

# Investigations into the potential of classical biological control of the invasive aquatic weed, *Crassula* *helmsii*

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## Declaration of Authorship

I, Sonal Varia, hereby declare that this thesis and the work presented in it is entirely my own with the exception of the following:

- Marion Seier assisted in the design and assessment of the fungal natural enemies of *Crassula helmsii* as outlined in chapter 3
- Suzy Wood assisted in the host-range testing assessments of *Hydrellia perplexa* as outlined in chapter 3 and the host-range testing assessments of *Aculus crassulae* in chapter 4, under my supervision
- Robert Allen assisted in the host-range testing of *Aculus crassulae* and assessment of the impact study assessments and data analysis as outlined in chapter 4 under my supervision
- Tim Beale developed the maps of the potential distribution of *Aculus crassulae* in chapter 4
- Corin Pratt assisted in the assessment of the application of glyphosate and *Aculus crassulae* as outlined in chapter 5 under my supervision
- Aylin Kerim assisted in the data collection of the mite phenology experiment as outlined in chapter 6 under my supervision

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## Abstract

*Crassula helmsii*, a semi-aquatic plant native to Australia and New Zealand, was first introduced to the UK over a hundred years ago. Since then, it has spread across much of the UK and is present in parts of Western Europe. *Crassula helmsii* can dominate aquatic habitats and there are limited options for its management, particularly in protected habitats. Consequently, alternative methods of control have been encouraged by the UK government. The potential for using classical biological control for *C. helmsii* was investigated and the results are presented in this thesis. Assessments of the plant and its associated natural enemy complex in south-eastern Australia were made and several natural enemies with high potential as biological control agents were selected and underwent further investigation to assess their suitability. The gall-forming mite, *Aculus crassulae* (Eriophyidae) was found to be the most promising natural enemy based on its high host specificity and the damage inflicted on *C. helmsii*. Further laboratory-based assessments predicted its ability to tolerate the low temperatures experienced during winters in the UK confirming that there was potential for the mites to survive in the UK. Field experimentation with *A. crassulae* was also undertaken and mite populations and *C. helmsii* growth were monitored, verifying predictions that the mites were able to survive natural conditions in the winter and develop robust populations in the spring and summer. Furthermore, the effect of shade was assessed and it was found that winter survival of mites increased under these conditions.

With herbicide application being the main control measure employed against *C. helmsii*, the impact of the use of a low dose of glyphosate in combination with the mites was also investigated. Findings were varied although the trend for increased plant decay when both were applied together warrants further study into the interactions between the mite and the herbicide.

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# 1 General Introduction

The spread of invasive non-native aquatic and riparian plants is a growing problem in Europe with wide-ranging ecological, economic and social impacts. In recent years, the need to work collectively to mitigate the threat of invasive species in Europe has been recognised, culminating in the adoption of European Union (EU) regulation 1143/2014 in 2014, aimed at preventing the entry and spread of invasive alien species into the EU (Genovesi *et al.*, 2015). Specifically in the UK, in 2003 the government established the “Great Britain Invasive Non-native Species Strategy”, outlining a plan to deliver a coordinated approach for addressing the threats posed by invasive non-native species (Great Britain non-native species secretariat, 2015). As part of this long-term strategy, the UK government has funded research into the biological control of several aquatic and riparian weeds, including *Crassula helmsii* (Kirk) Cockayne, commonly known as Australian swamp stonecrop.

Since its introduction from Australia over a hundred years ago, *C. helmsii* has spread across much of the UK and is also present in parts of Western Europe. This weed can dominate aquatic habitats, alter native plant species composition (Smith & Buckley, 2015) and affect recreational activities at infested sites. The current control of *C. helmsii* has been found to be challenging as the limited options for management can be expensive and damaging to the environment; this is of particular concern in protected habitats. There is a strong desire and need for an alternative solution.

The overall aim of the research presented here was to find classical biological control agents suitable for release against *C. helmsii* in the UK. Earlier studies had indicated that there was potential for the classical biological control of *C. helmsii* (Varia & Shaw, 2013). The investigations in this thesis outline the process from the natural enemy surveys in the native range of the weed to identify putative biological control agents, through to the trial release of the selected biological control agent. The agent selected was the gall-forming mite *Aculus crassulae* Knihinicki & Petanović (Eriophyidae).

The content of the thesis is as outlined: following on from the general introduction, chapter 2 gives a literature review of the field of invasive non-native species and biological control of weeds, before reviewing the literature on *C. helmsii* and the research into the biological control of *C. helmsii*. At the end of this chapter, the specific objectives of this thesis are given.

The natural enemy surveys that took place in Australia from 2011 to 2013 are described in chapter 3. Here, assessments of the plant and its associated natural enemies are made. Several natural enemies with high potential as biological control agents were selected and underwent further investigation including host-specificity testing, to assess their suitability. The mite, *A. crassulae* was prioritised as having the most potential based on the biology of mites in this superfamily (Eriophyoidea) and observations made in the field in Australia.

Further laboratory-based experiments were undertaken and are presented in chapter 4, based on the findings in chapter 3. Investigations into the effect of temperature on the development of *A. crassulae* took place with the aim of determining the degree day requirement of the mite and therefore the potential number of generations *A. crassulae* could complete in the field in the UK. The ability of this mite to tolerate the low temperatures experienced during winters in the UK was also evaluated. Host-specificity testing and the impact of *A. crassulae* feeding on *C. helmsii* growth was investigated to give an overall assessment of *A. crassulae* as a biological control agent.

In chapter 5, a low-dose application of glyphosate, the current main control measure used against *C. helmsii*, in combination with *A. crassulae* was investigated, including the identification of the most suitable dose of glyphosate for use in combination with the mite.

Field experimentation with *A. crassulae* was undertaken in chapter 6, where mite populations and *C. helmsii* growth were monitored over 10 months following government approval to release *A. crassulae* from quarantine conditions. This study investigated the population dynamics of mites under semi-natural conditions between winter and the following autumn and the impact of shade on both, mite populations and *C. helmsii* growth. The aim of these studies was to provide information for a release strategy of future field releases.

Finally, chapter 7 discusses the factors affecting the performance of *A. crassulae* as a biological control agent and the extent to which the mite could contribute to the successful management of *C. helmsii* in the future.



## 2 Literature Review

### 2.1 Invasive non-native species

Invasive non-native species (INNS) also termed invasive alien species (IAS), have been listed as one of the main threats to global biodiversity second only to habitat loss, and it is widely accepted that invasive non-native species can cause irreversible damage to the environment (IUCN, 2017). It was estimated in 2001 that damage from INNS worldwide totalled more than \$1.4 trillion - five percent of the global economy (Pimentel *et al.*, 2001). More recently for Great Britain, the annual cost of INNS was calculated to be £1.7 billion with almost a third of these costs attributed to invasive non-native plants (Williams *et al.*, 2010). With increased trade and movement of people, organisms are now more easily transported around the globe than ever before resulting also in the increased risk of invasion by INNS (Hulme, 2009; Seebens *et al.*, 2015; Seebens, 2019).

Invasive non-native species have wide ranging ecological impacts at the species, community and ecosystem level through competition, hybridisation or by causing major ecosystem changes (Pimentel *et al.*, 2001; Vilà *et al.*, 2011). Some of the most environmentally damaging and widespread INNS inhabit aquatic systems and community level effects can be vast (Gallardo *et al.*, 2016). Freshwater habitats in particular are heavily invaded by non-native species (Strayer, 2010) and some of the most damaging invasive aquatic species are plants. In aquatic systems native plant species play an important role as “ecosystem engineers”, regulating the availability of resources for other species by causing physical changes in abiotic and biotic materials (Jones *et al.*, 1994). Not only do these natives provide food and habitats for other organisms, they can also help control erosion and play a role in nutrient cycling. Any establishment of invasive non-native plants can therefore transform such ecosystems by disrupting these processes (Strayer, 2010). This problem is illustrated by water hyacinth, *Eichhornia crassipes* (Mart.) Solms, one of the world’s most destructive invasive aquatic plant species; the presence of this invader has been shown to reduce water quality, decrease phytoplankton availability and impact the abundance of fish (Villamagna & Murphy, 2010).

Many hypotheses have been proposed to explain why some non-native species become invasive in their introduced range. One of the major hypotheses is the Enemy Release Hypothesis (ERH) (Keane & Crawley, 2002) which suggests that on

introduction to an exotic region, plants experience a decrease in regulation by herbivores and other natural enemies, resulting in a rapid increase in distribution and abundance. This theory is based on the assumptions that host specific natural enemies will be absent from the new region, host switching by specialist enemies of native congeners will be rare and generalists will have a greater impact on native competitors (Keane & Crawley, 2002). A related theory, the Evolution of Increased Competitive Ability (EICA) suggests that in the absence of its natural enemies, selection will favour individuals that are more competitive, i.e. have higher reproductive success and reduced allocation to herbivore defence (Blossey & Nötzold, 1995). Consequently, over time plants will evolve to be more competitive in their introduced range than in their native range. Enemy release is not hypothesised to be the dominant mechanism behind all plant invasion however, the biotic resistance hypothesis predicts that more diverse communities have greater resistance to invasion by non-native species (Levine *et al.*, 2004) and the physical environment may also play a role. In addition, the production of allelopathic chemicals in the invader as described in the Novel Weapons Hypothesis (NWH) could also contribute to invasion success (Callaway & Ridenour, 2004). More recent studies have further developed concepts of invasion, showing for example that native and invasive plant species can occupy the same niche but invasive species are more competitive and are associated with productive habitats which are more likely to be invaded than resource-poor habitats (Dalle Fratte *et al.*, 2019). Many of the theories of invasion ecology go some way in explaining how and why a plant becomes invasive, however invasion is likely to involve multiple mechanisms (Blumenthal, 2005; Warren *et al.*, 2018) and there have been efforts to integrate these into conceptual frameworks (Gurevitch *et al.*, 2011). However, the ERH and EICA underpin the ecological theory behind the use of classical biological control by reuniting the natural enemies which contribute to the regulation of pest populations in their native range, with the pest in the introduced range, and biological control researchers continue to use these theories as guiding principles today.

## **2.2 Biological control of weeds**

Biological control has been defined as “the study and utilisation of parasites, predators and pathogens for the regulation of host population densities” (DeBach, 1964). There are three types of biological control: (a) “conservation” is the

protection or maintenance of existing populations of natural enemies, (b) “augmentation” involves artificially increasing populations of biological control agents at times when the pest population begins to grow; this can be done by periodic releases or by environmental manipulation, and (c) “classical biological control” which is the importation and release of exotic biological control agents, to exert permanent control in the introduced area of the pest (McFadyen, 1998). In practice various combinations of these core strategies are used.

Classical biological control of weeds has a long history; one of the most successful and well-known cases is the control of *Opuntia* spp. in eastern Australia in the 1920s. Prior to the release of biological control agents, over 60 million acres were infested by *Opuntia* spp. cacti (Dodd, 1959). The cochineal insects, *Dactylopius* spp. were released and successfully provided some control. Later in the 1920s, the South American moth, *Cactoblastis cactorum* Berg was released and by 1933 it was estimated that 90% of the *Opuntia* spp. in Queensland had been destroyed by *C. cactorum* (Walton, 2005). These agents have since been used successfully in other parts of the world. The benefit-cost ratio, the ratio between the value of the benefits arising from the use of biological control to the value of the costs, was calculated as 312.3:1 (Page & Lacey, 2006). The earliest examples of successful weed biological control programmes involved insects, but it was not until 1971 that a plant pathogen was used as a classical biological control agent (Winston *et al.*, 2014). That year, the rust fungus, *Puccinia chondrillina* Bubák & Syd. was introduced from Italy to Australia to control rush skeletonweed, *Chondrilla juncea* L. marking the first time that a plant pathogen had been deliberately imported from one country to another and studied in detail for this purpose (Hasan, 1974). Within three or four seasons, death of the rootstock of the plants was recorded and farmers began to report increases in wheat yield in previously infested plots (Cullen, 2012). The benefit-cost ratio of the whole program has been estimated to be 112:1 (Marsden *et al.*, 1980). Since then, the use of fungal plant pathogens as biological control agents has become more common and there have been many cases of successful introductions.

The success of rate of weed biological control agents has been assessed on several occasions (Crawley, 1989; Heimpel & Mills, 2017; Schwarzländer *et al.*, 2018). Using data published in the latest catalogue of biological control agents it was estimated that globally, 63% of all biological agents released established, increasing to 71% if the calculation included cases where the same agents were released in

different countries. It was also found that 66% of releases resulted in some level of control and this varied between regions, with the Caribbean experiencing the most impact. This was attributed to the fact that this region has released biocontrol agents against weeds in the Cactaceae family and aquatic weeds, both of which have had high success rates (Winston *et al.*, 2014; Schwarzländer *et al.*, 2018).

There are many factors which can affect the success of a biological control agent including predation, competition with native species or other agents, climate and poor release procedures (Harms *et al.*, 2020). The significance of predation and parasitism in weed biological control were first highlighted by Goeden and Louda (1976). Particular insect orders are known to be especially affected and it has been demonstrated that of the main insect orders released as biological control agents, Diptera have the highest parasitism rates followed by Lepidoptera (McFadyen & Spafford Jacob, 2004). The presence of closely related native insects in the introduced area also increases the likelihood of parasitism (McFadyen & Spafford Jacob, 2004). The dipteran control agents released against hydrilla, *Hydrilla verticillata* (L.f.) Royle in the USA, *Hydrellia pakistanae* Deonier and *H. balciunasi* Bock are known to be parasitised by a native parasitoid which is thought to have negative impacts on fly populations although this is currently unproven (Coon *et al.*, 2014). Incompatibility between the target weed and biological control agent is another important factor particularly with plant pathogens but is becoming less prevalent as new molecular and modelling techniques have become more widely available (Harms *et al.*, 2020). For example it was found with the bridal creeper rust, *Puccinia myrsiphylli* (Thüm.) Wint., that populations of the host, *Asparagus asparagoides* (L.) Druce in Australia were only susceptible to rust strains from the winter rainfall region in South Africa and not other regions (Morin & Edwards, 2006). Incompatibility of climatic conditions between the area of origin and the area of introduction of the agent is accepted as one of the main reasons why some agents fail to establish (Robertson *et al.*, 2008). Precipitation can affect agents by influencing humidity, physically damaging individuals or affecting plant quality (Harms *et al.*, 2020). Climate matching using software such as CLIMEX is now recommended for identifying the most climatically suited region to search for natural enemies (Hoelmer & Kirk, 2005). Despite numerous examples of the successful utilisation of classical biological control globally (Schwarzländer *et al.*, 2018), its use is a relatively new concept in Europe. One early experience in the UK in 1969 involved the release of the leaf beetle, *Altica carduorum* Guérin-Méneville from

France to control the native European weed, *Cirsium arvense* (L.) Scop., but the beetles did not overwinter successfully (Baker *et al.*, 1972). Another attempt in the 1970s and 1980s involved the introduction of the leaf beetle *Zygogramma suturalis* F. against *Ambrosia artemisiifolia* L. in Georgia, Ukraine and the former Yugoslavia, again with little or limited success (Igrc *et al.*, 1995; Reznik *et al.*, 2008; Winston *et al.*, 2014). More recently, there have been deliberate releases of four classical biological control agents to control their respective invasive weed hosts in Europe and these are largely still in the experimental phase; 1) the psyllid, *Aphalara itadori* Shinji for the control of Japanese knotweed, *Fallopia japonica* (Houtt.) Ronse Decr. in the UK (Shaw *et al.*, 2011), 2) the rust fungus, *Puccinia komarovii* var. *glanduliferae* R. A. Tanner, C. A. Ellison, L. Kiss and H. C. Evans for the control of Himalayan balsam, *Impatiens glandulifera* Royle in the UK (Ellison *et al.*, 2020), 3) the gall wasp *Trichilogaster acaciaelongifoliae* (Froggatt) for the control of *Acacia longifolia* (Andrews) Willd. in Portugal (Marchante *et al.*, 2017) and most recently 4), the mite, *A. crassulae* for the control of *C. helmsii* in the UK (Varia *et al.*, 2019); which is the subject of this thesis.

Prior to the release of a biological control agent, certain procedures are recommended in the International Plant Protection Convention (IPPC) “Code of Conduct for the Import and Release of Exotic Biological Control Agents”, International Standards for Phytosanitary Measures (ISPM) No.3 (Nowell & Maynard, 2005). Determining the host range of the agent is considered to be one of the most important of these procedures. The practice is based on the centrifugal phylogenetic method proposed by Wapshere (1974) and has been used successfully for several decades and continues to serve as the basis of current host-range testing protocols. Plants are selected based on their phylogenetic relatedness to the target weed, with those that are the most closely related being considered the species at higher risk and those that are more distantly related at lower risk. Biogeographic overlap, ecological and morphological similarity and the economic importance of plants in the region where the agent will be released are also considered when selecting a test plant list (Briese, 2005). As molecular methods have developed, it has been suggested that more emphasis is placed on molecular phylogeny rather than the traditional taxonomic nomenclature in choosing test plant species (Briese & Walker, 2008). Host-specificity testing generally takes place under quarantine conditions in the laboratory, and can be complemented with open field tests in the native range of the agent under consideration. In addition to host-specificity testing,

investigations into the taxonomy, biology and life history as well as the establishment potential of the biological control agent are undertaken. This information is then compiled in a Pest Risk Analysis (PRA) and submitted for evaluation to the appropriate national regulators. In the UK, an application for the release of an arthropod agent is made under the Wildlife and Countryside Act 1981, which ordinarily restricts the release of organisms into the wild. The PRA is also reviewed by the devolved governments, scientific and non-scientific independent reviewers as well as subject to public consultation and final ministerial approval before any release can occur (Shaw *et al.*, 2016). Under special circumstances, the PRA may be reviewed by stakeholder groups rather than the public consultation (M. Everatt, pers. comm.). Microorganisms are not regulated by the Wildlife and Countryside Act 1981 and as a result, the process of releasing a plant pathogen as a biological control agent is slightly different to arthropods. In this case, the PRA is also reviewed by the EU Standing Committee on Plant Health (Shaw *et al.*, 2016). Researchers petitioning for the release of an exotic biological control agent internationally undergo a similar process to that in the UK (Government of Canada, 2015; USDA, 2017).

Although the importance of proper regulation is recognised, biological control researchers have found that increasingly stringent risk assessments can become constrictive, particularly under highly bureaucratic systems (Barratt *et al.*, 2018; Messing & Brodeur, 2018), and could overestimate the risks associated with the release of some potential agents, leading to missed opportunities (Hinz *et al.*, 2014). Increased regulation regarding Access and Benefit Sharing (ABS) of genetic resources is also becoming more restrictive for biological control researchers. In the past, free exchange of biological control agents was possible and benefitted many in both developing and developed countries. However, where governments have adopted the regulatory requirements of the Convention on Biological Diversity (CBD) without strategic planning, or clear allocation of regulatory responsibility, the overseas activities of many biological control programmes worldwide have been affected (Silvestri *et al.*, 2020). Another challenge for biological control researchers in some regions is the reduction in educational and governmental support. Despite major successes, institutional support from some countries implementing weed biological control has significantly reduced in recent years, leading to a potential loss of capability in the future (Palmer *et al.*, 2014; Moran & Hoffmann, 2015; Messing & Brodeur, 2018).

## 2.3 *Crassula helmsii*

### 2.3.1 Taxonomy and nomenclature

*Crassula helmsii*, also known as Australian swamp stonecrop or New Zealand pigmyweed, is a member of the Crassulaceae family. This is the largest in the order Saxifragales, and is a family of succulent species, many of which are ornamental. The genus *Crassula* is one of over 30 genera within Crassulaceae. The Crassulaceae family includes over a thousand species, occurring worldwide but predominantly in dry or rocky habitats with centres of diversity in Mexico and South Africa (Thiede & Eggli, 2007).

### 2.3.2 Introduction and distribution

*Crassula helmsii* is native to Australia and New Zealand and is most common in the south-eastern Australian states of New South Wales, Victoria and Tasmania, although it has also been recorded in Western and South Australia. In New Zealand, it is only found on the South Island (Webb *et al.*, 1988). It was first imported to the UK from Australia sometime before 1914 (Swale & Belcher, 1982) and from 1927 sold commercially as an oxygenating plant for aquaria and ponds (Laundon, 1961). Early investigations into the genetic variation of *C. helmsii* as expressed in isoenzymes indicated that there was only one introduction of the species into the UK originating from the Murray River (Dawson, 1994); this river forms the border between New South Wales and Victoria, draining a considerable area of south-east Australia. A more recent preliminary study using molecular methods also indicated that plants in the UK were more similar to plants originating from Australia rather than New Zealand (G. Houliston, unpublished). *Crassula helmsii* was first found in the wild in Essex in 1956, most likely as a garden escapee (Laundon, 1961), and has since gradually spread throughout the country and is now naturalised in all parts of the UK (Figure 2.1). Its European distribution includes Germany, Belgium, Ireland, the Netherlands, Denmark, France, Spain, Italy and Austria, however it is most widespread and is expanding its range in the temperate regions of Northwest Europe, particularly in lowland regions (Denys *et al.*, 2014; EPPO, 2016). It has also been recorded in the USA (EPPO, 2016). In the UK, until recently, *C. helmsii* was sold in plant nurseries as a pond plant and was only banned from sale after legislation came into force in April 2014. It is also listed on Schedule 9 of the Wildlife and

Countryside Act 1981 making it an offence to plant or otherwise cause it to grow in the wild.

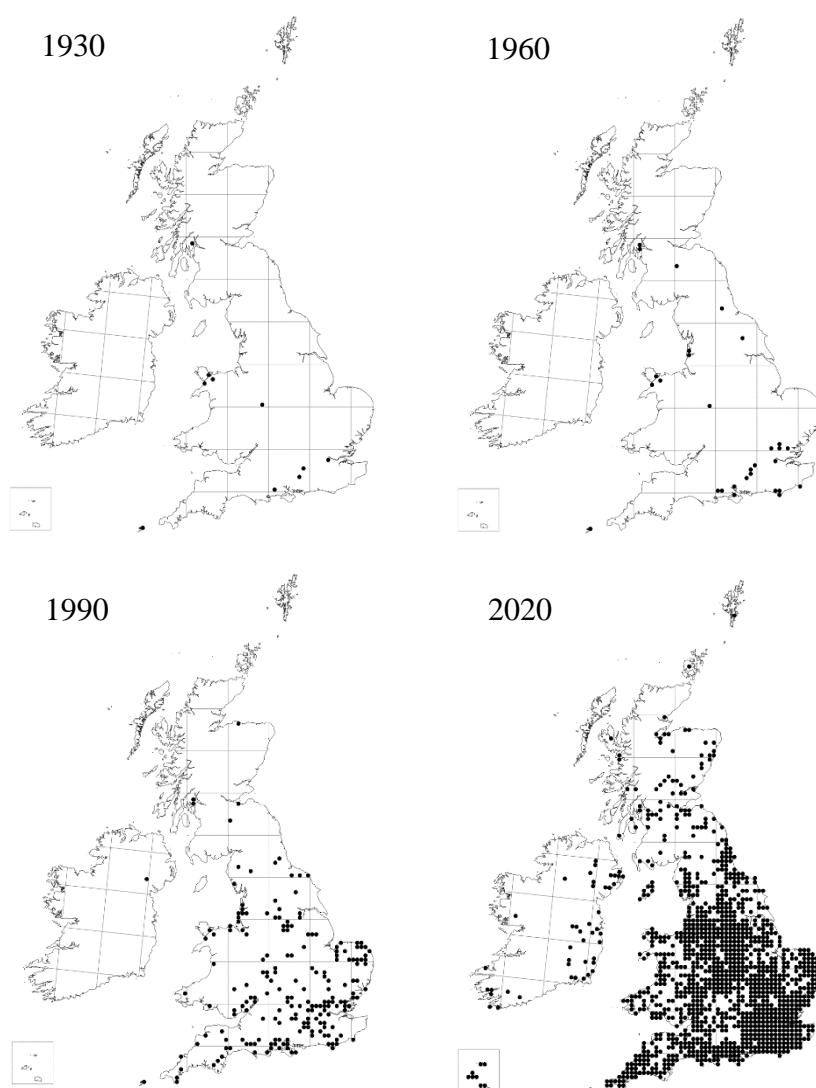


Figure 2.1: The distribution of *Crassula helmsii* in the UK between 1930 and 2020, each dot represents at least one record in each 10km x 10km square of the national grid (map courtesy of Botanical Society of Britain and Ireland, accessed April 2020).

### 2.3.3 Biology of *Crassula helmsii*

*Crassula helmsii* is a semi-aquatic, succulent, perennial herb that occurs in three growth forms; terrestrial, emergent and submerged. The growth form exhibited is dependent on the depth of the water in which the plant is found. The submerged growth form is generally more elongated than the other two forms and can be found in water up to 3m deep (Dawson & Warman, 1987). In the terrestrial habitats of



damp margins of water bodies, growth is creeping or erect. An inhabitant of still and slow-moving water bodies, *C. helmsii* is very tolerant of extreme environmental conditions including temperatures below 0°C, extended periods of drying, shade, and it can grow in both fresh and brackish water (Dean *et al.*, 2013). *Crassula helmsii* displays a preference for substrates with higher nutrients in the laboratory (Hussner, 2009), however this was not replicated in a field study by Dean (2015), where no preference was recorded. The plant is winter green providing a competitive advantage against other aquatic species that die back in the winter. Flowers found on emergent and terrestrial plants are borne singly on axils and are pale pink to white in colour (Figure 2.2A) (Dawson & Warman, 1987). Roots form at the nodes. Reproduction is mainly vegetative and spread is by fragments; although, there is some evidence that *C. helmsii* can also reproduce by seed in the introduced range. However, seed development from fruits and germination is known to be low, so dispersal by seed is regarded as a minor mode of spread (D'hont *et al.*, 2016). Movement of viable fragments to new sites is mainly anthropogenic, via boating equipment, on clothing or machinery for control, and by wildfowl (Lansdown, 2015). In its introduced range, *C. helmsii* grows in thick, impenetrable mats, dominating the waterbody it inhabits (Figure 2.2B). In the native range however, populations rarely form these dense mats and the plant is an ordinary member of the plant community (pers. obs.) although Dawson (1989) noted that at some sites in Australia the density of plants was comparable to that found in Britain. *Crassula helmsii* possesses the ability to use the Crassulacean Acid Metabolism (CAM) which allows plants to take up CO<sub>2</sub> during the night as an evolutionary response to drought. The presence of CAM in *C. helmsii* allows the plant to minimise carbon loss during respiration and as such provides the plant with a competitive advantage in growth over other aquatic plants in the same environment (Klavsén & Maberly, 2009).

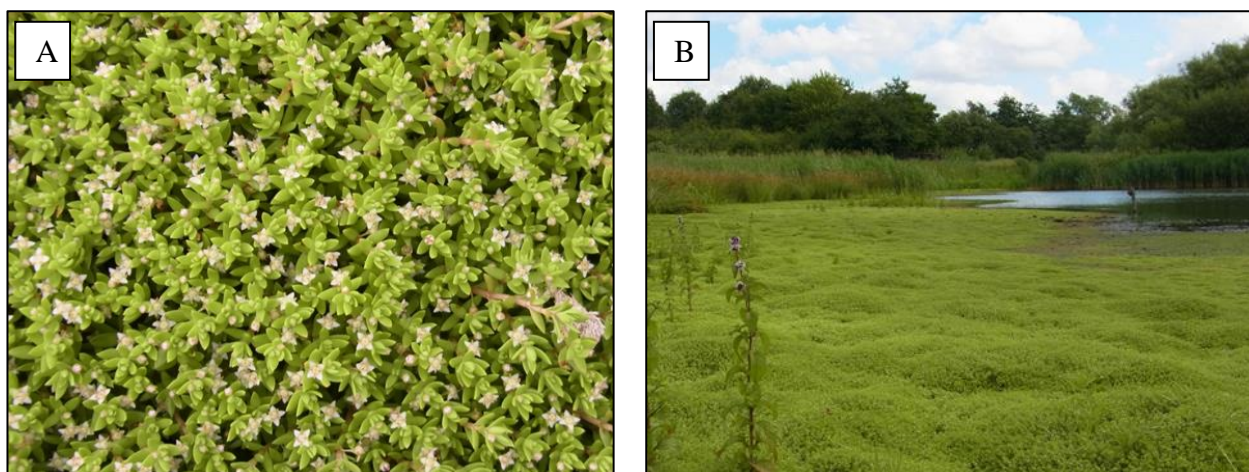


Figure 2.2: A; *Crassula helmsii* in flower, B; an infestation of *C. helmsii* in the West Midlands, UK.

### 2.3.4 Impact of *Crassula helmsii*

*Crassula helmsii* is considered an invasive non-native species and thus a threat in the UK (Dawson & Warman, 1987). When *C. helmsii* grows in dense, monospecific mats, there may be negative impacts on the environment, recreation, and blocking of filters essential for water treatment. There are also concerns of the water industry that the presence of *C. helmsii* in reservoirs may either directly or indirectly affect the quality of drinking water (K. Hills, pers. comm.). *Crassula helmsii* is capable of causing fluctuations in dissolved oxygen, carbon dioxide and nutrient levels in infested water bodies which can have wide-ranging impacts on aquatic species (Diaz, 2012). There is little empirical evidence in the literature showing that *C. helmsii* has a negative impact on native biodiversity which has led to the conservation community questioning its impact (Lockton, 2010). Much of the evidence is anecdotal with reports of reduced abundance of several aquatic or marginal plant species where the weed has established including *Damasonium alisma* Mill. (Watson, 2001), *Nymphaea* spp. (Swale & Belcher, 1982), *Elodea* spp. (Leach & Dawson, 1999), *Ludwigia palustris* (L.) Elliott and *Galium constrictum* Chaub. (Dawson & Warman, 1987). Ewald (2014) found that although the diversity of native plant species was not affected by the presence of *C. helmsii*, the abundance was significantly reduced in invaded ponds. It has been suggested that species that are particularly vulnerable to competition from *C. helmsii* are likely to be small, low growing plant species which are specialists of open, bare ground habitats (Dean, 2015). Perennial plants spreading by rhizome that can grow through the mats may be

less affected than annual species that require bare ground for germination (Dean, 2015). Other studies have demonstrated that the presence of *C. helmsii* can alter plant species composition in aquatic habitats and Smith & Buckley (2015) found that more rare species were recorded at sites invaded by *C. helmsii*. It was speculated that this could be due to *C. helmsii* modifying the habitat making it more favourable for other plant species. There is evidence that *C. helmsii* can significantly suppress the germination of other plant species which may have impacts further up the food chain. In the same study, the eggs of smooth newts, *Lissotriton vulgaris* (L. 1758), were found to hatch at a later developmental stage when laid on *C. helmsii* compared to their preferred substrate, *Nasturtium officinale* R. Br., but it is not known how this could affect newt populations in the long-term (Langdon *et al.*, 2004). A small number of studies have investigated the impact of *C. helmsii* on macroinvertebrate species but no impacts of significance have been detected (Ewald, 2014; Smith & Buckley, 2015)

### **2.3.5 Current control methods**

Control of *C. helmsii* is very difficult owing to the plant's tolerance of extreme conditions including temperatures as low as -6°C (Leach & Dawson, 1999). Manual control such as cutting is generally not recommended as the small fragments produced could aid dispersal to new sites. However, dredging of marginal and emergent plant material can be effective providing that plant fragments are prevented from spreading to new sites (Environment Agency, 2010). Shading with black polythene sheets can successfully control small infestations but should be in place for a significant period of time (Centre of Aquatic Plant Management, 2004; Environment Agency, 2010). Inundation with seawater has also been trialled with some success although the use of this technique is restricted to sites where seawater can be held at a site with minimal effect on non-target species (Charlton *et al.*, 2010; Dean *et al.*, 2013). The application of liquid nitrogen has also been found to successfully manage small infestations (EPPO, 2007). Chemical treatment is the most commonly used management tool practiced (van der Loop *et al.*, 2018) but only glyphosate is permitted for applications near water bodies in the UK and its future use is uncertain following an assessment by the International Agency for Research on Cancer (IARC) stating that glyphosate is "probably carcinogenic" (Kudsk & Mathiassen, 2020). Current good practice advice from the Environment

Agency is to apply a highly diluted solution of glyphosate to emergent growth to allow percolation through the *C. helmsii* matrix, rather than causing mortality of the top layer of plants (T. Renals, pers. comm.). Alternative methods of control have been trialled with little success; these include hot foam treatment and applying photosynthesis-limiting, dark coloured dyes to the water (Ewald, 2014). Since *C. helmsii* has invaded important nature reserves of conservation value, these non-selective methods of treatment are not favoured and there is the possibility that using these techniques could lead to *C. helmsii* recolonising bare substrates faster than native species (Diaz, 2012). Control is often expensive and time-consuming, and with the likelihood of recolonisation high if control is not maintained, land managers are left with limited options and often no management is carried out. As a result, in large infestations, current management practices do not provide sufficient control to reduce the population below an ecologically damaging threshold long-term. There are few cases where the weed has been eradicated and those cases where eradication has been possible, have been on the local scale (Charlton *et al.*, 2010; van der Loop *et al.*, 2018).

