend it for use as a biological control agent against *Pe. aculeata* in Australia.

Only three of the 25 test plant species were damaged by *Ph. guerini* larvae. These three test plants were *Portulaca oleraceae* (native), *Talinum caffrum* (ornamental), and *Anredera cordifolia* (invasive), all of which are from different families to the target weed, and from one another, but come from the same suborder as *Pe. aculeata*. The larvae successfully developed to and beyond the second instar on these three test plants although the percentage of larvae that survived beyond the second instar on these three plants was significantly smaller than those that survived on *Pe. aculeata*. No larvae successfully pupated after feeding on any of the test plants.

Adult *Ph. guerini* did not feed on any of the test plant species. The adults were most often on the cage when in the presence of a test plant whereas if they were placed in a cage with a control plant they were almost always on the plant when checked. There were very few batches of eggs (either fertile or infertile) oviposited on, or in the presence of, any test plant species, and these test plant species were from the same group that the larvae fed on. There was a much higher average of fertile batches and infertile batch of eggs oviposited on the target plant, *Pe. aculeata*. This is slightly surprising as adult *Ph. guerini* are not always careful in their choice of where they lay their eggs, with some laying batches of (fertile) eggs on cages, pots, branches, and container lids (pers. obs.). Therefore it was expected that they might oviposit or 'dump' eggs on the test plant species, even if it is not the ideal host for their larvae. However, when put in a no-choice environment where they were not feeding, only a small number of the test plants had any eggs oviposited on or around them. This may have been as a result of the lack of feeding by the adults on the test plants which may have reduced their energy and likelihood of mating and expending energy on reproduction when they didn't have a suitable food source to replace energy. Alternatively, this may have been due to a lack of secondary plant volatiles that may be required to stimulate oviposition (Bruce & Pickett 2011; Nishida 2014).

The test plants on which the larvae successfully fed and some adults oviposited eggs either on or in the presence of was surprising as they are not the most closely related species to the target plant. This would seem to go against the degrees of phylogenetic separation method of determining possible plants for an insect's host specificity (Briese 2003). If any plants were fed on, it was expected that these would be within the genus *Pereskia*, which are not only the closest relatives, but are also structurally similar in that they have well developed leaves (Leuenberger 1989). However, the plants that did experience some form of feeding by the larvae all fall within families that are closely related to the Cactaceae which were chosen according to the degrees of phylogenetic separation method.

Recently it has been argued that for some agents, plant chemistry, or plant metabolomics, could be a better predictor of host range than the degrees of phylogenetic separation method (Paynter *et al*. 2020b; Barrett *et al*. 2021; Wheeler *et al*. 2021). Paynter *et al*. (2020b) demonstrated how a larva's use and survival on a plant can be related to plant chemistry, even within the same species of plant, with the larvae they tested feeding on young saplings of the non-target plant but being unable to survive on older plants due to heteroblasty within the plant species. It is possible that the test plants that experienced larval feeding in my study may have been due to plant chemistry. It is possible that the other two *Pereskia* species, which would have been the most likely to be fed on based on phylogenetic relatedness, were less palatable, or even toxic, to the *Ph. guerini* larvae while *Po. oleraceae*, *A. cordifolia*, and *T. caffrum* were more palatable to the larvae, but this would have to be confirmed by testing the plant chemistry for each of the species.

Wheeler *et al*. (2021) showed that plant volatile profiles were a stronger predictor than phylogeny for the likelihood of oviposition on a test plant by the agent they studied. They studied the host range of *Neomusotima conspercatalis* (Warren) (Lepidoptera: Crambidae) and found that the similarity of volatile profiles to the target plant *Lygodium microphyllum* (Cav.) R. Br. (Lygodiaceae) was a stronger predictor of oviposition by the moth on other *Lygodium* species than phylogenetic relatedness to the target plant (Wheeler *et al*. 2021). As *Ph. guerini* adults have been observed laying eggs on cages or branches alongside *Pe. aculeata* this could be an indication that the adults require some sort of volatile stimulation from the plant to oviposit. It is possible that the plant chemistry of the species on which the larvae showed some feeding cause similar volatiles to *Pe. aculeata* and this could explain why the adults were observed ovipositing batches of eggs either on the same test plant species the larvae fed on or on the cage with one of these test plants. It is also possible that the beetles can only oviposit if

they have fed on something or as a response to damage or frass by other adult *Ph. guerini* which could stimulate oviposition. If paired or multiple choice tests had been used then false positives could have been experienced for the species that did not have any adults ovipositing on them (Paynter *et al*. 2015). This could have happened as the volatiles produced by the target plant, or feeding on the control plant, could have stimulated oviposition and, as the adults are not always careful about where they lay their eggs, they may have laid the eggs on a plant they would not have oviposited on if the volatiles had not been present (Bruce & Pickett 2011; Sutton *et al*. 2017). Based on the larval no-choice tests, these larvae would have fed but not survived to pupation unless they were able to migrate to *Pe. aculeata* plants during larval development.

The leaf morphology of the three test plant species was more similar to the target plant and so could suggest that the larvae would be more likely to feed on those particular test plant species. Morphological similarity should be considered in host specificity testing as leaf morphology, especially the structure of the surface of the leaf, can restrict insect use of a plant (van Lenteren & de Ponti 1990), although phylogeny is still the primary determinant of test plants (Sutton *et al*. 2021). *Pereskia aculeata* is one of the few cacti with true leaves and as such it would be expected that the larvae would have mouthparts better adapted to feeding on leaves rather than succulent stem segments or cladodes of other Cactaceae. Many of the test plants, both within the Cactaceae and in other families, had succulent leaves, or lacked leaves and had succulent stems that the *Ph. guerini* larvae are not adapted to feeding on. This seems to be supported by these data, as occasionally on a test plant species, such as *D. lydenbergense*, there would be up to eight larvae all gathered and appearing to be feeding around a little scar on a plant's leaf in the first 24 hours of testing. It seemed like they would try and feed if they found an area that was more suitable where they could get through the waxy epidermis of the test plant. This has been shown in the first instar of other insects which have underdeveloped mouth parts making the breaking of the first leaf layer difficult and so once the leaf layer has been broken the larvae gather in that area to feed (Hochuli 2001). However, none of the *Ph. guerini* larvae which demonstrated this behaviour survived to the second instar, even after potential exploratory feeding in this manor, and so it is unlikely that the leaf morphology and structure were the only aspects inhibiting their successful development. The two other *Pereskia* species that were tested both have a very similar leaf morphology to *Pe. aculeata* and are both cacti, but the larvae could not survive on those species either, which was unexpected because feeding was

observed on the three test plants that were fed on, which are much more distantly related to the host plant and not morphologically similar either.

Catorhintha schaffneri is the other agent that has been shown to be suitably host specific and was subsequently released against *Pe. aculeata* in South Africa (Paterson *et al*. 2014b). The test plant list used by Paterson *et al*. (2014b) was very similar to the list used in this study. There were a few similarities in the host testing results between *Ph. guerini* and *C. schaffneri*, although there were also a few notable differences.

Catorhintha schaffneri has four nymphal instars before becoming an adult, compared with *Ph. guerini* which has five larval moults (Paterson *et al*. 2014b). *Catorhintha schaffneri* and *Ph. guerini* both had a few juveniles survive to the fourth instar on *T. caffrum*, which is the final instar for *C. schaffneri*. Both agents survived beyond the first instar on *Po. oleraceae* but this was the test plant that *Ph. guerini* was the most successful on, surviving to its final instar, whereas *C. schaffneri* only survived to the second instar on *Po. oleraceae*. *Catorhintha schaffneri* did not survive on *A. cordifolia* although *Ph. guerini* did survive beyond the first instar on this test plant species. *Catorhintha schaffneri* survived to or beyond the second instar on four test cactus species, *Pe. grandifolia*, *O. aurantiaca*, *H. undatus*, and *R. bacciferra* (Paterson *et al*. 2014b), whereas *Ph. guerini* did not survive beyond the first instar on any cactus species, other than the target plant.

The host specificity test results for *C. schaffneri* seemed to follow the degrees of phylogenetic separation model for host choice by an agent (Briese 2003) as the test plant that the bug was the most successful on was the plant that is most closely related to the target plant. It also showed some levels of feeding and survival on various other species of the Cactaceae which was not observed for *Ph. guerini*. There does, however, also appear to be some other form of host choice selection being exhibited by *C. schaffneri* that is more similar to that of *Ph. guerini* because out of the non-Cactaceae species they successfully fed and survived to a degree on similar plants which may be due to some similarities in the chemistry or volatile profiles of these test plant species to the target weed.

Although there was feeding on three test plant species by *Ph. guerini*, this is not a cause for concern, and the beetle is suitably host specific for release in Australia. Only 6% of larvae survived to the third instar on *A. cordifolia*. This is a low survival rate and shows that they could not make it past the half way point of their larval development on this plant, so it is not a suitable host. Even if there was some damage and feeding caused on this plant by early instar

Ph. guerini larvae, it would still be acceptable, as *A. cordifolia* is an invasive species in Australia (Vivian-Smith *et al*. 2007). There was only 1% survival to the fourth instar (or 3% survival to third instar) on *T. caffrum* with minimal damage observed on these plants. *Talinum caffrum* is not native to Australia, and there are no native Australian *Talinum* species, with two *Talinum* species being pan-tropical weeds that are present in Australia (Nyffeler & Eggli 2010), so any minimal feeding on this plant should not be a risk for Australian biodiversity.

Portulaca oleraceae was the only test plant that had larvae feed and successfully go through all larval stages. This is also the only test plant that had larvae feed on it that is native to Australia. The feeding damage caused by *Po. oleraceae* was significantly higher than on the other two test plants that experienced feeding but was still significantly lower than that experienced by the larvae on the target weed, *Pe. aculeata*. The larvae did not successfully pupate after feeding on *Po. oleraceae,* with most of them going onto the pot or stem of the plants as late fifth instar larvae and dying in that place. No larvae were observed attempting to pupate after feeding on *Po. oleraceae* and, therefore, although there was some significant feeding observed on the plant, the insect cannot complete its lifecycle on the plant making *Po. oleraceae* an unsuitable host. The adult host specificity testing also showed that eggs were not oviposited onto *Po. oleraceae* so it is unlikely that eggs would be oviposited onto *Po. oleraceae* in the field which means it is extremely unlikely that larvae would be on the plant to feed in the first place. This shows the importance of selecting the correct life stage when conducting host specificity testing so that the "deciding" life stage is tested in the study (Marohasy 1998; van Klinken 2000). If *Pe. aculeata* and *Po. oleraceae* were growing together and the agents' population size became large enough it is possible that some sort of spill-over effect may be observed. There is also the potential for the adaptation of the beetle to the point where *Po. oleraceae* is a suitable host if *Pe. aculeata* and *Po. oleraceae* are growing together and the beetle begins to feed more on *Po. oleraceae*. However, this is extremely unlikely to happen and this level of shifts in host use patterns and host range expansion has never been observed in biological control agents despite many of the agents that have been released globally being far less host specific than *Ph. guerini* (Müller-Schärer *et al*. 2020).

The host specificity testing used was no-choice testing which is extremely conservative and as a result can sometimes over-state potential non-target attack (Paynter *et al.* 2015; Downey & Paterson 2016; Schaffner *et al*. 2018). Therefore it can be assumed that the non-target attack on these species in the field would be either to the level seen in these tests or lower. Further testing is not required to determine whether these species are at risk of non-target attack as it

is clear that under extreme conditions where they have no alternative option, they cannot complete a life cycle and feed extensively on these plants. The results also confirm that it is suitably host specific in South Africa, and would be released by the South African regulatory authorities based on these host specificity data under the current criteria for release of biological control agents. *Phenrica guerini* has successfully established in many places in South Africa (Mnqeta & Paterson 2019; King *et al*. 2021) and no non-target impacts have been recorded (Iain Paterson, pers. comm.).

Phenrica guerini is a suitably host specific agent for the biological control of *Pe. aculeata* in Australia. It has been in South Africa as an agent against the target weed for more than two decades and no expansion of host range has been reported. No-choice tests using the degrees of phylogenetic separation to choose test plants (Briese 2003) were used in this study, and therefore the results from this study should represent this agent's fundamental host range (Fowler *et al*. 2012) and should not have produced any false negative results (Marohasy 1998). This agent has shown that it is almost strictly monophagous with minimal feeding observed on three test plants, all of which were not able to support a full life cycle of the insect. Based on the host specificity results, *Ph. guerini* is recommended for release as a biological control agent of *Pe. aculeata* in Australia.

Chapter 3

Pre-release Efficacy Assessment

3.1 Introduction

Ensuring the host specificity and safety of biological control agents is regarded as the most important aspect of a biological control programme, however, the efficacy of the agent is in many ways equally important (Müller-Schärer & Schaffner 2008). Although host specificity testing, which is used to determine whether a candidate agent has a suitable host range to be safe for release has received much attention in biological control, other aspects of pre-release testing, including those that determine the efficacy of an agent, have historically received far less (van Klinken & Raghu 2006). Host specificity testing can accurately predict direct non-target attack prior to release, however, there are other forms of non-target impacts that cannot be determined using host specificity testing, including indirect non-target impacts to food-webs (Paynter *et al.* 2020a). Indirect non-target impacts are more likely to be caused by ineffective agents because they will not reduce the density of the target weed and therefore maintain high population densities of the agent for long periods of time, while effective agents reduce the target weed density, which results in a reduction in agent densities over time (McClay & Balciunas 2005; Paynter *et al.* 2020a). It has therefore been argued that pre-release efficacy assessments are an important aspect of testing before an agent is released (Goolsby *et al*. 2004; Balciunas & Smith 2006).

3.1.1 The importance of releasing effective biological control agents

McClay & Balciunas (2005) have argued that the release of ineffective agents should be avoided where possible, as the release of these ineffective agents adds to the costs and risks of biological control without adding to its benefits. They argue that the only reason to release an ineffective agent should be due to an unavoidable inability to predict the agent's efficacy in pre-release testing. It is however very difficult to predict how successful an agent will be prior to its release because there are so many ecological interactions, including biotic and abiotic factors, that determine success in the field that cannot be replicated under quarantine conditions (McClay & Balciunas 2005; Morin *et al*. 2009; van Driesche *et al*. 2009; Schaffner *et al*. 2018).

Releasing ineffective agents increases the risk of potential negative indirect non-target effects that are a result of abundant, host specific but ineffective agents (van Driesche *et al*. 2009; Paynter *et al*. 2020a). This can result in indirect impacts within the region that the agent is released (McClay & Balciunas 2005; Balciunas & Smith 2006). These indirect impacts are due

to interspecies interactions, particularly those that cause changes in food-web interactions (Pearson & Callaway 2005; Balciunas & Smith 2006; Fowler *et al*. 2012). If a released, host specific agent is effective and successfully suppresses its target weed then the agent will reduce its own population density through density-dependent feedbacks (Pearson & Callaway 2005). This will reduce the number of agents in the area and therefore reduce their potential indirect non-target impact (van Driesche *et al*. 2009; Fowler *et al*. 2012).

One well-known example of an indirect non-target interaction causing changes to food-web interactions due to a biological control agent is from the biological control programme of spotted knapweed (*Centaurea maculosa* Lam (Asteraceae)) in western North America (Pearson *et al*. 2000). Two gall fly biological control agents (*Urophora* spp. (Diptera: Tephritidae)) were successfully released against spotted knapweed although they didn't significantly reduce the population sizes of the target plant (Pearson *et al*. 2000). The native deer mouse (*Perosmyscus maniculatus* Wagner (Rodentia: Cricetidae)) subsequently changed its diet and habitat selection as a result of the two gall flies becoming its primary food item (Pearson *et al.* 2000). The more consistent winter food source provided by the gall fly larvae and pupae caused deer mouse population increases and subsequent unwarranted concern (Myers & Cory 2017) over the increase in the number of human cases of Hanta virus as a result of this increased deer mouse population (Pearson & Callaway 2006).

All biological control agents have an associated inherent risk with their release, even if it is small (van Klinken & Raghu 2006). If agent selection is done effectively and all releases are properly justified, this will reduce the number of agents needed for release for successful control and could increase project success rates (Sheppard 2003; van Klinken & Raghu 2006). Multiple insects are often released as part of a biological control programme against a particular weed, however, the successful control of a weed is usually only as a result of one or a small number of the released agents (van Klinken & Raghu 2006). Therefore, some biological control practitioners have argued that the only case in which more than one agent should be released is when the agent does not occupy the entire environmental range of the target weed (Myers 2008). However, Hoffmann & Moran (1998) showed that the reduction of the invasive tree *Sesbania punicea* (Cav.) Benth. (Fabaceae) to levels where it is almost irrelevant as an invasive plant was due to all three agents released against the tree acting together. They argued that control of the tree would not have been achieved if any one of the agents was acting on its own (Hoffmann & Moran 1998). This is an example of a biological control programme that was made successful due to cumulative stress and the agents successfully attacking different parts

of the plant. So in at least some cases, agents that are not sufficiently damaging to result in complete control alone should be released, as they will result in complete control in combination with other agents.

There are cases, however, in which the release of an ineffective agent may have a negative interaction with more effective agents, thus reducing the overall success of a biological control programme (van Driesche *et al*. 2009). This can be as a result of competitive exclusion. Crowe & Bourchier (2006) studied the interspecific competition between a seedhead weevil (*Larinus minutus* Gyll. (Coleoptera: Curculionidae)) and a gall-inducing fly (*Urophora affinis* Frfld. (Diptera: Tephritidae)), both of which are biological control agents against spotted knapweed. The weevil is the more effective agent but the gall-inducing fly was a better competitor resulting in the overall seed destruction being lower when both agents were together than if the weevil were attacking the plant on its own (Crowe & Bourchier 2006). Weyl & Hill (2012) also showed that there were some forms of competitive exclusion between three of the agents released against water hyacinth in South Africa. The presence of the mirid, *Eccritotarsus catarinensis* Carvalho. (Hemiptera: Miridae), caused significant reductions in the feeding scars and petioles mined by the weevil, *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae), compared with the weevil's impact when it was on its own (Weyl & Hill 2012). In these cases, avoiding the release of less damaging agents could reduce the potential for negative interactions in the field and therefore result in greater levels of control of the target weed.

Trait-mediated effects could also cause negative interactions between agents. He *et al*. (2021) studied the impacts of a flea beetle (*Agasicles hygrophila* Selman & Vogt. (Coleoptera: Chrysomelidae)), a biological control agent of alligator weed (*Alternanthera philxeroides* (Mart.) Griseb. (Amaranthaceae)), on a native tortoise beetle (*Cassida piperata* Hope (Coleoptera: Cassididae)). The flea beetle was shown to also feed on the native sessile joyweed (*Alternanthera sessilis* (L.) R.Br. ex DC. (Amaranthaceae)), a food source for the tortoise beetle (He *et al*. 2021). Where there was feeding by the flea beetle on sessile joyweed there was a notable decline in the tortoise beetle populations, and this was attributed to changes in the plant nutrition and induced repellent volatile production of the sessile joyweed due to the feeding by the flea beetle (He *et al*. 2021). Although this is not an example of a negative interaction between two agents against the same weed it does give an indication of how an agent's feeding can influence the feeding of another insect and it is possible that a similar trait-mediated effect could occur between two agents.

Efficacy assessments can help predict which of the candidate agents is likely to be most effective and should therefore be prioritised and released. This will ensure that only the most effective agents are released and that the presence of other less effective agents will not jeopardise the release and establishment of more effective agents (van Driesche *et al*. 2009).

3.1.2 Considerations for pre-release efficacy assessments

Predicting the efficacy of an agent prior to release can be difficult to do as the establishment and efficacy of an agent depends on many biotic and abiotic factors (McClay & Balciunas 2005; van Klinken & Raghu 2006; Harms *et al*. 2020). These factors include the type of damage caused by the agent, the interactions between the agent and other flora and fauna in the introduced range, and various climatic and habitat related differences between the agent's native range and the introduced range (van Klinken & Raghu 2006; Harms *et al.* 2020). These factors can be difficult to predict in quarantine situations and it is sometimes not possible to effectively assess the efficacy of an agent in its native area (van Driesche *et al*. 2009; Schaffner *et al*. 2018).

Pre-release efficacy assessments done in the laboratory or greenhouses can incorrectly predict the effectiveness or ineffectiveness of a candidate agent due to the confined conditions that are often not representative of the field (Morin *et al*. 2009). This could result in the predicted efficacy being inflated or decreased causing an agent to be incorrectly rejected or accepted. This was a concern that McClay & Balciunas (2005) assumed was a reason for some of the hesitation to conduct pre-release efficacy assessments, as they thought that some biological control practitioners were worried that an agent would be wrongly rejected, based on efficacy assessment results, when the agent may have been successful if it had been released. Although laboratory tests can cause inaccurate results due to the confined nature of the tests, Balciunas & Smith (2006) demonstrated that it is possible to quantify the efficacy of an agent under strict quarantine conditions. Pre-release efficacy studies done in the field in the agent's country of origin can help overcome the issues of confinement (Sutton *et al.* 2021) but are not always possible to do if there is a lack of research facilities, or adequately skilled and reliable researchers in the native range at the time (van Driesche *et al*. 2009). It may also be difficult to perform these tests in the field if the insect's native range is in a politically unstable area or if the agent is not sufficiently abundant in the native range due to parasitism and predation (Schaffner *et al*. 2018).

Even if it is not possible to know if the agent will establish and if it will obtain high enough densities, it is suggested to do efficacy testing with the candidate agents at a high enough density to represent the best case scenario. This at least ensures that it is possible for the agent to be damaging under ideal conditions (McClay & Balciunas 2005). It is also important to try provide optimal conditions for the agents when they are tested. This was demonstrated by Dhileepan *et al*. (2000a), where the plants in their field trials lasted for longer than the greenhouse trials and they suggested that it could have been due to a different soil type making the penetration of the larvae and subsequent survival of the pupae more difficult in field trials.

The experimental design used in pre-release efficacy assessments is dependent on the agent's mode of damage. In host specificity testing all life stages of the insect that can cause damage to a plant are tested (van Klinken 2000) and since the efficacy of the agent against the target weed depends on all damaging life stages it is best to test all damaging life stages in pre-release efficacy assessments for insects that do not have a distinctively more damaging life stage. If an insect is a galler then the life stage that forms a gall must be tested and the effects of the gall on the plant quantified (Balciunas & Smith 2006). However, if a candidate agent is a defoliator and both its larvae and adults cause defoliation, then both stages should be tested on the plant and the impacts of the defoliation measured (Dhileepan *et al*. 2000a; 2000b) unless one stage clearly causes much greater impacts.

3.1.3 Examples of pre-release efficacy assessments

Pre-release efficacy assessments can be done using various methods in the field in the native range of the agent. Zapater *et al*. (2004) studied the impacts of a flea-beetle being considered for release in Australia by doing a field study with some plants left to grow under natural conditions with natural attack whilst control plants were sprayed with insecticide to ensure that there was no attack experienced on those plants. The plant growth, damage experienced, and mortality of the plants was compared for plants that had natural attack from the agent and those that had no insect feeding (Zapater *et al*. 2004). Sutton *et al*. (2021) used data collected from field surveys in the native range to model the probability of the survival and reproduction of rat's tail grass based on the abundance of the three host-specific species on that target plant. These models were used to predict the relative efficacy of each of the agents against rat's tail grass.

Laboratory or quarantine based efficacy assessment studies are more common than field based studies. For defoliators, these studies are often done using caged potted plants of similar size

that are inoculated with different densities of the potential agent or left with no agents as a control (Brockerhoff *et al.* 1999; Weed & Casagrande 2010; Wang *et al*. 2010). The damage caused by the different densities of agents is then compared with the control and the impact of the insects on various plant parameters. Brockerhoff *et al.* (1999) did a pre-release efficacy assessment, as described above, on the agent, *Cleopus japonicus* Wingelmüller (Coleoptera: Curculionidae). It was subsequently released in New Zealand in 2006, has successfully established and appears to be an effective agent if integrated with complementary weed management techniques (Watson *et al.* 2011). Weed & Casagrande (2010) did a pre-release laboratory based efficacy assessment on the agent, *Hypena opulenta* (Christoph) (Lepidoptera: Noctuidae) and predicted that it would be damaging. *Hypena opulenta* was released in Canada in 2014 and successfully established, however, it has not yet reached a high enough density to determine whether it is an effective agent or not (Bourchier *et al.* 2019). Some efficacy assessments can incorporate the use of simulated defoliation, without the use of insects. These can involve manual defoliation which is done to represent insect herbivory but without having to use insects (Wirf 2006). This can be done to better understand if defoliation will have a desired impact on a plant. However, insects often do more damage than just feeding as there can be microorganisms associated with the insects that get introduced to the plant through feeding by the insect and so simulated herbivory can often underestimate the impact an agent will have (Venter *et al*. 2013).

3.1.4 Efficacy of Phenrica guerini *as a biological control agent of* Pereskia aculeata

Phenrica guerini has established in South Africa (Paterson *et al.* 2014a; Mnqeta & Paterson 2019) and is resulting in a reduction of *Pereskia aculeata* in some areas in which it has established (Mnqeta 2017; King *et al*. 2021). More than a decade after its release in South Africa, Paterson *et al*. (2014a) applied the same criteria used to prioritise new biological control agents for the control of *Pe. aculeata* to *Ph. guerini* and concluded that the flea beetle was promising for biological control in South Africa in terms of host specificity, climatic matching, and genetic matching but not in terms of its impact due to its feeding and resultant damage being restricted to the leaves. This agreed with the few post-release evaluations that had been done at the time which indicated that the impact of *Ph. guerini* on *Pe. aculeata* in South Africa was trivial, although possibly underestimated (Klein 1999; Paterson *et al.* 2011a). However, a more detailed post-release impact assessment showed that *Ph. guerini* is providing some form of control in some of the areas in which it has established and is an effective agent at some sites (Mnqeta 2017; Mnqeta & Paterson 2019; King *et al*. 2021).

Mnqeta (2017) conducted an insect-exclusion post-release evaluation of the impact of *Ph. guerini* at a site where it has established in South Africa to determine whether *Ph. guerini* was reducing *Pe. aculeata* density at that site. It was found that the mean number of leaves at the end of the experiment (100 days) was significantly less on plots with *Ph. guerini* than plots where the beetle was excluded by regular applications of insecticide (Mnqeta 2017). Paterson (2010) did a greenhouse study looking at the impact of the beetle on the target plant and found that there was no significant relationship between feeding by the beetle and various plant growth parameters. However, these results may have been impacted by the plants facing a high nutrient, low stress environment and the experiment being terminated too soon for significant effects to be seen (Paterson 2010). Based on the field data from South Africa (King *et al.* 2021) it is thought that *Ph. guerini* may be an effective biological control agent against *Pe. aculeata* in Australia. In this study, a laboratory based impact assessment was done to determine how *Ph. guerini* effects the growth of *Pe. aculeata* over multiple generations of the agent. This was used alongside the field assessments done by Mnqeta (2017) and laboratory based assessments (using different methodology) done by Paterson (2010), to predict the efficacy of *Ph. guerini* as a biological control agent for *Pe. aculeata* in Australia.

3.2 Materials and Methods

3.2.1 Experimental Design

3.2.1.1 Plant Selection

Seventy plants of *Pe. aculeata* from the Waainek Research Facility at Rhodes University were selected. These plants had been growing in the same pots for over a year and were therefore well-established with woody stems and well developed root systems. This helped overcome the limitation faced by Wang *et al*. (2010) who could not measure the impact of their insects on plants with well-established root systems as they used newly planted plants. The root systems were also not large enough for the plants to be pot-bound. The plants were of a similar size but the number of stems and leaves was counted for each plant and the plants were then pruned so that the number of leaves was between 60 and 80 for each plant. This number was chosen to match the upper limit of the size used by Mnqeta & Paterson (2019) as the insect densities used for this experiment were chosen based on this previous work (Paterson 2010; Mnqeta 2017; Mnqeta & Paterson 2019). The plants were then left for two weeks to recover from pruning. The plants were watered once a week and no fertiliser was given to the plants in order to simulate slightly stressed conditions to more accurately mimic the condition most

plants face in the field. The plants were sorted into ten groups of seven similar sized plants and assigned to different treatments.