### **2.3.6 Research on the biological control of *Crassula helmsii***

In 2000, EU legislation in form of the Water Framework Directive (WFD) was introduced in the UK, stating that waterbodies were required to reach a “good ecological status” by 2015. This included aims to reduce the abundance of invasive non-native species and the use of harmful chemicals. In order to achieve the WFD objectives, in 2010 the Department for the Environment, Food and Rural Affairs (Defra) and the Environment Agency in the UK, commissioned CABI to investigate the potential of controlling several non-native aquatic and riparian weeds using classical biological control, including *C. helmsii*. While an earlier study had failed to identify any natural enemies causing damage to the plant in the native range (Dawson, 1989), more recent scoping studies revealed additional organisms with potential for further study (Varia & Shaw, 2013) including arthropod species in the genera *Aculus* (Acari: Eriophyidae), *Hydrellia* (Diptera: Ephydriidae) and fungal pathogen species in the genus *Colletotrichum* (Glomerellales: Glomerellaceae). The relevant literature on the biology of these genera and their previous use in biological control programmes was used to inform the selection process for potential biological control agents and is presented in chapter 3. As the results of this and other research

presented in this thesis will show, subsequent work focussed on the mite *A. crassulae*.

## **2.4 Aims and objectives**

The overall aim of this research was to investigate the potential for the classical biological control of *C. helmsii* in the UK using natural enemies from Australia, from initial natural enemy surveys to eventual field release. This aim was divided into the following specific objectives:

- 1) To assess the suitability of natural enemies from Australia as potential classical biological control agents of *C. helmsii*
- 2) To investigate the suitability of the mite *A. crassulae* as a biological control agent, in particular, its host specificity, ability to cause damage to its host *C. helmsii* and ability to establish under UK environmental conditions
- 3) To assess the potential of combining the use of the mite with conventional control measures, namely the use of glyphosate, and the impact on *C. helmsii*
- 4) To assess *A. crassulae* population dynamics under field conditions

### **3 An assessment of the Australian natural enemies of *Crassula helmsii* as potential biological control agents**

#### **3.1 Introduction**

##### **3.1.1 Native range surveys**

One of the first stages in a classical weed biological control programme is to determine the natural enemy community of the target weed in its native range. A comprehensive review of the literature surrounding the natural enemies of the weed and of co-occurring, closely related species is carried out including information regarding the distribution, taxonomy and biology of the target plant (Julien & White, 1997). Herbarium specimens may also be examined for collector's notes on natural enemies, for signs of natural enemy damage and to note the locations of historic plant populations.

It is important to establish the native origin of the invasive population targeted for control and to focus natural enemy surveys in that region. A combination of historical records and molecular techniques are used to determine this and how many introductions there may have been (Gaskin *et al.*, 2011). The highest diversity of natural enemies of a plant is widely accepted to be found in the evolutionary centre of origin or the Pleistocene refugia of a genus (Wapshere, 1974b; Müller-Schärer *et al.*, 1991) and for this reason, this is generally where native range exploration is initiated. Natural enemies from the centre of origin are likely to be more closely associated with the target pest, having co-evolved over a long period of time (Hoelmer & Kirk, 2005). Climate matching increases the likelihood of agent establishment and can be particularly important where the native range of the target plant is broad. Although there have been many examples of non-climate matched agents performing poorly following release, non-climate matched biological control agents have also performed well outside of their native climate range (McFadyen, 1998). Therefore, the native range should be surveyed as widely as possible to identify a diverse range of species. Despite the importance of the aforementioned criteria, in practice foreign exploration might be dictated by logistics including access and safety (Dhileepan *et al.*, 2006). In other situations, the centre of diversification may not be known (Schroeder & Goeden, 1986). Field observations

and further investigations of natural enemies upon return can provide guidance on subsequent agent selection and prioritisation.

### **3.1.2 Agent prioritisation**

Following native range surveys, organisms that may have potential to be host specific must be separated from those with a known broad host range. With host specificity generally regarded as the most important factor for selection, knowledge of the host specificity of a taxon is first assessed, closely followed by its potential as an effective and damaging control agent. A systematic approach for agent selection results in less risk to native species by only introducing host-specific species which have the best chance of reducing weed populations, and reduced costs involved in research and development of potential agents (Morin *et al.*, 2006). Historically scientists have attempted to develop scoring systems to aid the selection of the most effective arthropod agents (Harris, 1973; Goeden, 1983), however, these have mostly not been adopted due to the oversimplification of the criteria used (Cullen, 1995; Sheppard, 2002). By reviewing weed biological control successes and failures, Crawley (1989) found particular taxa (i.e. beetles) to be more successful biological control agents than others, but concluded that the biological attributes associated with the success of these groups were not yet understood. Furthermore, the interaction of the biology of the agent, either arthropod or pathogen, with environmental factors such as climate further complicates the ability to predict which agents may be more successful (McFadyen, 1998). A more recent analysis found that Hemiptera and Coleoptera agents had the highest likelihood of success in terms of establishment and damage caused to the host (Schwarzländer *et al.*, 2018). The behaviour of an organism in its native range can also be useful in helping to prioritise agents including the level of damage caused to the target weed and its impact on plant fitness. It has also been suggested that a wide geographic range could indicate an organism's ability to expand its range and quickly increase in density (Harris, 1991).

In contrast to arthropods, there are several different important criteria for the selection of fungal plant pathogens. Of these, ensuring there is a compatible match between the host plant and the pathogen is crucial (Morin, 2020), although it is also becoming clear that such a match is also important for arthropods, particularly mites (Boughton & Pemberton, 2011; English *et al.*, 2019). Ellison *et al.* (2004)

highlighted this issue with respect to *Mikania micrantha* Kunth and the wide range of rust pathotypes and plant biotypes found in the native range. As with arthropods, the impact and aggressiveness (i.e. amount of damage caused to the host) of strains is also considered (Morin *et al.*, 2006). As obligate parasites, rust and smut fungi are preferred groups of agents due to their host specificity, frequently because of the existence of *formae speciales* adapted to single host species, and their ability to cause significant damage to their hosts (Barton, 2004; Morin *et al.*, 2006).

Facultative pathogens have also been used in weed biological control, particularly as bioherbicides (Cordeau *et al.*, 2016), although less successfully than obligate pathogens (Morin *et al.*, 2006). Furthermore, there has been a preference towards foliar fungal pathogens as classical agents due to their increased host specificity and dispersal via wind or rain splash (Morin, 2020). In the 1980s, “Collego™” became one of the first bioherbicides registered using *Colletotrichum aeschynomenes* (previously known as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. f. sp. *aeschynomene*) against *Aeschynomene virginica* (L.) Britton, Sterns & Poggenb., in the USA (Bowers, 1986) and has more recently been re-registered as “Lockdown™”. The pathogen causes the development of lesions on the stems of *A. virginica* and wilt thereby reducing competitive ability (Bailey, 2014). The most successful bioherbicide is “DeVine®” which used chlamydospores of *Phytophthora palmivora* (Butler) Butler to target *Morrenia odorata* (Hook. & Arn.) Lindl. in the USA. It was so successful that the soil-borne pathogen re-infected the target weed years after treatment (Kenney, 1986).

### 3.1.3 Host-range testing

Host-range testing aims to determine the range of plants which the agent under consideration will accept as a host for feeding, oviposition and development (for arthropods) or infection and disease development (for pathogens). This process becomes the main area of research for biological control scientists and can help distinguish which species are most suitable for further study from those species presenting a wide host range, which can be quickly rejected from further study. Host-range testing of arthropods for the classical biological control of weeds usually involves testing for feeding preference. Oviposition tests using adults may also be carried out. No-choice tests, where arthropods are exposed to test plants only and not their hosts, are considered the most conservative type of test and are used to



determine the fundamental host range - the plant species upon which the arthropod is physically able to successfully feed and develop. The main difficulty with no-choice tests is that they can overestimate the risk to non-target plant species and there have been many documented cases where biological control agents which were released proved to be more host specific in the field than predicted from host-range testing in the laboratory (as reviewed by Hinz *et al.* 2014). Most scientists consider no-choice tests as an important initial screening to reject those species unlikely to be accepted due to their feeding and development behaviour and characteristics (Cullen, 1989; Hill, 1999). Choice tests involve offering the arthropod the target weed and one or several other test plant species for oviposition and feeding. The realised or ecological host range can be more realistically predicted from these tests. Both choice and no-choice tests are generally carried out under controlled conditions in cages which can affect insect host selection behaviour, resulting in feeding or oviposition that would not ordinarily occur in the wild thus leading to ambiguous results (Clement & Cristofaro, 1995; Marohasy, 1998). One way of minimising these issues is to carry out additional open-field tests in the native range although this is not always possible due to political or logistical reasons (Schaffner *et al.*, 2018). For fungal pathogens, the testing procedure is more strict than for insects (Evans, 1995). Following pathogen inoculation of test plants under optimal conditions for infection, disease severity is assessed and scored by examining plants macroscopically and microscopically (Barton, 2004), often involving specific staining methods to assess the development of the agent within the plant (Bruzzese & Hasan, 1983). Although laboratory testing is generally sufficient and the few non-target effects recorded following agent release were always predicted by pre-release testing (Barton, 2012), field testing can also help to elucidate any potential ambiguous results (Baudoin *et al.*, 1993; Evans, 2000).

#### **3.1.4 Natural enemies of *Crassula helmsii***

The natural enemy complex of *C. helmsii* was previously investigated by Dawson (1989) who found no microorganisms or invertebrates damaging the plant during an extensive study of *C. helmsii* across southern Australia. It is very unlikely that any plant species would have no natural enemies in their entire native range although the main focus of that study was to determine the environmental range of the plant. *Crassula helmsii* has been highlighted as a good target for classical biological

control in Europe as a species with only a few European congeneric species and little knowledge of associated natural enemies in its native range (Gassmann *et al.*, 2006; Sheppard *et al.*, 2006). In addition, the biological control of emergent and free-floating aquatic plant species provide some of the most successful examples of classical biological control, particularly using genus-specific beetles from Chrysomelidae and Curculionidae families. A pilot study had previously been carried out in Australia and New Zealand confirming the presence of insect herbivory and infection by fungal pathogens on *C. helmsii* prompting the need for further surveys in the region (R. Shaw, unpublished data).

## **3.2 Objectives**

The objective of the studies presented here was to assess the suitability of *C. helmsii* as a target for classical biological control. This was achieved by:

- 1) Describing the invertebrate and fungal pathogen natural enemies present in the native range of *C. helmsii* in southern Australia and prioritising those species with the most potential as classical biological control agents
- 2) Establishing the infection parameters of the prioritised fungal natural enemies as a prerequisite to commence host-specificity testing
- 3) Characterising the host range of the prioritised natural enemies

The section that follows includes the materials and methods and results of the native range surveys. The material and methods and results of the subsequent investigations into the biology of the prioritised natural enemies is presented subsequently.

## **3.3 Native range surveys**

### **3.3.1 Materials and methods**

Between 2011 and 2013, four surveys were carried out for natural enemies of *C. helmsii* throughout Victoria and Tasmania, Australia. Herbarium records were examined at Kew herbarium prior to undertaking the surveys to identify target regions for surveying *C. helmsii*. These records served as a starting point for locations to search for the plant. The herbarium specimens were also examined for evidence of natural enemy damage, in particular fungal disease symptoms that may

have been preserved. Online databases were used to search for fungal natural enemies that had been previously recorded on *C. helmsii* including the USDA fungal database, <https://nt.ars-grin.gov/fungaldatabases/> (Farr & Rossman, 2020), the Herb IMI database, <http://www.herbimi.info/herbimi/home.htm> (Kew) and the Australian Plant Pest Database <https://appd.ala.org.au> (Plant Health Australia, 2001). Literature was searched for records of arthropod natural enemies.

In total, 36 field sites were surveyed; 10 in Tasmania and 26 in Victoria (Figure 3.1, Appendix 1). In addition to the natural enemy assemblage observed at each site, specific site data including water pH, temperature and depth, *C. helmsii* growth type and type of water body were noted. Surveys took place throughout the main growing season of the plant (November 2011 to April 2013) to ensure that all life stages of natural enemies would be observed. Regions at higher altitude were also identified as areas to survey for climatically matched natural enemies of *C. helmsii*.



Figure 3.1: Map of Australia, the sites surveyed for natural enemies are marked in orange. The map was created using QGIS (QGIS.org, 2020).

### **3.3.1.1 Surveys for invertebrate natural enemies**

At each site, plants were sampled from the margins of the waterbody to depths of around 0.5m, as the limit of where *C. helmsii* was found growing, and were visually examined for symptoms of damage caused by arthropod feeding. When stem mining was observed, whole plants were removed from the root and kept under observation for adult emergence. Any larvae that were collected feeding on the plant were reared through to adult. Live specimens were maintained in ventilated containers and sustained with *C. helmsii* as a food source until export to the CABI quarantine facilities in the UK. Adult specimens of invertebrates with high potential as biological control agents, based on observed field host specificity and damage in the field were preserved in 70% alcohol or pinned for taxonomic identification.

### **3.3.1.2 Surveys for fungal natural enemies**

Plants were sampled from the shallow edges of the waterbody to depths of around 0.5m, as the limit of where *C. helmsii* was found growing, and examined using a x10 magnification hand lens for symptoms of pathogen infection such as necrosis and/or the presence of reproductive structures. Specimens of pathogens that appeared to be damaging in the field were pressed and dried in a plant press for taxonomic identification. Each specimen was given a unique identifying number. Live plants, both healthy and diseased, were maintained during the survey by wrapping their roots in moist tissue before placement in plastic tubes. They were then exported to the CABI quarantine laboratory in the UK.

## **3.3.2 Results**

### **3.3.2.1 *Crassula helmsii* sites surveyed**

*Crassula helmsii* was observed growing in a range of different types of freshwater habitats namely ponds, creeks, ditches, wetlands and the edges of large lakes - both artificial and natural. Plants were observed growing in the terrestrial or emergent growth form in still water at all sites except for at one site where it was found growing in slow flowing water in the submerged form. The pH of the water *C. helmsii* was growing in ranged from pH 6-7.5 and the maximum water depth was 0.5m. Several sites supported a greater diversity of natural enemies than other sites, namely Lake Colac and the Cranbourne site in Victoria and Big Waterhouse Lake in Tasmania. Lake Colac is a large but shallow, freshwater lake while the site at

Cranbourne consists of a series of artificial ditches. Big Waterhouse Lake is a coastal deep-water lagoon. The diversity of natural enemies appeared not to be linked to the size of the site, and may have been instead been related to the environmental conditions.

### 3.3.2.2 Surveys for invertebrate natural enemies

Several invertebrate species were regularly recorded during the surveys in Australia (Table 3.1). Of those, *Hydrellia perplexa* Bock (Diptera: Ephydriidae) was found to be the most widespread and abundant (Figure 3.2A). The larvae of these flies were frequently observed mining stems in emergent *C. helmsii* plants in both Victoria and Tasmania, causing stem death. *Hydrellia perplexa* is distributed across Southern Australia (Bock, 1990). Flies of the *Hydrellia* genus have been released as biological control agents for the management of several aquatic weeds before, most notably against hydrilla, *H. verticillata* in the USA (Buckingham *et al.* 1989); they have also been considered as candidates against *Lagarosiphon major* (Ridl.) Moss (Mangan & Baars, 2016), *Egeria densa* Planch. Casp. (Smith *et al.*, 2019) and *Elodea canadensis* Michx. (Pratt *et al.*, 2019). *Hydrellia* flies are leaf and stem miners and can cause significant damage to their host plants leading to a reduction in host plant fitness (Doyle *et al.*, 2002; Grodowitz *et al.*, 2007).

*Steriphus* spp. (Coleoptera: Curculionidae) were also collected at several sites in Victoria and Tasmania. Larvae and adults were observed feeding on *C. helmsii* (Figure 3.2B), however, species belonging to this genus are also known to feed on the roots of grasses and are unlikely to be host specific. Further experimentation in the laboratory proved this to be true (unpublished data).

*Clepsis* sp. (Lepidoptera: Tortricidae) (Figure 3.2C) belongs to the Archipini tribe which includes many oligophagous and polyphagous species and as such is unlikely to be suitable for biological control. Subsequent feeding tests confirmed this (unpublished data).

*Aculus crassulae* (Acari: Eriophyidae) (Figure 3.2D) was found at only two sites although it is possible that the subtle symptoms could have been missed during the early surveys, before galls were identified as caused by *A. crassulae* feeding and colonisation. *Aculus crassulae* was a species new to science prior to this study (Knihinicki *et al.*, 2018) and as such there were no records of its distribution or biology. Eriophyoid mites have a reputation for being highly host specific and have

been used in weed biological control globally. A more detailed review of eriophyoid mites is presented in chapter 4.

Table 3.1: Arthropod species observed feeding on *Crassula helmsii* during surveys in south-eastern Australia.

Arthropod species	Taxonomic group	Initial host specificity assessment	Number of sites recorded	Action
<i>Aculus crassulae</i> Knihinicki & Petanović	Acari (Eriophyoidea)	Potentially monophagous	2	Imported for further study
<i>Hydrellia perplexa</i> Bock	Diptera (Ephydriidae)	Potentially monophagous	6	Imported for further study
<i>Clepsis</i> sp.	Lepidoptera (Tortricidae)	Polyphagous	4	Imported for further study
<i>Steriphus</i> spp.	Coleoptera (Curculionidae)	Potentially monophagous	3	Imported for further study
unknown sp.	Lepidoptera (Larentiinae)	Polyphagous	1	No further study
<i>Epiphyas postvittana</i> Walker	Lepidoptera (Tortricidae)	Polyphagous	1	No further study
<i>Mythimna (Pseudaletia) convecta convecta</i> Walker	Lepidoptera (Noctuidae)	Polyphagous	1	No further study

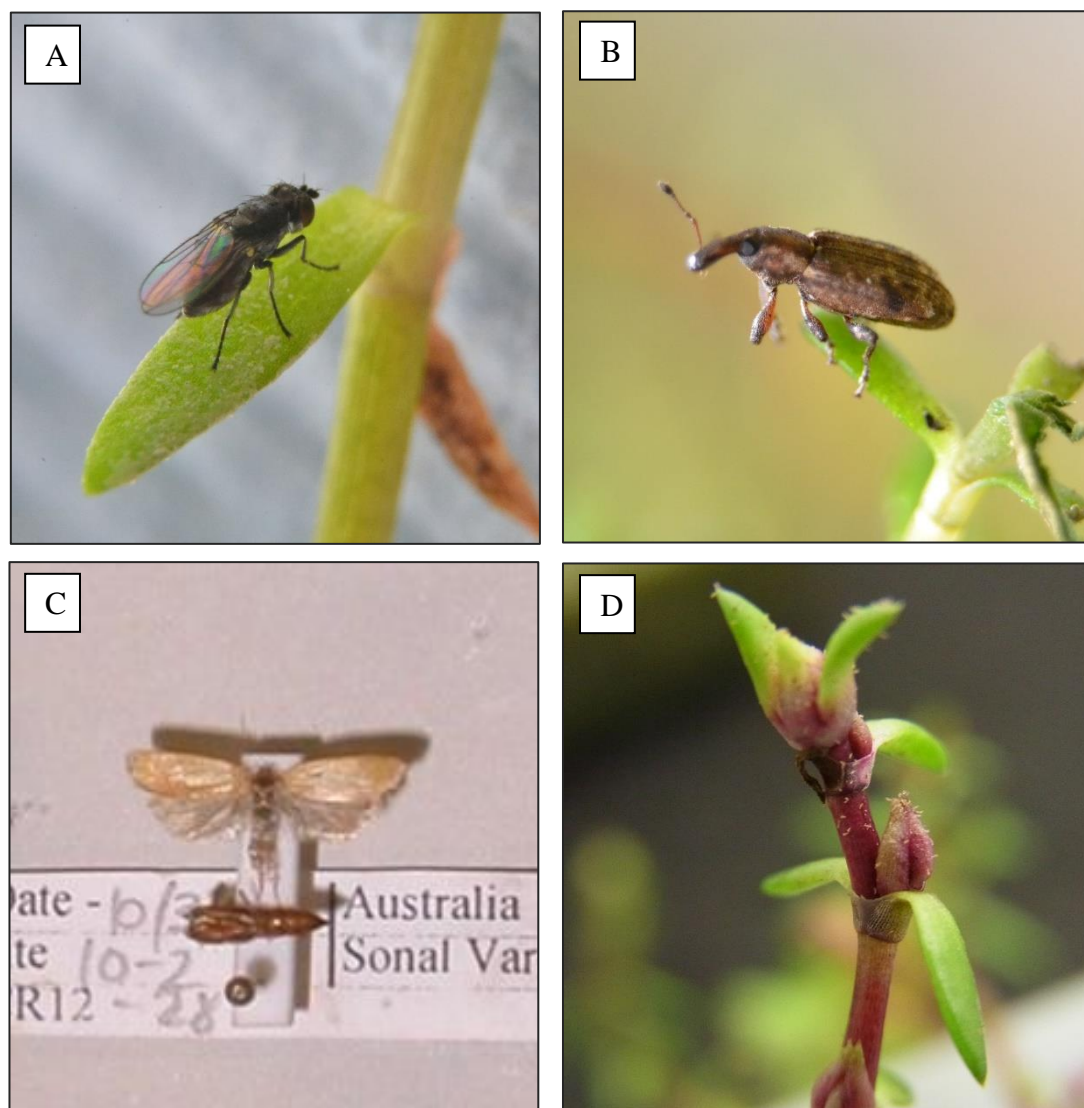


Figure 3.2: A-D Arthropods collected from *Crassula helmsii* in south-eastern Australia, A, *Hydrellia perplexa*; B, *Steriphus* sp.; C, *Clepsia* sp.; D, *C. helmsii* bud infested by *Aculus crassulae*.

### 3.3.2.3 Surveys for fungal natural enemies

There were no records of fungal species on *C. helmsii* in any of the online databases searched. Only one species of interest was recorded on *Crassula* sp. in Australia, the rust fungus, *Uredo tillaeae* McAlp., recorded in the Australian Plant Pest Database (Plant Health Australia, 2001); however, no rust species were found infecting *C. helmsii* in the field. A range of fungal plant pathogens was collected during surveys (Table 3.2). Apart from the *Colletotrichum* spp. which were found at multiple sites, the majority of fungal pathogen species collected were only found at one or two sites, thus were not found to be widespread.

At least two *Colletotrichum* species were collected (Figure 3.3A); one characterised by falcate conidia and one by rounded conidia. Both species were observed causing necrotic lesions on the stems and leaves of emergent *C. helmsii*. *Colletotrichum* species have previously been used both as classical biological control agents and as bioherbicides, as briefly described in section 3.1.2. *Colletotrichum gloeosporioides* f. sp. *miconiae* Killgore & Sugiyama was successfully introduced as a classical agent in Tahiti to control *Miconia calvenscens* D.C. It's infection leads to the partial defoliation of the *M. calvenscens* canopy and its release enabled the recruitment of native plants by enhancing the light availability in the understory (Meyer *et al.* 2007).

Other species which were considered to have potential for further study were two *Alternaria* spp. (Figure 3.3C) which caused shoot die back. A *Stemphylium* species (Figure 3.3B) causing similar symptoms and a *Cercospora* species causing leafspot symptoms were also collected. *Alternaria* and *Cercospora* species have previously been under consideration in various biocontrol programmes and have had some success as biological control agents for water hyacinth, *E. crassipes* (Cilliers, 1991). A powdery mildew species was also found infecting *C. helmsii* plants in the field (Figure 3.3D).



Table 3.2: Fungal pathogen species observed infecting *Crassula helmsii* during surveys in Australia.

Species	Family	ID number	Number of sites recorded	Action
Powdery mildew	Erysiphaceae	W2605	1	Imported for further study
Cercosporoid fungus	Mycosphaerellaceae	W2606	1	Imported for further study
<i>Cercospora</i> sp.	Mycosphaerellaceae	W2607A, W2611	2	Imported for further study
<i>Colletotrichum</i> sp. round spores	Glomorellaceae	W2607B, W2630, W2643	2	Imported for further study
<i>Colletotrichum</i> sp. falcate spores	Glomorellaceae	W2607C, W2642	2	Imported for further study
<i>Fusarium</i> sp.	Nectriaceae	W2612	1	No further study
<i>Fusarium</i> sp.	Nectriaceae	W2613	1	No further study
<i>Stemphylium</i> sp.	Pleosporaceae	W2628	1	Imported for further study
<i>Alternaria</i> sp.	Pleosporaceae	W2631A	1	Imported for further study
<i>Alternaria</i> sp.	Pleosporaceae	W2631B	1	Imported for further study
<i>Alternaria</i> sp.	Pleosporaceae	W2644	1	Imported for further study
cf. <i>Ramularia</i> sp.	Mycosphaerellaceae	W2645	1	No further study



Figure 3.3: A-D *Crassula helmsii* infected by fungal plant pathogens in south-eastern Australia: A, plant with necrotic leaf tissue caused by *Colletotrichum* sp.; B, plant with necrotic leaf tissue caused by *Stemphylium* sp. infection; C, necrotic leaf tissue caused by *Alternaria* sp. infection; D, Powdery mildew infection on leaves and stem surface.

Specimens of the isolates of *Colletotrichum* spp. and *Alternaria* sp. in Table 3.2 were confirmed morphologically and molecularly at the genus level by fungal taxonomists. Identification to the species level would have required a thorough molecular analysis due to the complexity of the taxonomy in both the *Colletotrichum* and *Alternaria* genera. Such analysis was not deemed to be appropriate in this study as neither of the fungal agents was subsequently prioritised for detailed evaluation as a biocontrol agent (see section 3.5).

Plants infected with *Colletotrichum* sp. were collected from several sites. While it could be assumed that isolates with the same spore type and causing the same symptoms on *C. helmsii* belong to the same *Colletotrichum* species, for reasons outlined before a detailed molecular study, required to state with confidence that samples are identical, was not possible for this study. The different *Colletotrichum* species isolated from *C. helmsii* in Australia are detailed in Table 3.3 and the studies described in section 3.4.1.4 involved several of these isolates.

Table 3.3: *Colletotrichum* spp. isolated from *Crassula helmsii* in Australia.

Species	ID number	Conidium type	Collection site
<i>Colletotrichum</i> sp.	W2607B	Rounded	Lake Colac
<i>Colletotrichum</i> sp.	W2607C	Falcate	Lake Colac
<i>Colletotrichum</i> sp.	W2614B/ W2643	Rounded	Cranbourne
<i>Colletotrichum</i> sp.	W2642	Falcate	Cranbourne

### 3.3.2.4 Conclusion of native range survey

Based on literature documenting host specificity of these genera, impact on the host, and field observations in Australia, *A. crassulae*, *H. perplexa* and the fungal pathogens, *Colletotrichum* spp. and *Alternaria* sp. were selected for further study. Experimentation with *H. perplexa*, *Colletotrichum* spp. and *Alternaria* sp. are discussed further in section 3.4 that follows while studies with *A. crassulae* are investigated in more detail in chapter 4.

## 3.4 Investigations into the biology of the natural enemies of *Crassula helmsii*

### 3.4.1 Materials and methods

#### 3.4.1.1 Assessment of *Hydrellia perplexa* as a biological control agent

##### Host-range testing

The *H. perplexa* culture originated from field collections made in 2011 and 2012 at Big Waterhouse Lake in Tasmania, Australia. Flies were reared in clear-plastic, ventilated boxes containing UK grown *C. helmsii* and tap water. Cultures were maintained in Perspex cages in a controlled temperature (CT) room running at

23°C/18°C, on an 18 hour light:6 hour dark cycle and 70% relative humidity under quarantine conditions. Host-range testing of 29 plant species from the test plant list developed for the biological control project of *C. helmsii* was carried out by oviposition and subsequent development tests as no-choice tests (Table 3.4). Further details on the selection of species in the full test plant list are described extensively in studies on the mite covered in chapter 4 (section 4.4.2.1). Ten adults of mixed sex were added to a ventilated container containing the test plant and allowed to oviposit for four days. Preliminary life history studies had demonstrated this period of time as sufficient for oviposition. A cotton wool ball soaked in a yeast hydrolysate-sugar solution was also provided for adult flies to feed on in each replicate. Corresponding *C. helmsii* control plants were set up in the same manner. Between three and six replicates per species were tested. After four days, the adult flies were removed from the containers and the numbers of eggs laid on each test plant were counted under a stereomicroscope. Detailed observations on the subsequent development and feeding by larvae were recorded every seven days over 42 days using a 0-5 scale estimating percentage of overall plant damage; 0=No feeding, T=trace or “minor” levels of feeding, 1= 0-5%, 2=6-10%, 3=11-25%, 4=26-50%, 5=over 50%. This method was chosen over larval or egg transfer due to high mortality observed using these latter methods.

Additional choice tests were carried out to determine whether larvae would feed to the same extent when provided with a choice between the rare and closely related plant species, water pigmyweed, *Crassula aquatica* (L.) Schönland and *C. helmsii* in a more realistic situation. In the choice test, small containers of *C. aquatica* and *C. helmsii* were exposed to twenty flies of mixed sex in the Perspex cage. A longer exposure time was used in this experiment to increase the opportunity for oviposition. After seven days, the flies were removed. Development of the larvae was recorded every seven days using the same scale as with the no-choice tests and any emerging adults were removed.

Table 3.4: Plant species tested against *Hydrellia perplexa* in host-range testing

Plant family	Plant species
Crassulaceae	<i>Crassula helmsii</i> (Kirk) Cockayne <i>Crassula aquatica</i> (L.) Schönland <i>Crassula tillaea</i> Lest.-Garl. <i>Crassula ovata</i> (Mill.) Druce <i>Crassula perforata</i> Thunb. <i>Crassula sarcocaulis</i> Eckl. & Zeyh. <i>Crassula lycopodioides</i> Lam. <i>Kalanchoe blossfeldiana</i> Poelln. <i>Aeonium arboreum</i> (L.) Webb & Berthel. “Zwartkop” <i>Rhodiola rosea</i> L. <i>Echeveria elegans</i> Rose <i>Sedum acre</i> L. <i>Sedum anglicum</i> Huds. <i>Sedum album</i> L. <i>Sedum forsterianum</i> Sm. <i>Sedum villosum</i> L. <i>Sedum spectabile</i> Boreau <i>Umbilicus rupestris</i> (Salisb.) Dandy <i>Sempervivum arachnoideum</i> L. “Rubin”
Haloragaceae	<i>Myriophyllum spicatum</i> L. <i>Myriophyllum aquaticum</i> (Vell.) Verdc. <i>Chrysosplenium oppositifolium</i> L.
Lentibulariaceae	<i>Utricularia vulgaris</i> L.
Callitrichaceae	<i>Callitriche stagnalis</i> Scop.
Alismataceae	<i>Alisma plantago-aquatica</i> L. <i>Luronium natans</i> (L.) Raf.
Hydrocharitaceae	<i>Hydrocharis morsus-ranae</i> L.
Typhaceae	<i>Typha latifolia</i> L.
Potamogetonaceae	<i>Potamogeton natans</i> L.
Marsileaceae	<i>Pilularia globulifera</i> L.

### **3.4.1.2 Assessment of fungal pathogens as biological control agents of *Crassula helmsii***

#### **Isolation from plant material**

Field collected *C. helmsii* plants exhibiting symptoms of fungal infection were assessed under a stereomicroscope and selected, avoiding any hyperparasites, to be incubated in a humid chamber to induce fungal sporulation. Individual infected plants were placed in a sealed petri dish containing damp filter paper for 24 hours. Subsequently, freshly produced conidia were dislodged from fungal bodies or conidiophores under a stereomicroscope using a sterile needle and inoculated onto tap water agar containing chloramphenicol and penicillin G. antibiotics to reduce bacterial contamination. Agar plates were incubated under a 12 hour light:12 hour dark cycle in an incubator set at 19°C for two to four weeks until sporulation occurred *in vitro*.

#### ***Colletotrichum* spp. studies**

##### **Germination of *Colletotrichum* spp. on agar**

To determine the optimal temperature for conidial germination on agar as a guidance for the optimal infection temperatures of two *Colletotrichum* species collected from Australia, the effect of five temperatures on spore germination was investigated. The isolates tested using the set temperatures of 4, 10, 15, 21 and 25°C were W2607B (rounded conidium type, ex Lake Colac) and W2607C (falcate conidium type, ex Lake Colac).

Conidia were dislodged from four-week old agar cultures using a sterile needle and suspended in 0.3ml sterile distilled water. Spores were inoculated onto fresh tap water agar using a sterile syringe and plates were maintained in incubators at the desired temperature for 48 hours. Each temperature test was carried out six times on two separate occasions and following incubation, one hundred randomly selected spores were assessed for germination after 24 and 48 hours, respectively, and the percentage germination was calculated. Germination was assessed as emerging germ-tubes longer than half the conidial length. Percentage germination for the two time periods was analysed using Wilcoxon signed-rank test which was carried out in R Studio (R Core Team, 2019).

### **Inoculation techniques for *Colletotrichum* spp.**

Several different inoculation methods were trialled with two of the *Colletotrichum* isolates; W2607B (rounded conidium type, ex Lake Colac) and W2642 (falcate conidium type, ex Cranbourne) in order to establish which of these isolates causes the most consistent expression of disease symptoms on *C. helmsii*. The following inoculation techniques were trialled to investigate the requirements to achieve consistent and reliable infection. Unless otherwise stated, following inoculation, plants were maintained in Perspex cages in a controlled temperature room held at 23°C/ 18°C and 70% relative humidity, on an 18 hour light:6 hour dark cycle.

To account for potential differences in viability, spores were harvested from a minimum of three different agar cultures that were two to four weeks old. Spores were suspended in sterile distilled water and, using a haemocytometer, adjusted to a concentration of  $1 \times 10^6$  conidia ml<sup>-1</sup>. Using a fine paintbrush, the inoculum was then applied onto the leaves and stem of single *C. helmsii* plants held in universal tubes with tap water. This test was carried with six replicates and repeated six times. As a second inoculation method, the stem and leaves of individual *C. helmsii* plants were also inoculated by applying conidia directly from cultures onto plant surfaces with a fine needle. *Crassula helmsii* plants originating from both Australia and the UK were tested. There were three replicates and this test was repeated four times. All inoculated plants were lightly sprayed with sterile distilled water and placed in a dew chamber set at 19°C for 48 hours. Plants were monitored for symptoms of infection every seven days for six weeks.

Additionally, plants were inoculated by allowing potential uptake of the spores via the plant roots through placing whole plants in a spore suspension with a concentration of  $1 \times 10^6$  conidia ml<sup>-1</sup> in sterile distilled water, for 48 hours. Uptake via plant roots has been recorded in several fungal pathogens including species in the *Colletotrichum* genus (Sukno *et al.*, 2008) and therefore this method was trialled with the *Colletotrichum* isolates here. The plants were then removed from the inoculum and maintained in fresh water for observation of symptoms. There were six replicate plants and this test was carried out three times. Plants were monitored every seven days for disease development over six weeks.

### **Host-range testing of *Colletotrichum* sp. W2642**

Some preliminary host-range testing was carried out with *Colletotrichum* sp. isolate W2642 (falcate conidia type, ex Cranbourne) in order to assess its suitability as a biological control agent. Using the inoculation method which resulted in the most consistent infection, test plants were held in glass universal tubes with their roots placed in a spore suspension of  $1 \times 10^6$  conidia ml<sup>-1</sup>. The spores used were harvested from cultures that were two to four weeks old. Plants were held in the spore suspension for 48 hours in a CT room maintained at 23°C by day and 18°C at night, at 70% relative humidity and 18 hour light: 6 hour dark, light regime. Plants were held in place with Parafilm. Plants were subsequently kept in sterile distilled water under the same conditions to maintain humidity around the lower portion of the stem not submerged in water. Plants were assessed every seven days for six weeks for symptoms of infection. The non-target species tested were *C. aquatica*, *Crassula tillaea* Lest.-Garl. and *Callitriche stagnalis* Scop. Six replicates of each species were tested on two separate occasions and each test included three *C. helmsii* plants as a control. The suspension was also inoculated onto tap water agar plates to confirm the viability of the spores used in each test through germination on agar.

### ***Alternaria* sp. studies (W2631A)**

The following preliminary studies were carried out to investigate the requirements to achieve consistent and reliable infection of *C. helmsii* by *Alternaria* sp. Spores used for all experiments were harvested from agar cultures that were two weeks old. Plants inoculated with *Alternaria* sp. were incubated in a dew chamber set at 19°C for 24 hours unless otherwise stated. This temperature was selected based on a high percentage germination of spores on agar at this temperature. Following inoculation, plants were maintained in Perspex cages in a CT room held at 23°C/ 18°C, on a 18 hour light:6 hour dark cycle and 70% relative humidity.

### **Plant wounding**

Wounded plant tissue, for example caused by insect feeding, can be more susceptible to infection by *Alternaria* species as the germ tube can penetrate plants via such wounds (Thomma, 2003). Therefore, the effect of wounding of different plant tissues prior to inoculation was compared. Leaves in the shoot tips and stems were lightly scraped with a sterile needle and inoculated with spores taken directly from cultures. Control plants were also wounded with the needle. Plants were then lightly sprayed



with sterile distilled water before placing in a dew chamber. The spores were also inoculated onto a tap water agar plate to confirm viability of the spores. This experiment was carried out once with two replicates for each wounded tissue. Plants were monitored every seven days over six weeks for disease development.

### **Spore concentration**

The effect of spore concentration on infection was assessed using suspensions of *Alternaria* sp. spores scraped from cultures adjusted to  $0.15 \times 10^6$  and  $0.25 \times 10^6$  spores  $\text{ml}^{-1}$  sterile distilled water. Two *C. helmsii* leaves per plant were lightly scraped with a sterile needle and then inoculated with 3  $\mu\text{l}$  inoculum per leaf using a micropipette. Plants were also inoculated directly by transferring spores from growing cultures onto the previously wounded tissue using a fine needle. Inoculated plants were then sprayed with sterile distilled water and placed in the dew chamber for 24 hours. Three replicate plants were used for each of the two spore concentrations and the test was carried out once. Plants were monitored every seven days over six weeks for disease development.

### **Humidity**

Regular humidity is required to progress *Alternaria* infection and therefore different post-inoculation humidity regimes were investigated. Plants were inoculated by first scraping the leaves with a sterile needle and subsequently inoculating spores directly from the culture plate onto the wounded plant tissue. Following 24 hours in the dew chamber, plants were assigned one of three humidity regimes; 10 hours at 90% humidity once every seven days, 10 hours at 90% humidity once every three days and 24 hours at 90% humidity once every seven days. Three replicate plants were used for each humidity regime. The humidity treatment continued for 21 days and the presence or absence and severity of the *Alternaria* sp. infection was recorded. This test was carried out twice.

### **Host-range testing**

Preliminary host-range testing was carried out with *Alternaria* sp. for the closely related species, *C. aquatica*. Four *C. helmsii* and six *C. aquatica* plants were each inoculated with a spore suspension of  $1 \times 10^6$  spores  $\text{ml}^{-1}$  in sterile distilled water following leaf wounding as described above. This concentration was used as it is the standard spore concentration used in the host-range testing of fungal pathogens. Plants were then placed in the dew chamber for 48 hours and again every 7 days for

24 hours to aid disease development. Plants were assessed every seven days for six weeks for symptoms of infection.

### 3.4.2 Results

#### 3.4.2.1 Assessment of *Hydrellia perplexa* as a biological control agent

Oviposition by *H. perplexa* was recorded on 24 of the 29 plant species tested in no-choice tests. Females appeared to oviposit randomly (Figure 3.4).

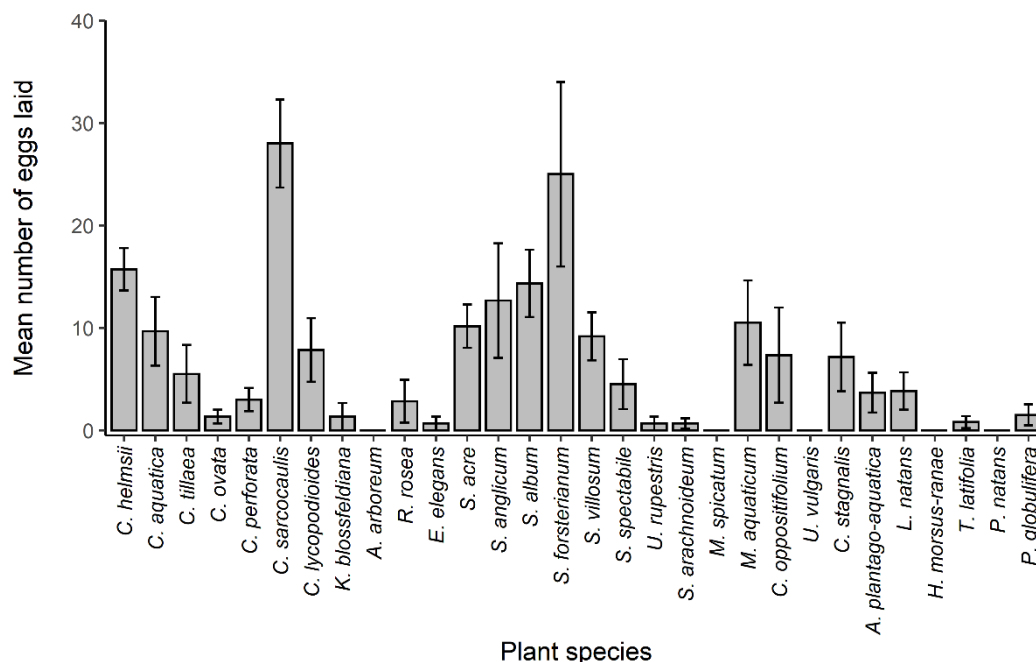


Figure 3.4: The mean number of eggs ( $\pm$  SE) laid on test plants after 4 day exposure to adult *Hydrellia perplexa* flies in no-choice tests.

In no-choice tests, feeding damage was recorded on four of the non-target species tested; *C. aquatica*, *Sedum anglicum* Huds., *Sedum album* L. and *Sedum villosum* L. (Figure 3.5). Development did not progress past the larval stage in *S. villosum* or *S. album* plants nor past the pupal stage in *S. anglicum* plants. *Hydrellia perplexa* was able to complete development in the native congener *C. aquatica* in both no-choice and choice tests (Figure 3.6) resulting in viable progeny, in addition to causing extensive feeding damage (Figure 3.7). In the choice tests, the mean damage score for both *C. aquatica* and *C. helmsii* was 4.

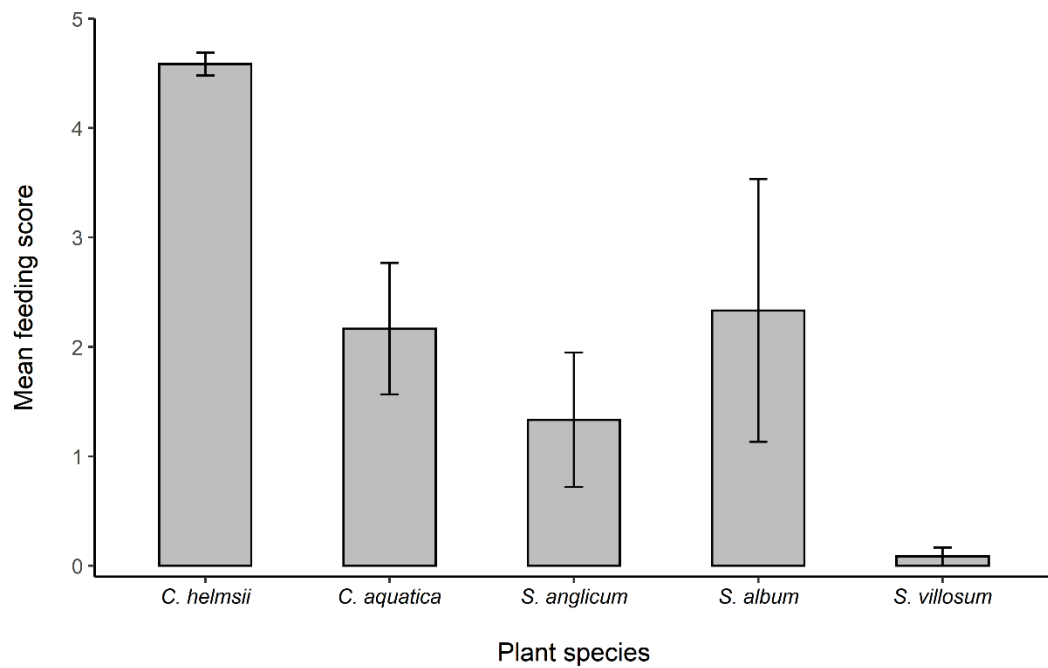


Figure 3.5: The mean feeding score ( $\pm$  SE) recorded on test plants 42 days after feeding by *Hydrellia perplexa* larvae in no-choice tests. Feeding score was measured on a 0-5 scale; 0=No feeding, T=trace, 1= 0-5%, 2=6-10%, 3=11-25%, 4=26-50%, 5=over 50%.

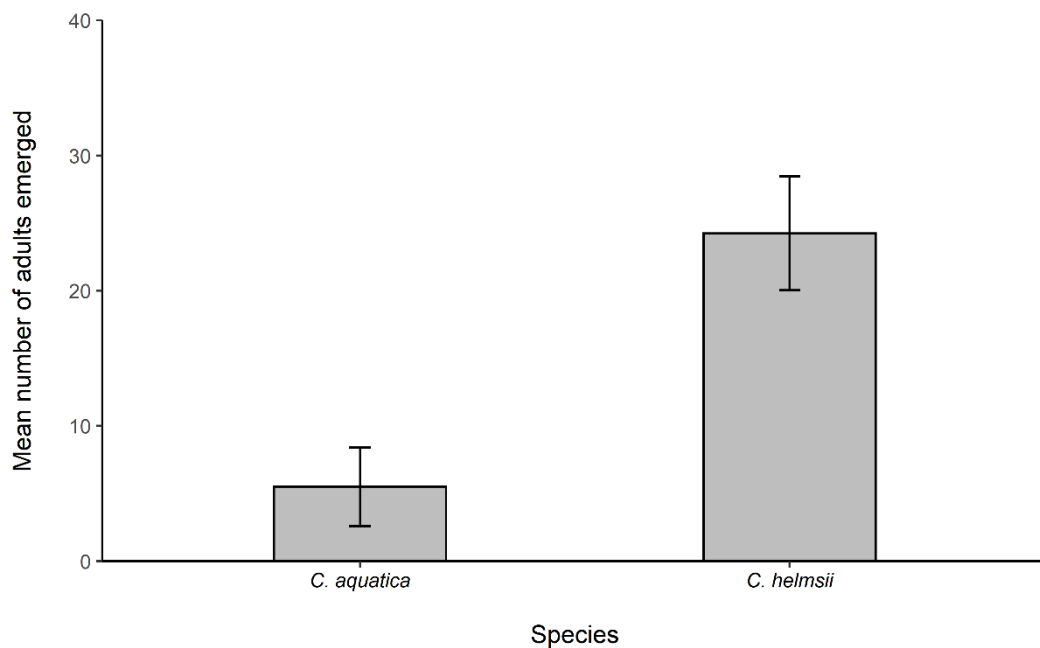


Figure 3.6: Mean number of *Hydrellia perplexa* adults ( $\pm$  SE) emerged from *Crassula aquatica* and *Crassula helmsii* in choice tests.



Figure 3.7: *Crassula aquatica* plant with stem infested by 14 day old *Hydrellia perplexa* larvae. Arrows indicate larvae within stem.

#### 3.4.2.2 Assessment of fungal pathogens as biological control agents of *Crassula helmsii*

##### *Colletotrichum* spp. studies

##### *Colletotrichum* spp. infection parameter and inoculation methods

Percentage germination of spores on agar was much lower for W2607B than for W2607C. There was very little germination below 21°C and after 24 hours the highest mean percentage germination was 12% at 25°C. Germination did not significantly increase after 48 hours except at 21°C (Wilcoxon signed-rank test,  $p < 0.05$ ) (Figure 3.8).

Spores of W2607C germinated well at all temperatures tested (Figure 3.9). Spore germination increased with time at temperatures tested between 10°C and 21°C (Wilcoxon signed-rank test,  $p < 0.05$ ). Below 10°C and above 20°C there was no increase in germination from 24 hours to 48 hours (Wilcoxon signed-rank test,  $p > 0.05$ ).

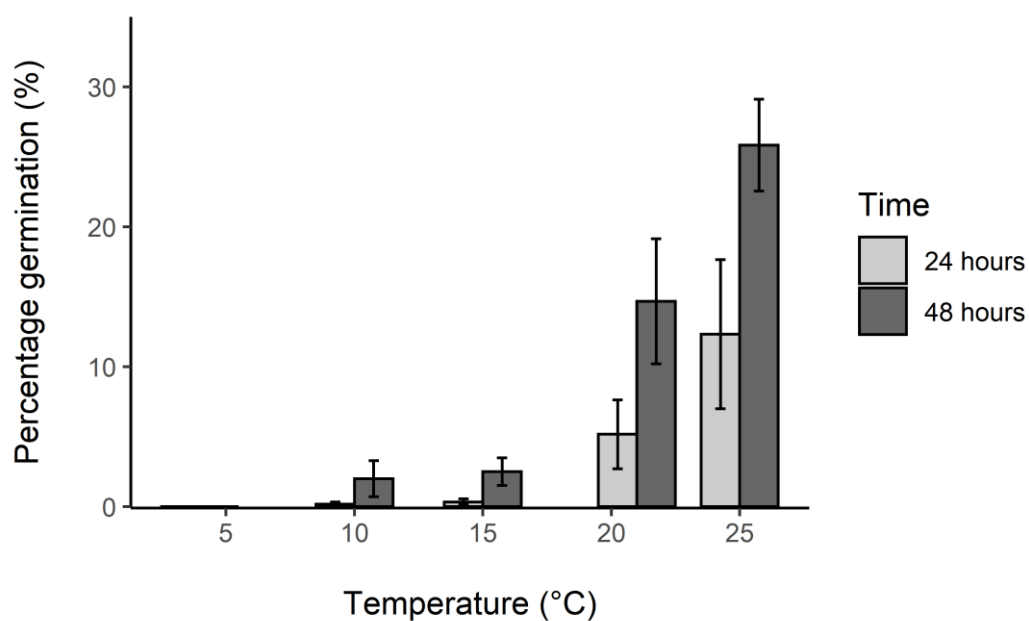


Figure 3.8: Percentage germination ( $\pm$  SE) of *Colletotrichum* sp. (W2607B) spores after 24 and 48 hours.

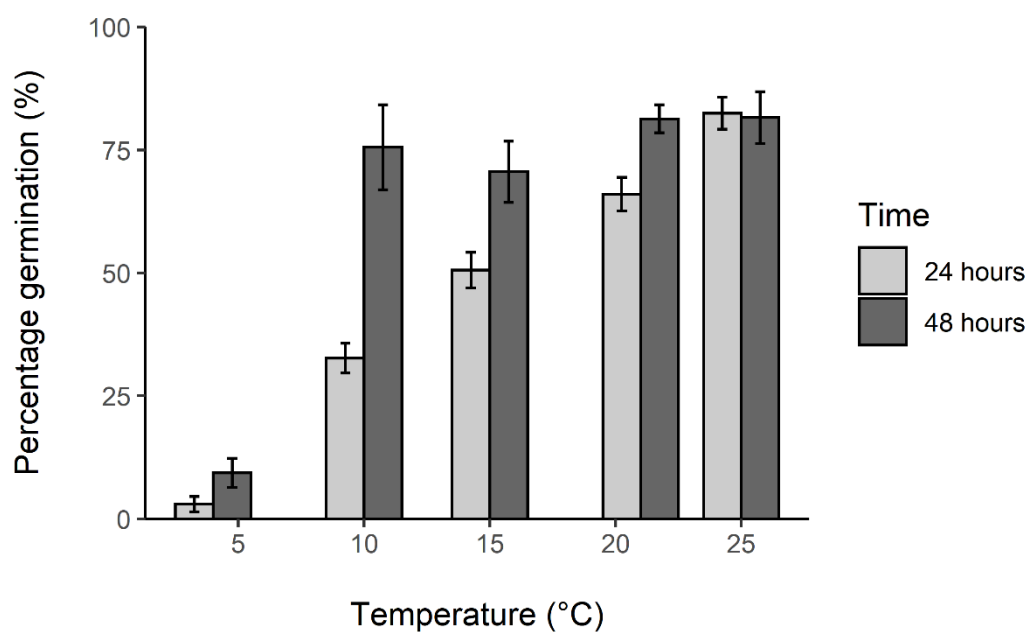


Figure 3.9: Percentage germination ( $\pm$  SE) of *Colletotrichum* sp. (W2607C) spores after 24 and 48 hours.

#### Inoculation techniques for *Colletotrichum* spp.

Both inoculation with the spore suspension and the direct inoculation of spores onto plant tissue were unsuccessful with isolate W2607B. Submersion in a spore

suspension led to infrequent and inconsistent development of disease symptoms. The results were the same for both Australian and UK-grown plants.

Inoculation with the spore suspension and direct inoculation of spores were more successful with W2642, but disease development on treated plants was inconsistent. Inoculum uptake through submersion in a spore suspension was observed to be the more reliable method resulting more consistently in the development of disease symptoms.

#### **Host-range testing of *Colletotrichum* sp. W2642**

All three non-target species tested were susceptible to *Colletotrichum* sp. infection (Figure 3.10A-C). Infection was observed on four of the six replicates of *C. aquatica*, five of the six replicates of *C. tillaea* and two of the six replicates of *C. stagnalis*. Setae and acervuli producing conidia developed on the leaves of the non-target species; 14-22 days after inoculation on *C. aquatica*, after 15-16 days on *C. tillaea* and on *C. stagnalis* after 13 days. Setae and acervuli were also recorded on *C. helmsii*, as the positive controls, with the earliest development recorded 7 days after inoculation.

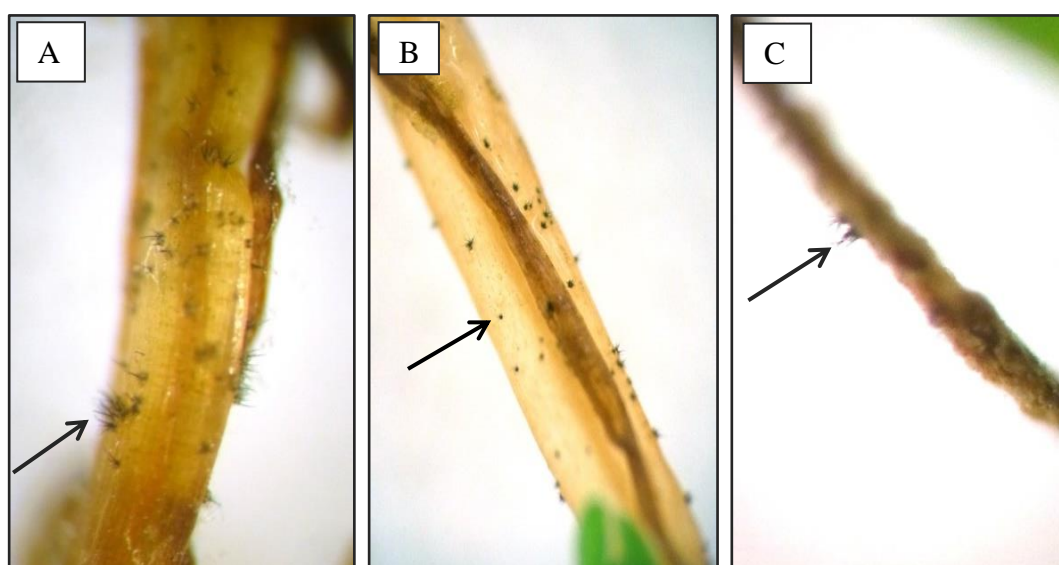


Figure 3.10: Three *Crassula* species showing symptoms of *Colletotrichum* infection on the stem following inoculation, arrows indicate setae and acervuli on A, *Crassula tillaea*; B, *Crassula helmsii* and C, *Crassula aquatica*.

It was critical to establish whether the infection observed was due to the *Colletotrichum* sp. isolate under evaluation or results from the expression of an endophytic *Colletotrichum* species naturally present in those non-target plants. To determine the identity of the sporulating *Colletotrichum* species on these non-targets

and thereby to confirm or reject Koch's postulates, re-isolations from infected tissues of the test species were undertaken and the respective obtained *Colletotrichum* sp. cultures underwent molecular characterisation. The results of this, which are not presented here, showed that all re-isolated fungal samples were the same species, *Colletotrichum* sp. that was originally inoculated onto these plants proving Koch's postulates.

#### ***Alternaria* sp. studies (W2631A)**

Both leaves and stems of *C. helmsii* that were wounded prior to inoculation developed necrosis with the pathogen sporulating on necrotic tissues 17 days after inoculation.

Plants inoculated with a higher concentration of spores showed more severe disease symptoms (Figure 3.11 A-B) and the most prolific infection occurred where spores were inoculated directly from cultures onto plant tissue. All humidity regimes tested resulted in *Alternaria* sp. disease development but the treatment with more regular humidity (high humidity every three days) resulted in more severe symptoms on the plants.

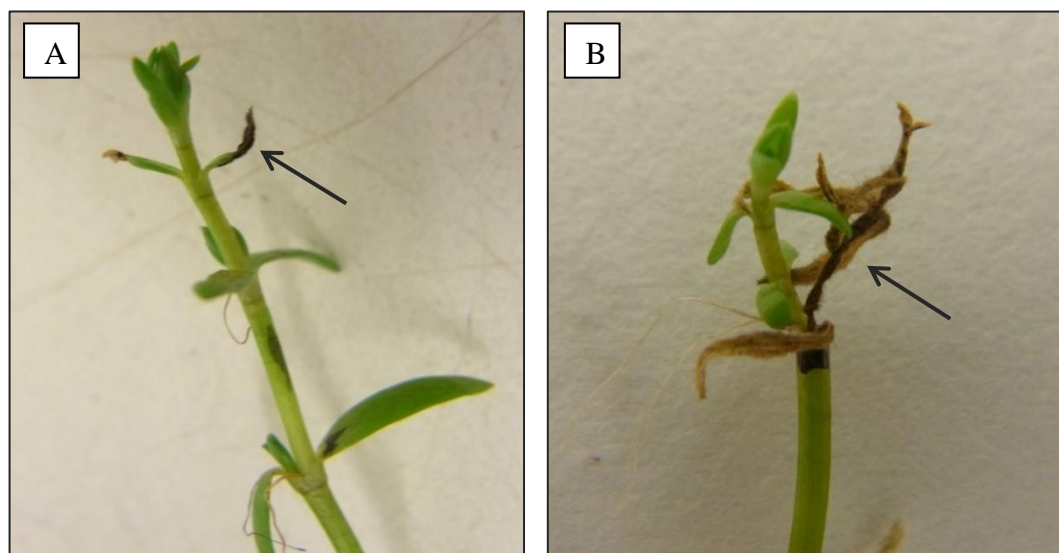


Figure 3.11: *Crassula helmsii* infected with *Alternaria* sp. (W2631A) 17 days after inoculation with different conidial concentrations, A,  $0.15 \times 10^6 \text{ ml}^{-1}$  and B,  $0.25 \times 10^6 \text{ ml}^{-1}$  in sterile distilled water. Arrows indicate necrotic tissue.

### **Host range of *Alternaria* sp.**

*Alternaria* sp. infection was observed on *C. aquatica* on three of the six replicates tested. Necrosis and sporulation was recorded on both *C. helmsii* and *C. aquatica* leaves 15 days after inoculation. Microscopic evaluation of the spore morphology indicated that the disease symptoms observed were caused by the *Alternaria* sp. originally inoculated onto these plants. However, definite confirmation that the species was not an endophyte already present in the inoculated plants would have required molecular analysis. This was not undertaken at this point as the biocontrol potential of this agent was not considered to be high enough to be pursued due to the likely non-target infection, and other natural enemies of greater potential were prioritised for further work.

### **3.5 Discussion**

The natural enemy assemblage of *C. helmsii* in south-eastern Australia was described, revealing a variety of arthropod and fungal species feeding on and infecting the plant. Many of these warranted further study as potential biological control agents, contradicting what was previously found (Dawson, 1989). Members of several invertebrate families renowned for their potential as biological control agents including weevils (Curculionidae), shore flies (Ephydriidae) and eriophyid mites (Eriophyidae) were found, although the majority of species observed did not demonstrate the required level of host specificity. Of the plant pathogens collected, the species with the most potential lacked host specificity and as a result did not warrant further study.

It is known that biodiversity is higher in the tropics than in temperate regions. With the survey area being classed as temperate, it was expected that the abundance and diversity of natural enemies associated with *C. helmsii* would be lower than for tropical aquatic plants, however the abundance was still lower than anticipated. For example, over 70 invertebrate species were recorded from the tropical species, water hyacinth in its native and invasive range (Perkins, 1974). Twenty invertebrate herbivore species were recorded on the more temperate species, *Hydrocotyle ranunculoides* L.f., in its native range (Cabrera Walsh *et al.*, 2013), although a large proportion of these had little potential for classical biological control. Far fewer invertebrate herbivores were recorded on *C. helmsii* in this study. The difference observed could partly be explained by the size and variability of the area surveyed in



Australia being less extensive than for water hyacinth and *H. ranunculoides*. The surveys for water hyacinth were also conducted over a much longer timeframe than the surveys in Australia. During 2011-2013 when the surveys took place, south-eastern Australia had just emerged from the Millennium Drought, considered to be one of the worst droughts experienced in the region in the last 200 years. The drought had caused declines in a range of aquatic species (Bond *et al.*, 2008) and populations were recovering. Although *C. helmsii* and its associated co-evolved fauna are likely to have developed some drought-resistant or resilience traits having evolved in a drought-prone environment, the duration and severity of the drought might have affected the number of species observed during that period (Boulton, 2003; Brock *et al.*, 2003). As suspected by Dawson (1989), it appears that drought is important in limiting populations of *C. helmsii* in its native range. However, the fact that natural enemies may not be the main factor limiting *C. helmsii* populations in its native range does not necessarily mean that they would not exert control of *C. helmsii* in the introduced range.

In contrast to the UK, *C. helmsii* was generally not found growing in its submerged form in Australia, probably due to reduced rainfall. As a result, the majority of species observed were feeding or infecting the aerial portion of the plant, with the exception of *H. perplexa* larvae, which fed within the submerged stems. Based on field observations the likelihood of finding potential agents that cause damage to all growth forms present in the invasive range of *C. helmsii* was therefore low.

*Hydrellia perplexa* was prioritised for further study; the damage observed in the field was significant and *Hydrellia* spp. have been previously released successfully against hydrilla, *H. verticillata* in the USA (Winston *et al.*, 2014). The oviposition tests showed that *H. perplexa* laid eggs on a wide range of non-target species which also occurred with the two species released against *H. verticillata*; *Hydrellia pakistanae* (Buckingham *et al.*, 1989) and *Hydrellia balciunasi* (Buckingham *et al.*, 1991). Eggs were laid on almost every non-target species tested. Like other *Hydrellia* species, *H. perplexa* larvae appear to be mobile and relatively selective. In this study, the use of oviposition tests and subsequent development allowed the assessment of larval development on plants which had received eggs, but those which did not receive eggs were not additionally tested for larval development. Larval or egg transfer tests would have been useful in completing the full risk assessment and understanding the fundamental host range of *H. perplexa*. The four

non-target species that supported larval feeding were all in the Crassulaceae plant family and *C. aquatica*, the most closely related plant on the test plant list, supported complete development. The F1 generation was found to be viable, however, the productivity or fitness of future generations were not tested. Bownes (2014) found that the number of adults produced in the F2 and F3 generations of *Hydrellia* sp. reared on non-target *Lagarosiphon* spp. was lower than the F1 generation and, on some test plant species, the fly died out. If the future generations of *H. perplexa* were tested for viability, it would have been possible to understand the long-term risk posed to *C. aquatica* over several generations. However, even minor or temporary damage caused to this rare, native species is highly likely to have been deemed too much of a risk by regulators in the UK and EU to consider the release of this agent into the wild and further experimentation was discontinued.

The fungal species with the most potential for classical biological control were the *Colletotrichum* species because they can be host specific, particularly if *formae speciales* exist. As well as being host specific, *Colletotrichum* species can be damaging to their host plant and have previously been used successfully in weed biological control as demonstrated by the introduction of *Colletotrichum gloeosporioides* f. sp. *miconiae* Killgore & Sugiyama against *Miconia calvescens* D.C. in Tahiti (Meyer & Fourdrigniez, 2011). Infection studies with isolate W2607B did not result in the expression of disease symptoms within the timeframe of the assessment. Furthermore, germination of this isolate on agar was poor. Inoculations with W2642 resulted in more consistent development of damaging disease symptoms and germination on agar was higher. The apparent poor performance of isolate W2607B could be linked to a more endophytic status of the pathogen which could reside asymptotically in the plant, only becoming pathogenic after time. *Colletotrichum* species are well known for being endophytes as well as disease causing agents and most major groups of angiosperms are known to host them (Cannon *et al.*, 2012). It would have been valuable to confirm the identity of the *Colletotrichum* species from different sites which superficially looked identical, in order to make a more complete assessment of the fungal natural enemies of *C. helmsii*. However, as the results of the experiments demonstrated, none of the *Colletotrichum* species were suitable as biological control agents of *C. helmsii* and this research was not prioritised. Host-range testing of *Alternaria* sp. (W2631A) established that this species was also unsuitable to be considered further as a classical biological control agent against *C. helmsii*.

However, native fungal pathogens found to attack weeds in their invasive ranges have been successfully utilised as biological control agents in an inundative approach in the past (Martínez Jiménez & Gómez Balandra, 2007). Thus, the possibility of using a UK-native pathogen which attacks *C. helmsii* without causing severe damage remains of interest, if it could be developed into a bioherbicide as an alternative control option.

### **3.6 Key conclusions**

The studies described in this chapter demonstrate that *C. helmsii* is a suitable target for investigating biological control as the natural enemy community of *C. helmsii* in Australia is broader than previously thought. *Crassula helmsii* was not found growing in its submerged form in Australia as it does in the UK in large and deep waterbodies, and most natural enemies were found on the emergent parts of the plant. This indicates that classical biological control is not an option for the submerged type of growth. Many of the fungal and insect species collected from *C. helmsii* in Australia which were prioritised for further study were found to be unsuitable as classical biological control agents due to the potential non-target effects if released. As a result, subsequent studies focussed on *A. crassulae* given its apparent specificity from field observations in Australia coupled with the generally host specific nature of the Eriophyidae mite superfamily.