3.2.1.2 Non-destructive Sampling

A feeding experiment was run to determine the impact that *Ph. guerini* larvae feeding had on the growth and leaves of *Pe. aculeata* plants. Plants were either assigned to be a control plant or to an herbivory treatment and placed in a cage in the same laboratory with grow lights, and labelled randomly according to their treatment and replication number. The sample size decreased throughout the experiment due to the destructive sampling (see Results section). The number of leaves, the length of the main stem, and the number of additional stems were counted for every plant at the beginning of each new stage of the experiment. At the start, all plants that had been assigned to the herbivory treatment were inoculated with 12 two-day old larvae. Only larvae were used for this assessment as they cause more damage to the newly growing, more important parts of the plant than the adults which generally feed on the older leaves and cause less damage. The density of larvae was chosen as Paterson (2010) found that using 20 larvae did not significantly increase damage compared to using 10 larvae and Mnqeta & Paterson (2019) found that 12 larvae did cause significantly more damage than six larvae on the same size plants. This density is also higher than the average densities found in most of the places in which the beetle has established in South Africa (Mnqeta 2017) so should represent the best case scenario that McClay & Balciunas (2005) recommended for efficacy assessments. Once all of the larvae were fifth instars (about one month) they were removed from the plants, to prevent them from pupating in the cages, and because the second half of the fifth instar stage do not feed and so will not inflict further damage to the plants. A day after all of the larvae were removed, a new batch of larvae were placed on the herbivory treatment plants for a second feeding event, and all of the non-destructive sampling measurements were taken, and the percentage damage was visually estimated for the herbivory treatment plants by comparing the percentage of leaves that had significant feeding to leaves without feeding, or with limited feeding. This was repeated for a third feeding event. When the larvae were removed after the third feeding event all of the plants were left without any larvae on them for the equivalent time of one larval cycle. This enabled the plants a brief period of recovery that would somewhat mimic the period in the field in which the beetle populations decrease as they disperse to find better food that could maintain their offspring due to the badly damaged plants, allowing the plants a time for recovery. At the end of this time another set of larvae were inoculated onto

the herbivory treatment plants for a fourth feeding event and a fifth feeding event. The total time from the start to the end of the fifth feeding event was just under six months.

3.2.1.3 Destructive Sampling

To determine the impact of feeding by *Ph. guerini* larvae on the biomass of *Pe. aculeata* destructive sampling was also done on a subset of the plants at various stages throughout the experiment. Destructive sampling occurred at the beginning of the set up for ten control plants and then for ten control and ten herbivory treatment plants at the end of the third feeding event, after the feeding break, and at the end of the fifth feeding event. The destructive sampling involved separating the plants into leaves, green stems, woody stems, and roots. The wet mass of each of these was weighed. They were then placed in a brown paper bag in an oven set at 50°C with the fan on to dry. The dry mass of each was weighed once it was clear that the mass was no longer decreasing (about two weeks in the oven, determined by weighing a subset of samples every second day until the weight remained constant). The dry mass for the plants after the first feeding destructive sampling event was calculated using the relationship between the wet mass and dry mass of the plants at all other destructive sampling events due to a lack of oven space.

3.2.2 Statistical analyses

All statistical analyses were run in RStudio (R Core Team 2021). Residual diagnostics were run for each model tested, and only the best model fit is reported.

For the destructive sampling data, an Analysis of Variance (ANOVA) was run for the wet and dry masses of the woody stems, green stems, roots, and leaves (package = stats, function = aov). The p values were calculated using a type II Sum of Squares ANOVA (package = car, function = Anova, type = II). A Tukey's post hoc test was run to check where the significant differences were if there were any significant results (package = emmeans, function = emmeans, adjust = tukey).

For the non-destructive sampling various analyses were run for each of the variables, using random effects for the different sampling events to account for the repeated measures effect. For the length of stems a log transformed generalised linear mixed effects model was run (package = lme4, function = lmer, REML = FALSE). The model fit was checked using residual diagnostics (package = DHARMa, function = simulateResiduals). An ANOVA of the model was run to calculate the significance (package = car, function = Anova, test = "Chisq") and a

Tukey's post hoc test was run to check whether there was any significant difference between treatments (package emmeans; function; emmeans, adjust = tukey). A post hoc test could not be run on the random effect (Time) so a 95% Confidence Interval graph was plotted to determine if there were any clear significant differences between time periods.

For the number of leaves and the number of stems, Generalised Additive Models (GAMs) were run (package = mgcv, function = gam, method = REML). The models were checked for their fit (package = gratia, function = appraise, qq_plot). The p values were calculated using an ANOVA of the model (package = mgcv, function = anova.gam). As a post hoc test was not possible for these models, 95% Confidence Interval graphs were plotted to check for significant differences in the data.

The total change in the number of leaves, number of additional stems, and the length of the main stems was calculated for the plants that were measured from start to the end of the fifth feeding event. A Generalised Linear Model (GLM) was run for each of these parameters (package = stats, function = glm). The model fit was checked using residual diagnostics (package = DHARMa, function = simulateResiduals). An ANOVA of the model was run to calculate the significance (package = car, function = Anova).

A Kruskal-Wallis test was run on the percentage damage data (package = stats, function = kruskal.test). A Pairwise Wilcoxon Rank Sum Test was run to check where the significance was found (package = stats, function = pairwise.wilcox.test, p.adjust.method = "BH").

3.3 Results

The feeding of *Ph. guerini* larvae on *Pe. aculeata* in this experiment did not have a significant impact on all plant parameters, although there were some significant impacts observed on some parts of the plants. There was only a significant difference observed in the mass of the leaves and woody stems, but these were mostly due to changes over time and not differences between treatments at the same sampling events (Fig 3.2 and 3.4), and no significant change was observed in the mass of the green stems or roots. There was no significant difference in the number of additional stems on the plants as a result of feeding by the larvae (Fig 3.7a and 3.7b), however, there was a surprising significant difference in the length of the main stem between the control plants and those with larval feeding (Fig 3.6). The number of leaves increased significantly over time and there were some significant differences between the number of leaves on the control plants and larval feeding plants (Fig 3.8) which seemed to follow the trend in percentage damage (Fig 3.5).

3.3.1 Destructive Sampling

There was only a significant difference observed in one of the four biomass parameters observed. There was a significant increase in the mass of the leaves over time and the mass of the leaves was significantly higher for some of the control plants compared to the treated plants. There was no significant difference in the change of mass for the roots, woody stems, and green stems over time and between treatments.

The wet mass and dry mass of the roots, woody stems, green stems, and leaves showed similar patterns to one another. The wet mass of the roots, woody stems, and green stems for the plants at each destructive event showed no significant difference between treatments and no significant change between sampling events. However, the dry mass of the woody stems did show some significant differences between sampling events. There was some significant increase observed over time and between sampling events in the wet mass of the leaves and some significant difference observed between sampling events for the dry mass of the leaves.

The wet mass of the roots was fairly consistent across all sampling periods with the median mass occurring around 100-130 g for the control plants and treated plants throughout the experiment (Fig 3.1(a)). There was no significant difference between treatments ($F = 0.0307$, d.f. = 1, $P = 0.8614$) and across time for the wet mass of the roots ($F = 0.8796$, d.f. = 3, $p = 0.4564$). There was an outlier for the control plants at the start, first feeding destructive event and feeding break, and two outliers for the treated plants at the final feeding destructive sampling event (one lower and one upper outlier). The dry mass of the roots was, on average, 44.1% of the wet mass of the roots (S.E. ± 0.11). The dry mass of the roots showed the same lack of change as the wet mass of the roots with no significant difference between treatments ($F = 0.2408$, d.f. = 1, $p = 0.6253$) and across time ($F = 0.8699$, d.f. = 3, $p = 0.4613$) for the dry mass of the roots (Fig 3.1(b)).

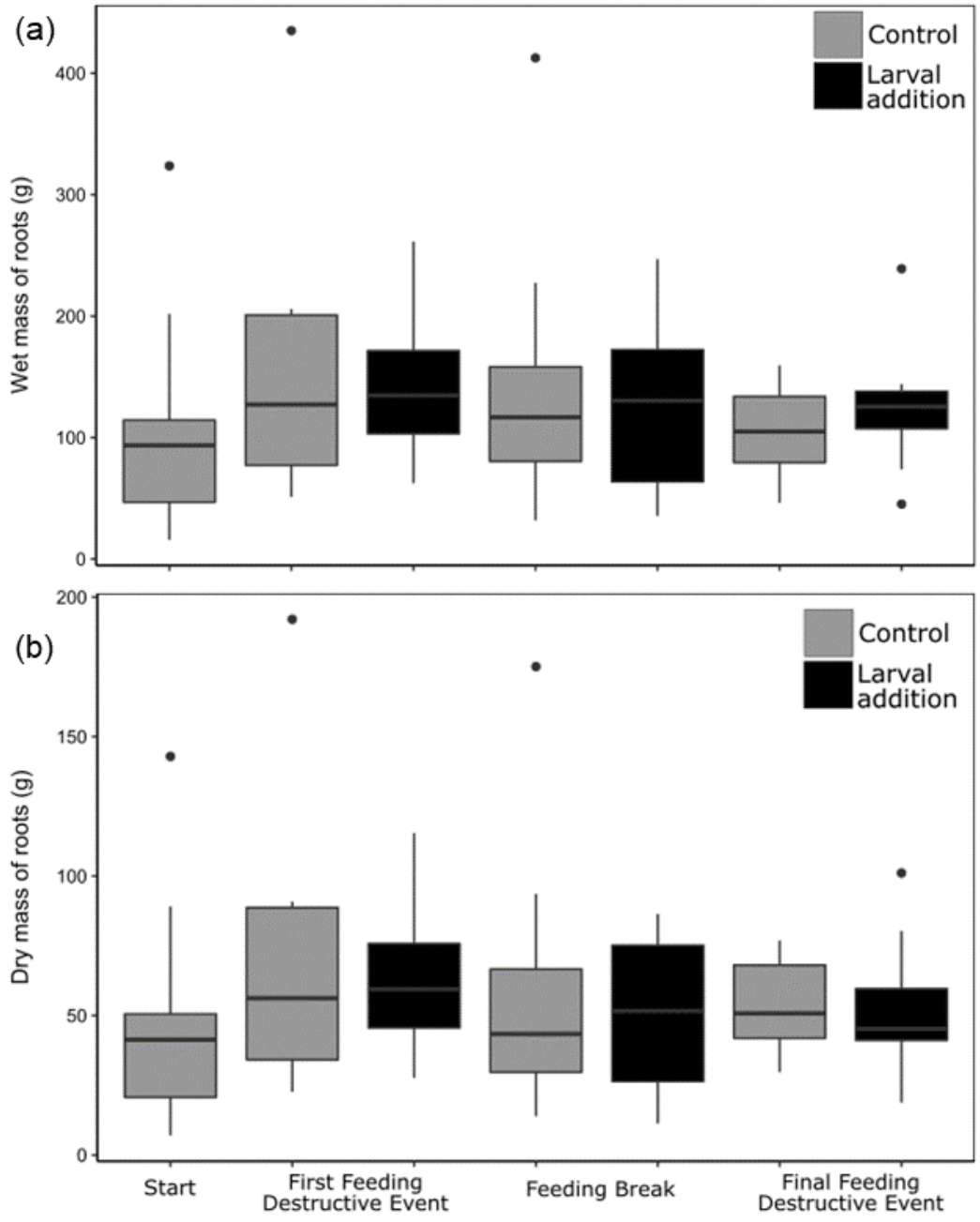

Figure 3.1: The change in (a) wet mass and (b) dry mass of the roots of *Pereskia aculeata*, as a result of feeding by *Phenrica guerini* larvae, observed at each destructive sampling event. No significant differences were found in the data (n=10). (The dry mass for the plants at the first feeding destructive event was calculated from the relationship between the wet mass and dry mass for the plants at all other sampling events).

The median for the wet mass of the woody stems varied between 80 and 120 g with a fair bit of variation across treatments and sampling events (Fig 3.2(a)). There were outliers for three treated plants across two sampling events and two control plants across two sampling events. There was no significant difference in the wet mass of the woody stems between treatments ($F = 0.0248$, d.f. = 1, $P = 0.8750$) and sampling events ($F = 0.7766$, d.f. = 3, $P = 0.5113$). The dry mass of the woody stems was, on average, 25.6% of the wet mass of the woody stems (S.E. ± 1.1). The pattern observed for the dry mass of the woody stems was similar to the wet mass of the woody stems (Fig 3.2(b)), with no significant difference between treatments ($F = 0.1768$, d.f. = 1, $p = 0.6755$), although there was a significant difference between sampling events ($F = 4.4675$, d.f. = 3, $p = 0.0060$) which was not observed in the wet mass. There was a significant increase in the dry mass of the woody stems between the control plants from the feeding break to the final feeding, and a significant increase between the dry mass of the woody stems of the treated plants from the feeding break to the final feeding sampling event (Fig 3.2(b)).

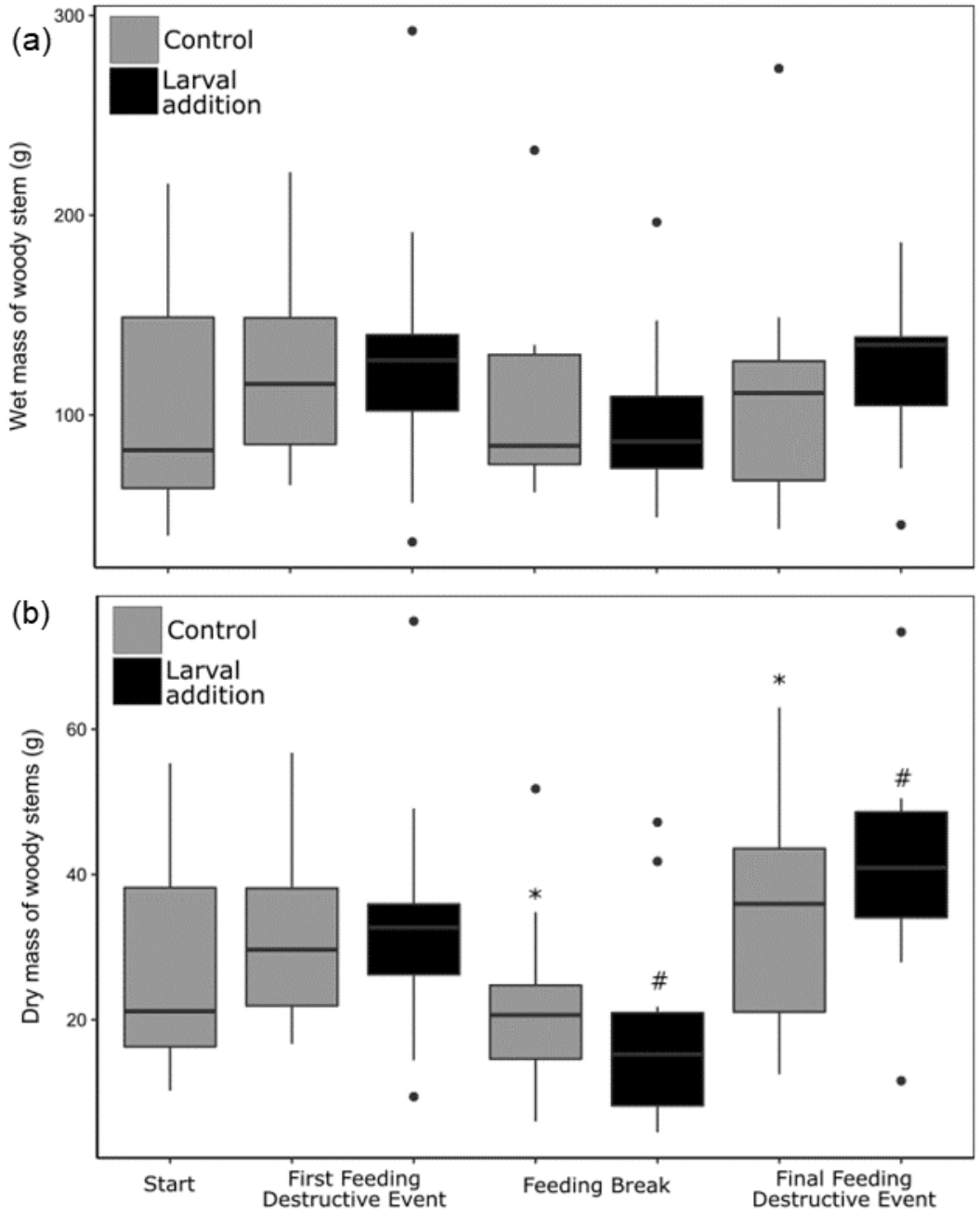

Figure 3.2: The change in (a) wet mass and (b) dry mass of the woody stems of *Pereskia aculeata*, as a result of feeding by *Phenrica guerini* larvae, observed at each destructive sampling event. No significant differences were found in the data for the wet mass. For the dry mass the "*" and "#" show the two groups that were significantly different to one another (e.g. the treated plants after the feeding break were significantly different from the treated plants after the final feeding destructive event) (n=10). (The dry mass for the plants at the first feeding destructive event was calculated from the relationship between the wet mass and dry mass for the plants at all other sampling events).