## **4 A pre-release assessment of the suitability of *Aculus crassulae* as a classical biological control agent of *Crassula helmsii***

### **4.1 Introduction**

The mite, *A. crassulae* (Eriophyidae) was considered to have the most potential as a classical biological control agent against *C. helmsii*, as concluded in chapter 3. Specifically, the criteria used to assess this potential were the highly host specific nature of mites from this family and also the damage caused by this particular species in its native range in Australia.

The aim of the studies reported in this chapter were to understand various aspects of the biology of *A. crassulae* in order to further assess its suitability as a biological control agent for *C. helmsii* in the UK. To make this assessment, three aspects were investigated, 1) the potential of *A. crassulae* to establish under UK environmental conditions, 2) the host range of *A. crassulae* and 3) the ability of *A. crassulae* to cause sufficient damage to the host plant to contribute to its control. First, an overview of the literature concerning eriophyoid mites is given, followed by a review of the general literature of each topic under investigation.

#### **4.1.1 Eriophyoid mites**

Mites from the superfamily Eriophyoidae are tiny plant-feeding mites ranging in size from 80-500µm (Lindquist, 1996) and possessing only two pairs of legs rather than the usual four pairs observed in mites. Dispersal is mainly passive and is primarily wind-borne although they can also be spread via animal carriers or by rain (Michalska *et al.*, 2010). Mites in this superfamily are known to be very closely associated with their host; the available data show that 80% of eriophyoid mites are reported from one host species only (Vacante, 2015). Indeed, some species are specific to host plant biotype (Caresche & Wapshere, 1974; Jupp *et al.*, 1997). There is limited data available in the literature regarding eriophyoid mites, mainly as their minute size makes detailed studies difficult and most of the available data in the literature refers to pest species on important crop species such as *Aceria guerreronis* Keifer, a pest of coconut and *Phyllocoptruta oleivora* (Ashmead), a pest of citrus fruits. Mites from the Eriophyidae family are known vectors of plant pathogens

including viruses; for example the mite *Cecidophyopsis ribis* (Westwood), transmits blackcurrant reversion virus which can reduce fruit yield in blackcurrant, *Ribes nigrum* L. (Oldfield & Proeseler, 1996). The relationship between eriophyoid mite and plant pathogen is highly specific and no plant pathogen is known to be transmitted by more than one mite species (Oldfield & Proeseler, 1996).

#### **4.1.1.1 The use of eriophyid mites in biological control**

The use of mites from the Eriophyidae family in weed biocontrol has been gaining more attention in recent years, with several species currently under consideration as biological control agents for weeds in North America (CABI, 2016). There have been nine different species of eriophyoid mites intentionally released as biocontrol agents globally between 1971 and 2016 (Winston *et al.*, 2014; Weyl *et al.*, 2019) and perhaps the most successful of these is *Aceria malherbae* Nuzzaci which was released in the USA to control the perennial weed, *Convolvulus arvensis* L. This species, commonly known as field bindweed, is an aggressive weed of Eurasian origin, globally found infesting cultivated and pasture land, gardens and roadsides. Large crop losses have been reported from infestations (Boldt *et al.*, 1998) and long-lived seeds and an extensive root system make this weed difficult to control. *Aceria malherbae* was introduced from Greece to Texas, USA in 1989. Colonies of the mite form galls and malformation of leaves, stems and buds, causing stunting and a reduction in flower production and root biomass (Boydston & Williams, 2004). The mite is now established in several regions in the USA and has resulted in more manageable populations although its establishment appears to depend upon humidity (Cortat *et al.*, 2012).

#### **4.1.1.2 Impact of feeding on the host**

Eriophyoid mites can be refuge-seeking or refuge-creating where mites either seek protection from existing plant structures such as within bud scales, or create a refuge such as leaf or flower galls or erineae. Conversely, free-living or vagrant species can be found across the whole leaf surface (Petanović & Kielkiewicz, 2010b) and may cause symptoms such as russetting or silvering of leaves. Galls can be so distinctive that they can be used to identify the mite causing the damage (Westphal & Manson, 1996). The piercing and sucking mouth parts of eriophyoid mites are used to feed primarily on cells in the epidermal layer. This feeding can cause alterations in the morphology of mesophyll cells which can lead to the development of abnormalities in plant growth including galls. Damage caused by eriophyoid mites may also

develop as a result of salivary toxins from feeding (Oldfield, 1996). The damage caused by eriophyoid mites can impact the fitness of the host in several ways, for example by reducing photosynthesis and therefore growth, seed production, and biomass accumulation. They have been shown to successfully reduce growth in weeds when released as classical biological control agents. For example, *Aceria chondrillae* (Canestrini) which was first released in Australia as a biological control agent against rush skeletonweed, *C. juncea*, has been known to reduce flowering and seed production by 50–90%, depending on plant size and environmental conditions (E. Coombs pers comm. in Smith *et al.*, 2010). Another eriophyoid mite, *Aceria acroptiloni* Shevchenko & Kovalev which is under consideration as a classical biological control agent against *Rhaponticum repens* (L.) Hidalgo, was shown to reduce the biomass of *R. repens* shoots by 40–75% in field experiments in its native range in Iran (Asadi *et al.*, 2014).

#### **4.1.1.3 Biology of eriophyoid mites**

Mating by eriophyoid mites is dissociated and male mites deposit a spermatophore on the plant surface which is then picked up by female mites (Michalska *et al.*, 2010). Arrhenotokous parthenogenesis can take place, whereby male progeny are produced from unfertilised eggs allowing single females to reproduce in the absence of males. Generally, the egg passes through two immature stages before the mite emerges as an adult. The first instar is commonly known as a larva and the second instar is known as a nymph. Quiescent stages occur between the larva and nymph stage and between the nymph and adult stage. In gall-forming eriophyoid mite populations that exhibit arrhenotoky, there is usually a high female to male sex ratio (Sternlicht & Goldenberg, 1971).

Some eriophyoid mite life cycles involve deuteroecy; the presence of two female forms; the deutogyne and the protogyne. The protogyne is the female form that structurally corresponds to the male and the deutogyne is the overwintering form. Morphology of the protogyne differs from the deutogyne in several anatomical characteristics which require experienced taxonomists to detect (Manson & Oldfield, 1996). This strategy is an evolutionary adaptation for survival on deciduous plants in regions with clearly defined winter periods although there are also examples of deuteroecy in tropical species (Michalska *et al.*, 2010).

#### 4.1.1.4 *Aculus crassulae* Knihinicki & Petanović

*Aculus crassulae* (Figure 4.1) is a mite in the Eriophyidae family from Australia and has been under investigation as a biological control agent of *C. helmsii* since 2013 following the surveys described in chapter 3. Its taxonomy was recently described by Knihinicki *et al.* (2018) and after undergoing the Pest Risk Analysis process, was approved by Defra for release as a biological control agent of *C. helmsii* in 2018.

*Aculus crassulae* is a gall-forming species causing infested plants to develop the “big bud” symptom. In this type of gall, infested buds enlarge, the leaves become thickened and there is a failure to produce new leaves (Westphal & Manson, 1996). Feeding by a single mite can induce gall development which in this case is initially observed by a deep pink colouration and leaf curl as the gall develops on the growing shoot, preventing the normal growth of shoots (Figure 4.2). The mite utilises terrestrial and emergent growth forms of *C. helmsii*, and although it can survive significant periods under water (pers. obs), it does not colonise the submerged form of its host. No symptoms of plant pathogens have been found associated with symptoms caused by the mite in Australia so it is assumed that they do not vector any diseases. The overwintering strategy of *A. crassulae* in the native range is not clear, however it is possible that deuteroecy exists.

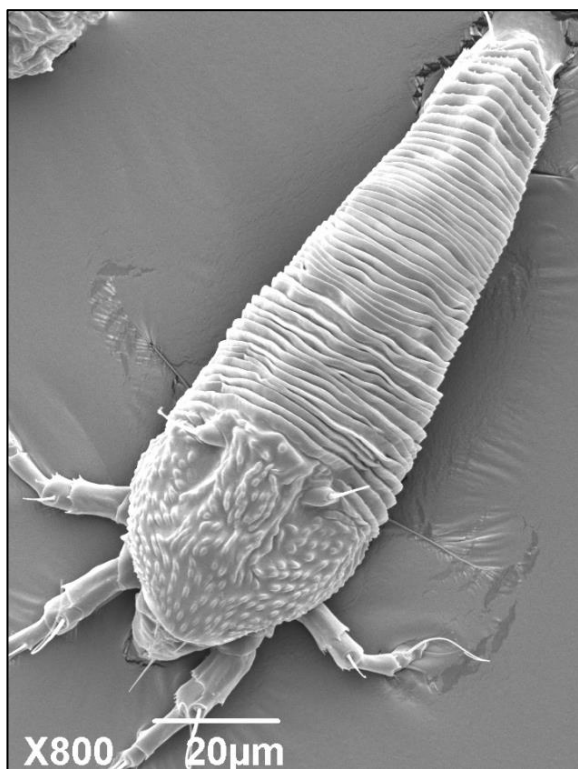


Figure 4.1: Scanning electron micrograph of adult *Aculus crassulae* at x800 magnification (Image credit: Radmila Petanović).



Figure 4.2: *Crassula helmsii* stem infested by *Aculus crassulae*. Arrows indicate big bud galls induced by *A. crassulae*.

## 4.1.2 Establishment potential

### 4.1.2.1 Cold Tolerance

To provide an assessment of cold tolerance and therefore overwintering potential of the biological control agent, lower lethal temperature (LTemp), lower lethal time (LTime) and supercooling points (SCP) are often studied (Hart *et al.*, 2002; Hatherly *et al.*, 2004). These experiments can help to establish whether arthropods under investigation possess the necessary cold hardiness to survive winter temperatures and can provide information on the ability of invasive non-native pests or non-native biological control agents to establish and spread in a new region.

To examine lower lethal temperature, individuals are exposed to a range of cold temperatures for a pre-determined period of time and mortality is monitored. The lower lethal temperature is the temperature at which all individuals are killed. This can be highly variable among invertebrate species, ranging from -10°C to -40°C (Bale & Hayward, 2010). In lower lethal time experiments, individuals are exposed to set temperatures and monitored for mortality over longer periods of time. Ltime<sub>50</sub> is the time required to kill 50% of the population at set temperatures. Hatherly *et al.* (2005) suggested that the LTime<sub>50</sub> of different arthropod groups at 5°C, including mites, is positively correlated to the length of time that individual arthropods survive winter temperatures under field conditions in the UK; with a higher LTime<sub>50</sub> came a longer survival time under natural field conditions. It was concluded that studying



lethal time at similar temperatures could be an important initial assessment for establishment potential for non-native species which may be released as biological control agents. Investigating the lethal temperature and lethal time for *A. crassulae* is expected to provide evidence to whether these mites are able to tolerate cold temperatures and therefore survive and establish in the UK.

#### 4.1.2.2 Relationship between temperature and development rate

Investigating the effect of temperature on the development rate of arthropods is an important part of understanding the establishment potential of a biological control agent. The development rate can be used to develop phenological models which can predict adult emergence times, the number of generations possible under certain temperatures and the potential geographical distribution of an arthropod (Rebaudo & Rabhi, 2018). For many insects the relationship between development rate and temperature is approximately linear at moderate temperatures, and curved at the extremes. Linear models are the most commonly used models to describe the relationship between development rate and temperature (Quinn, 2017). They are used in order to calculate the lower developmental threshold ( $T_{\min}$ ) and the thermal constant ( $K$ ) of a particular species and they are the only models that can estimate  $K$  (Kontodimas *et al.*, 2004). Treating the response as linear however, can lead to underestimations of development rates and  $K$  at low temperatures and overestimations at high temperatures (Howe, 1967; Moore & Remais, 2014). The equation below describes the relationship between development rate and temperature; development rate is calculated as 1/number of days ( $D$ ),  $a$  is the intercept of the line,  $b$  is the slope of the line and  $T$  is the temperature (Campbell *et al.*, 1974; Rebaudo *et al.*, 2017):

$$1/D = a + b \times T$$

Many non-linear models have been proposed to make more accurate and realistic estimates and can provide additional parameters. One of the non-linear models that is commonly used is the Lactin-2 model (Lactin *et al.*, 1995). The Lactin-2 model is an adaptation of the Logan model (Logan *et al.*, 1976) and the formula is presented as:

$$1/D = e^{\rho \times T} - e^{\left(\rho \times T_m \frac{T_m - T}{\Delta T}\right)} + \lambda$$

where  $T$  is the rearing temperature,  $\rho$  is a constant defining the rate at optimal temperature,  $T_m$  or  $T_{max}$  is the upper temperature threshold,  $\Delta T$  is the temperature

range over which physiological breakdown becomes the overriding influence. The addition of  $\lambda$  forces the curve to intercept the y-axis.

#### **4.1.2.3 Degree day calculation using linear models**

Calculating the thermal constant allows scientists to determine the growth and development of the arthropod over the growing season by subsequent calculation of day degrees. Day degrees are used to calculate the accumulated heat required by an organism to complete a generation at a particular location and they are based on the upper and lower developmental thresholds between which development can occur. The relationship between development rate and temperature as illustrated in Figure 4.3, is linear in the middle range of temperatures (range B). Therefore, when linear models are used for this purpose, only the middle portion of values is used to extrapolate the line to determine the lower developmental threshold, thermal budget and therefore degree day value (Campbell *et al.*, 1974). When combined with daily temperature data, the potential number of generations an arthropod can complete in a given region can be estimated which can provide important supporting evidence in the Pest Risk Analyses required for releasing a biological control agent. Insect biological control researchers regularly use these methods when assessing risk and establishment potential of biological control agents in the glasshouse (Hart *et al.*, 2002; Hatherly *et al.*, 2004; Tullett *et al.*, 2004).

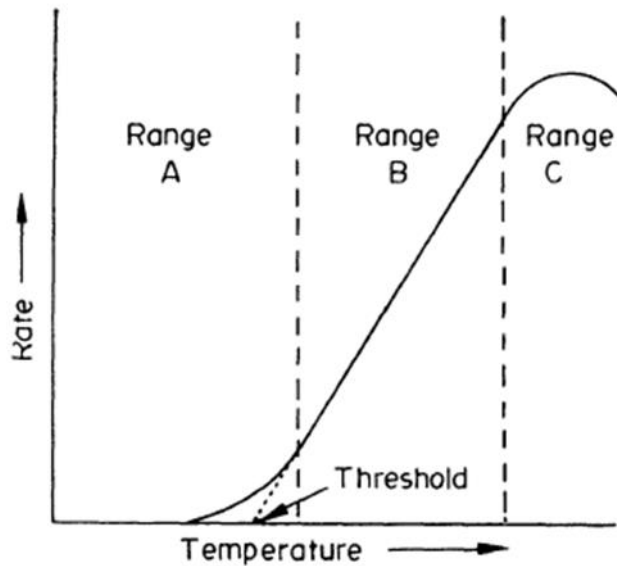


Figure 4.3: The relationship between development rate and temperature (from Campbell *et al.*, 1974). The lower developmental threshold can be estimated by extrapolation of the linear portion (range B) of the fitted line.

By following the law of total effective temperatures, the thermal budget can also be estimated using the equation;

$$K = D (T - T_0)$$

where  $K$  is the thermal constant,  $D$  is the duration of development in days,  $T$  is the temperature and  $T_0$  is the lower temperature threshold (Damos & Savopoulou-Soultani, 2012). The thermal constant cannot be derived from non-linear models.

### 4.1.3 Host range for phytophagous mites

#### 4.1.3.1 Determination of the test plant list

Developing a suitable test plant list forms the foundation of risk assessments for weed biological control. As described in section 2.2, the methodology used to compile this list is based on the centrifugal phylogenetic method of Wapshere (1974). Today however, there is more emphasis on the degree of phylogenetic relatedness between plant species rather than taxonomy circumscriptions (Briese, 2005). For arthropods, the type of species under investigation as a biological control agent and its life history should not significantly affect which plant species are included in the test plant list. The biology of the organism may need to be considered however when composing the list for organisms such as heteroecious rust fungi

which include an alternative host in their life cycle (Morin, 2020). The test plant list is primarily based upon the target plant rather than the agent. One situation where the agent under investigation affects the test plant list is if host acceptance behaviour of the organism is associated with a particular plant biochemistry. In this case biocontrol researchers would include those species which are part of that plant family but potentially geographically distant (Sheppard *et al.*, 2005). There are no known specific biochemical associations of mites in the *Aculus* genus and the Crassulaceae plant family.

#### **4.1.3.2 Host-range testing of mites**

As outlined in section 3.1.3, the host-range testing of insect agents involves choice and no-choice tests. In the case of eriophyoid mites, generally only no-choice tests are carried out. This is partly due to the fact that these mites disperse by wind currents with minimal active movement and therefore it is difficult to replicate the natural spread by wind currents (Skoracka *et al.*, 2010). Some studies have involved choice tests by allowing test plants to touch thereby permitting ambulatory movement between plants (Goolsby *et al.*, 2005; Stoeva *et al.*, 2012) and these choice tests were used specifically with plant species that supported reproduction in no-choice tests or for locally important plant species (Goolsby *et al.*, 2005).

#### **4.1.4 The impact of biological control agents on host plants**

Alongside investigating the host range of a potential biological control agent, an important part of pre-release experimentation involves studying the impact of the agent under consideration. Ensuring a biological control agent is safe to release is of the highest priority for regulators and biological control scientists alike and an agent's impact on a target plant should not overshadow the focus on its host specificity. However, it is recognised that selecting agents that cause the greatest impact on the target weed will have the best potential to succeed as a biological control agent thereby reducing ecologically damaging densities of the weed. Where the use of classical biological control has successfully contributed to control of the pest, there have been major cost-savings, but initial costs can be substantial, particularly in the early stages of a programme, involving extensive overseas surveys and long periods of testing in costly quarantine facilities. Therefore, efforts should be made to focus studies on potential agents with a high likelihood of establishing and reducing the fitness of the target weed.

Damage caused by some weed biological control agents is clearly evident and may even cause the direct death of the plant. One such example is the effect of the weevil, *Cyrtobagous salviniae* (Calder & Sands) against the aquatic plant, *Salvinia molesta* D. Mitch., which has been released with great success across the world. However, species including sap-sucking insects and plant pathogens may display a more subtle impact on the target weed which can become more noticeable when assessing the effect in combination with competition from other plant species and over time under natural conditions (Coetzee *et al.*, 2005). Such impacts may involve a reduction in plant growth rate or in seed production, thereby reducing plant fitness at a population level. Feeding by eriophyoid mites in particular, might appear inconspicuous, despite the presence of distinctive galls. Since eriophyoid mite infection does not generally result in plant death, or in some cases may not even seem to significantly affect the host, it is important to assess the impact in more detail.

Pre-release impact studies often take place in a quarantine laboratory, under favourable conditions and free from the external factors that will influence the performance of the biological control agent including competition and predation. In addition, these organisms do not experience the normal fluctuations in temperature and humidity that would occur in a natural setting. Therefore, it is acknowledged that these studies are highly simplified and may overestimate the potential impact there may be under natural conditions.

## 4.2 Objectives

### 1) Investigation into the establishment potential of *A. crassulae*

- Explore linear and non-linear models to examine the relationship between development rate and temperature to establish the extent *A. crassulae* can survive and spread during the main growing season under UK climatic conditions.
- Investigate the ability of *A. crassulae* to survive winter conditions in the UK by examining the effect of cold temperature on its survival.

### 2) Investigation into the host range of *A. crassulae*

- Develop the test plant list for host-specificity testing of non-target species against *A. crassulae* for the UK and Western Europe.

- Understand the host-specificity of *A. crassulae* by host-range testing, using choice and no-choice tests with non-target plants under quarantine conditions.

### 3) Investigation into the impact of *A. crassulae* on the growth of *C. helmsii*

- Examine the impact of *A. crassulae* colonisation on *C. helmsii* by comparatively assessing the change in growth characteristics in plants with and without mite colonisation.

## 4.3 Materials and Methods

### 4.3.1 Establishment potential of *Aculus crassulae*

#### 4.3.1.1 Effect of cold temperature on *Aculus crassulae* survival

Mites were exposed to 5°C, 0°C and -5°C to assess survival at cold temperatures. Mites were also exposed to 15°C to compare survival at cold temperatures to survival at a temperature closer to ambient levels. For all treatments, five adult mites were collected from mature galls and hand transferred (with a hooked pin attached to a long handle) to Eppendorf tubes containing a single *C. helmsii* leaf for humidity. There were 10 replicates per temperature. Prior to treatment, survival of the transfer was recorded. The tubes were sealed and incubated at 10°C for 30 minutes prior to exposure to cold treatment to prevent shock. They were then divided between three glass boiling tubes plugged with cotton wool and held in a programmable low temperature alcohol bath filled with anti-freeze pre-cooled to 10°C. The temperature was gradually reduced to the experimental temperature with a cooling rate of 0.5°C per minute. Once the desired temperature was reached, the temperature was held for 24 and 48 hours respectively. Data loggers (Logtag HAXO-8) were placed in the incubator and in the alcohol bath and recorded the temperature once a minute. Following the cold treatment, the alcohol bath was gradually returned to 10°C at a rate of 0.5°C per minute. The tubes were then incubated at 10°C. After 12 hours the number of surviving mites was recorded under a stereomicroscope as indicated by movement.

This experiment was repeated using eggs. Mites were allowed to oviposit on fresh *C. helmsii* growing shoots for 24 to 48 hours in a CT room at 23°C/18°C under an 18 light: 6 dark, light regime and at 70% relative humidity. Following oviposition, the

top 5mm of the plant including the growing shoot was removed from the plant and transferred to an Eppendorf tube. This allowed for minimal handling of the egg and the nature of the particular protective structure of the plant. The tubes were exposed to 5°C, 0°C and -5°C using the same methodology as for adult exposure. The eggs were incubated at 10°C for two hours prior to incubation at 22°C. This temperature was determined to be optimal for mite development. The assessment of egg hatch and thus survival of the eggs, took place after six days.

#### **4.3.1.2 Investigation into the $LTime_{50}$ of *Aculus crassulae***

Five to eight individual adult mites were each hand-transferred using a fine needle to 30 Eppendorf tubes. Survival of the transfer was recorded for each tube. The tubes were first incubated at 10°C for two hours to prevent cold shock, and then placed in a 4°C incubator with a light regime of 12 hours light: 12 hours dark. One tube was removed every two to three days and assessed for survival until 100% mortality was observed.

#### **4.3.1.3 Effect of temperature on the development of *Aculus crassulae***

Individual adult mites were hand transferred using a fine needle to the apical shoot of individual *C. helmsii* stems which was held in tap water within a Perspex cage in a CT room set at 23°C day and 18°C night, with an 18 hour light: 6 hour dark, light regime and 70% relative humidity for 24 hours for oviposition. The number of replicates for each temperature is presented in Table 4.1 (section 4.4.1, page 74). When oviposition was observed, the adult was removed and the plant and its egg were transferred to individual 30ml universal tubes each held within a small ventilated container. The lower stem of each plant was wrapped in damp tissue to provide moisture to the plant and to prevent excessive humidity in the container. The ventilated containers were then placed in a larger ventilated container with a data logger (LogTag, HAXO-8) recording temperature and humidity once every 30 minutes. The containers were placed in lit incubators with a 12 hour light: 12 hour dark regime, 70% relative humidity at constant temperatures: 4, 10, 12, 15, 17, 20, 22, 25 and 30°C. The development of the egg was monitored every two to three days. Following the emergence of the nymph, development was monitored daily until the final moult and the adult mite emerged. The total duration of the life cycle from egg to adult was recorded at all temperatures, but not the duration of the immature life stages since they are difficult for non-specialists to distinguish and the more regular handling of the gall was found to cause higher levels of mortality. The

duration of the egg stage was recorded for temperatures between 15 and 25°C but not for the lower temperatures where development was much slower.

#### **4.3.1.4 Data analysis**

To assess the effect of cold temperature on mite survival, data were analysed using a generalised linear model (GLM) using a binomial error structure. A logistic regression was carried out on  $L_{time50}$  data. The rate of development data was analysed using a one-way ANOVA and further analysed using Tukey's HSD.

The rate of development was calculated and plotted against temperature as displayed in Figure 4.7. As described in section 4.1.2.2, for arthropods, the relationship between development rate and temperature is not completely linear, particularly at the lower and higher end of the temperature scale where minimum and maximum biological thresholds are reached. Therefore, in this particular study, the middle range of the data series (10-22°C), as the linear portion, was used to estimate the lower developmental threshold. Using simple linear regression, the lower developmental threshold was determined by extrapolation of the fitted line.

All models were fitted using R version 3.4.1 (R Core Team 2019). The Lactin-2 model was fitted using the devRate package in R (Rebaudo, 2018).

#### **4.3.1.5 Distribution mapping using degree days**

The annual degree days across the UK were calculated using monthly average temperatures from 2014-2018 obtained from the UK Met Office. Since *A. crassulae* only utilises emergent and terrestrial forms of its host plant, *C. helmsii*, using air temperature rather than water temperature was deemed to be the most appropriate. A base temperature of 10°C was subtracted from the mean daily temperature and multiplied by 30.5 to achieve a monthly degree day total. The mean monthly degree days totals were then combined to obtain the total degree days per year. These data were then used to calculate the likely number of generations possible in the UK. A threshold of 10°C rather than the calculated lower development threshold of 8.7°C was used in this case because the true development threshold is likely to be between 10 and 12°C as demonstrated by the results of the study into the effect of temperature on development of *A. crassulae*. Using this higher temperature provided a more conservative estimate of the number of generations likely to be achieved in the UK each year.



### **4.3.2 Host range of *Aculus crassulae***

#### **4.3.2.1 Test plant list**

The test plant list was developed from the latest phylogenetic data on the Saxifragales order and Crassulaceae family using the Angiosperm phylogeny website (Stevens, 2019). The list was developed and agreed with advice from the Defra-headed biological control project board and botanist, Dr Mark Spencer (formerly of the Natural History Museum, London). Plants were sourced primarily from nurseries or field collected. Kew's Millennium Seed Bank and collaborators around the UK supplied seeds and plant material of test species that were otherwise difficult to source.

#### **4.3.2.2 No-choice tests**

The technique used in these tests involved attaching one mature, mite-infested bud with an average of 50 mites to the potted test plant and to positive controls of the host plant, *C. helmsii*, using fine entomology pins. The attachment of the bud allowed the mites to transfer independently onto the new plant as the infested bud desiccated. The plants were held within a Perspex cage in a CT room set at 23°C/18°C, 18 hour light: 6 hour dark, light regime and 70% relative humidity. Every seven days the plants were monitored for symptoms and the number of live mites per plant was estimated under a stereomicroscope as indicated by movement of the mites. Damage was scored on a 0-5 scale (0=No symptoms, T=trace or "minor" feeding, 1= 0-5%, 2=6-10%, 3=11-25%, 4=26-50%, 5=over 50%) and the number of live mites was estimated using a scale (less than 10, 10-50, or more than 50). Feeding damage was observed by reddening and swelling of the growing shoots as manifested in *C. helmsii*. Under optimal conditions, these symptoms on *C. helmsii* start to develop two to four days after the application of mites. The "big bud" symptom is clearly evident after 14 days, producing the gall-like structure. After 42 days, mites were extracted from the plants by washing them in a diluted soap solution and all mobile stages were counted under a stereomicroscope at x10 magnification. The tests were run for 42 days to allow sufficient time for mites to develop on non-target plants, should they have the ability to.

Forty species were exposed to the mites in no-choice tests and tests with each species were replicated a minimum of eight times. Parallel negative control test runs without

the attachment of mite infested buds, but healthy buds attached instead, were set up for each test species to compare and account for natural plant variation.

#### **4.3.2.3 Choice tests**

Choice tests were carried out where a non-target species supported oviposition or feeding damage, and was only undertaken for the native congener *C. aquatica*. This test was set up for *C. helmsii* and *C. aquatica* plants to touch in order to allow mites to transfer between the two species. There were eight replicates for this test and plants were maintained under the same conditions as the no-choice tests.

#### **4.3.3 Impact of *Aculus crassulae* on *Crassula helmsii***

Plants were subjected to three treatments: a high or low density of mites and control with no mites. The densities of mites were classified in these categories rather than an exact number due to the fragility of the mite; the transfer of large numbers of mites was likely to result in high levels of mortality. The high density treatment corresponded to approximately 40 mites and the low density corresponded to approximately 20 mites. These densities were chosen as likely densities naturally occurring under field conditions as observed in Australia. Each replicate consisted of an individual plant with no secondary shoot or flower growth and a standardised number of roots. Each treatment consisted of 10 replicates. Plant height, number of nodes and number of leaf pairs in all plants were recorded prior to treatment with mites. Mites were applied to plants by attaching infested buds onto the plant using fine entomology pins allowing the mites to transfer independently onto the new plant as the infested bud desiccated. Control plants also had pins attached to them. The plants were held within a Perspex cage in a CT room set at 23°C/18°C, 18 hour light: 6 hour dark, light regime and 70% relative humidity.

Plant parameters including plant height, leaf pair number, number of nodes, number of nodes with roots on primary stem, number of flowers, number and length of secondary and tertiary shoots were measured 7, 21 and 44 days after experimental set up.

##### **4.3.3.1 Data analysis**

For plant height, node number and leaf pair number, the data reflected relative changes over a period of 44 days rather than a direct comparison between treatments. The rest of the plant parameters measures were compared directly. Analysis of the

number of flowers was not possible due to insufficient data over the course of the study. Data were analysed using generalised linear models (GLM), using a Poisson error structure for count response variables (number of secondary and tertiary shoots, nodes and leaf pairs) and a Gaussian error structure where the response variable was continuous (plant height and secondary shoot length). Data were tested for normality, heterogeneity and overdispersion where necessary to meet with the assumptions of the relevant tests. Where count variables were overdispersed, a quasi-Poisson structure was used. The Benjamini-Hochberg (BH) procedure was used to correct for multiple testing where necessary. All analyses were carried out in R Studio (R Core Team, 2019).

## **4.4 Results**

### **4.4.1 Establishment potential of *Aculus crassulae***

#### **4.4.1.1 Effect of cold temperature on *Aculus crassulae* survival**

There was no difference in adult mite survival between any of the temperatures tested and no difference in adult survival between 24 and 48 hours ( $p > 0.05$ , Figure 4.4). Overall temperature had a significant effect on egg viability ( $z = 2.167$ ,  $p < 0.05$ ) and there was greater viability of eggs at  $0^{\circ}\text{C}$  than at all other temperatures (Figure 4.5). Viability of eggs was also higher after 24 hours than after 48 hours ( $z = 0.4888$ ,  $p < 0.001$ ). The viability of eggs was lower than the survival of adults ( $t = -3.103$ ,  $p < 0.01$ ).

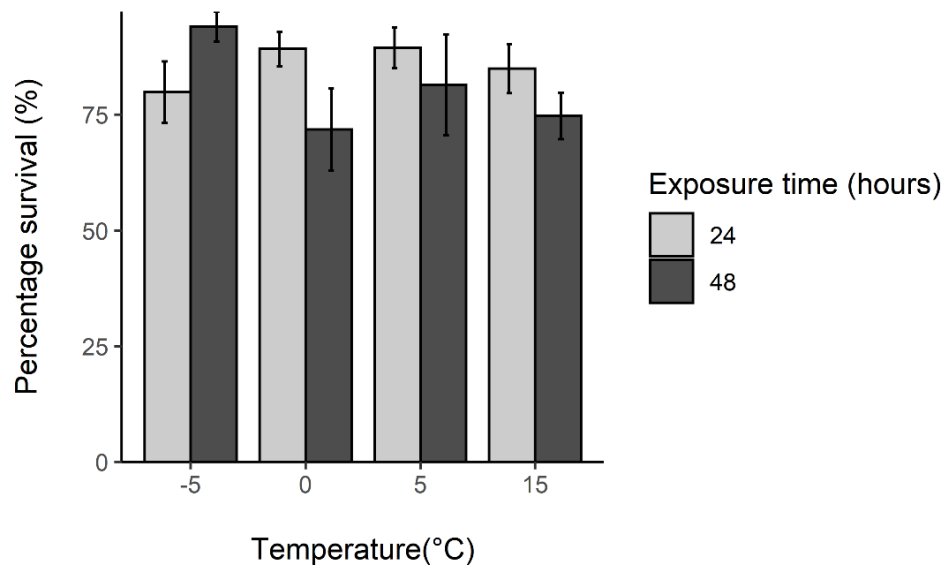


Figure 4.4: The mean proportion ( $\pm$  SE) of adult *Aculus crassulae* mites surviving exposure to -5°C, 0°C 5°C and 15°C after 24 and 48 hours.

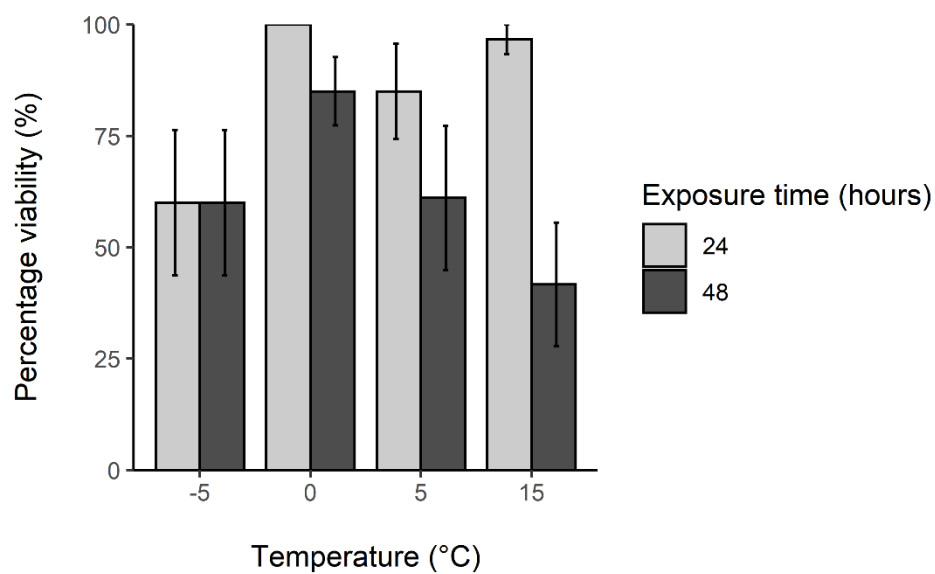


Figure 4.5: The mean proportion ( $\pm$ SE) of eggs of *Aculus crassulae* surviving exposure to -5°C, 0°C 5°C and 15°C after 24 and 48 hours.

#### 4.4.1.2 Investigation into the $LTime_{50}$ of *Aculus crassulae*

The proportion of mites surviving the treatment over time was calculated; there was 10% mortality at 14 days, 50% at 46 days and 90% at 78 days (Figure 4.6).

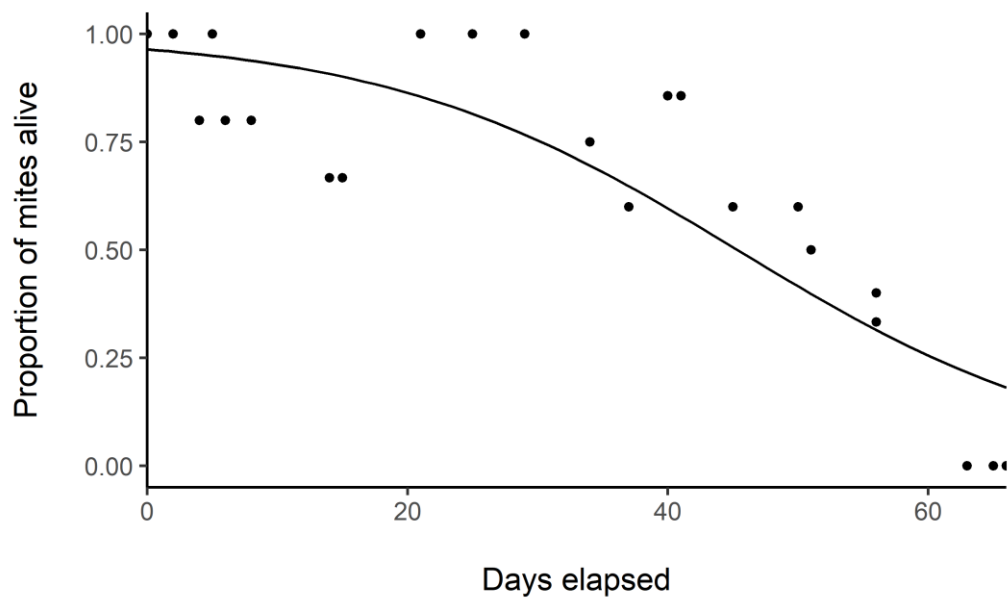


Figure 4.6: Survival of adult *Aculus crassulae* mites after exposure to 4°C. The line is fitted using the calculated proportions from the logistic regression.

#### 4.4.1.3 Effect of temperature on the development of *Aculus crassulae*

No development took place at 4°C or 30°C and incomplete development took place at 10°C (Figure 4.7, Table 4.1). Developmental rate increased as the temperature increased ( $F_{1,8}=703$ ,  $p<0.01$ ). There was no difference in development rate between 17°C and 20°C, but development rate increased between 12°C and 15°C ( $p<0.01$ ), 15°C and 17°C ( $p<0.01$ ) and 20°C and 22°C ( $p<0.01$ ). Developmental rate decreased between 22°C and 25°C ( $p<0.01$ ). The result was reflected in the analysis for the number of days for complete development ( $F_{1,8}=1117$ ,  $p<0.01$ ) except there was no difference between 22°C and 25°C.

Table 4.1: Effect of temperature on development of *Aculus crassulae* (mean days  $\pm$  SE).

Temperature/ °C	Egg hatch/ days	Total time egg to adult/ days ( $\pm$ SE)	Number of individuals	Mean development rate (1/days)
4	0	0	15	0
10	Data unavailable	0	15	0
12	Data unavailable	40.8 $\pm$ 0.6	10	0.025
15	10.8 $\pm$ 0.3	30.9 $\pm$ 0.9	7	0.033
17	7.5 $\pm$ 0.5	18.4 $\pm$ 0.3	17	0.056
20	6.2 $\pm$ 0.2	17.6 $\pm$ 0.5	12	0.057
22	4.5 $\pm$ 0.3	13.1 $\pm$ 0.3	13	0.075
25	4.6 $\pm$ 0.3	14.6 $\pm$ 0.4	9	0.069
30	0	0	20	0

The rate of development was calculated and plotted against temperature (Figure 4.7). Using simple linear regression, the lower developmental threshold was determined by extrapolation of the fitted line and was estimated to be 8.7°C. The thermal budget of *A. crassulae* was calculated to be 175 degree days above the threshold temperature to complete development from egg to adult. This value is calculated from the linear regression and is the reciprocal of the slope of the line (1/0.0057). The  $R^2$  value indicates that 91% of the variation can be explained by assuming the relationship at these temperatures is linear.

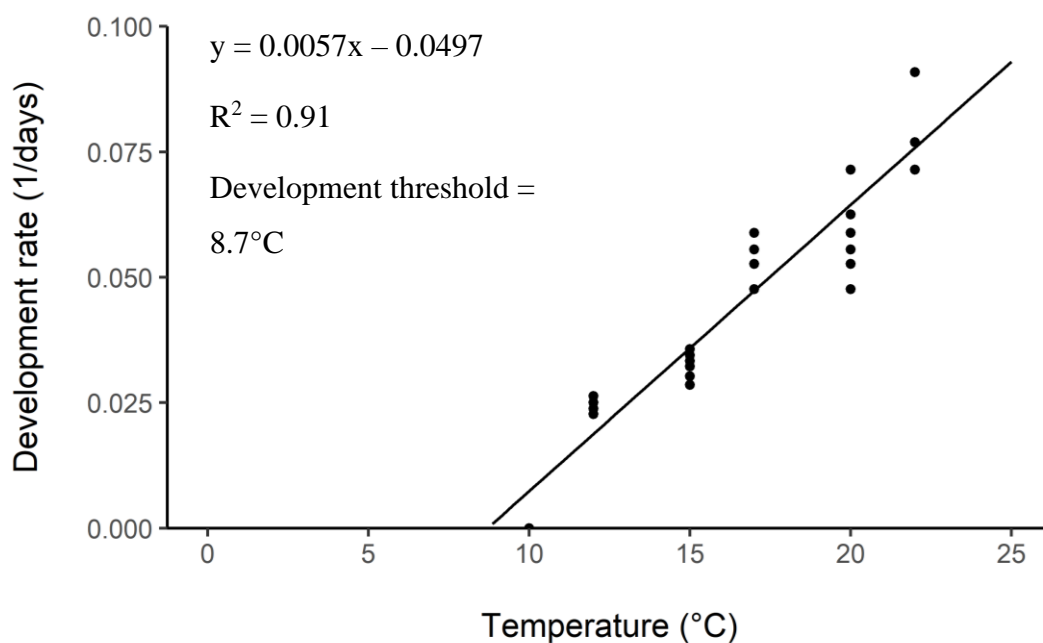


Figure 4.7: Development rate of *Aculus crassulae* from egg to adult at five constant temperatures with the line fitted by simple linear regression. The developmental threshold is determined by extrapolation of the fitted line to the x axis where development rate is 0. Each point on the graph represents more than one data point if several replicates had the same developmental rate. ( $y=0.0057x - 0.0497$ ,  $R^2 = 0.91$ ,  $p<0.01$ ).

#### 4.4.1.4 Non-linear models

The parameters estimated by the Lactin-2 model and linear model are shown in Table 4.2. Figure 4.8 shows the non-linear curve as estimated by the Lactin-2 model.

Table 4.2: Parameters as estimated by the linear model and Lactin-2.

Model	Parameters	Total (from egg to adult)
Linear	K (days)	175
	$T_{min}$	8.7°C
	$R^2$	0.91
Lactin-2	$T_{max}$	39.79°C
	$T_{min}$	8.93°C
	$\Delta T$	4.80
	Optimal temperature/ $\rho$	24.36°C
	$R^2$	0.26

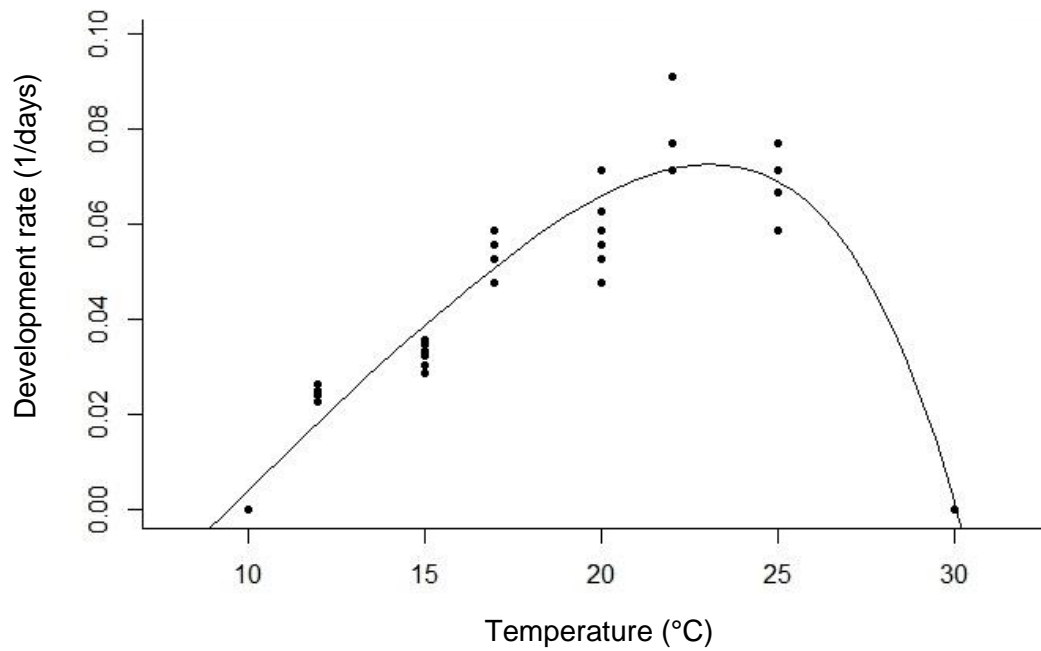


Figure 4.8: Development rate of *Aculus crassulae* from egg to adult at eight constant temperatures with the line fitted by the Lactin-2 equation.

#### 4.4.1.5 Distribution mapping using degree days

Figure 4.9 shows the estimated range of generations possible in different parts of the country; the mite may achieve one generation per year in high altitude regions but there could potentially be 4 or more generations in some parts of the Midlands, southern England and the southern coast of Wales.



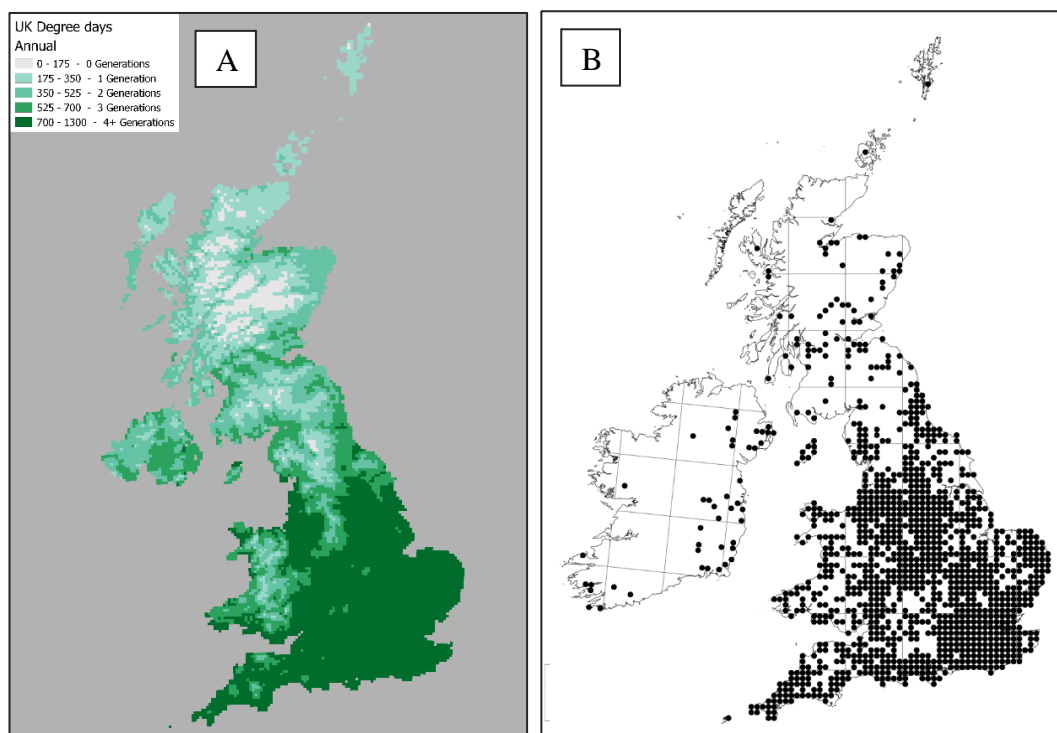


Figure 4.9: A, map of the predicted number of generations possible in the UK based on the degree day figure of 175 required for *Aculus crassulae* to complete development; B, distribution of *Crassula helmsii* in the UK, each dot represents at least one record in each 10km x 10km square of the national grid (map courtesy of Botanical Society of Britain and Ireland, accessed April 2020).

#### 4.4.2 Host range of *Aculus crassulae*

##### 4.4.2.1 The test plant list

The target weed, *C. helmsii* belongs to the plant order Saxifragales, which although relatively small, is a morphologically highly diverse group. The order includes annual and perennial herbs, succulents, aquatics, shrubs, vines and large trees (Jian *et al.*, 2008). Molecular techniques have established the basic relationships of the families within this order, but deeper associations within the clades are not as well defined (Jian *et al.*, 2008). The phylogram (Figure 4.10) based on the Bayesian consensus of total evidence data, was produced by Jian *et al.* (2008) and shows the relationship between the families in the Saxifragales. The “Woody Clade” consists of the families; Paeoniaceae, Altingiaceae, Hamamelidaceae, Cercidiphyllaceae and Daphniphyllaceae. The “Core Saxifragales” include the families Crassulaceae, Haloragaceae, Iteaceae, Pterostemonaceae, Saxifragaceae and Grossulariaceae. Species from all of these families are represented in the test plant list.

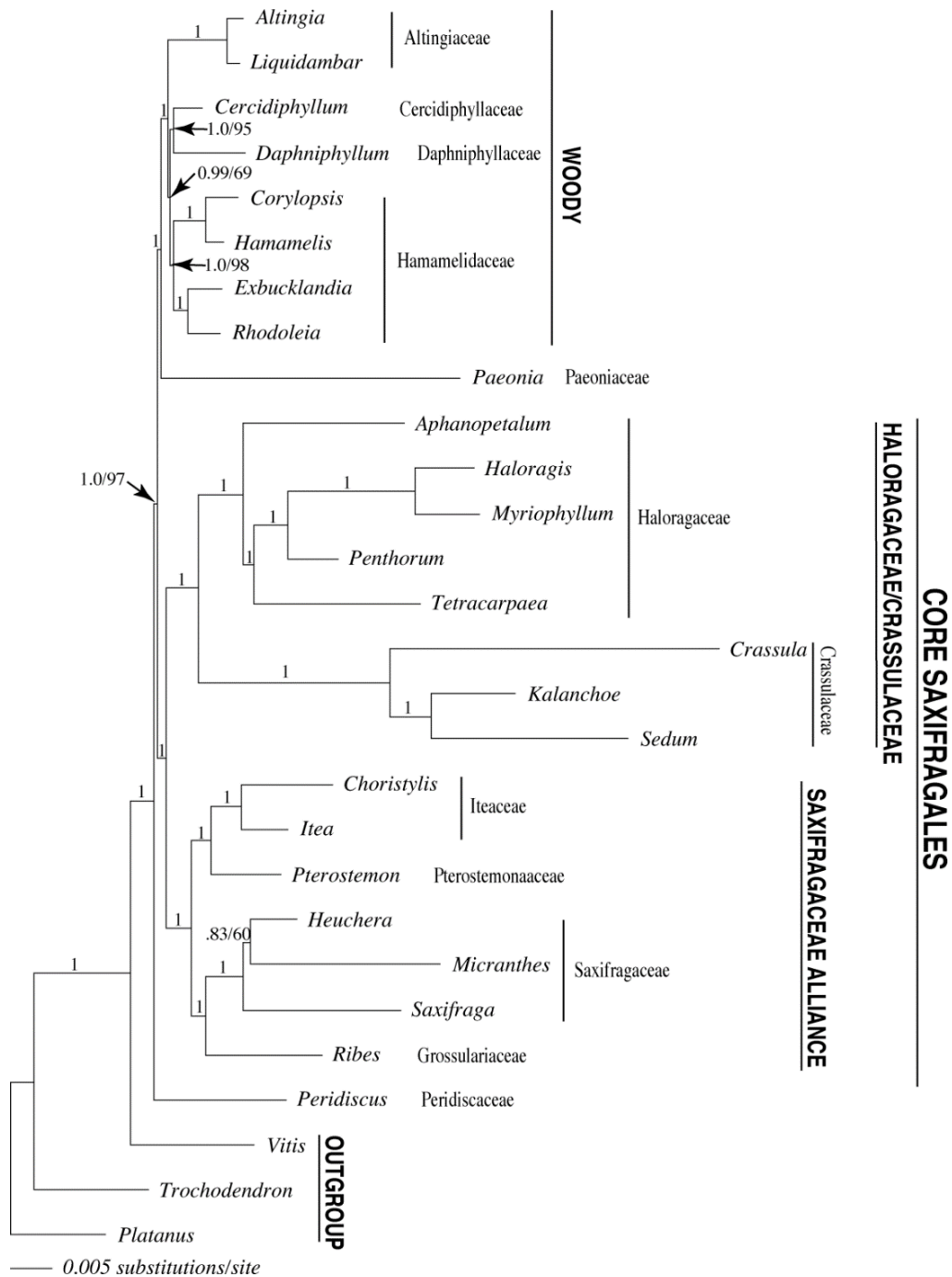


Figure 4.10: Phylogram of the Saxifragales order based on the Bayesian consensus of total evidence as produced by Jian *et al.* (2008).

The Crassulaceae family is the largest in the order Saxifragales, and is a family of succulent species. The genus *Crassula*, to which the target plant belongs, is one of over 30 genera within Crassulaceae, many of which are well-known to the public; for example, *Sedum*, *Kalanchoe* and *Sempervivum*. The majority of plants in this family are recognised as being particularly tolerant of cold climates and as a result are favourites in rock gardens. Consequently, representatives from these groups are

included in the test plant list. The UK is home to two native *Crassula* species; *Crassula aquatica* and *Crassula tillaea*. *Crassula aquatica* is an aquatic species with a similar morphology to *C. helmsii*, and for which there is only one record in the UK, located in western Scotland (Biological Records Centre, 2020). Its European distribution is primarily in Scandinavia with disjunct populations in other parts of Western Europe. In the UK its conservation status is “vulnerable”. *Crassula tillaea* is a terrestrial annual plant with a western European/ Mediterranean distribution. *Crassula decumbens* Thunb. is a non-native *Crassula* species that is present on the Scilly Islands, and *Crassula pubescens* Thunb. is found on the Channel Islands. *Crassula decumbens* and *C. pubescens* have not been included in the test plant list as they are not found on the mainland. Most of the ornamental Crassulaceae which are popular in the UK are native to the South African region. Encouragingly, no ornamental or otherwise desirable non-native *Crassula* species present on the UK mainland are from Australasia.

The Saxifragales order contains several families which are important in the horticultural industry, particularly Paeoniaceae, Crassulaceae and Saxifragaceae. Species belonging to the economically significant genus *Ribes* are abundant in Western Europe and therefore have also been included.

The *Myriophyllum* genus in the Haloragaceae family contains some native water plants which share a similar habitat to *C. helmsii* and could regularly be exposed to the potential biological control agent. The non-native aquatic weed, *Myriophyllum aquaticum* (Vell.) Verd., is also included here.

Finally, safeguard species which are unrelated but share a habitat with *C. helmsii* have been included in this test list due to the likelihood of contact with a potential biological control agent and the risk-averse nature of UK regulators. Given the target weed’s different growth forms, both marginal and submerged aquatic plant species from genera such as *Potamogeton* and *Alisma* were included. *Damasonium alisma* Mill. populations have suffered significant decline and inhabit a similar environment to *C. helmsii*. This species therefore was also included in the test plant list.

The proposed test plant list was composed of 40 species (Table 4.3).

#### **4.4.2.2 No-choice tests**

Low numbers of mites were found on 22 of the 40 test plant species 42 days after initial exposure (Table 4.3). There was no apparent taxonomic relationship between

the species that supported mite survival for 42 days. The abundance of mites recorded on test plants at the end of the test were significantly lower on the non-target plants than on the target plant and no damage was recorded on any non-target plants after 42 days except for minor trace feeding on two replicates of *C. aquatica*.

Nine of the 22 replicates of the closely related plant, *C. aquatica* that were tested experienced trace feeding in no-choice tests after 14 days of exposure. This manifested itself as a curve in the apical leaves which was more apparent than in control plants (Figure 4.9A). The symptoms were not visible during later assessments. Between one and six eggs were laid in five replicates of the 22 tested in no-choice tests but no further development took place. Adult mites were the only life stage recovered from the plants at the end of the experiment.

#### **4.4.2.3 Choice tests**

In choice tests, no symptoms of feeding damage were apparent and no eggs were laid on *C. aquatica*. The presence and absence of mites on *C. aquatica* was inconsistent throughout the duration of the test. *Crassula helmsii* displayed the typical symptoms associated with *A. crassulae* feeding.

Table 4.3: Host-range testing data showing the number of *Aculus crassulae* individuals present on the plant and the mean damage score after 42 days. Damage was scored using a scale; 0=No symptoms, T=trace or “minor” feeding, 1= 0-5%, 2=6-10%, 3=11-25%, 4=26-50%, 5=over 50%. Plant families are shown in bold.

\*Scored 0 in 20 replicates and T (trace) in two replicates

Test plant	Number tested	Number mites present after 42 days ( $\pm$ SE)	Plant damage score after 42 days
<b>Crassulaceae</b>			
<i>Crassula helmsii</i> (Kirk) Cockayne	154	612 $\pm$ 41	4
<i>Crassula aquatica</i> (L.) Schönland	22	3 $\pm$ 2	0*
<i>Crassula tillaea</i> Lest.-Garl.	17	2 $\pm$ 1	0
<i>Crassula ovata</i> (Mill.) Druce	8	0	0
<i>Crassula perforata</i> Thunb.	8	4 $\pm$ 1	0
<i>Crassula sarcocaulis</i> Eckl. & Zeyh.	8	1	0
<i>Crassula lycopodioides</i> Lam.	8	5 $\pm$ 1	0
<i>Kalanchoe blossfeldiana</i> Poelln.	8	0	0
<i>Aeonium arboreum</i> (L.) Webb & Berthel. “Zwartkop”	8	19 $\pm$ 10	0
<i>Rhodiola rosea</i> L.	8	4 $\pm$ 2	0
<i>Echeveria elegans</i> Rose	8	0	0
<i>Sedum acre</i> L.	11	6 $\pm$ 4	0
<i>Sedum anglicum</i> Huds.	8	0	0
<i>Sedum album</i> L.	8	1 $\pm$ 1	0
<i>Sedum forsterianum</i> Sm.	8	1	0
<i>Sedum villosum</i> L.	8	2 $\pm$ 1	0
<i>Sedum spectabile</i> Boreau	8	1 $\pm$ 1	0
<i>Umbilicus rupestris</i> (Salisb.) Dandy	8	0	0
<i>Sempervivum arachnoideum</i> L. “Rubin”	8	3 $\pm$ 1	0

Test plant	Number tested	Number mites present after 42 days ( $\pm$ SE)	Plant damage score after 42 days
<b>Haloragaceae</b>			
<i>Myriophyllum spicatum</i> L.	8	0	0
<i>Myriophyllum aquaticum</i> (Vell.) Verdc.	8	$2 \pm 1$	0
<b>Grossulariaceae</b>			
<i>Ribes nigrum</i> L.	10	$9 \pm 5$	0
<i>Ribes rubrum</i> L.	8	$3 \pm 2$	0
<b>Saxifragaceae</b>			
<i>Saxifraga granulate</i> L.	8	0	0
<i>Saxifraga hypnoides</i> L.	8	0	0
<i>Chrysosplenium oppositifolium</i> L.	17	$1 \pm 1$	0
<b>Paeoniaceae</b>			
<i>Paeonia officinalis</i> L.	8	$4 \pm 2$	0
<b>Altingiaceae</b>			
<i>Liquidambar styraciflua</i> L.	8	0	0
<b>Hamamelidaceae</b>			
<i>Hamamelis mollis</i> Oliv.	8	$1 \pm 1$	0
<b>Cercidiphyllaceae</b>			
<i>Cercidiphyllum japonicum</i> Siebold & Zucc.	9	$1 \pm 1$	0
<b>Daphniphyllaceae</b>			
<i>Daphniphyllum macropodum</i> Miq.	8	8	0
<b>Lentibulariaceae</b>			
<i>Utricularia vulgaris</i> L.	8	0	0
<b>Callitrichaceae</b>			
<i>Callitriche stagnalis</i> Scop.	8	$1 \pm 1$	0
<b>Alismataceae</b>			
<i>Damasonium alisma</i> Mill.	8	0	0
<i>Alisma plantago-aquatica</i> L.	9	0	0

Test plant	Number tested	Number of mites present after 42 days ( $\pm$ SE)	Mean plant damage score after 42 days
<i>Luronium natans</i> (L.) Raf.	8	0	0
<b>Hydrocharitaceae</b>			
<i>Hydrocharis morsus-ranae</i> L.	8	0	0
<b>Typhaceae</b>			
<i>Typha latifolia</i> L.	9	0	0
<b>Potamogetonaceae</b>			
<i>Potamogeton crispus</i> L.	8	0	0
<i>Potamogeton natans</i> L.	8	0	0
<b>Marsileaceae</b>			
<i>Pilularia globulifera</i> L.	8	0	0

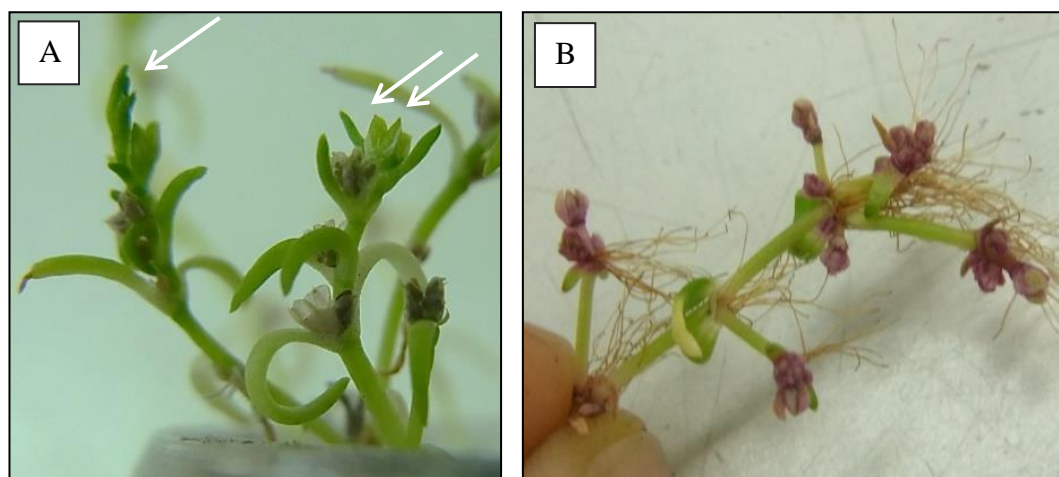


Figure 4.11: Symptoms of feeding by *Aculus crassulae* on A, *Crassula aquatica* and B, *Crassula helmsii* in host-range testing. Single arrow points to curve in leaf on *C. aquatica* caused by *A. crassulae* in a no-choice test. Double arrow points to adjacent unaffected leaf, B; enlarged buds in host *C. helmsii*.

#### 4.4.3 Impact of *Aculus crassulae* on *Crassula helmsii*

When data across all dates was compared, a significant interaction was observed between time and the density of mites applied for plant height, but not for any of the other growth parameters measured. Over the course of the experiment, plant height increased over time within each mite treatment; control ( $F_{2,27} = 115.6$ ,  $p < 0.001$ ), low ( $F_{2,27} = 30.1$ ,  $p < 0.001$ ) and high treatment ( $F_{2,27} = 18.71$ ,  $p < 0.001$ ).

At the first assessment, after seven days, mites had no effect on any of the plant growth traits measured. However, after 21 days, where mites were applied, there was a reduction in several of growth parameters. The change in plant height (linear model,  $t = -6.667$ ,  $p < 0.01$ ), number of nodes (GLM,  $z = -3.593$ ,  $p < 0.01$ ), the number of new leaf pairs (GLM,  $z = -4.438$ ,  $p < 0.01$ ) and number (GLM,  $z = -2.694$ ,  $p < 0.05$ ) and length of secondary shoots (linear model,  $t = -5.967$ ,  $p < 0.01$ ) were lower in the high treatment than in control plants without mites.

Plants under the low treatment had shorter stems than the control plants (linear model,  $t = 4.376$ ,  $p < 0.05$ ). There were also differences between the high and low treatments; there were fewer new leaf pairs in the high treatment than the low (GLM,  $z = -3.424$ ,  $p < 0.01$ ) and secondary shoot length was shorter in high treatment plants than in the low treatment plants (linear model,  $t = 4.915$ ,  $p < 0.01$ ). Tertiary growth was not sufficient for statistical analysis at this stage in the experiment.



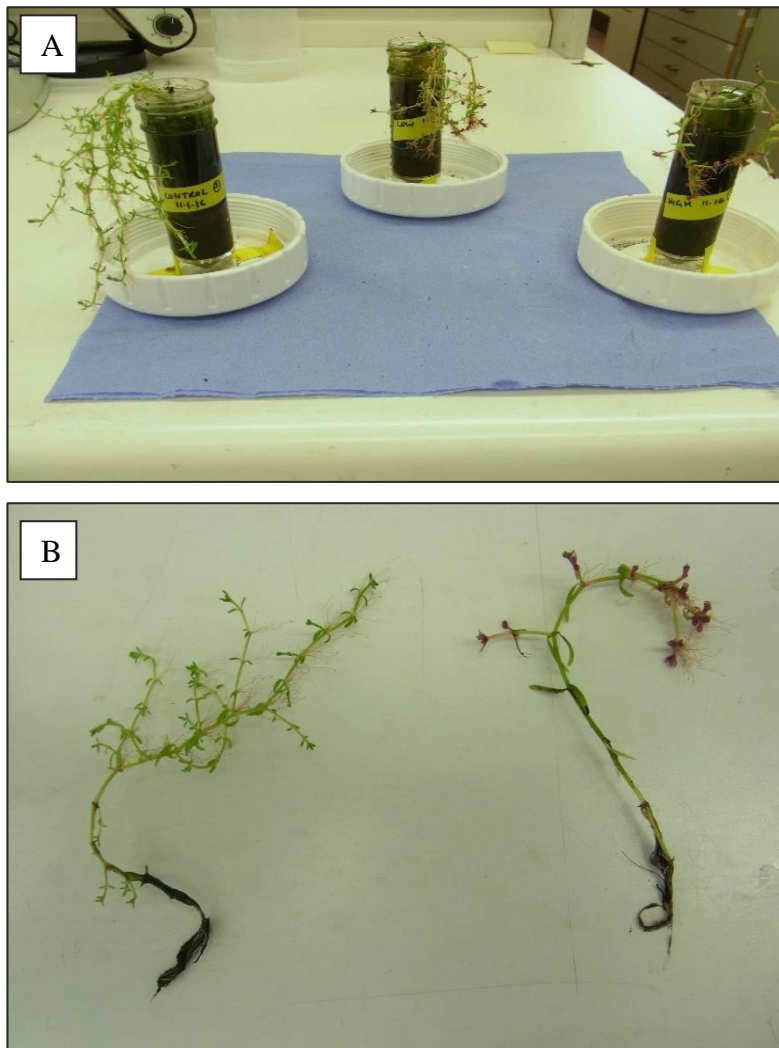


Figure 4.12: A, Plants of all three treatments at day 44 assessment; B, control plant (left) compared to high treatment plant (right) at day 44 assessment.