The median for the wet mass of the green stems ranged between about 17 and 35 g (Fig 3.3(a)). There was greater variation in the wet mass of the green stems at the feeding break destructive sampling event than the other sampling events. There was an outlier for the control plants and the treated plants at the first feeding destructive sampling events. There was no significant difference between treatments ($F = 1.9332$, d.f. = 1, $p = 0.1690$) and across sampling events ($F = 1.4713$, d.f. = 3, $p = 0.2310$) for the wet mass of the green stems. The dry mass of the green stems was, on average, 22.6% of the wet mass of the green stems (S.E. ± 0.4). The dry mass of the green stems showed a similar pattern to the wet mass of the green stems but with less variation observed in the dry mass of the green stems (Fig 3.3(b)). There was no significant difference for the dry mass of the green stems between treatments ($F = 2.9475$, d.f. = 1, $p = 0.0908$) and between sampling events ($F = 0.7666$, d.f. = 1, $p = 0.5169$).

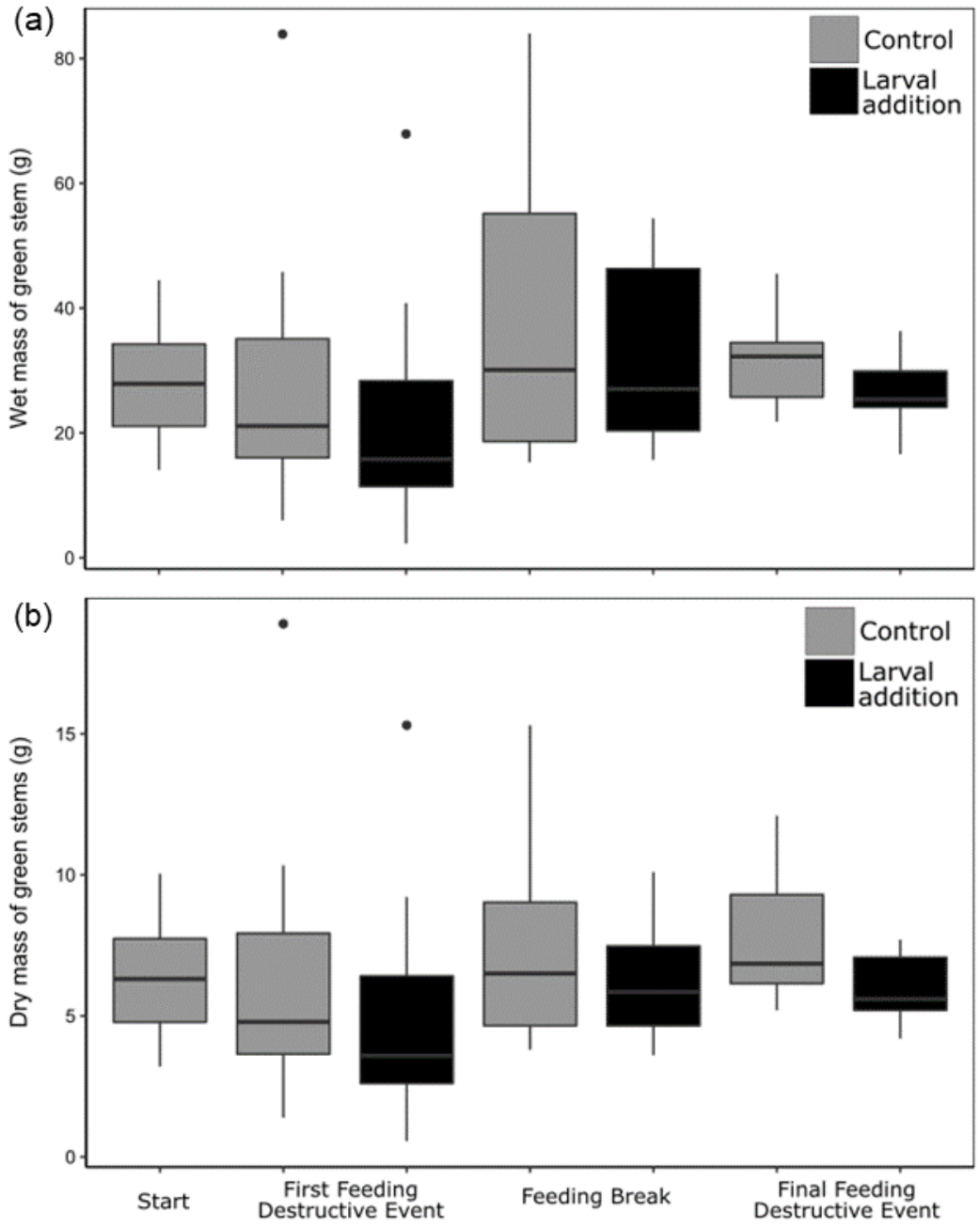

Figure 3.3: The change in (a) wet mass and (b) dry mass of the green stems of *Pereskia aculeata*, as a result of feeding by *Phenrica guerini* larvae, observed at each destructive sampling event. No significant differences were found in the data (n=10). (The dry mass for the plants at the first feeding destructive event was calculated from the relationship between the wet mass and dry mass for the plants at all other sampling events).

The wet mass of the leaves increased over time from about 70 g to about 200 g for the control plants (Fig 3.4(a)). Although the wet mass of the leaves is lower for the larval feeding plants than the control plants at each sampling event this was not a significant result ($F = 2.0018$, d.f. $= 1$, $P = 0.1620$). There was a significant difference for the wet mass of the leaves between the sampling events ($F = 8.24$, d.f. $= 3$, $P = 0.0001$). The wet mass of the leaves for the control at the start was significantly lower than the wet mass of the leaves for the control after the first and final feeding destructive sampling events, and for the treated plants at the final feeding destructive sampling event (Fig 3.4(a)). The wet mass of the leaves for both treatments was significantly different between the final feeding events and the start and first feeding events. There was no significant difference between the wet mass of the leaves for both treatments for the feeding break destructive sampling events and all of the other sampling events (Fig 3.4(a)).

The dry mass of the leaves was, on average, 5.9% of the wet mass of the leaves (S.E. ± 0.3). The pattern observed for the dry mass and wet mass of the leaves was similar (Fig 3.4). There was not a significant difference between treatments for the dry mass of the leaves ($F = 0.3949$, d.f. $= 1$, $p = 0.5139$). However, there was a significant difference between the sampling events for the dry mass of the leaves ($F = 13.8883$, d.f. $= 3$, $p < 0.0001$) and there was a significant difference between the dry mass of the leaves for the plants at the final feeding sampling event and the plants at all of the other sampling events (Fig 3.4(b)).

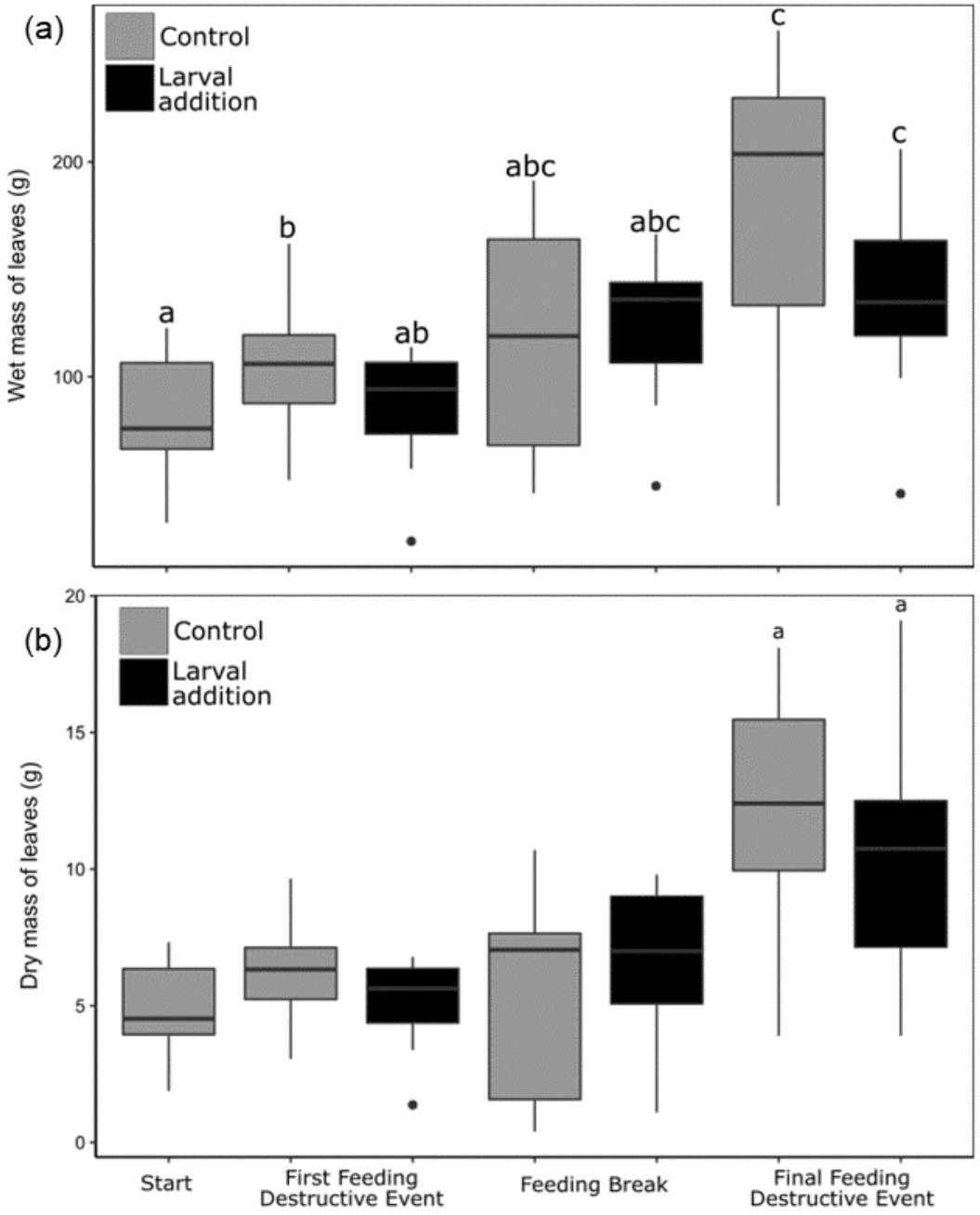

Figure 3.4: The change in (a) wet mass and (b) dry mass of the leaves of *Pereskia aculeata*, as a result of feeding by *Phenrica guerini* larvae, observed at each destructive sampling event, with significant differences represented by letters a, b, and c (n=10). (The dry mass for the plants at the first feeding destructive event was calculated from the relationship between the wet mass and dry mass for the plants at all other sampling events).

3.3.2 Non-destructive Sampling

Feeding by *Ph. guerini* on *Pe. aculeata* had a significant impact on the leaves and the main stem of the plant but not on the number of additional stems. There was a general increase over time in the number of leaves, number of additional stems and length of the main stem. Treated plants had fewer leaves and less growth in the main stem by the end of the experiment.

In general, there was a difference between the number of leaves, the length of the main stem, and the percentage damage for the control plants and plants with larvae, with more leaves on the control plants than on the treated plants, a longer main stem on control plants than treated plants and higher percentage damage on the treated plants. There was an increase observed in the number of leaves, length of the main stem, and number of additional stems over time although this was not significant for all of the variables.

There was a significant difference between the treatments and across sampling events for the percentage damage observed on the plants ($\chi^2 = 236.28$, d.f. = 11, $p < 0.0001$) (Fig 3.5). There was a significant difference between all of the control plants (no damage) and all of the treated plants. There was a significant increase in the percentage damage from the first feeding event (n = 30) to the third feeding event (n = 30) of the treated plants, increasing to a median of about 10% feeding damage (Fig 3.5). The percentage damage then decreased significantly during the feeding break (n = 20) to about 7% damage, so that it was not significantly different from the percentage damage after the second feeding event (n = 30). When feeding started again the percentage damage increased significantly to about the same as what it was after the third feeding event and although it increased further at the fifth feeding event (n = 10), to a median of about 12% damage, this difference was not significant (Fig 3.5).