By the final assessment after 44 days, the difference between the increase in plant height in high treatments compared to control plants was 36.7mm (Figure 4.12 - 4.14, linear model,  $t = -7.217$ ,  $p < 0.01$ ).

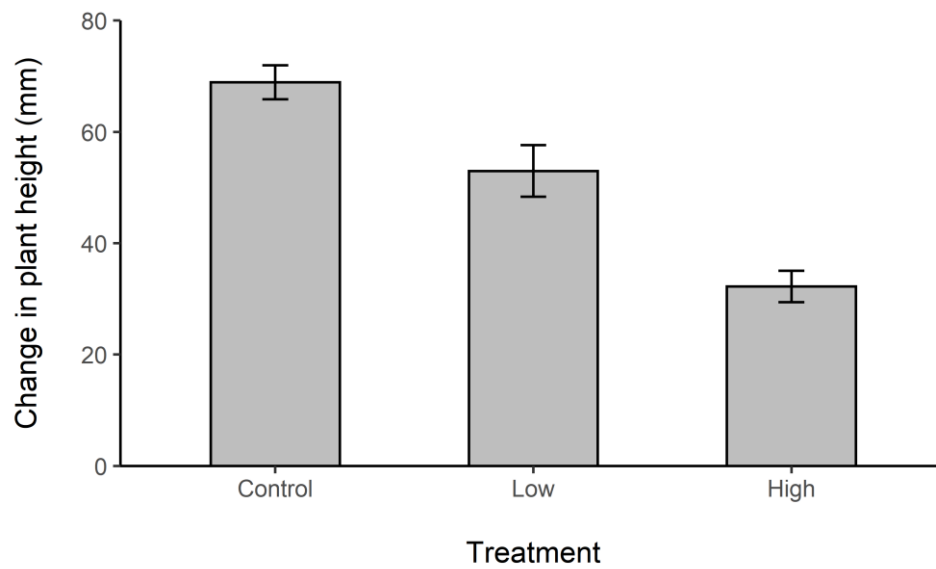


Figure 4.13: The mean ( $\pm$ SE) increase in plant height after 44 days of *Aculus crassulae* infestation at three densities.

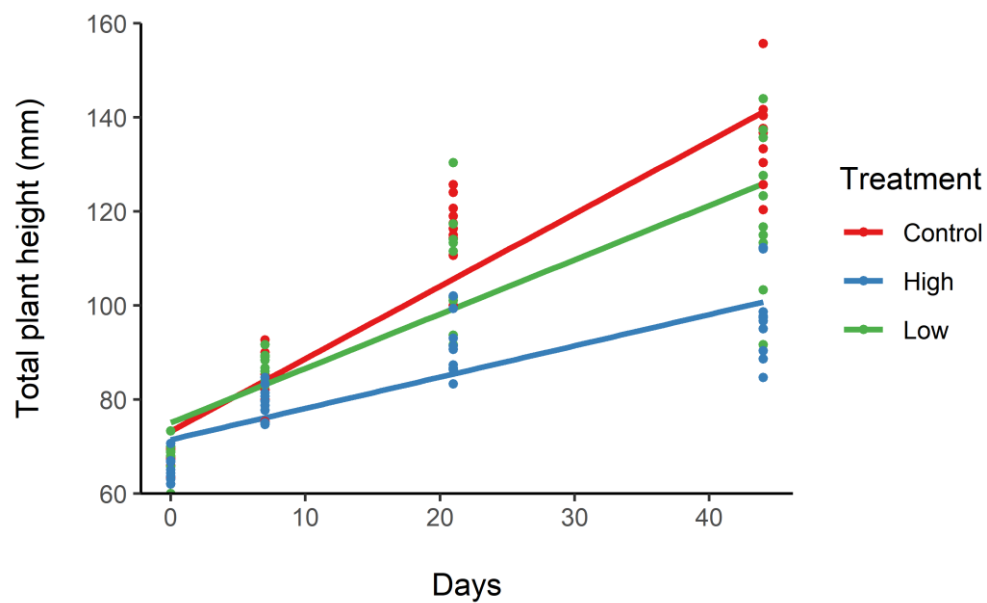


Figure 4.14: Total growth in plant height ( $\pm$ SE) for the duration of the experiment at the three treatments, control (no mites) and a low and high density of *Aculus crassulae*.

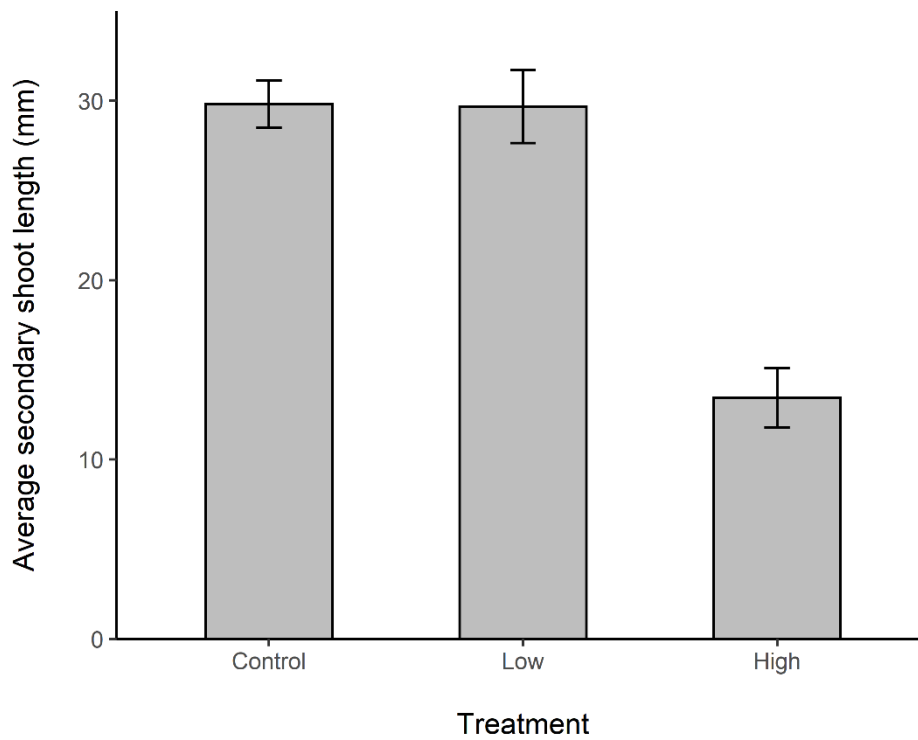


Figure 4.15: The mean length ( $\pm$ SE) of secondary shoots after 44 days of *Aculus crassulae* infestation at three mite densities.

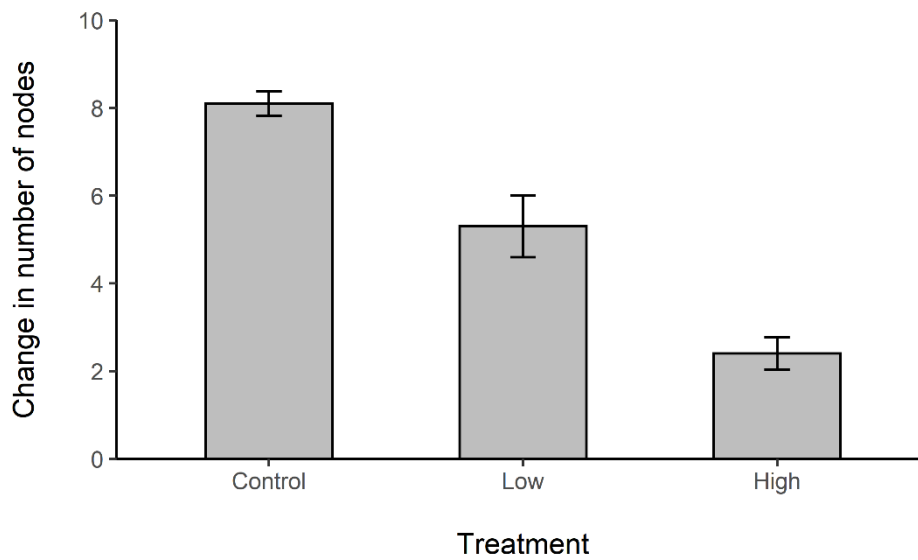


Figure 4.16: The average number of nodes ( $\pm$ SE) after 44 days of *Aculus crassulae* infestation at three mite densities.

The average increase in the number of nodes was lower in high treatments compared to controls (GLM,  $z = -5.234$ ,  $p < 0.001$ ) and the low treatment (GLM,  $z = 3.220$ ,  $p < 0.01$ ).

The reduction in secondary shoot number observed between mite-treated plants and control plants earlier in the experiment was no longer apparent by the assessment at 44 days (GLM,  $p > 0.05$ , Figure 4.15). However, the lengths of the secondary shoots in the high treatment remained shorter than in the control (linear model,  $t = -6.825$ ,  $p < 0.01$ ) and low treatment plants (linear model,  $t = 6.768$ ,  $p < 0.01$ ). The greatest impact on plant height and node number was observed after 44 days of exposure (Figure 4.13 and 4.16). Only plant height (linear model,  $t = -3.136$ ,  $p < 0.01$ ), number of nodes (GLM,  $z = -2.401$ ,  $p < 0.05$ ) and the number of nodes with roots (GLM,  $z = 0.1265$ ,  $p < 0.01$ ) were reduced with a low dose of mites and for all other growth parameters, the low dose had no effect by this stage of the experiment. More tertiary shoots developed with exposure to mites in both high (linear model,  $t = -6.320$ ,  $p < 0.001$ ) and low mite treatments (linear model,  $t = -3.011$ ,  $p < 0.01$ ).

The overall growth rate (linear model,  $F_{2,27} = 26.19$ ,  $p < 0.001$ ) was also slower in the high treatment plants than the low (Tukey test,  $p < 0.01$ ) and control plants (Tukey test,  $p < 0.001$ ).

## 4.5 Discussion

### 4.5.1 Establishment potential

#### 4.5.1.1 Cold temperature exposure

These studies have shown that adult *A. crassulae* mites have the ability to survive moderate time periods under cold temperature and therefore have the potential to survive the mild climate in the UK.

High proportions of adult mites survived for at least 48 hours at temperatures as low as  $-5^{\circ}\text{C}$  and there was no significant difference between survival at the temperatures tested. Exposure to a longer period of time at cold temperatures did not affect the adults significantly. The eggs were more sensitive and fewer were viable after a second day under cold treatment. However, eggs had the same reduction in viability at the control temperature so it is possible that the treatment was unfavourable to egg survival. The reduction in egg survival at  $-5^{\circ}\text{C}$  compared to adult survival at the same temperature indicates that this life stage is more sensitive to low temperature and provides support that the overwintering life stage is most likely to be the adult, which is also the most common stage for eriophyoid overwintering stated in the

literature (Lindquist & Oldfield, 1996). Average winter temperatures in Great Britain rarely reach lower than -5°C for longer than one day without increasing during the day and so it is unlikely that the mites would experience this type of constant cold temperature. Since mites reside within the gall, they would be further protected from temperature extremes. In addition to this, the buffering effect of water in aquatic habitats means that temperatures that the mites are likely to experience in those habitats are expected to be less extreme than in terrestrial habitats.

It has been suggested that the survival of arthropod biological control agents at 5°C was positively correlated to persistence in the field and therefore could be a reliable predictor of field survival in the UK (Hatherly *et al.*, 2005). The predatory mirid, *Macrolophus caliginosus* Wagner and the predatory mite, *Neoseiulus californicus* McGregor, which both had lower LTime<sub>50</sub> values than *A. crassulae*, showed that when not provided with food, field survival was 75 and 100 days respectively and when provided with food, the survival was significantly longer (Hart *et al.*, 2002; Hatherly *et al.*, 2005). If following a similar pattern, this indicates that *A. crassulae* could also potentially survive long periods under UK winter conditions. Assuming that *A. crassulae* does not possess an overwintering life stage, as the host plant of *A. crassulae* is winter green and will be available as a food source and habitat during the winter, adult mites would be able to also feed during this time, increasing their chances of survival. These studies indicate that *A. crassulae* is not adversely affected by short periods of cold temperatures that are experienced under UK conditions and has the ability to survive at low temperatures.

#### **4.5.1.2 Thermal budget**

The thermal budget for one generation was calculated to be 175 degree days based on the lower developmental threshold of 8.7°C calculated using linear regression. Experimental data demonstrated that *A. crassulae* was in fact unable to complete development at 10°C, a temperature above the estimated lower developmental threshold calculated in this model and therefore the model is not completely accurate; this is also reflected by the R<sup>2</sup> value for the model (0.91). Estimating the parameters by extrapolating the line from the linear portion, in a region in which the relationship is not linear, is likely to result in inaccuracy as demonstrated here for the lower developmental threshold. Using only the linear portion of data can also be problematic where temperatures are known to increase beyond the linear portion. In the UK, summer temperatures regularly exceed the linear portion of 22°C so using

this model it is not possible to make predictions at those higher temperatures realistically experienced in the UK.

The Lactin-2 model estimated the  $T_{max}$  to be 39°C. However, in the experimental studies mites were unable to develop at 30°C showing that this model clearly also has its inaccuracies. Nevertheless, the estimated line visually appears to fit the data closely when all data is included although the  $R^2$  value is low at 0.26. The lower developmental threshold estimation was similar to the linear model estimation at 8.9°C.

Despite these limitations, using several models is common due to the ease of use with simple data requirements and the ability to calculate different model parameters; for this purpose, both models are useful for *A. crassulae*. For example, Pakyari *et al.* (2011) used linear and four non-linear models to estimate the thermal thresholds and optimal temperature for the predatory thrips species, *Scolothrips longicornis* Priesner for use against the two-spotted spider mite, *Tetranychus urticae* Koch.

The lower developmental threshold for the map of the estimated number of generations in the UK was chosen to be 10°C (Figure 4.9A), since the experiment to assess development at different temperatures indicated that the actual lower developmental threshold lies between 10 and 12°C. The range of generations possible in different parts of the country, as estimated using degree days, showed that the mite could achieve one generation per year in high altitude regions but there could potentially be four or more generations in some parts of southern England. The distribution map of the host plant *C. helmsii* shown in Figure 4.9B demonstrates that this plant is less well distributed in those high-altitude regions and suggests that host plant and mite have similar environmental requirements. This analysis is based upon fewer replicates than would usually be used in such a study due to the difficulties in handling the delicate, gall-inhabiting mites and the associated high mortality rate; however, the results provide sufficient evidence to indicate that the mite can establish and spread through most parts of the UK under the current climate and most importantly in parts of the UK where *C. helmsii* is abundant. It is accepted that there would be fewer generations than in the area of origin where temperatures are higher.

#### 4.5.2 Host range of *Aculus crassulae*

The host-specificity testing provides evidence that *A. crassulae* is host specific to *C. helmsii*. Mites were present on several non-target species for the duration of the no-choice tests, but no symptoms were observed for the majority of species tested. As eriophyoid mites, especially those that form galls, are renowned for their host specificity (Skoracka *et al.*, 2010) it would be highly unusual for *A. crassulae* to feed on unrelated plant species. Eriophyoid mites whose hosts are perennial and therefore represent a stable, reliable, food source are also likely to be host specific (Skoracka *et al.*, 2010). *Crassula helmsii* is perennial in both its native and introduced range and as such it can be expected that *A. crassulae* would also be host specific. The results of these tests indicate that the mite is specific to its host.

It is not known whether all of the recovered mites from non-target plants were alive or not, and therefore it is possible that the mites had died at some stage during the experiment, and the structure of those plants could have been more favourable for retaining the desiccated remains as suggested by Smith (2005). Eriophyoid mites are sensitive to desiccation and can be sensitive to light (Sternlicht, 1969) and in this case mites may have been using the plant structure for protection when unable to develop their protective galls in non-host plants. It was also not possible to determine whether the recovered mites had been feeding on the non-target plants; however, no gall-like symptoms established on any species, with the exception of *C. aquatica*, where symptoms resembling partially developed galls were observed. The feeding symptoms observed on *C. aquatica* demonstrated that it was possible for *A. crassulae* to make attempts to feed and reproduce on this species. Since these symptoms were no longer evident after 14 days, it appears that *C. aquatica* was unpalatable and the plant was able to outgrow the symptoms. This suggests that *C. aquatica* is unlikely to be a natural or sustainable host and is thus unlikely to come under attack in the field.

It is not known how long *A. crassulae* can survive without feeding. However, Wosula *et al.* (2015) showed that wheat curl mite, *Aceria tosichella* Keifer survived up to 7.4 days off host at 10°C. Valenzano *et al.* (2019) found that *Aceria caulobia* (Nalepa) could survive in water for 11 days at 25°C and for six weeks at 5°C, although in this study, mites were treated individually and therefore didn't have the protective structure of a plant to support their survival. If *A. crassulae* could survive for a significant amount of time on non-host species and no negative impacts were

observed on these species, their presence on this species would have little impact. Willis *et al.* (2003) demonstrated this in their study with eriophyoid mite *Aculus hyperici* Liro. Despite the ability of *A. hyperici* to colonise the native species, *Hypericum gramineum* L., it had a negligible impact on the plant.

Michalska *et al.* (2010), described the behaviour of eriophyoid mites on suitable or unsuitable hosts suggesting that mites can quickly discriminate between acceptable and non-acceptable hosts. In laboratory experiments, the grain rust mite, *Abacarus hystrix* (Nalepa) was more active on non-hosts than hosts, and found on parts of the plant other than their usual location on the host. The mites also exhibited dispersal behaviour on non-hosts (Skoracka *et al.*, 2007). The eriophyoid mite under investigation as a biocontrol agent against *Salsoa tragus* L., *Aceria salsolae* de Lillo & Sobhian also displayed more prevalent dispersal behaviour on non-host plants than on host plant (Smith, 2019). The result of the choice tests with *C. aquatica* were consistent with these findings; mites were observed in different locations on the non-host plants between the assessments rather than settling as they did on *C. helmsii* in this test.

#### **4.5.3 Impact of *Aculus crassulae* on *Crassula helmsii***

*Aculus crassulae* caused a significant negative impact on the growth of *C. helmsii*. The rates of increase in important growth characters were significantly reduced in plants with well-established mite colonies compared to control plants. Mite impact developed over time and as the population grew and the galls matured, the effect on some plant growth parameters also increased. By the end of the experiment, some of the parameters which were earlier affected by mites, including the number of secondary shoots, were no longer affected. Plant height, number of nodes and the number of nodes with roots were the only parameters measured which were consistently negatively affected by both doses of mites. This suggests that mite feeding can delay plant development, however as time progresses, the plant can compensate in some but not all growth traits. It would be interesting to observe how the response of the plant may have changed over a longer period of time and if the mites had not been restricted from dispersing to new plants which would be likely under natural conditions.

The reduction in overall vegetative growth particularly in primary stem and secondary shoot growth in plants colonised by mites under laboratory conditions



suggests that *A. crassulae* could have a significant impact on the growth of *C. helmsii* field populations by reducing the number of vegetative propagules available to spread to new uninvaded sites. If the impact observed in single plants demonstrated here would be replicated in a population of plants, localised mite infection could potentially lead to less dense *C. helmsii* infestations due to the reduced overall height of plants and number of branches. This kind of impact could open the dense matrix *C. helmsii* produces to allow small patches of open ground on pond margins; the habitats which are being lost to *Crassula* invasion.

The study demonstrated that in the mite-colonised plants the number of leaves reduced over time, and it appeared that there was increased leaf senescence in these plants compared to control plants. A similar impact was found with the eriophyoid mite, *Floracarus perrepae* Knihinicki & Boczek, a biological control agent released in Australia against Old World climbing fern, *Lygodium microphyllum* (Cav.) R. Br. Goolsby *et al.* (2004) found during a long-term study, the longevity of leaves on mite-infested *L. microphyllum* was significantly lower than in control plants and these leaves underwent rapid necrosis. Fewer leaves would lead to a reduction in photosynthesis and therefore reduce further growth. Although the mechanisms by which *A. crassulae* causes these impacts on the host are not known, there is some evidence to show that feeding by eriophyoid mites can reduce photosynthesis not only in galled leaves but also in neighbouring ungalled leaves on the same shoot (Petanović & Kielkiewicz, 2010a). A change in photosynthetic ability (increase or decrease) is a common result of eriophyoid mite feeding and it may be possible to speculate that the reduction in growth may be caused by a reduction in photosynthetic ability.

The application of the higher dose of mites had a greater and faster impact than the lower dose. As the experiment progressed and the number of mites grew, the plants treated with the low dose of mites were also increasingly negatively affected by the mites, albeit to a lesser degree than in the high dose. These results suggest that even at the lower abundance, mites were still able to have a significant negative impact on host fitness but to have the greatest impact on the plant, higher numbers of mites are required. This was also the case with *A. chondrillae*, the mite released to control rush skeletonweed, *C. juncea* in Australia and elsewhere. *Aceria chondrillae* caused the most severe negative impacts on its host plant at higher densities, and mite density was found to be influenced by the size of the host population (Cullen *et al.*, 1982).

In this type of study at the individual plant scale, there may be a greater observed impact observed than under natural conditions due in part to the restriction of the herbivore to their containers and the prevention of dispersal. Experimentation in this study took place under optimal conditions. In nature, there are many factors influencing the growth of plants and of course, the impact of a biological control agent on its host would not be independent of these. However such studies are important in providing evidence that the biological control agent under consideration has the potential to cause significant damage in field conditions.

#### **4.6 Key conclusions**

The studies described in this chapter provide evidence, that *A. crassulae*, prioritised for research, has a good potential as a weed biological control agent in the UK. *Aculus crassulae* is highly host specific to the target weed, *C. helmsii* with no significant non-target feeding and development. *Aculus crassulae* can develop at and tolerate temperatures occurring in the UK and inflict sufficient damage resulting from feeding to potentially negatively affect plant growth under field conditions.

## 5 Studies on the integration of biological and chemical control of *Crassula helmsii*

### 5.1 Introduction

Where biological control is successful, the pest can be described as being under “complete” or “substantial” control (Hoffman, 1995). Complete control of a target weed is where no other control measure is needed in the area where the biological control agent has established. This does not mean that the weed is no longer present in or part of the flora in its invasive range but rather implies that the target weed is no longer the leading cause of economic or ecological loss and additional control measures are no longer required against the invasive pest (McFadyen, 1998). Even when complete control is achieved, it can be a long process taking up to 20 years to attain (McFadyen, 2000). Although complete control as described above is the most desired outcome, more often substantial or partial control is the result of the release of one or more biocontrol agents, where the weed is controlled in part of its invasive range or under particular environmental conditions but still requires additional control measures (Hoffman, 1995). Therefore, it is important to consider if conventional methods of control can be integrated with biological control if successful control is to be achieved. Methods frequently integrated with biological control include physical control such as mowing, plant competition, prescribed burning and herbicide application. However combining the use of herbicide application with weed biological control is the most regularly used approach as an integrated control measure (Lake & Minter, 2018).

As detailed in section 2.3.5, there are few conventional techniques available to successfully manage *C. helmsii* infestations long-term and implementing these techniques often results in regrowth over time, particularly in large, established populations (Ewald, 2014). The application of herbicides, specifically glyphosate, is considered to be the most effective for *C. helmsii* control and a highly dilute, high volume solution of glyphosate (5ml/l) is generally recommended. When applied at a walking rate of 6 seconds per metre, this provides a treatment of 6l/ha (Environment Agency, 2010) which equals 2.16 kg ha<sup>-1</sup> of active ingredient. (J. Newman pers. comm). Its use in combination with the biological control agent, *A. crassulae* will be investigated in this chapter. Glyphosate acts by inhibiting an enzyme, 5-

enoylpyruvylshikimate-3-phosphate synthase (EPSPS) necessary for the production of essential aromatic amino acids eventually leading to plant death.

Integrating biological and chemical methods can result in successful control as demonstrated by Lym and Nelson (2002) in the USA. It was found that integration of *Aphthona* spp. flea beetles with herbicides resulted in greater control of leafy spurge, *Euphorbia esula* L., than each of the methods alone. Where a combination of chemical and biological control is used, the timing of herbicide application is an important consideration to avoid negatively affecting the more sensitive stages of lifecycle of the biological control agent (DiTomaso, 2008). In the case of *E. esula*, herbicide treatment in spring eliminated the adult food source and had a detrimental impact on establishment of the agent whereas an autumn application led to an increase in *Aphthona* spp. (Lym & Nelson, 2002).

For integration with biological control, a herbicide should be applied at a sub-lethal dose rather than at the recommended rate required to kill the weed. A sub-lethal dose should retard the growth of the plant but not cause mortality and should also limit the negative impact of the herbicide on the biological control agent (Ainsworth, 2003). This approach has been used successfully for fungal pathogen biological control agents, particularly in aquatic systems (DiTomaso, 2008). For example when sub-lethal doses of the herbicide endothall were applied with the pathogen *Mycoleptodiscus terrestris* (Gerd.) Ostazesk to the submerged aquatic weed hydrilla, *H. verticillata*, plant biomass was reduced more significantly than by using either herbicide or pathogen alone (Shearer & Nelson, 2002). While there are fewer studies involving arthropod herbivores and sub-lethal doses, Jadhav *et al.* (2008) identified a sub-lethal dose of glyphosate that did not negatively affect *Neochetina* spp. weevils feeding on water hyacinth, *E. crassipes*. In a subsequent study, Katembo *et al.* (2013) observed more feeding on water hyacinth when using such sub-lethal doses compared to untreated plants at one field site. Another example used mites from the Eriophyidae family; Boydston and Williams (2004) found that when *A. malherbae* was applied in combination with glyphosate or 2,4-DB, shoot and root biomass of the host, *C. arvensis* was greatly reduced although the herbicide and mite were thought to act independently of each other. However, these results were not replicated in a more recent experiment, potentially due to differences in the plant stages used in each experiment; plants grown from seed and plants grown from rhizome can react differently to herbicides. The stage of gall development also

varied between the two experiments, with the younger galls supporting the synergistic response (Konigsberg, 2014).

Arthropod herbivores can be directly affected by the presence of herbicides due to the toxicity of the chemicals. Although glyphosate is marketed as less toxic than many herbicides currently registered for use, the toxicity of glyphosate and its surfactant can have wide-ranging negative impacts across many taxa from unicellular organisms to humans (Gill *et al.*, 2018) and as a result its continued widespread use is under question. The toxicity of glyphosate on invertebrates has been widely reported and honeybees in particular, as major pollinators of crops, have been under investigation. Studies have shown how glyphosate can affect navigation, gut microbiota and appetitive behaviour in honeybees (Herbert *et al.*, 2014; Balbuena *et al.*, 2015; Motta *et al.*, 2018). Research has also shown that molluscs, amphibians and earthworms can be directly and indirectly impacted negatively by glyphosate (Tate *et al.*, 1997; Relyea, 2005; Gaupp-Berghausen *et al.*, 2015). In the context of weed biological control, Hill *et al.* (2012) found that direct application of 2,4-D amine and diquat caused significant mortality in the biological control agents of water hyacinth. Here, mortality of the mirid, *Eccritotarsus catarinensis* (Carvalho) and the weevil, *Neochetina eichhorniae* (Warner), was higher than when glyphosate was applied and increased further with the addition of surfactants used to enhance herbicide efficacy. Indirect toxicity may also occur as a result of the reduction in food quality caused by the herbicide (Messersmith & Adkins, 1995).

Studies in chapter 4 of this thesis established that *C. helmsii* plants colonised by *A. crassulae* developed into smaller plants. The aim of the studies reported here were therefore to investigate whether there is a potential for integrated management of *C. helmsii* using glyphosate. This was achieved by investigating if in the presence of both herbicide and *A. crassulae*, the fitness of the host *C. helmsii*, is reduced more than if plants are exposed to the herbicide and *A. crassulae* independently.

## 5.2 Objectives

1) Assess the suitability of the use of a sub-lethal dose of glyphosate for integration with the biological control agent, *A. crassulae*.

- Examine the direct effect of glyphosate on *A. crassulae* survival

- Identify a suitable sub-lethal dose of glyphosate that will retard the growth but not cause mortality of *C. helmsii*.
- 2) Investigation into the integration of *A. crassulae* and glyphosate
- Evaluate the combined impact of *A. crassulae* and the selected sub-lethal dose of glyphosate on the growth of *C. helmsii* and assess its potential in the management of *C. helmsii*.

## 5.3 Materials and methods

### 5.3.1 Preliminary studies

#### 5.3.1.1 Herbicide concentration

*Crassula helmsii* plants were treated with six concentrations of glyphosate, formulated as Roundup® Pro Biactive® (Monsanto, Cambridge, UK), hereafter referred to as Roundup, to give an indication of the most suitable concentration range to use in determining the sub-lethal dose. This product contains a soluble concentrate of 360g/l glyphosate, present as 441g/l (35% w/w) of the potassium salt of glyphosate (a.i. glyphosate). The concentrations used were 1, 2, 3, 4, 5 and 6ml/l and were based on the field recommended dose of 5ml/l. Concentrations between 3 and 6ml/l were found to be too high, causing rapid plant mortality, so these concentrations were excluded from the later study.

Plants were also treated with glyphosate with and without the addition of an adjuvant, Topfilm™ (BioSorb Inc, UK), to investigate whether the performance of the herbicide increased with the application of the adjuvant. Visual assessments of the plants showed that plant growth appeared to be affected equally between the two treatments so no adjuvant was used in subsequent studies to avoid potential toxicity of the adjuvant to the mites.

#### 5.3.1.2 Toxicity of glyphosate on *Aculus crassulae*

Some preliminary experimentation was undertaken to ensure mites were not seriously affected by the herbicide treatment. The survival of mites on single plants treated with Roundup and concentrations ranging from 0.2ml/l to 2ml/l, were trialled. These concentrations were based on the preliminary work described in 5.3.1.1. Individual mites were applied to plants and the number of mites surviving

were regularly monitored and recorded following herbicide treatment. Survival of the mites was similar across the treatments and as a result these informal assessments demonstrated that that survival was sufficiently adequate to conclude that adult mites were not seriously adversely affected by the treatment.

Direct toxic effects of glyphosate were investigated by spraying individual adult mites with Roundup. Five mites were held in 6cm diameter petri dishes on a filter paper disc moistened with sterile distilled water to prevent desiccation and sprayed with a set volume of 0.5ml of herbicide using a hand-held sprayer. One pump of the spray equated to 0.5ml. A control treatment was sprayed with distilled water. There were five petri dishes per treatment. Mite survival was monitored daily for seven days as indicated by movement under a stereomicroscope. Mortality of the mites sprayed with glyphosate was similar to control mites and it was concluded that the toxicity of glyphosate to *A. crassulae* was acceptable for the continuation of the investigation.

### **5.3.2 Determination of a sub-lethal dose of glyphosate**

Single *C. helmsii* stems of a standardised length of 140mm with no secondary shoots or flowers were inoculated with seven different concentrations of Roundup or tap water as a control. The concentrations used were 0.1ml/l, 0.2ml/l, 0.4ml/l, 0.6ml/l, 0.8ml/l, 1.0ml/l and 2.0 ml/l and six replicates were set up for each concentration. The concentrations chosen were based on the results of the preliminary studies which indicated that the chosen concentrations did not cause rapid mortality of plants. *Crassula helmsii* stems were laid flat and sprayed with the solution on both sides until runoff using a hand-held pressurised sprayer. Plants were left to dry and then placed in separate universal tubes containing 2cm tap water in a heated greenhouse with natural light supplemented by high intensity discharge (HID) lamps and a temperature set at a minimum of 15°C. The control plants were sprayed with distilled water. After 14 days, plant height and the number and length of secondary shoots were measured.

### 5.3.3 Impact of the integration of glyphosate and *Aculus crassulae* on *Crassula helmsii*.

Single *C. helmsii* plants of a standardised length of 140mm were prepared and inoculated as in the dose identification study, with 10 replicates per treatment. Twenty individual plants were sprayed with a single dose of 0.5ml of herbicide as described above at 0.6ml/l concentration, 20 were sprayed with 0.2ml/l and 20 were sprayed with distilled water using a hand-held pressurised sprayer. These concentrations were chosen based on the results of experiment in section 5.3.2. Plants were left to air dry and then placed in separate universal tubes containing 2cm tap water and maintained in a heated greenhouse with natural light supplemented by HID lamps, set at a minimum temperature of 15°C for seven days. Subsequently half of the plants in each treatment were infested with approximately 50 mites each by attaching a mite-colonised bud to a terminal leaf using a single fine entomology pin. The other half of the plants did not receive mites, and had a fine entomology pin attached to a terminal leaf. All plants were held separately in small ventilated pots and randomly placed in Perspex cages in a controlled temperature room held at 23°C on a 18 hour light:6 hour dark cycle and 70% relative humidity. Plants were assessed 14, 28 and 42 days after the application of mites. The following plant parameters were measured: primary plant height, number of secondary shoots, length of secondary shoots, length of decaying stem and number of tertiary shoots. The number of mites per shoot was estimated on a 0-3 ordinal scale; 0 = 0 mites, 1 = 1-10 mites, 2 = 11-50 mites and 3 = over 50 mites, and the number of mite-infested shoots was also recorded.

### 5.3.4 Data analysis

Data were analysed using generalised linear models, using a Poisson error structure for count response variables (number of secondary or tertiary shoots) or a binomial error structure for proportion data (proportion of decayed stem). Linear models with a Gaussian error structure were used where the response variable was continuous (plant height and length of secondary shoots). The experiment across all dates was assessed using a generalised linear mixed model with plant ID as a random factor. Data were transformed to meet assumptions of the test where required. Where linear models were used, if residuals were not normally distributed, data were transformed by squaring or cubing data. When proportion data were overdispersed, a quasi-



binomial error structure was used instead and where count data were overdispersed a negative binomial error structure was used. Post-hoc analyses were conducted using Tukey's HSD and corrected for multiple testing using the Benjamini-Hochberg correction. All analyses were carried out in R Studio (R Core Team, 2019).

## 5.4 Results

### 5.4.1 Determination of a sub-lethal dose of glyphosate

Application of glyphosate had a significant effect on overall plant height (linear model,  $F_{7,40} = 10.15$ ,  $p < 0.01$ ). Herbicide-treated plants were shorter than control plants at all concentrations except 0.1ml/l (Figure 5.1). Only concentrations above 0.4ml/l had a negative impact on the number of secondary shoots produced; 0.6ml/l ( $z=4.37$ ,  $p < 0.001$ ), 0.8ml/l ( $z=3.16$ ,  $p < 0.01$ ), 1ml/l ( $z=-3.93$ ,  $p < 0.001$ ), 2ml/l ( $z=-4.9$ ,  $p < 0.001$ ) (Figure 5.2), although all treatments caused a reduction in the length of secondary shoots compared to the control treatment (linear model,  $F_{7,40} = 10.90$ ,  $p < 0.001$ ) (Figure 5.3). Plants treated with 1ml/l and 2ml/l had signs of premature death with substantial chlorosis and stem decay.

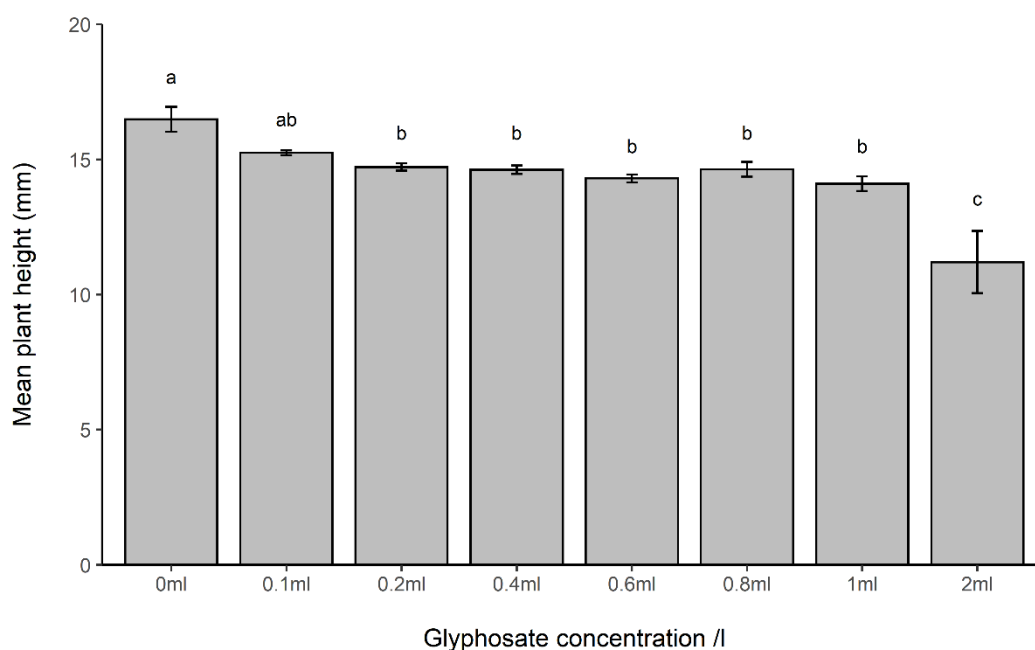


Figure 5.1: Mean plant height ( $\pm$ SE) of *Crassula helmsii* plants 14 days after glyphosate application at concentrations from 0 –2ml/l. Bars with a different letter above are significantly different to each other ( $p < 0.05$ ).

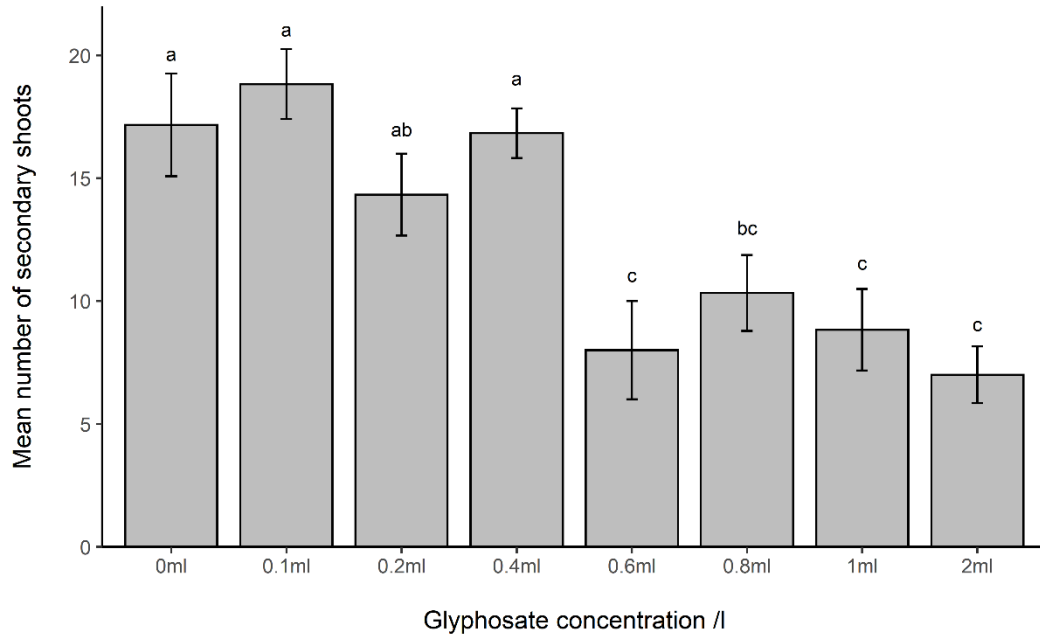


Figure 5.2: Mean number ( $\pm$ SE) of secondary shoots on *Crassula helmsii* plants 14 days after glyphosate application at concentrations from 0 – 2ml/l. Bars with a different letter above are significantly different to each other ( $p<0.05$ ).

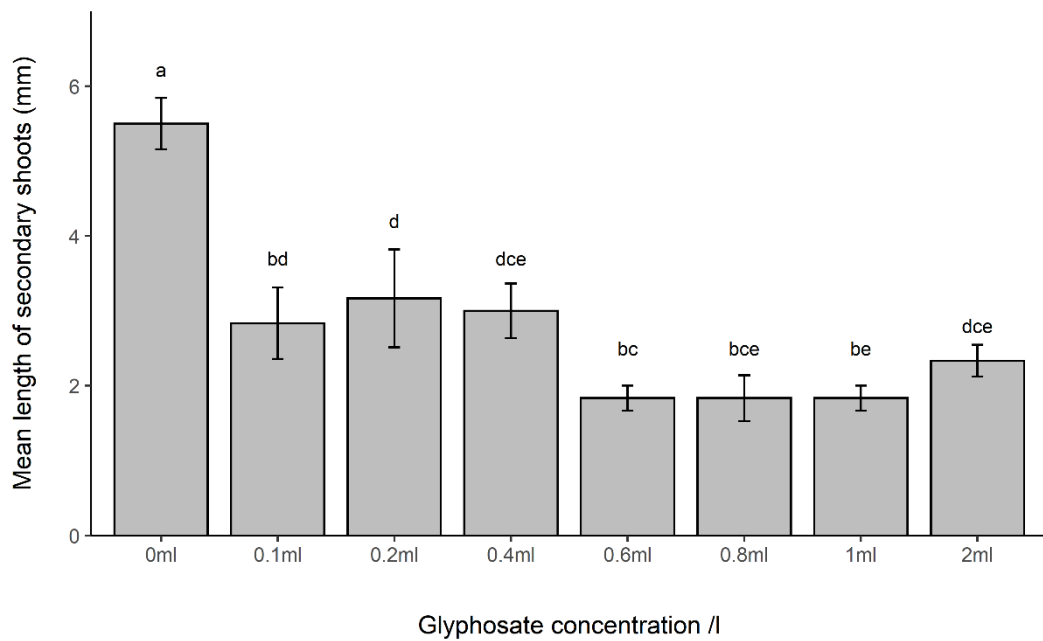


Figure 5.3: Mean length ( $\pm$ SE) of secondary shoots on *Crassula helmsii* plants 14 days after glyphosate application at concentrations from 0 – 2ml/l. Bars with a different letter above are significantly different to each other ( $p<0.05$ ).

#### 5.4.2 Impact of the integration of glyphosate and *Aculus crassulae* on *Crassula helmsii*.

For the duration of the experiment, plant height was reduced by the application of the herbicide (linear mixed model,  $\chi^2 = 27.26$ , d.f. = 1,  $p < 0.001$ ) and by the presence of mites (linear mixed model,  $\chi^2 = 7.71$ , d.f. = 1,  $p < 0.01$ ) but time had no effect on plant height. When data across the three assessment dates was compared together, no interaction was observed between herbicide concentration and mite presence for any of the growth parameters measured. There were observable differences between the assessment dates as the plants grew and mite populations increased.

A significant interaction was observed between herbicide concentration and mite presence regarding plant height in the early stages of infestation i.e. after 14 days (Tukey test,  $p < 0.05$ ); plants treated with 0.6ml/l, without mites were shorter than the plants in all other treatments (Tukey test,  $p < 0.05$ ), including 0.6ml/l with mites. Plants treated with 0.6ml/l and mites were also shorter than control plants (Tukey test,  $p < 0.05$ ). At the 28 day assessment, plants treated with 0.6ml/l with and without mites were shorter than all other treatments. All other treatments had no impact on plant height at this stage. After 42 days, the main difference between treatments was that plants treated with herbicide and *A. crassulae* were shorter than treatments without mites (Figure 5.4).

Overall when the data across all dates was combined, the number of secondary shoots was also reduced by increased herbicide concentration (GLMM,  $\chi^2 = 16.61$ , d.f. = 1,  $p < 0.001$ ) and by the presence of mites (GLMM,  $\chi^2 = 4.71$ , d.f. = 1,  $p < 0.05$ ) but not by time. The average length of secondary shoots reduced with increasing herbicide concentration (GLMM,  $\chi^2 = 46.29$ , d.f. 1,  $p < 0.001$ ) and time (GLMM,  $\chi^2 = 132.32$ , d.f. 1,  $p < 0.001$ ) but not by mite presence. At the 14 day assessment, plants treated with herbicide and mites had fewer and shorter secondary shoots than control plants (Tukey test,  $p < 0.05$ ) and the higher the glyphosate concentration, the fewer secondary shoots were produced. However, after 28 days, only the treatment with 0.6ml/l with mites had fewer and shorter secondary shoots than control plants (Tukey test,  $p < 0.01$ ) and after 42 days, 0.6ml/l treated plants with and without mites still had the fewest secondary shoots (Tukey test,  $p < 0.05$ ) (Figures 5.5 and 5.6).

There appeared to be a trend for a greater proportion of stem decay in plants treated with herbicide and mites (Figure 5.7) however only the 0.6ml/l treatment had a higher proportion of decayed stem, statistically (Tukey test,  $p < 0.01$ ). There was no

difference between the proportion of shoots infested by *A. crassulae* at any date (Figure 5.8). The number of tertiary shoots was not affected by any of the treatments, mites nor herbicide concentration.

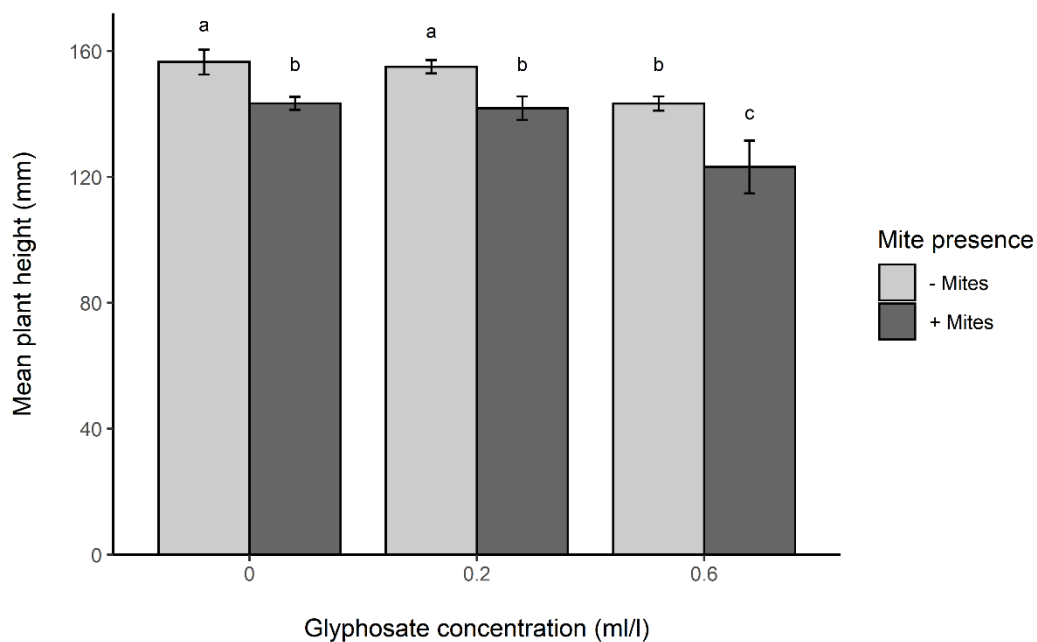


Figure 5.4: Mean plant height ( $\pm$ SE) of *Crassula helmsii* plants 42 days after treatment with *Aculus crassulae* and/or glyphosate. Bars with a different letter above are significantly different to each other ( $p < 0.05$ ).

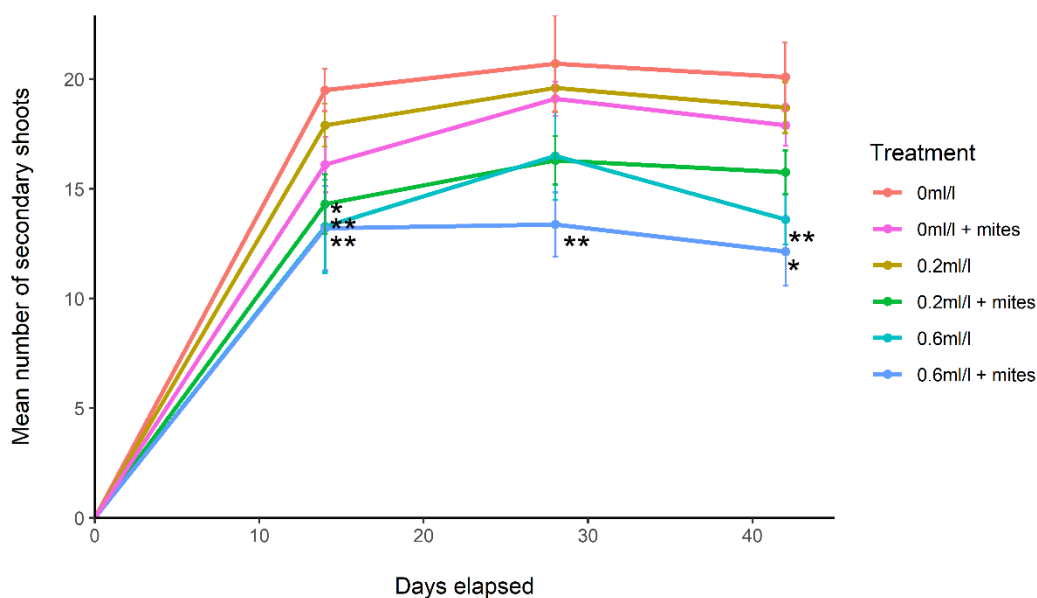


Figure 5. 5: Mean number ( $\pm$ SE) of secondary shoots on *Crassula helmsii* over 42 days following treatment with *Aculus crassulae* and/or glyphosate. Asterisks mark significant (\*) or highly significantly (\*\*) differences between the treatment and the control.

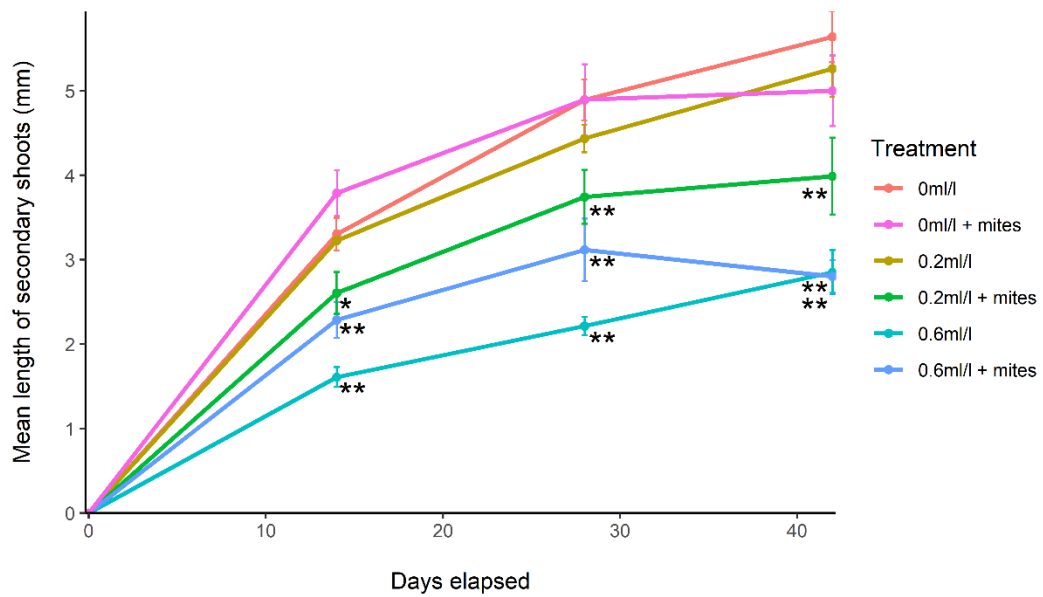


Figure 5. 6: Mean length ( $\pm$ SE) of secondary shoots on *Crassula helmsii* over 42 days following treatment with *Aculus crassulae* and/or glyphosate. Asterisks mark significant (\*) or highly significantly (\*\*) differences between the treatment and the control.

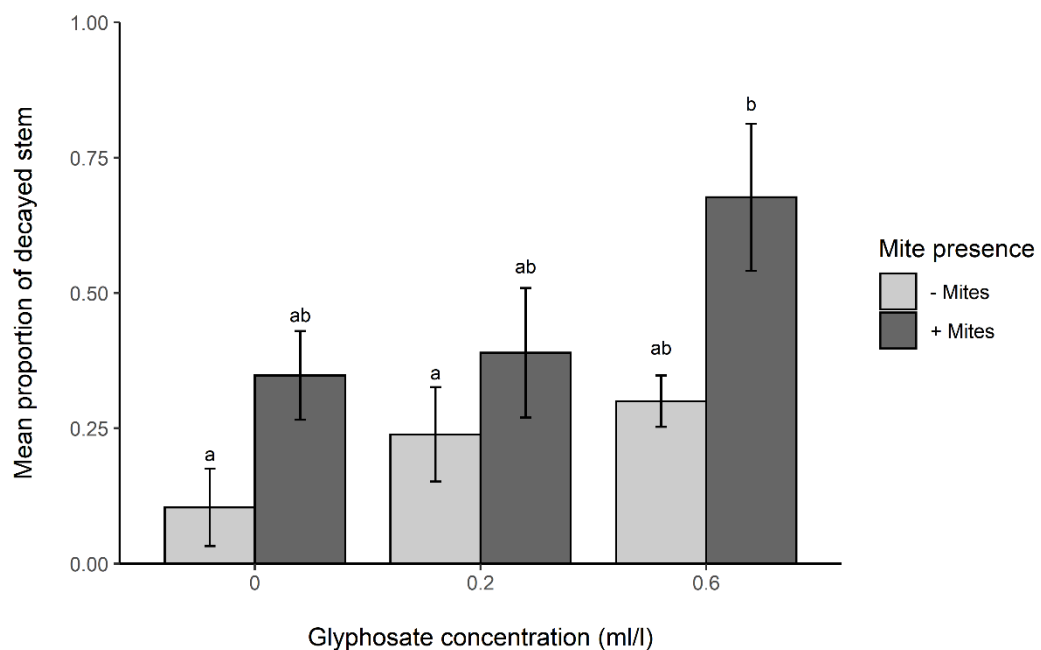


Figure 5.7: Mean proportion ( $\pm$ SE) of stem decay of *Crassula helmsii* plants 42 days after treatment with *Aculus crassulae* and/or glyphosate. Bars with a different letter above are significantly different to each other ( $p < 0.05$ ).

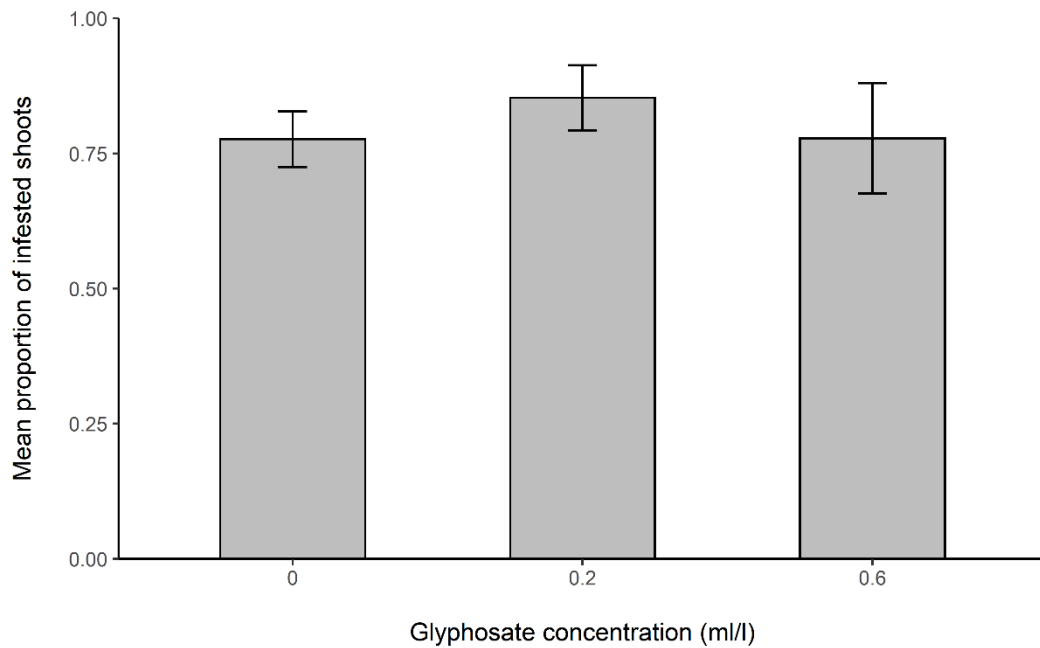


Figure 5. 8: Mean proportion of *Crassula helmsii* shoots ( $\pm$ SE) infested by *Aculus crassulae* 42 days after treatment with *Aculus crassulae* and glyphosate.

## 5.5 Discussion

Studies investigating the sub-lethal dose demonstrated that a much lower concentration than that used to control *C. helmsii* in the field (5ml/l) is required if chemical and biological control are to be integrated. The sub-lethal dose identified here, 0.6ml/l was found to reduce plant height and the number and length of secondary shoots without appearing to significantly affecting mite survival. The application of concentrations lower than 0.6ml/l caused a reduction in some but not all of the plant parameters measured and therefore the lower concentration of 0.2ml/l was also considered in further studies. The application of concentrations above 0.6ml/l led to accelerated plant death leading to a lack of plant resources for mites and as such were not considered suitable for further work. The sub-lethal dose identified here equates to a 0.000026g/m<sup>2</sup> of active ingredient, which is much more dilute than that identified for water hyacinth; of 0.11g/m<sup>2</sup> (Jadhav, 2011; Katembo *et al.*, 2013).

In the main experiment, plant growth parameters were generally reduced with increasing glyphosate concentration, whether mites were present or not. Often the glyphosate treatment alone was most effective at reducing plant growth, particularly early in the experiment before mite colonies had established robust populations.

However, plant height in particular was shortest when plants were treated with both glyphosate and mites and the reduction in plant height associated with mite infestation reflects earlier results in chapter 4. The impact of *A. crassulae* at higher glyphosate concentrations may be restricted by the action of the herbicide reducing new shoot growth. *Aculus crassulae* feeds exclusively on the meristematic tissue in new shoots and a reduction in secondary shoot growth represents a reduction in potential resources for *A. crassulae*, limiting population expansion and the potential impact on the *C. helmsii*. The results showed that fewer secondary shoots were produced in plants treated with 0.6ml/l (both with and without mites) compared to other treatments. The effect of mites on secondary shoot number and length on plants not treated with glyphosate, however was not replicated in this study. These differences could potentially be due to a greater abundance of mites on plants as the experiment progressed in the study in chapter 4.

When both mites and glyphosate were applied there was a higher proportion of stem death and this stem death also occurred across all treatments including controls. However, with a high degree of variability between replicates no statistically significant relationship could be found between treatment and plant death. While not significant, this warrants further study to understand whether there is a true relationship between plant decay, mite presence and herbicide application and its potential in the management of *C. helmsii*. The action of glyphosate can negatively affect the production of compounds used in a defence response against herbivory or disease (Sharon *et al.*, 1992). It may be that the potential disruption in the production of these compounds could lead to a general weakening of plant defences, increasing the impact of natural enemies on the host plant, despite results not evidently showing this for *A. crassulae* during this experiment. Increased vulnerability could lead to further infection by facultative or opportunistic plant pathogens however, and it seems possible that there could have been plant pathogens present in the water which could have contributed to stem death here. Indeed, plants that were not treated with glyphosate also had high levels of stem decay.

The effect of glyphosate on *A. crassulae* is not clear and this evidently needs to be clarified before further work continues. Although there was no difference between the proportion of shoots infested by *A. crassulae* in herbicide-treated plants compared to untreated control plants and during preliminary studies glyphosate seemed not to visibly impact mite survival, there appeared to be fewer mites within

the galls in plants treated with 0.6ml/l as time increased. During this study it was not possible to record the actual numbers of mites and their life stages due to their fragility; however the potential development of a more refined methodology to assess such numbers would allow a more detailed evaluation of the impact of the herbicide on mite populations and therefore of the effect on plant growth. De Saraiva *et al.* (2016) showed that glyphosate can affect oviposition in mites. Oviposition in the mites, *Polyphagotarsonemus latus* Banks and *Tetranychus bastosi* Tuttle, Baker & Sales on *Jatropha curcas* L. first increased with a sub-lethal dose (0.36 kg ha<sup>-1</sup> a.i.) of glyphosate. However, as residual glyphosate in the plant sap was fed on over time, reproductive potential reduced and this increased with dose. Hislop and Prokopy (1981) also observed significant mortality in the predatory mite, *Neoseiulus fallacis* following treatment with glyphosate in laboratory tests. Glyphosate may have a direct toxic effect on *A. crassulae* particularly considering the wide-ranging impacts found in other invertebrates, even at low doses. By contrast, Boydston and Williams (2004) found no toxic effect of glyphosate on the eriophyoid mite, *A. malherbae* when fed glyphosate-treated plants. It is also possible that as plant death increased, host material became less attractive for inhabiting mites, and this may have also restricted mite impact on the host plant. Paynter (2003) described how development of the moth, *Neurostrota gunniella* Busck reduced after feeding on herbicide-treated *Mimosa pigra* L. plants, and this was attributed to declining plant quality rather than toxicity of the herbicide.

The mites in this experiment were applied post herbicide treatment when any herbicide induced changes in the plant would have already been initiated. Infesting plants with *A. crassulae* initially to allow colonies to develop robust populations and mature, protective galls prior to herbicide application may be a practical approach to trial in the future. The data suggests that mites prefer healthy plant material so if colonies were initially established on healthy plants, the interaction of the herbicide and mite may result in an alternative result.

These studies demonstrate that there is a potential for integrated control of *C. helmsii* that includes biological and chemical components. Replicating this experiment using a greater amount of plant material and under natural field conditions would be valuable in determining how effective the combination of mites and glyphosate could be under more realistic conditions, and whether the plant death observed here



would be repeated. Determining the direct and indirect effect of the sub-lethal dose on *A. crassulae* would need to be clarified before further research was carried out.

## **5.6 Key conclusions**

A suitable sub-lethal dose of glyphosate for use on *C. helmsii* was identified as 0.6ml/l, which resulted in reduced plant growth. With the addition of *A. crassulae*, plant height was reduced further. The acceleration of plant decay was also observed and although this was not statistically significant, warrants further study. *Aculus crassulae* demonstrated some tolerance to low doses of glyphosate but this should be investigated further.

## **6 Seasonal population dynamics of *Aculus crassulae* and the effect of shade**

### **6.1 Introduction**

Understanding key aspects of the ecological requirements of a biological control agent in the research phase of a biological control programme is of utmost importance if the successful establishment and control of the target weed are to be achieved at an early stage. Historically, the failure to conduct relevant studies has led to a delay in finding suitable ecologically matched agents which often results in more financial input (Compere, 1961; McFadyen, 2002). However, in modern biological control, ecological studies now commonly form part of the research phase of programmes (McClay & Balciunas, 2005). One important aspect of ecology are the effects of abiotic factors such as local environment or climate and these can be investigated to some extent under laboratory conditions. Conducting field-based studies on these factors however may not always be possible in the native range and the full understanding of these factors under natural conditions may only occur post-release. This is especially true of organisms with complex or cryptic lifecycles such as rust fungi or gall-forming insects.

Even when an agent has been selected from a climatically matched region of the target's native range, the biology of either the biological control target or the agent may differ in the introduced range. Overwintering behaviour is one particular area of ambiguity that is important to elucidate if the biological control agent is to complete its lifecycle under local conditions, establish populations and have an impact on the target weed. The leaf beetle, *Trirhabda bacharidis* Weber, a species introduced into Australia in 1969 for the control of *Baccharis halimifolia* L. was found to have failed to establish largely due to changes occurring in beetle phenology following its introduction. The beetles overwinter as eggs in the native range but as pupae in the introduced Australian range due to differences in climate. The duration of the pupal stage is longer in Australia and there is greater vulnerability to predators and adverse soil conditions (Palmer & Haseler, 1992). A full understanding of the overwintering behaviour of *T. bacharidis* in both regions supported the understanding of the failure of this programme.

There may also be subtle variation in plant physiology in the target between native and introduced host plant populations which could affect biological control agent lifecycle completion such as in the case of the mite *A. chondrillae*, used for the control of *C. juncea* in the western USA. Milan *et al.* (2006) found that *A. chondrillae* required the development of autumn rosettes to overwinter and as a result, *C. juncea* populations in the intermountain region were unable to support overwintering populations of *A. chondrillae* due to the inability of north-facing populations to produce these rosettes.

Similarly, the importance of specific habitat requirements may not be fully understood prior to release, including specific water level requirements. The weevil, *Bagous affinis* Hustache was introduced to the USA as a biocontrol agent for hydrilla, *H. verticillata* in the 1980s but the weevils failed to establish due to their requirement of extended dry periods and waterbody drawdown to complete the lifecycle on shore which were not present in the region of release (Purcell *et al.*, 2019). The leaf beetle *Galerucella pusilla* (Duftschmidt), one of the successful biological control agents against *Lythrum salicaria* L. in wetlands in USA and elsewhere, was also found to have difficulty overwintering particularly in tidal wetlands because of the negative impact of regular tidal inundation (Ferrarese & Garono, 2011).

Apart from establishment, the extent of arthropod biological control agent feeding and its ability to reduce plant fitness may also vary under different environmental conditions. For example *Agasicles hygrophila* Selman and Vogt, a biological control agent for the semi-aquatic plant alligator weed, *Alternanthera philoxeroides* (Mart.), was found in laboratory experiments to have less of an impact under drought conditions due to reduced palatability and egg hatch failure (Wei *et al.*, 2014). Host plant growth was also suppressed under flooded conditions and as a result, the beetles also had limited impact in these circumstances due to the reduction in plant resource.

Little is currently known about the overwintering behaviour of *A. crassulae* in its native range or in its potential introduced range. In general, leaf-inhabiting eriophyoid mites may move to the buds, under loose bark or in old leaf scar crevices during winter (Easterbrook, 1979; Herbert, 1979). Others may migrate to other parts of the plant such as rosettes (Caresche & Wapshere, 1974) or root buds (Schaffner *et al.*, 2012). It is expected that *A. crassulae* mites would overwinter within the galls

however the behaviour of the host plant, *C. helmsii* differs between the native and invasive range (pers. obs), particularly in the winter. Winters in the UK are known to be more severe than in south-east Australia, with frost and snowfall occurring throughout the season. In Victoria, winters are warmer with a mean minimum temperature in July of 3.9°C in Colac where the strain of *A. crassulae* was collected (Australian Government, Bureau of Meteorology, 2019) and as a result *C. helmsii* plants are generally present all year round (Dawson, 1989). In the UK, *C. helmsii* stems growing above the water level can die back when plants are exposed to the severe weather conditions, leaving live plant material surviving only under the water. Rainfall is also higher in the UK. These differences between the UK and Australia may have an impact on mite survival throughout winter, either directly as a result of exposure to lower temperatures or indirectly due to fewer plants surviving the cold temperatures thus being available for overwintering. As described in section 2.3.3, the host plant of *A. crassulae*, *C. helmsii* can tolerate a wide range of environmental conditions including varying levels of light and exposure and as such can be found in varying types of habitat.