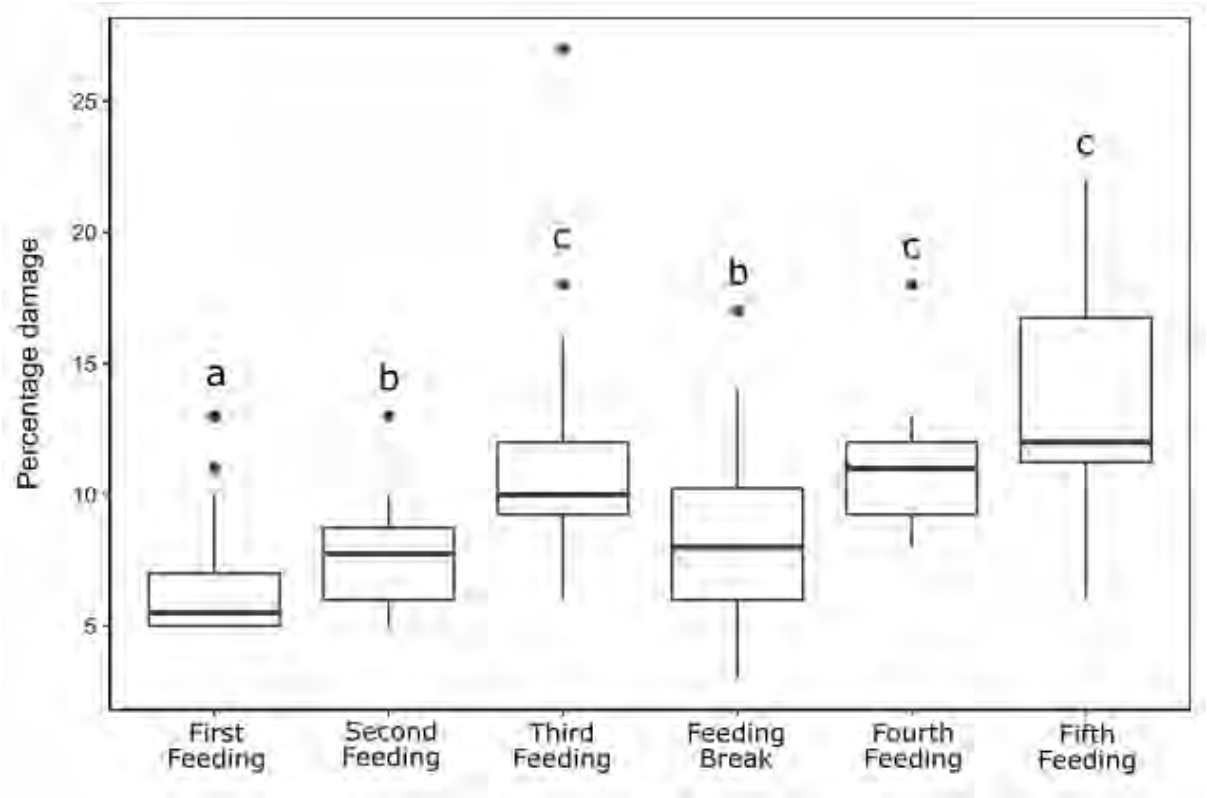

Figure 3.5: Percentage of damaged leaves of *Pereskia aculeata* due to feeding by *Phenrica guerini* larvae, at each sampling event, for the plants that had larvae. The significant differences are represented by the letters a, b, and c. The sample size changed from 30 (first to third feeding breaks) down to 20 (feeding break), and finally 10 (fourth and fifth feeding breaks).

The length of the main stems increased overtime for both the control plants and the treated plants (Fig 3.6a). There was a significant difference between the length of the main stems for the control plants and the treated plants ($\chi^2 = 27.066$, d.f. = 1, $P < 0.0001$). The 95% confidence interval graph (Fig 3.6b) showed significant difference between the length of the main stems for the control and treated plants for all sampling events after the third feeding event (n = 30 for third feeding event; n = 20 for feeding break; n = 10 for fourth and fifth feeding event).

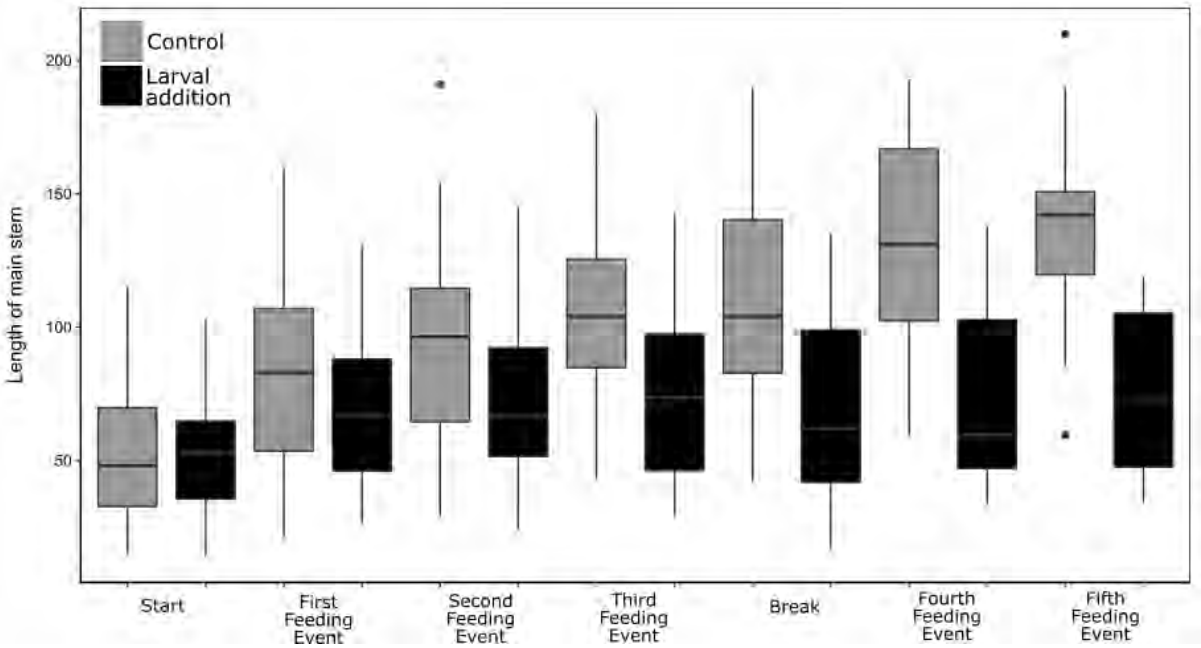

Figure 3.6a: The change in the length of the main stems of *Pereskia aculeata* control plants and plants that had *Phenrica guerini* larval feeding at each non-destructive sampling event. The sample size changed from 40 control plants and 30 larval feeding plants at the start to 30 plants for both treatments (first to third feeding breaks) down to 20 plants for both treatments (feeding break), and finally 10 plants for both treatments (fourth and fifth feeding breaks).

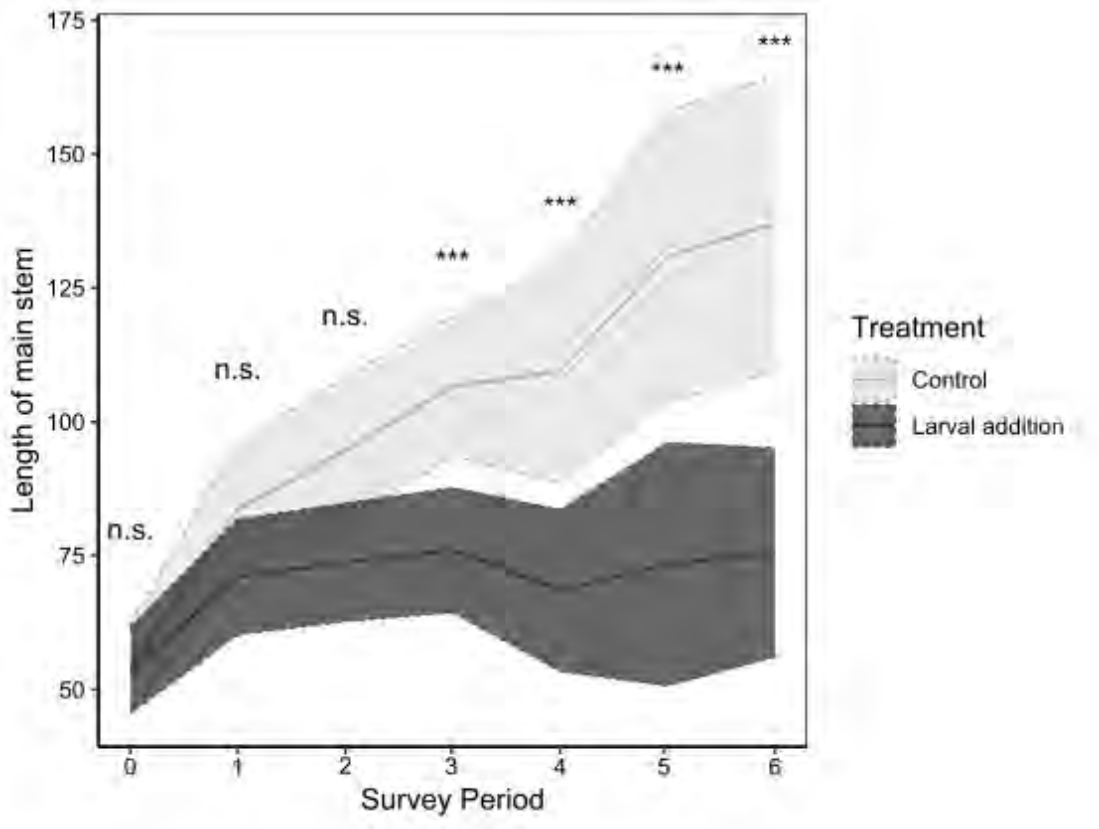

Figure 3.6b: The 95% Confidence Intervals for the length of the main stems of the control plants and plants with larval feeding used to determine statistical significance of the results in Figure 3.6a. Survey period 0 is the start. Survey periods 1, 2, and 3 are the first three feeding events. Survey period 4 is the feeding break, and survey periods 5 and 6 are the final feeding events. The time periods with "***" show significant differences and the time periods with "n.s" show no significant difference.

The number of additional stems increased slightly for both treatments from a median of around 5 to 7 at the start to a median of about 12 to 15 at the end, with the greatest increase seen from the start (n = 40 for control and 30 for treated plants) to the first feeding event (n = 30). There was no significant difference for the number of additional stems between treatments (F = 3.154, d.f. = 1, P = 0.0767) (Fig 3.7a and 3.7b). There was a lot of variation in the number of additional stems for most of the plants from the first feeding event onwards, with quite a lot of the sampling events having an outlier or two.

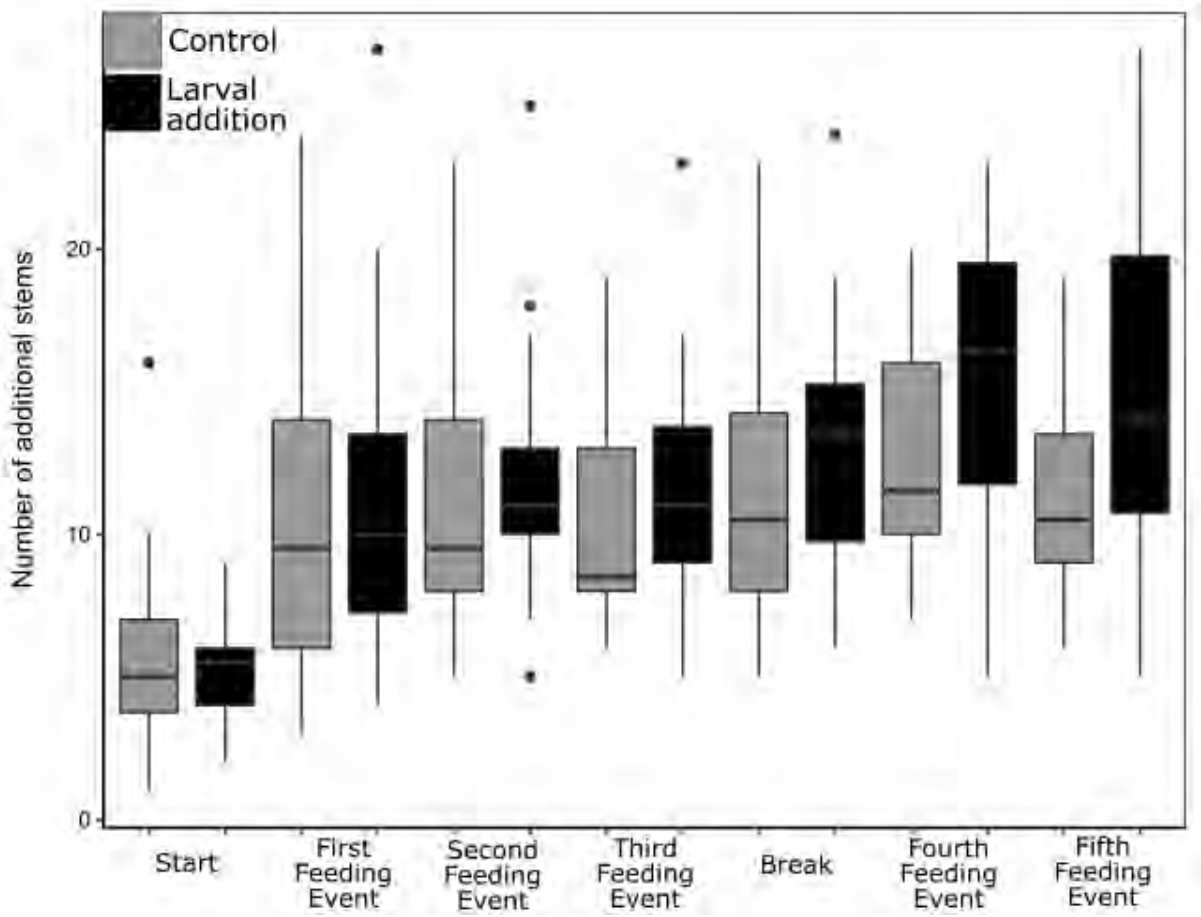

Figure 3.7a: The change in the number of additional stems on *Peresekia aculeata* control plants and plants that had *Phenrica guerini* larval feeding at each non-destructive sampling event.

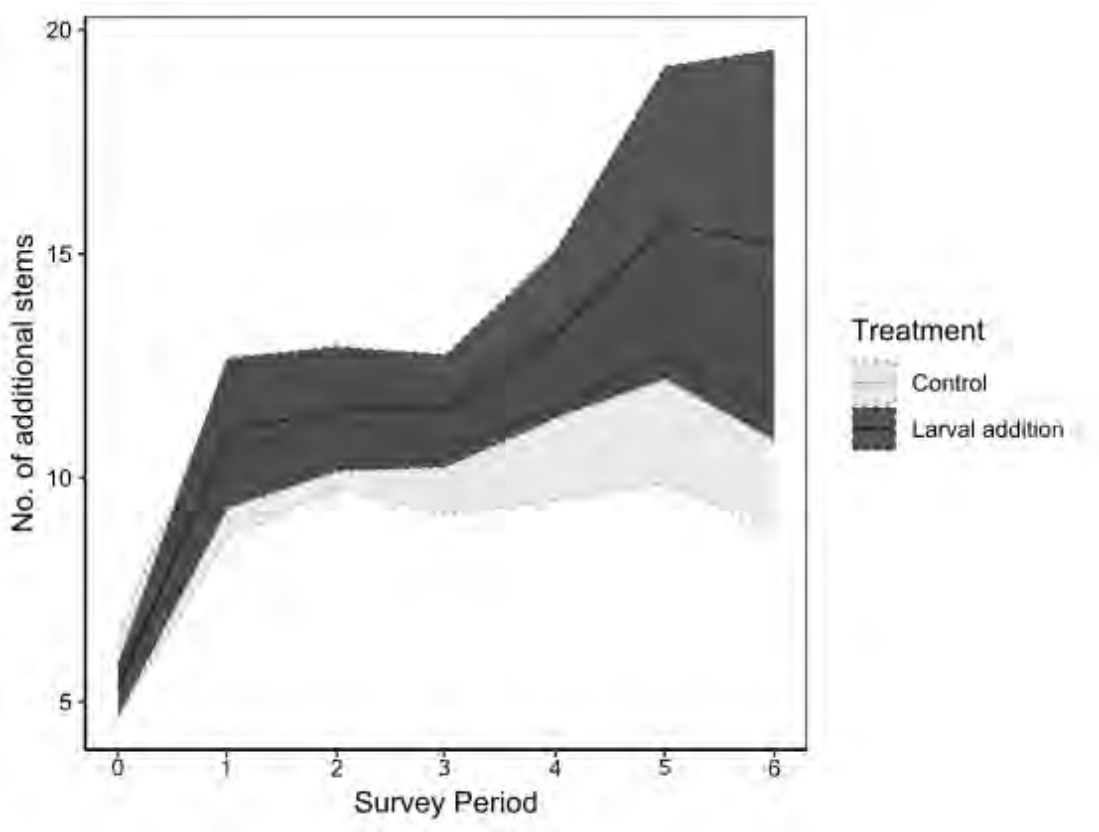

Figure 3.7b: The 95% Confidence Intervals for the number of additional stems of the control plants and plants with larval feeding used to determine statistical significance of the results in Figure 3.7a. Survey period 0 is the start. Survey periods 1, 2, and 3 are the first three feeding events. Survey period 4 is the feeding break, and survey periods 5 and 6 are the final feeding events. There was no statistically significant differences.

The number of leaves increased over time with a greater increase observed in the control plants than in the treated plants (Fig 3.8a). The control plants increased from a median of about 70 leaves at the start (n = 40) to around 230 leaves at the end (n = 10). The treated plants increased from a median of about 70 leaves at the start (n = 30) to a median of about 180 leaves after the feeding break (n = 20) with a decrease in the number of leaves to a median of about 140 at the end (n = 10). There was a significant difference in the number of leaves between the treatments ($F = 34.59$, d.f. = 1, $p < 0.0001$) and across time ($F = 65.55$, d.f. = 6, $p < 0.0001$). The 95% confidence interval graph showed that there was a significant difference in the number of leaves between the control and treated plants at the third (n = 30) and fifth (n = 10) feeding events (Fig 3.8b).

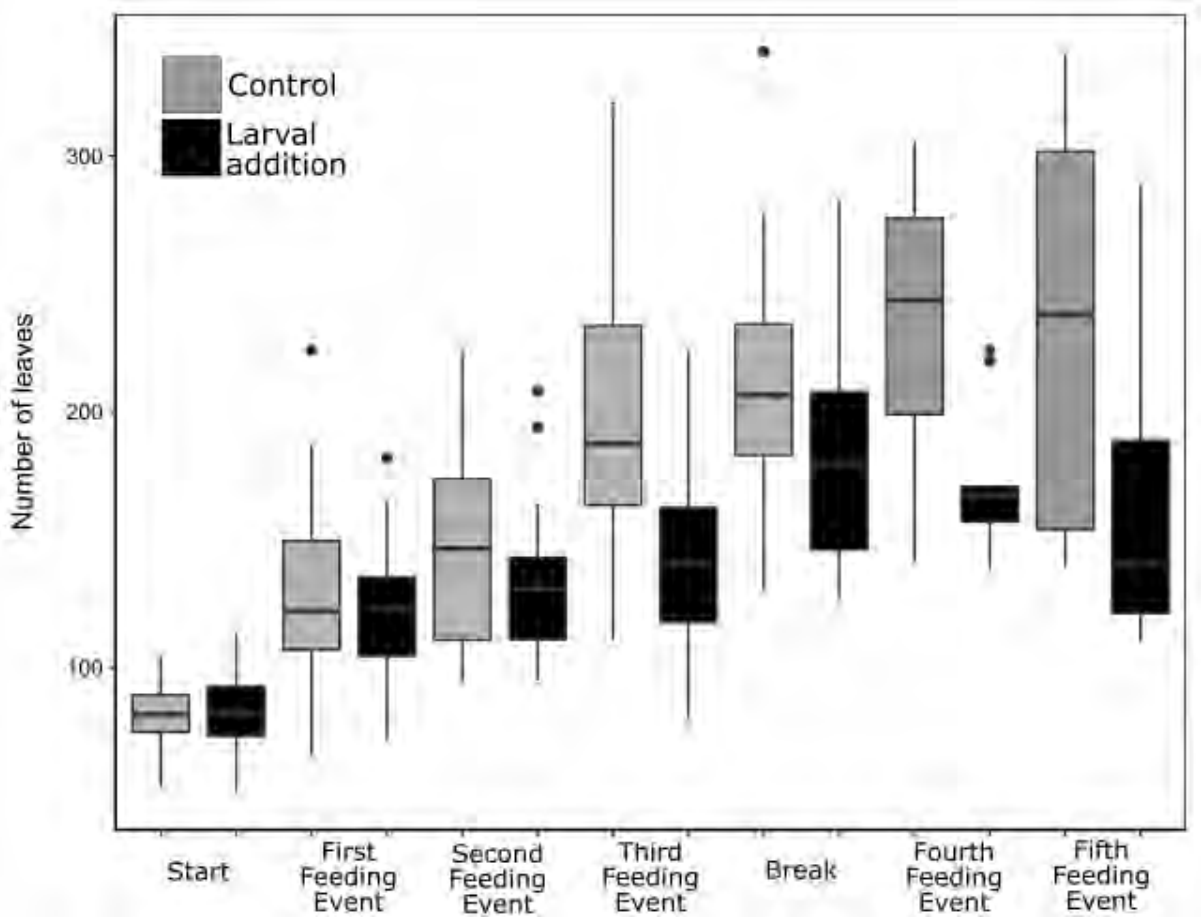

Figure 3.8a: The change in the number of leaves of *Pereskia aculeata* control plants and plants that had *Phenrica guerini* larval feeding at each non-destructive sampling event.

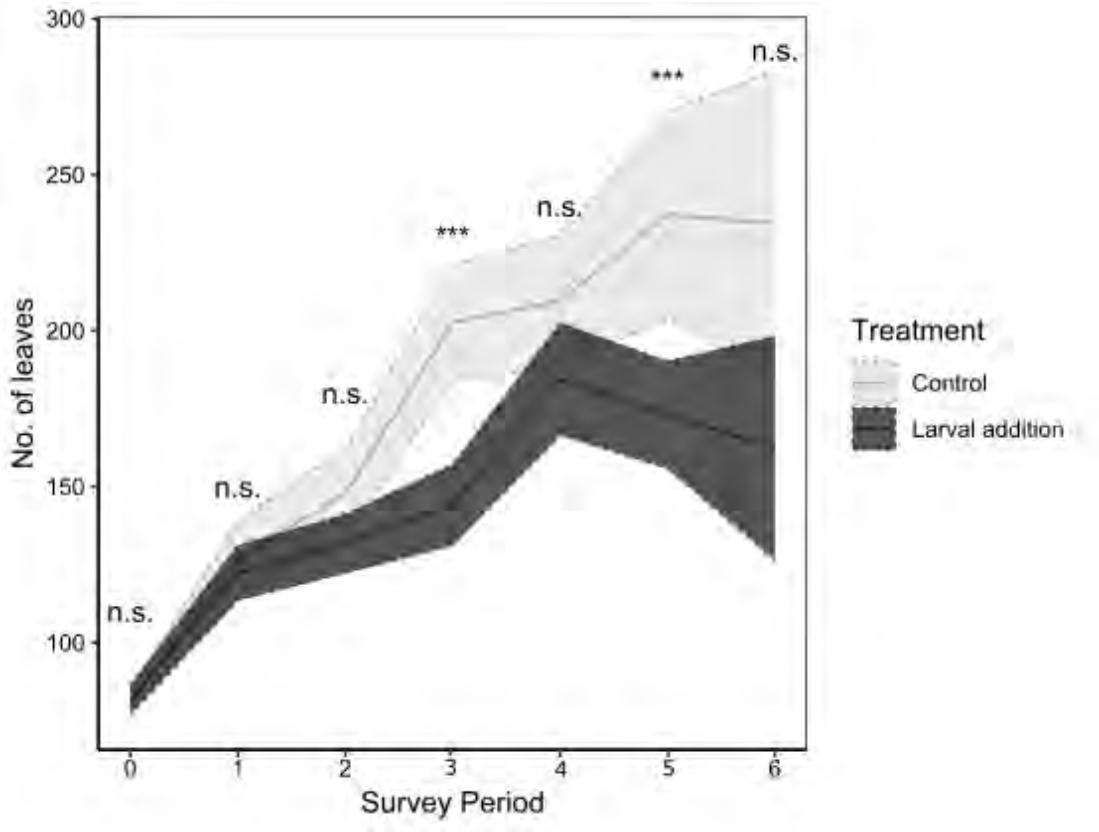

Figure 3.8b: The 95% Confidence Intervals for the number of leaves on the control plants and plants with larval feeding used to determine statistical significance of the results in Figure 3.8a. Survey period 0 is the start. Survey periods 1, 2, and 3 are the first three feeding events. Survey period 4 is the feeding break, and survey periods 5 and 6 are the final feeding events. The time periods with "***" show significant differences and the time periods with "n.s" show no significant difference.

The average total change in the number of leaves and the length of the main stem were significantly lower in the treated plants than the control plants (Table 3.1). On average there were 72 (S.E. ± 12.33) leaves less on the treated plants than on the control plants and the main stem was 11.1 cm (S.E. ± 7.17) shorter on the treated plants than on the control plants. There was no significant difference for the number of stems (Table 3.1).

Table 3.1: The mean (± S.E) total change and statistical difference in the number of leaves, number of additional stems, and length of the main stems of *Pereskia aculeata* control plants and plants that had *Phenrica guerini* larval feeding observed for the entire sampling period (n = 10).

Total change in:	**Treatment**	**Mean (± S.E)**	χ^2	**d.f.**	**p**
No. of leaves	Larval addition	78±17.28	6.6417	1	0.0010
	Control	152.4±23.13			
No. of additional stems	Larval addition	9.8±2.04	1.2229	1	0.2688
	Control	7.1±1.35			
Length of the main stem (cm)	Larval addition	21.6±8.44	11.738	1	0.0006
	Control	80.5±14.98			

3.4 Discussion

The feeding of *Ph. guerini* larvae on *Pe. aculeata* in this experiment had a significant impact on two of the seven plant parameters looked at. There was a significant difference observed in the mass of the leaves and woody stems, but these were mostly due to changes over time and not differences between treatments at the same sampling events, and no significant change was observed in the mass of the green stems or roots. There was no significant difference in the number of additional stems on the plants as a result of feeding by the larvae, however, there was a significant difference in the length of the main stem between the control plants and the treated plants, with the length of the main stem being significantly longer for the control plants. The number of leaves increased significantly over time and there were some significant differences between the number of leaves on the control plants and treated plants which seemed to follow the trend in percentage damage. There is a clear trend that the number of leaves, number of additional stems, and the length of the main stem reduced over consecutive feeding events by the beetle. If the experiment had been continued it is likely that the difference between the controls and treatments would become greater and the agent would start to show a significant overall impact.

The plants used in my study all had well established root systems to try overcome one of the limitations experienced by Wirf (2006), as the agents will mostly be attacking plants with well-established root systems in their released environment. As this was a short term study, it is possible that the lack of significant difference in the wet and dry mass of the roots was because

any impact on root biomass caused by feeding of the larvae would not be seen in the short period of time.

As the plants were well established potted plants they had fully developed woody stems. It was expected that there would be no significant change observed in the biomass of the woody stems as that part of the plant takes a long time to develop and be impacted, and is not damaged by the agent directly. There was no significant change observed in the wet mass of the woody stems. However, there was a surprising significant difference observed in the dry mass of the woody stems. This difference was observed for the control plants sampled after the feeding break and the final feeding and then another significant difference was observed for the treated plants after the same sampling periods. There was no significant difference between treatments at the same sampling period, however. The significant difference observed for the dry mass of the woody stems for some of the sampling events was surprising as the same difference was not observed for the wet mass. This may be a reflection of slightly different conditions that the plants were sampled in, causing a greater difference in the change from wet mass to dry mass, although this change was not observed in any other plant measurements at the same time period. This result does not show anything related to the impact of the agent on the plant, however, as the significant difference was observed between the same treatments across time, rather than the between different treatments across the same sampling events.

The length of the main stem was significantly different between treatments and across time, with the control showing a greater increase in length of main stem while the length of the main stems did not increase as much over time for the treated plants. Some plants store energy in their stems (Chapin *et al.* 1986; Slewinski 2012) and as the target weed was compensating for the herbivory it is possible that reserves in the stem were reducing, resulting in the difference in stem length between the control and larval treatment plants. However, this result may also just be due to the nature of the main stems. Although the control plants and treated plants were matched to have similar plant structure and size, including type of main stem, it is possible that there were more treated plants that had a main stem that did not grow as it was the base for other stems whereas, the control plants had less of those types of main stems, with more main stems simply being the main growing stem.

As the leaf-feeding flea beetle is a defoliator, the damage caused by the beetle can be seen most clearly by the percentage damage caused on the leaves and the number of leaves and mass of the leaves of the plants. When the larvae were feeding there was a significant increase in the

percentage damage over time with a decrease in the percentage damage observed at the end of the feeding break. Although this does show that the larvae were causing significant cumulative increases in damage levels on the plants when feeding, it also shows that the plants were able to recover fairly well and quickly as the percentage damage was significantly lower after the feeding break. This is important to note as it is likely that the plants will undergo periods of time without much feeding due to temperatures in the field being lower and the insect populations decreasing which has been observed in the field in South Africa. Although there are fluctuations in beetle population sizes due to winter, the growth rate of the plants is also greatly reduced in winter in the field in South Africa with some plants losing most of their leaves by the end of winter (pers. obs.). This could mean that the plants do not have enough time to recover before the beetle populations increase again, therefore increasing the chances of control of the target weed.

The number of leaves significantly increased over time for both the control and treated plants. There was a significant difference between the control plants and the treated plants after the third feeding period and after the fourth feeding period, immediately after the feeding break. This difference, due to feeding by the larvae, was emphasized by the lack of significant difference for the number of leaves between the two treatments immediately after the feeding break. If there had been no feeding break, and time for the treated plants to recover, it is possible that the significant difference in number of leaves between the two treatments would have become clearer and greater over time. This shows that over time the larvae can significantly decrease the number of leaves on the plants, but again, the plants do appear to be able to recover fairly quickly when feeding is stopped. However, with enough feeding and a cumulative decrease in the number of leaves it is possible that there will be a slow reduction in the ability of the plants to recover and effectively compete with other plants over time, which could not be observed in my short term study.

The wet mass and dry mass of the leaves showed some significant changes over time but only one sampling event had a significant difference between the treatments for the wet mass. This does not support the number of leaves data which showed a significant impact of the beetles on the leaves. It is possible that this is due to the feeding of the larvae, which mostly feed on newer leaves and not on the older, larger leaves. This could have impacted the mass data as the larger leaves weigh a lot more than the smaller leaves and would not have been as impacted as the smaller leaves so a significant difference in mass may not have been observed even if there was a significant difference in number of leaves. This could be supported by the wet mass and

dry mass graphs which do show the treated plants have a generally lower mass of leaves than the control plants after feeding events.

Paterson *et al*. (2014a) suggested that *Ph. guerini* may not be the most effective biological control agent for *Pe. aculeata* compared with all of the natural enemies of *Pe. aculeata* in its native range that were potential agents at the time. However, there are only two current biological control agents of *Pe. aculeata* (*Ph. guerini* and *C. schaffneri*) and *Ph. guerini* does appear to be a potentially effective agent based on this study and field data in South Africa. There were many factors that were not taken into account in this study that may have confounded these results causing an underestimation of the efficacy of the agent.

At some stage within a biological control programme a decision is made to prioritise agents and historically this has had to do with their host-specificity in the field or ease of rearing rather than their efficacy (van Klinken & Raghu 2006). The efficacy of an agent in a country in which it has already been released and established can help with the prioritising of agents for release in a new country. In low-income and middle-income countries, although they only have a few biological control programmes, their programmes often have a high success rate, and this is attributed to their using only the effective agent, or agents, from programmes against the same weed in countries that have more active biological control programmes (Day *et al*. 2020). By using other countries' success data they can determine the efficacy of biological control agents prior to release in their country and improve the success, decrease the risks, and decrease the costs of their biological control programmes (McClay & Balciunas 2005; Balciunas & Smith 2006; van Klinken & Raghu 2006; Day *et al*. 2020). From field studies in South Africa we know that *Ph. guerini* can have an impact on *Pe. aculeata* as it has been shown to be effectively damaging *Pe. aculeata* infestations at some sites and new sites with large and damaging populations of the beetle have recently been found (King *et al*. 2021).

A common prerequisite for successful biological control is that the released biological control agents will reach high densities in their introduced range (Müller-Schärer & Schaffner 2008). This can be predicted using climate matching which can predict firstly whether an agent will be able to develop in the introduced climate and then whether it will be able to undergo enough generations in a year to successfully establish and become abundant (McClay & Hughes 1995; McClay 1996; Muskett *et al*. 2020). This can help avoid failures that have resulted due to agents being unsuitably matched to the climate they are released in (Byrne *et al*. 2002; Day *et al*. 2013; Cowie *et al*. 2016). The climate where the beetle has established in South Africa and the

invaded area in Australia are similar and therefore it can be assumed that the beetle should successfully establish in Australia. It is important to note, however, that there is variable efficacy of the beetle in South Africa and this does not appear to be restricted to certain climatic conditions (King *et al*. 2021), so there are likely to be factors other than climate that affect the efficacy of *Ph. guerini* as an agent against *Pe. aculeata*.

Although it is ideal for a biological control agent to have an impact on both the aboveground and belowground biomass of their target weed, it is possible for an agent to be successful without completely effecting the plant's biomass (Brockerhoff *et al*. 1999; Wirf 2006; Cowie *et al*. 2018). Defoliators can be important agents, even if they don't destroy a plant, as they can reduce growth and fitness of the plant making it more susceptible to interspecific competition and various other forms of control that may be used against it (Erneberg 1999; Wirf 2006). Huang *et al.* (2012) showed that the invasive populations of Chinese tallow (*Triadica sebifera* (L.)) successfully outcompeted native plant populations in terms of biomass production when there were no natural enemies present, even if there was a high level of competition with other plant species. However, when the invasive Chinese tallow was grown with a more competitive neighbour its tolerance to herbivory was reduced, with its lowest biomass being recorded when it was fed on by specialist herbivores and competing with a strong neighbour (Huang *et al.* 2012). It is possible that if *Pe. aculeata* is in the presence of other plants and is damaged by *Ph. guerini,* it will be more heavily impacted and a less successful competitor, making its control more effective.

It is also possible that a plant being attacked by a defoliator will compensate for the defoliation by reducing sexual reproductive output (Weed & Casagrande 2010). This study did not include the plant's sexual reproductive outputs and so the impact of defoliation on the plant's sexual reproduction could not be measured. *Pereskia aculeata* produces fruit sporadically in South Africa with the magnitude of fruiting events being difficult to predict. A reduction in fruit production would reduce the chances of new pristine sites being infested by *Pe. aculeata*, because fruits being eaten by birds and bats is likely to be one of the mechanisms by which pristine areas are invaded. However, by far the most important mechanism of reproduction is through asexual vegetative reproduction, so any impact to fruit production is relatively unimportant.

Defoliation reduces the photosynthetic output of a plant and can reduce the plant's water use efficiency which could have a longer term impact on the plants (Cowie *et al*. 2018) and it is

possible that defoliation by the beetle may impact the target weed in these ways. It has also been observed in other biological control agents that defoliation allows for the introduction of fungi or pathogens that negatively impact the plant, increasing the control of the plant (Moran 2005). However, this has not been observed for *Ph. guerini* on *Pe. aculeata*.

The timing of defoliation is also very important for plants because plants can compensate for defoliation more under optimal conditions and depending on the plant's growth stage (Prins & Verkaar 1992; Dhileepan *et al*. 2000a). If a plant is putting more effort into new growth or reproduction then it may not be able to compensate for defoliation and if the defoliation occurs at this growth stage of the plant and under stressed conditions, then it may be more affected by defoliation than at other stages (Prins & Verkaar 1992; Dhileepan *et al*. 2000a). This study did not induce herbivory during a specific stage of growth in the plants, although the plants were placed under semi-stressful conditions to try and better mimic field conditions. It is unlikely that this had an effect on the results of this study, as *Pe. aculeata* tends to reproduce during autumn and early winter when the beetle numbers, particularly of the larvae, are greatly reduced (pers. obs.) and so there is reduced herbivory during the time that they would be less likely to compensate for defoliation in the field.

Although this study has not shown a great impact of the beetle on *Pe. aculeata*, there is some evidence that it does have an impact on the plant. There were many limitations to this study and field studies in South Africa have shown that the beetle is probably having more of an impact than originally thought and predicted (Mnqeta & Paterson 2019; King *et al*. 2021). This study measured a reduction in the number of leaves and shoot lengths, which would give other plants in the environment a greater opportunity to compete with *Pe. aculeata.* The observations in the field in South Africa should be considered along with this study and other laboratory based studies when considering whether *Ph. guerini* is an effective agent that should be recommended for release. The beetle has shown some levels of damage to the plants in laboratory based studies and has been shown to be effective at reducing the plant density in some areas in which it has been released in South Africa. It has the potential to be an effective agent that could have some significant impacts in areas in Australia, so the release of the beetle in Australia is recommended.

Chapter 4

General Discussion

The studies in this thesis aimed to determine whether *Ph. guerini* is a suitable biological control agent against *Pe. aculeata* for release against the weed in Australia. This was done through host specificity testing to determine the host range of *Ph. guerini* (Chapter 2) and a pre-release efficacy assessment which was used in conjunction with field data from South Africa to determine whether the flea beetle could be a suitably effective agent (Chapter 3). This chapter will discuss (1) the goals of biological control and how success is measured for a biological control programme, (2) the biological control programme against *Pe. aculeata* in South Africa, and (3) which of the available agents on *Pe. aculeata* should be prioritised for release in Australia. These will be used, in conjunction with the results from chapters 2 and 3 to determine the potential of *Ph. guerini* as a biological control agent against *Pe. aculeata* and make a recommendation for its use in Australia.

4.1 The goals of weed biological control

Eradication of a target weed is not an expected outcome of biological control (Briese 2000; Paterson *et al*. 2011b). Rather biological control aims to reduce the negative impact of an invasive alien plant by reducing its density and limiting its range, or to reduce the health risks or commercial impacts associated with the target weed (Briese 2000; Denslow & d'Antonio 2005: Morin *et al*. 2009). Since total eradication is not the ultimate goal of biological control, practitioners and researchers need to carefully define what success in a biological control programme looks like so that this can be measured and the outcome of the programme can be determined (Morin *et al*. 2009).

Early biological control programmes set an unrealistically high standard for the outcomes of biological control with massive reductions in the target weed density in very short periods of time for some of the first weeds ever targeted (Hoffmann & Moran 2008). However, dramatic reductions in weed density within short periods of time are a rare outcome of biological control, so more realistic measures of success need to be used. In South Africa, the success of a biological control programme has been historically determined by looking at the combined impact of the established agents for a biological control programme and the amount of alternative control needed alongside the biological control programme to reduce the target weed (Hoffmann 1995; Klein 2011; Zachariades *et al.* 2017; Hoffmann *et al*. 2019). These categories were (1) complete control; no other measure of control was needed where the agents

were established, (2) substantial control; less effort was required in using other methods to reduce the invasive alien plant to acceptable levels, (3) negligible control; control of the target weed relied on other measures although the agent was causing some levels of damage, and (4) not determined; either due to a lack of evaluation of the programme or the agent being released too recently for any meaningful evaluation to be done (Hoffmann 1995). These categories have also been used to determine success of biological programmes in Australia, New Zealand, and Hawaii (Schwarzländer *et al*. 2018).

Hoffmann *et al*. (2019) proposed that a new and more standardized method to determine the success of a biological control programme was needed. The new method no longer relies on the relative importance of other forms of control and was improved in several other ways. The previous method assigned a descriptive term to measure success resulting in an average term which did not necessarily represent what was happening with the biological control programme, as in one area there could be complete control of the target weed due to biological control, whilst in another area there was negligible control, but when averaged out the category assigned to that programme would be substantial control (Hoffmann *et al*. 2019). The new method proposed by Hoffmann *et al.* (2019) assesses how the trajectory of an invasion, by an invasive alien plant, has changed through the use of biological control. Moran *et al*. (2021) applied this new method to 54 species of invasive alien plants in South Africa that have biological control programmes against them.

The method breaks down the level of control achieved by biological control in the different habitats the target weed is found in, which can be useful as control is not always the same across habitats (Hoffmann *et al.* 2019; Moran *et al.* 2021). In each habitat the density, biomass, area, and rate of spread of the invasive alien plant is reviewed and assigned to one of three broader categories that describe the level of control achieved and then made more specific within each of those categories. The three categories are; (1) Category A: the invasive alien plant has declined to a level below a "tolerable threshold" as a result of biological control, resulting in the extent and impact of the invasion becoming trivial or non-existent, (2) Category B: the degree of the invasion is reduced due to the use of biological control to levels below a "reversal threshold" but the populations of the target weed are still above a "tolerable threshold", resulting in the invasive alien plant still being problematic but to a lesser extent than if biological control were absent, (3) Category C: the populations of the target weed continue to increase over time despite the use of biological control, although they may be

increasing at a slower rate and to a lesser extent than if biological control was not implemented (Hoffmann *et al*. 2019).

In South Africa, the biological control programme against *Pe. aculeata* using *Ph. guerini* had initially been categorised as "trivial" with limited impacts (Klein 2011; Paterson *et al*. 2014a). However, Mnqeta & Paterson (2019) argued that in some areas of South Africa the beetle was having an impact and its control should rather be categorised as "moderate". Using the new method of determining the outcome of a biological control programme, Moran *et al*. (2021) also evaluated the programme for *Pe. aculeata* in South Africa. They said that the previously used description for control of *Pe. aculeata* was "substantial" and then determined the level of control reached using their new system (Moran *et al*. 2021). As the only other agent against *Pe. aculeata* in South Africa (discussed below) is still a relatively new agent the control levels determined by Moran *et al*. (2021) are representative of the impact that *Ph. guerini* has had on the target weed in South Africa, and not any other agents. They determined that in terms of the density and rate of spread of *Pe. aculeata*, the biological control programme outcome thus far was given a category "B-", meaning that it was "less favourably suppressed requiring enhanced biological control efforts or other management interventions continuously". The biomass of *Pe. aculeata* as a result of biological control was classified as a category "B", meaning that *Pe. aculeata* was at a level such that "the situation has been stabilized but still requires other interventions intermittently". The area of *Pe. aculeata* in South Africa was not determined to be impacted by biological control, however, as Moran *et al*. (2021) categorized it as "C-" which means that "the extent and invasiveness of the weed is unaffected by biological control". This is still a better situation than would be the case if *Ph. guerini* were not present as it is likely that the presence of *Ph. guerini* is slowing the spread of *Pe. aculeata*.

Methods of control other than biological control are much more difficult to use on vines and climbing plants. Their climbing habit and the way in which they grow intertwined with other vegetation makes manual clearing labour intensive and economically unfeasible (King *et al*. 2021). For a vine like *Pe. aculeata,* manual clearing is also made more difficult by their ability to reproduce asexually as any part of the stem left behind may later fall to the ground and grow as a new plant. Chemical control is also difficult for use against vines as general broad leaf herbicides can only be used in certain cases due to the risk of non-target effects on the plants on which the vines are growing (King *et al*. 2021). Therefore, biological control is the only viable and sustainable method of control for invasive vines such as *Pe. aculeata* (Moran & Zimmermann 1991a; Paterson *et al.* 2011a; King *et al*. 2021).

As South Africa is the only country in which biological control has been used against *Pe. aculeata* so far, the outcomes of their programme are a good predictor of what biological control against the target weed in Australia could look like. Paterson *et al.* (2011a) stated that in South Africa the ultimate goal of the biological control programme against *Pe. aculeata* was to reduce infestations to below 30% and maintain them at this level, as this was the level of infestations that was shown to not have a significant impact on native plant biodiversity. Thus far, this level has not been reached as a result of biological control against the weed in South Africa (Mnqeta 2017). There have, however, also been additional sites with large, damaging populations of *Ph. guerini* observed in South Africa that have not been included in any studies and there has recently been a notable increase in damage being observed as a result of the beetle. It is therefore possible that the full extent and level of control that the beetle is having in South Africa has been underestimated (King *et al*. 2021). Australia is in an earlier stage of invasion of *Pe. aculeata* than in South Africa, and it is possible that *Ph. guerini* could have a greater impact at this early stage of the invasion than what it is capable of at a later stage when the weed is abundant and widespread. If *Ph. guerini* is released in Australia whilst *Pe. aculeata* is still a relatively new and emerging weed it could be a more successful programme than has been observed in South Africa thus far.

Biological control has often been a last resort option when the populations of an invasive alien plant are too high for eradication or effective control using other methods, with very few biological control programmes being initiated, against emerging weeds or invasive alien plants in the early stages of invasion (Olckers 2004). In general, the two ways in which a biological control programme against an emerging weed has started have been either due to the presence of a programme against the plant in another country, or due to the opportunistic collection of natural enemies of invasive alien plants of lesser importance when doing surveys for target weed species of more importance (Olckers 2004). Transfer projects, in which the most suitable agents from an area in which they have been introduced and established are used in another country, are cost effective and save time and resources, and can be of great help to countries in which the target weed is only just becoming an issue (Olckers 2004; Zachariades *et al*. 2017). In South Africa, St John's wort (*Hypericum perforatum* L. (Hypericaceae)) was accidentally introduced in the 1940s and within 18 years of the weed's introduction into the country a successful biological control programme was started due to the programmes against the weed in other countries in the world (Gordon & Kluge 1991; Olckers 2004). The quick action against the plant and the use of agents from biological control programmes in other countries resulted

in this weed becoming one of minor importance and under complete control (Olckers 2004). Of the 25 biological control programmes against emerging weeds in South Africa reviewed by Olckers (2004) 36% of the target weeds have been brought under complete control and 24% have been brought under substantial control. The release of *Ph. guerini* in Australia would be a transfer project. There is therefore a good understanding of the levels of control that are expected compared to a novel programme, a better chance of success than a novel programme, and far less resources required to develop the biological control programme than for a novel target. Although complete control may not be expected, a reduction in the invasion potential is likely if *Ph. guerini* were released in Australia, and this is a reasonable goal for the biological control programme because even a small reduction in the level of invasion can result in significant benefits (Hoffmann *et al*. 2019)

4.2 The biological control programme against *Pe. aculeata* in South Africa

The biological control programme against *Pe. aculeata* in Australia is in its beginning stages and is largely reliant on the South African programme. The climate in the invaded regions in Australia and South Africa are similar (Tatem & Hay 2007; Bennett 2011). Therefore it is likely that successful biological control agents from South Africa could be useful and establish in Australia (McFadyen 1998) and so it is important to understand the programme in South Africa.

In South Africa there are two agents that have been released against the target weed, *Ph. guerini* and *C. schaffneri*, both of which have successfully established in some areas (Mnqeta & Paterson 2019; Muskett *et al.* 2020; King *et al*. 2021). Another potential agent, *P. brasiliensis*, which showed promise as an effective agent was rejected for release as it was not suitably host specific, causing significant levels of damage on a few species from the Basellaceae family (Paterson *et al*. 2019b). Its broad host range also most likely excludes it from release in Australia. There have been some other agents considered for the control of *Pe. aculeata* in South Africa but due to difficulty with rearing in quarantine or the likelihood of attack of similar parts of the plants of the other two established agents they have been shelved or are not currently being considered (Klein 2011; Paterson *et al.* 2014a). Therefore, the only two current options for biological control against the weed in Australia, based on the work done in South Africa, are *Ph. guerini* and *C. schaffneri*.

Catorhintha schaffneri was released in South Africa in October 2014 after being shown to be suitably host specific and effective as a potential agent against *Pe. aculeata* (Paterson *et al*. 2014b; King *et al*. 2021). *Catorhintha schaffneri* is a stem-wilting bug that causes the growing

tips of the plant to wilt and die (Paterson *et al*. 2014b). It was prioritised as it was shown to be damaging in its native distribution and because it attacks the vascular tissue of *Pe. aculeata* which was determined to be important, as a seed or fruit attacking agent would not be sufficient as a control agent on its own due to the ability of *Pe. aculeata* to reproduce asexually (Paterson *et al*. 2014a; 2014b).

Muskett *et al*. (2020) used climatic-matching studies to determine the places in South Africa in which *C. schaffneri* was likely to successfully establish. They also did a field survey of the establishment of the bug throughout areas in South Africa where they conducted controlled releases of the agent and conducted ground-truthing of the climate-matching data, in which they checked if the agent established or failed to establish as predicted by climatic suitability studies (Muskett *et al*. 2020). *Catorhintha schaffneri* has successfully established at a minimum of five sites in South Africa and it would appear that climate (namely, humidity and average winter temperatures) was a reasonable predictor of the establishment of the bug in South Africa (Muskett *et al*. 2020). Rates of successful establishment of *C. schaffneri* have been low and the levels of damage observed at sites where the bug has established are varied (King *et al*. 2021). However, it is still too early to fully assess whether *C. schaffneri* is a successful agent against *Pe. aculeata* in South Africa or not (Novoa *et al*. 2019; King *et al*. 2021).

The establishment and success of *Ph. guerini* as a biological control agent against *Pe. aculeata* in South Africa has been studied to determine whether it is an effective agent or not (Mnqeta 2017; King *et al*. 2021). Paterson *et al*. (2011a) studied the impact of *Pe. aculeata* on native plant biodiversity to determine thresholds that would be appropriate in reviewing the success of the biological control programme against *Pe. aculeata*. They found that at a density of *Pe. aculeata* of 30% or less there was no significant difference observed on the impact of native plant biodiversity compared with if the weed were not present, making this the ultimate goal of biological control against *Pe. aculeata* (Paterson *et al*. 2011b). However, they also found that at densities of 50% *Pe. aculeata*, or under, there was significantly higher native plant biodiversity recorded than at sites with a density of *Pe. aculeata* that was greater than 50%, making a density of 50% *Pe. aculeata* a reasonable alternate goal for biological control programmes (Paterson *et al*. 2011b).

Mnqeta (2017) did a field agent-exclusion study at one of the sites in which *Ph. guerini* has established in South Africa to determine the impact of the agent. Paired 1 m^2 plots were used with one plot in which insects were excluded through the use of an insecticide and another plot

in which insects were left to feed at field densities on the plants (Mnqeta 2017). At the end of the study it was found that the percentage cover was 98% for the plots in which the beetle was excluded, which was significantly greater than the mean 63% cover for the plots in which the agents were able to feed (Mnqeta 2017). Although this 63% cover at the end of the study does not show that the agent was able to reduce the cover to one of the thresholds mentioned by Paterson *et al*. (2011a), the field exclusion study was a short term study and was only run for 100 days, so it is possible that the percentage cover may have reduced to 50% or below for the plots with beetles had the study been continued.

Although the establishment and impact of *Ph. guerini* has been variable in South Africa (Mnqeta 2017), there were highly damaging populations of the agent found in South Africa and additional sites in the Eastern Cape Province in South Africa have now been observed with large and damaging populations (King *et al*. 2021). At sites in which the population density of the agent is high enough the agent has been shown to provide some level of control of *Pe. aculeata* in South Africa (King *et al*. 2021).

There are also potential factors that limited the initial establishment and success of *Ph. guerini* in South Africa that could be taken into account if the agent is released in Australia. The establishment of the biological control agent, *E. catarinensis* used against water hyacinth in South Africa was predicted to be limited by cold winters and this was initially supported by the distribution of the agent (Coetzee *et al*. 2007). However, the agent was later found at a site that was predicted to be too cold for it to have established (Griffith *et al*. 2019). Microevolutionary changes in the thermal physiology of *E. catarinensis* appeared to have occurred since their establishment (Griffith *et al*. 2019). Griffith *et al*. (2019) demonstrated that if an agent successfully establishes at a cold site, the insects can develop local adaptations that enable them to be better suited to the thermal environment they find themselves in. It is possible that *Ph. guerini* took a long time to show successful establishment and damage in South Africa as these sites were not climatically favourable for the agent but they may have now adapted to the climate in the same way as *E. catarinensis* has. If this is the case, it may be worth following the suggestion by Griffith *et al*. (2019) and sourcing populations of *Ph. guerini* from sites in which they have established successfully in South Africa that have similar climates to the sites in which they are to be released in Australia. This could help increase their chances of establishment and reduce the time it takes to see impact caused by the beetle.

Paterson *et al*. (2012) investigated whether the success of *Ph. guerini* was determined by the genotype of *Pe. aculeata* it was feeding on as different genotypes can sometimes influence the fitness of an insect herbivore. *Pereskia aculeata* has a large native distribution and the genotype of *Pe. aculeata* found in South Africa is genetically distant from those in the native range (Paterson *et al*. 2009). However, it was found that there was no difference in the fitness of *Ph. guerini* when developing on different genotypes of *Pe. aculeata* (Paterson *et al*. 2012). It is therefore unlikely that a genetic mismatch has resulted in reduced efficacy of *Ph. guerini* in South Africa and the plant genotype(s) present in Australia are also unlikely to influence the efficacy of *Ph. guerini* as an agent.

Mass-rearing can also be used to increase the chances of establishment of an agent, as well as to augment populations in the field and thus increase the level of control provided (Hill *et al*. 2021). Although mass-rearing requires some level of expertise, it can also be taught to non-Entomologists who can do the main work for mass-rearing and releases of agents at more sites without using expert's time and resources (Hill *et al*. 2021). Frequent and large releases of *Ph. guerini* are likely to have aided establishment and control in South Africa, and the same is likely to be true for Australia.

4.3 Which potential agent should be prioritised for release in Australia?

There are two general approaches to how many agents should be released against a target weed and therefore how to prioritise agents for release. One approach is the "cumulative stress model" in which multiple agents are needed in combination to control the weed through cumulative damage (Harris 1985). The other approach is the "lottery model" in which control of the target weed is achieved by one agent only, although the "correct" agent was usually only found through the release of multiple agents (Myers 1985). Biological control practitioners use efficacy assessments to try to predict which agents will be the most effective to ensure the release of the most damaging agent without having to release multiple agents where only one agent is needed for successful control of a target plant. It is also important to determine which agent is more effective and therefore worth prioritising as it is possible that there may be negative interactions between different agents on the same weed which would reduce the success of the biological control programme compared to if only one agent were present (Crowe & Bourchier 2006).

A greenhouse based experiment was done to determine the relationship between *C. schaffneri* and *Ph. guerini* and it was found that there was an antagonistic and inhibitory interaction

between the two agents with lower levels of damage experienced when the agents were both on the plant compared with plants that had only *C. schaffneri* on them (Mnqeta & Paterson 2019). The study also showed that *C. schaffneri* was more damaging on *Pe. aculeata* when on the plant alone than if *Ph. guerini* was feeding on the plant alone (Mnqeta & Paterson 2019). Although the two agents attack different parts of the plant, the wilting of the stems by *C. schaffneri* also resulted in the wilting of the leaves on the entire stem which resulted in less suitable food for the leaf-feeding flea-beetle (Mnqeta & Paterson 2019). This antagonistic relationship between the two agents means that they should not be released together as a combination of the two agents is not likely to be more damaging than if only one of the agents is present (Mnqeta & Paterson 2019). Both agents are relatively poor dispersers, so there is limited dispersal to sites where the agents have not been released. It is therefore recommended that only one agent is released at each *Pe. aculeata* infestation (Mnqeta & Paterson 2019).

Both agents have been shown to be suitably host specific as biological control agents against *Pe. aculeata* (*Ph. guerini* – chapter 2; *C. schaffneri* – Paterson *et al*. 2014b). Although Mnqeta & Paterson (2019) showed that *C. schaffneri* was more damaging than *Ph. guerini* in a greenhouse study, both field surveys in South Africa (Mnqeta 2017; King *et al*. 2021) and my study (chapter 3) have shown that *Ph. guerini* can be a damaging agent. Extrapolation of laboratory based or greenhouse studies to the field should be done carefully as there are many important limitations that these studies cannot take into account, such as different environmental conditions and the impact of the agents on more than just an individual plant (Morin *et al*. 2009). *Phenrica guerini* has successfully established in more places in South Africa than *C. schaffneri* and is proving to be more damaging than it was originally thought to be (King *et al*. 2021). Although it is still too early to fully determine the rates of establishment and damage caused by *C. schaffneri* in the field in South Africa (Novoa *et al*. 2019), the current situation indicates that *Ph. guerini* is the more damaging of the two agents in the field (King *et al*. 2021). An application for the release of *Catorhintha schaffneri* in Australia was submitted in January 2021 but as of October 2021 further testing was required before approval for release of the bug was given (CSIRO 2021). Based on the field results from South Africa it would seem that *Ph. guerini* is worth prioritising over *C. schaffneri* for release as a biological control agent against *Pe. aculeata* in Australia until further evidence of the success of *C. schaffneri* is recorded in South Africa.

4.4 Conclusion

Phenrica guerini is suitably host specific and potentially effective as a biological control agent against *Pe. aculeata*. It is recommended for release in Australia, where it is likely to reduce the invasion potential of *Pe. aculeata* with subsequent reductions to the costs of the invasion, even if it does not obtain levels of "complete control". A post-release evaluation should be conducted after the release of the flea-beetle in Australia to determine its establishment and success. These post-release data can also be used to determine whether *C. schaffneri* should be considered as an additional biological control agent.

Chapter 5

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