The studies in chapter 4 of this thesis demonstrated that *A. crassulae* is tolerant of a wide range of temperatures and has the potential to survive UK temperatures. However, the impact of other environmental conditions on *A. crassulae* is unknown, and particularly how these other factors may affect winter survival under natural conditions. It is uncertain how long-term submergence could affect *A. crassulae* winter survival although it is expected that the mites would have some tolerance in contrast to terrestrial mite species, having evolved in a marginal aquatic habitat. Shade could equally affect mite survival. Habitats offering protection from low temperatures or wind exposure may support increased winter survival of both *C. helmsii* and *A. crassulae*, but these habitats might also have higher levels of shade if they are sheltered by the presence of trees for example. *Crassula helmsii* can grow in both open and shaded habitats but shade can affect invertebrate feeding and abundance in different habitats positively or negatively (Guerra *et al.*, 2010; Salgado-Luarte & Gianoli, 2010). Due to the importance of the survival of *C. helmsii* plants in order for *A. crassulae* to survive winter, further investigation into the effect of shade on both *C. helmsii* and *A. crassulae* was considered most important factor to investigate at this stage.

## 6.2 Objectives

The aims of the studies in this chapter were to 1) Investigate the seasonal changes in *A. crassulae* abundance during winter, spring and summer and how this is affected by shade and 2) to investigate the associated changes in plants growth under both shade and non-shade conditions. In particular, the following questions were addressed:

- Under shade and no-shade treatments, does mite abundance reduce in the winter and could this leave the mite population vulnerable to extinction?
- Under shade and no-shade treatments, how fast does the mite population increase in the spring and is it enough to maintain a viable population?
- How do changes in the mite population relate to *C. helmsii* plant growth under shade and no-shade treatments?

## 6.3 Materials and methods

The most representative methodology to address the above objectives would have been to carry out studies in natural waterbodies infested with *C. helmsii* in the field. However due to practical difficulties with such a set-up, a mesocosm study was established under semi-natural conditions. This was considered to be the most appropriate way of making detailed assessments of mites and plants at regular intervals whilst maintaining a level of control over factors such as water level and shade.

Field collected *C. helmsii* plants were planted in heavy duty containers 34cm x 48cm x 20cm in size, in a 50:50 mixture of multipurpose and aquatic compost and filled with tap water until plants were covered with 5cm of water. Plants were left to grow for three months under natural conditions during the summer to ensure healthy plants in the most suitable growth form were available for mite colonisation. Each container had drainage holes in place 20cm from the bottom to ensure consistent water levels were maintained throughout the experiment. Prior to infestation with mites, the wet weight of the plant material in each container was standardised to weigh 2.5kg. To provide consistent shade, an enclosure measuring 14.5m x 2.6m x 1.9m was erected using standard greenhouse shade material with 80% density.



Figure 6.1: The containers within the shade treatment (background) and the no-shade treatment (foreground).

Mites were applied to plants in half of the containers under the both the shade and no-shade treatment, and plants in the other half of the containers had healthy shoot tips without mites attached to them. Containers were randomly assigned a treatment. Mites were applied by attaching mite-infested galls to the plants with each gall containing approximately 30 adult mites. Each mite-infested container was infested with 30 buds, spaced evenly in a grid, hosting approximately 900 mites. Each infested plant was marked with a wooden stick for future identification. The plants were maintained in an unheated building with a minimum temperature of 10°C for one week post infestation before being placed in their final positions outside.

The positioning of the containers was laid out as a two-factor experimental design with shade/ no-shade as one factor and mite treatment/ no mite treatment as the other (Figure 6.1) and there were nine replicates in each treatment. Half of the containers were placed inside the enclosure and half outside of the enclosure, all 0.5m apart from each other. Each container was placed within a custom-made polystyrene box with 5cm thickness to provide insulation from extreme temperature. By insulating the containers, the water would be less likely to freeze as often and therefore the water temperature of larger water bodies would be more closely resembled. Data loggers (Logtag HAXO-8 and Hobo MX2201) recording air and water temperature

once every hour and were placed in the centre of both treatment arenas and data was downloaded regularly. Wind speed and light intensity were also measured regularly to ensure the difference between the shade and no-shade conditions was maintained throughout the experiment.

Between November 2018 and September 2019, at each sampling date, three plants were randomly selected from each container, and in all mite-treated containers, all life stages of *A. crassulae* were counted under a stereomicroscope. In non-mite treated containers, plants were checked visually for symptoms of mite colonisation. Gall diameter, plant height and the number and length of side shoots were also recorded in plants from all containers. Between November and March, assessments took place approximately every four weeks but since the population was expected to rise as spring approached, assessments were increased to every two weeks between March and May. Following this period, recordings reduced to approximately once every four to six weeks.

These studies took place shortly after ministerial approval of *A. crassulae* for release from quarantine as a biological control agent was granted by Defra in August 2018.

## 6.4 Data Analysis

A preliminary analysis of data was undertaken to understand the nature of the mite abundance data over time. These graphs (Figure 6.2) show that there are two general time periods: 1) where abundance was static or decreasing in autumn/winter and 2) where abundance began to increase again in spring. Therefore, the data were split into two periods; the “winter” period, from November to the end of March (week 4 to week 21) and the “summer” period, from April to September (week 23-44). The winter period related to the perceived inactive period and the summer referred to the active growing period. The data could have been dealt with in its entirety however, activity during the summer or inactivity in the winter may have skewed results and splitting the data into the two periods was considered the most appropriate.

Data were analysed using generalised linear mixed models, using a Poisson error structure for count response variables (abundance of mites of all life stages and shoot number) and a Gaussian error structure where the response variable was continuous (plant height and secondary shoot length). Where count variables were overdispersed, a negative binomial error structure and a likelihood ratio test were

used. The explanatory variables in these analyses were shade treatment (shade or no-shade), mite presence and time. Container and plant ID were included as random effects to address any pseudoreplication in the data. Data were tested for significant interactions between shade treatment, mite presence and time, and were transformed to meet assumptions of the test where required. Models were compared using ANOVA and Chi-square tests. Immature mites in these analyses consisted of nymphs and larvae. All analyses were carried out in R Studio (R Core Team, 2019).

## **6.5 Results**

### **6.5.1 Seasonal mite abundance and the effect of shade**

#### **Winter period**

In the winter, there was no interaction between shade treatment and time for adult mites, immature mites nor eggs ( $p > 0.05$ ). However, there were more adult mites under the shade treatment than in the no-shade treatment, (GLMM,  $\chi^2 = 8.096$ , d.f. = 1,  $p < 0.01$ ) and under both treatments they reduced over time (GLMM,  $\chi^2 = 28.02$ , d.f.=1,  $p < 0.001$ ) but they did not go extinct (Figure 6.2). Adult mites were also observed moving further into the gall as the temperature decreased in winter. There was no effect of the shade treatment on the number of eggs but these also reduced over time (GLMM,  $\chi^2 = 28.13$ , d.f.=1,  $p < 0.0001$ ); the number of eggs dropped to almost zero by week 12 (late January) until week 19 (mid-March) when egg numbers began to increase again under both treatments (Figure 6.3). There was also no effect of shade on the number of immature mites, but as seen for eggs and adult mites, the number of immature mites did reduce over time (GLMM,  $\chi^2 = 75.60$ , d.f.=1,  $p < 0.0001$ ). The abundance of immature mites was low from the start of the experiment and then dropped further to close to zero until numbers started to increase from week 23 (mid-April) following egg hatch (Figure 6.4).



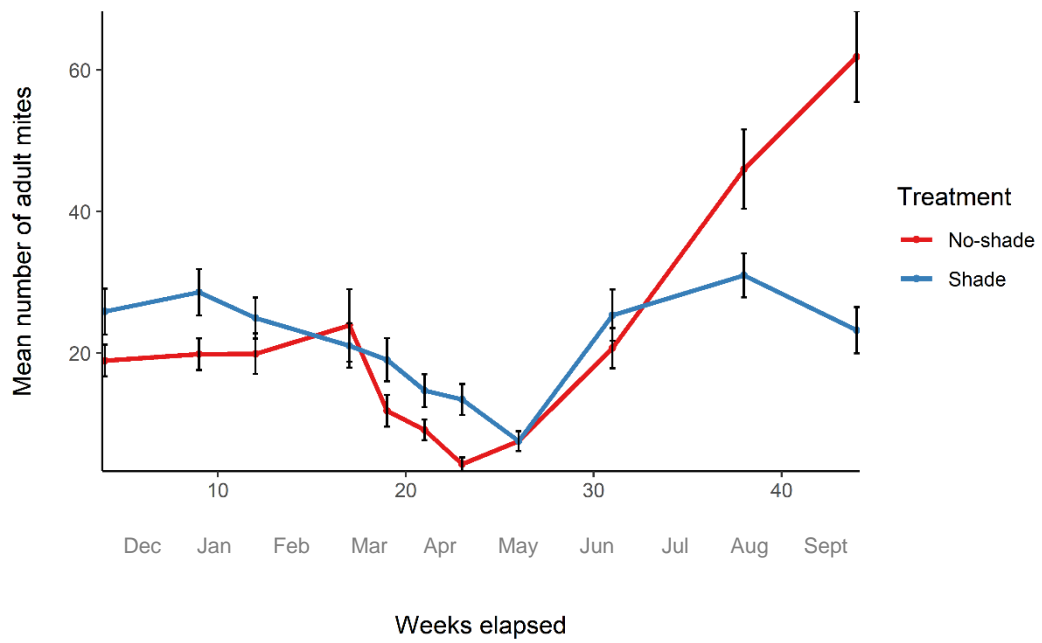


Figure 6.2: The mean number of adult *Aculus crassulae* mites ( $\pm$ SE) recorded under shade and non-shade conditions over a 44 week period.

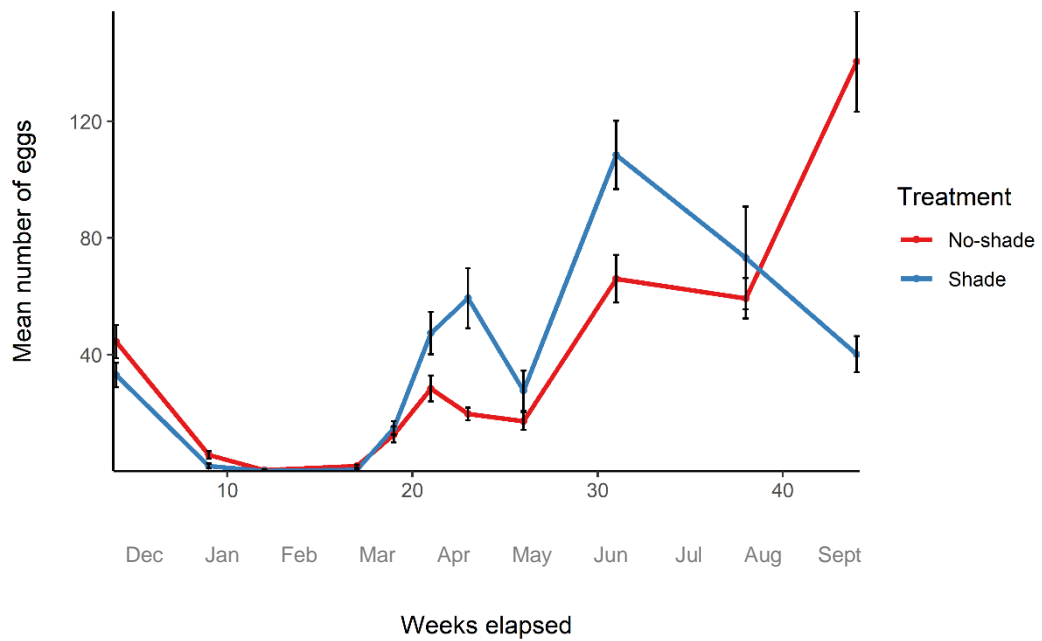


Figure 6.3: Mean abundance of eggs ( $\pm$ SE) recorded between under shade and non-shade conditions over a 44 week period.

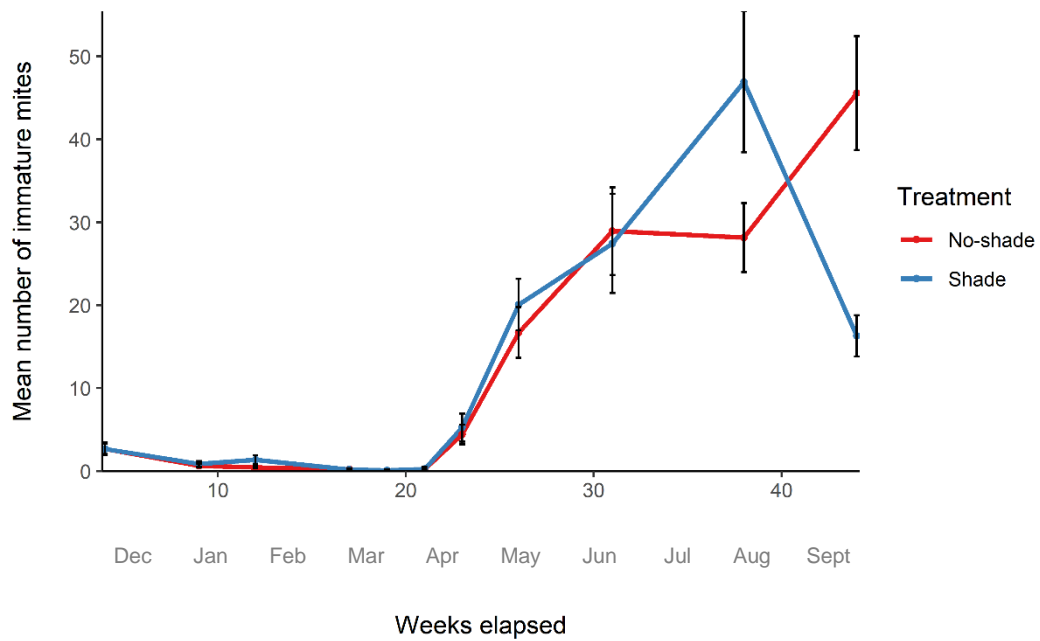


Figure 6.4: Mean abundance of immature mite stages (larvae and nymphs) ( $\pm$ SE) recorded between under shade and non-shade conditions over a 44 week period.

### Summer period

During the summer, there was a significant interaction between time and shade treatment for adults (GLMM,  $\chi^2 = 40.07$ , d.f. = 1,  $p < 0.001$ ), eggs ( $\chi^2 = 40.34$ , d.f. = 1,  $p < 0.001$ ) and immature mites (GLMM,  $\chi^2 = 4.11$ , d.f. = 1,  $p < 0.05$ ). There was a rapid increase in the number of adults in the no-shade treatment from week 26 (early May) as spring progressed (GLMM,  $\chi^2 = 130.85$ , d.f. = 1;  $p < 0.001$ ); this increase occurred two weeks earlier than under the shade treatment (GLMM,  $\chi^2 = 20.13$ , d.f. = 1,  $p < 0.001$ ) (Figure 6.2). Although the number of mites increased under both treatments, the extent of the increase in the number of adults was much higher outside of the shade. During the winter period, the difference in the number of adult mites between shade and no-shade treatments was generally consistent but after week 31 (early June) the difference dramatically increased. By the end of the experiment, there were on average, 40 more adults per plant in the no-shade treatment than in the shade, and there were three times as many than at the start of the experiment (Figure 6.2). In the shade, there was little difference in the number of adult mites recorded at the start and at the end of the experiment. Eggs were not affected by time under the shade but appeared to first increase and then decrease (Figure 6.3) however, in the no-shade treatment, eggs also quickly increased over time (GLMM,  $\chi^2 = 69.39$ ; d.f. = 1,  $p < 0.0001$ ) to much higher numbers by the end of

the experiment. The number of immature mites also increased with time under shade (GLMM,  $\chi^2 = 11.47$ , d.f. = 1,  $p < 0.001$ ) and in the no-shade treatment (GLMM,  $\chi^2 = 48.46$ , d.f. = 1,  $p < 0.0001$ , Figure 6.4). All life stages of mites; eggs, immature stages and adults, rapidly reduced in the shade treatment towards the end of the experiment, whereas in the no-shade treatments, numbers continued to rise.

The number of mite-infested plants in and out of the shade were generally consistent until week 31 in June (Figure 6.5). After this point in time mite dispersal between plants rose leading to an increased difference between these two treatments and a higher number of infested plants in the shade (GLMM, Likelihood ratio = 5.63, d.f. = 1,  $p < 0.001$ ) demonstrating that mites started to disperse in significant numbers between week 31 and 38 (early June and mid-July) under both treatments. There was a similar result at week 44 (GLMM, Likelihood ratio = 9.07, d.f. = 1,  $p < 0.01$ ). By the final assessment, plants in all nine containers in the shade enclosure that had not been infested with mites at the start of the experiment, were exhibiting symptoms of mite colonisation. Outside of the shade, only three non-mite infested containers had become infested by mites.

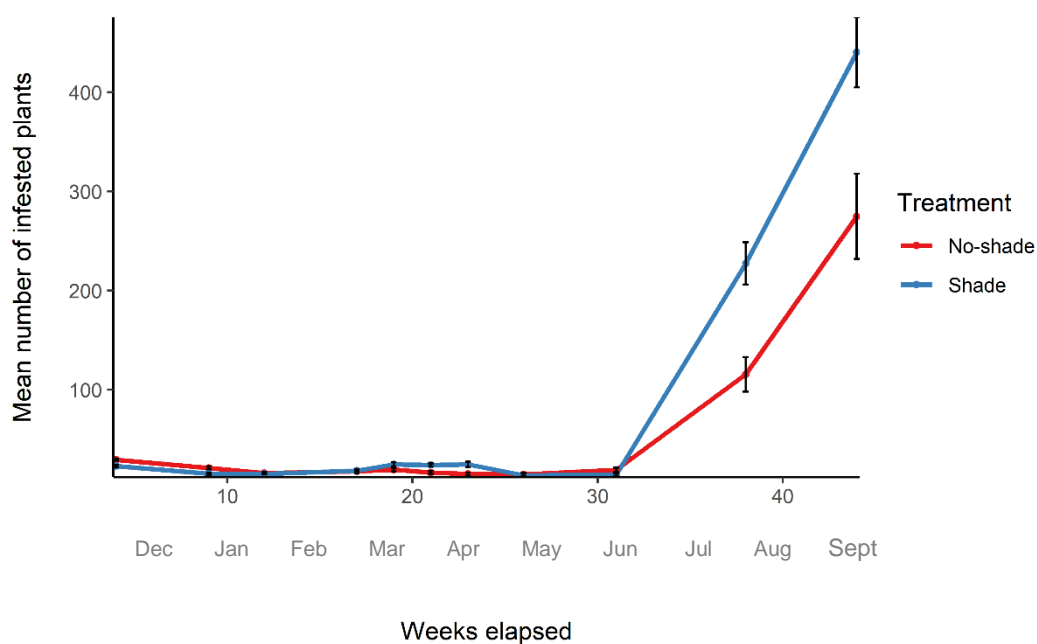


Figure 6.5: Mean number of *Aculus crassulae*-infested plants per container ( $\pm$ SE) in the shade and no-shade treatments recorded over a 44 week period.

## 6.5.2 Plant growth assessment

### Winter period

With respect to plant height, there was a significant interaction between shade treatment and mite presence (GLMM,  $\chi^2 = 5.63$ , d.f.=1,  $p < 0.05$ , Figure 6.6); mite-infested plants in the shade were shorter than plants without mites (GLMM,  $\chi^2 = 17.98$ , d.f.=1,  $p < 0.0001$ ) but there was no difference between the plants in the no-shade treatment. Plant height increased slightly with time in the winter (GLMM,  $\chi^2 = 9.04$ , d.f.=1,  $p < 0.01$ ). There were fewer secondary shoots produced by plants over time across all treatments (GLMM,  $\chi^2 = 35.43$ , d.f.=1,  $p < 0.001$ ) (Figure 6.7) but the length of the shoots increased (GLMM,  $\chi^2 = 8.15$ , d.f.=1,  $p < 0.01$ ) (Figure 6.8). Neither shade treatment nor presence of mites had any effect ( $p > 0.05$ ) on the number or length of secondary shoots.

### Summer period

In the summer, where both mites and plants were actively growing, there were more significant interactions between treatments. With plant height, there were significant interactions between shade treatment and time (GLMM,  $\chi^2 = 99.77$ , d.f.=1,  $p < 0.0001$ ); under the shade, plant height increased with time (GLMM,  $\chi^2 = 91.96$ , d.f.=1,  $p < 0.0001$ ) in contrast to plants decreasing in height in the no-shade treatment (GLMM,  $\chi^2 = 17.98$ , d.f.=1,  $p < 0.0001$ ). In mite-infested plants however, there was no effect of time on plant height but plants without mites did change in height over time in plants (GLMM,  $\chi^2 = 20.80$ , d.f.=1,  $p < 0.0001$ , Figure 6.6). There was also a significant interaction between shade treatment and time for the number of secondary shoots (GLMM,  $\chi^2 = 6.30$ , d.f.=1,  $p < 0.05$ ), which increased over time in both the shade (GLMM,  $\chi^2 = 58.618$ , df=1,  $p < 0.0001$ ) and the no-shade (GLMM,  $\chi^2 = 17.10$ , d.f.=1,  $p < 0.0001$ ). Interactions between shade treatment and mite presence (GLMM,  $\chi^2 = 4.94$ , d.f.=1,  $p < 0.05$ ) were also significant; there were more secondary shoots in mite-infested plants in the shade than in plants without mites (GLMM,  $\chi^2 = 4.58$ , d.f.=1,  $p < 0.05$ ), but there was no effect in the no-shade treatment ( $p > 0.05$ ). The interaction between mite presence and time (GLMM,  $\chi^2 = 4.41$ , d.f.=1,  $p < 0.05$ ) also demonstrated that both mite-infested plants (GLMM,  $\chi^2 = 65.57$ , d.f.=1,  $p < 0.0001$ ) and plants without mites (GLMM,  $\chi^2 = 14.617$ , d.f.=1,  $p < 0.001$ ) developed more secondary shoots over time. Across all treatments, the number of these shoots were low, ranging between one and three, and the numbers recorded over the duration of the experiment fluctuated over time (Figure 6.7). For secondary shoot

length there was an interaction between shade treatment and time (GLMM,  $\chi^2 = 6.72$ , d.f.=1,  $p < 0.01$ ); in the shade, the length of shoots was not affected by time but in the no-shade treatment, the lengths were smaller over time (GLMM,  $\chi^2 = 6.54$ , df=1,  $p < 0.01$ ). Mites had no effect on secondary shoot length (Figure 6.8).

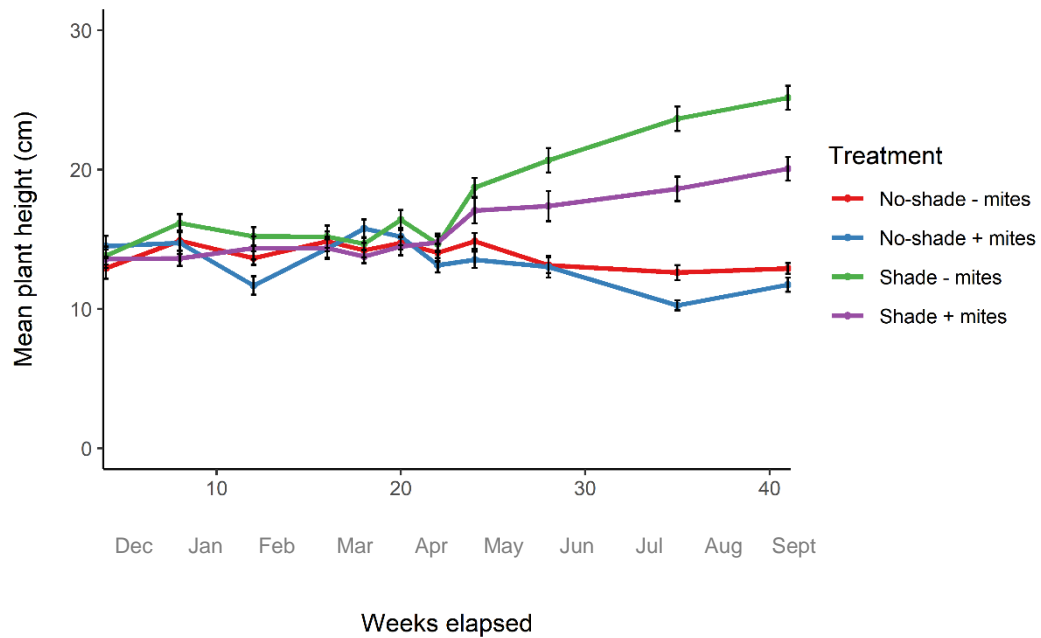


Figure 6.6: Mean plant height of *Crassula helmsii* plants ( $\pm$ SE) with and without *Aculus crassulae* and with and without shade treatment over 44 weeks.

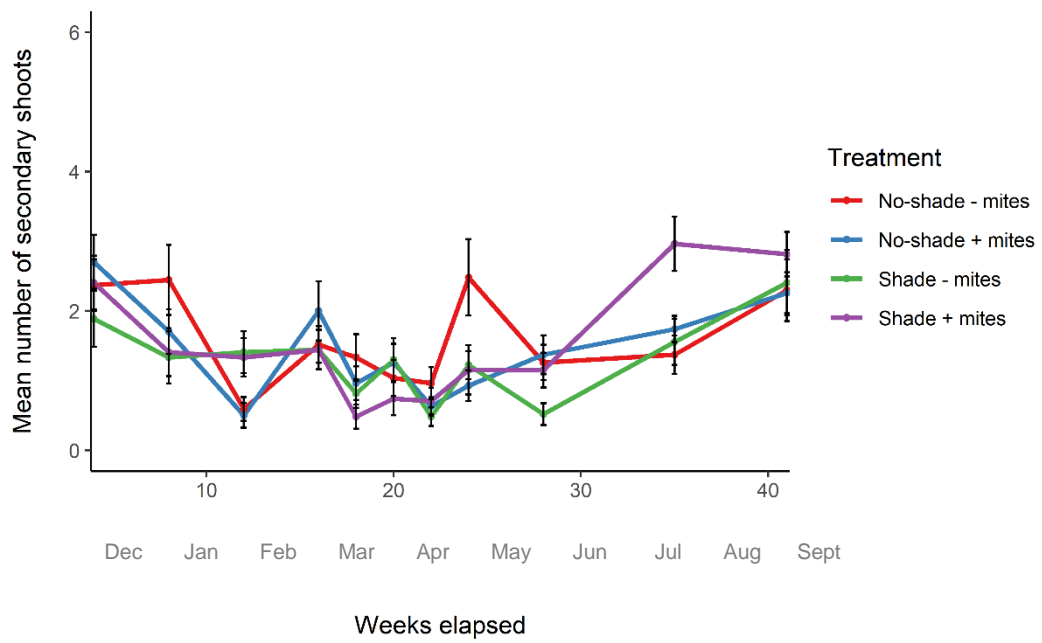


Figure 6.7: Mean number of secondary shoots of *Crassula helmsii* plants ( $\pm$ SE) with and without *Aculus crassulae* and with and without shade treatment over 44 weeks.

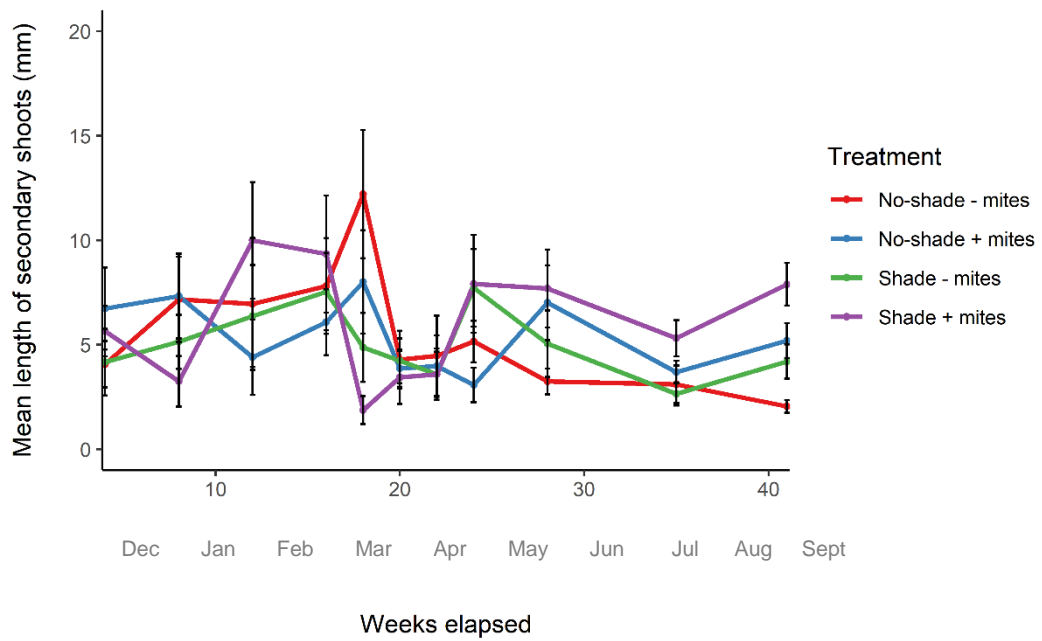


Figure 6.8: Mean length of secondary shoots on *Crassula helmsii* plants ( $\pm$ SE) with and without *Aculus crassulae* and with and without shade treatment over 44 weeks.

Throughout the experiment, no changes in gall diameter were detected, with measurements ranging from 1-3mm.

The water temperature and the temperature of the air at the interface of the plants and water were recorded during the experiment. The data showed that the minimum and maximum temperatures were much more severe under the no-shade treatment, recorded as -6°C and 43°C in the no-shade treatments and -4°C and 28°C under the shade. The average monthly temperatures were very similar throughout the experiment.

## 6.6 Discussion

This study clarifies the overwintering behaviour of *A. crassulae* that was previously unknown, and particularly that the mites can successfully overwinter under UK environmental conditions, and do so as adults and not as eggs. During the winter, the number of adult mites remained stable, averaging around 20 mites per plant as temperatures remained low, from a starting figure of approximately 30 per plant. In December 2018 the number of eggs and immature mites reduced to zero indicating that mites entered an inactive state between the first and second assessment during that month. It was not possible to determine the sex of the mites or whether deuteroynes were present but to date, deuteroynes have not been found in subsequent field experiments. With deciduous hosts, herbivorous mites must first migrate to their overwintering sites in preparation for hibernation. Factors including day length (photoperiod) and temperature are known to affect the timing of invertebrates entering hibernation. The most important factor in the migration of the grape rust mite, *Calepitrimerus vitis* (Nalepa), to its hibernation sites on grapevine in Korea is decreasing day length (Kyung *et al.*, 2018). Since the host plant is present over winter, there is no requirement for *A. crassulae* to move to another site for winter. Mites moved further into the gall and egg production and development stalled so rather than entering hibernation, it is possible that *A. crassulae* mites are simply less active. This preparation for winter seems later than in other species, for example, migration to hibernation sites by *C. vitis* and the apple rust mite, *Aculus schlechtendali* (Nalepa) is known to start in early autumn (Easterbrook, 1979; Kyung *et al.*, 2018). However, this is likely to be linked to changes in leaf nutrition in deciduous plant hosts (Easterbrook, 1978). Some herbivorous mites with evergreen hosts do not undergo hibernation. *Cecidophyopsis psilaspis* (Nalepa), an eriophyoid pest of Pacific yew was found not to hibernate at all in Canada, with all life stages present all year round (Marshall & Clayton, 2004). The same has been reported in

Italy with the bud mite *Trisetacus juniperinus* (Nalepa) but not the vagrant mite, *Epitrimerus cupressi* (Keifer) on evergreen cypress (Castagnoli & Simoni, 2000). In South Africa, the water hyacinth mite, *Orthogalumna terebrantis* Wallwork which is native to South America, can be found in the crown of the plant, close to the water surface and can survive winter this way without hibernation (Marlin, 2010).

The galls induced by *A. crassulae* remained intact and there was no significant plant mortality during the winter confirming that *C. helmsii* can indeed support mite inhabitation during this period, although the shape and colour of the galls became less pronounced over time as plants continued to grow when conditions were suitable. As the temperature decreased, mites moved further into the gall for protection. The first eggs observed after winter were recorded in mid-March. The increase in activity (oviposition and migration out of the innermost part of the gall) was not associated with major changes in plant growth and as such indicates that spring activity was triggered by temperature. Further evidence that that spring activity in *A. crassulae* is triggered by temperature could be seen when an unseasonal period of high day-time temperatures at the end of February 2019 (Met Office, 2019) lead to a gradual increase in immature and adult mites. Adult mites continued to decline in abundance, possibly as they survive long enough to oviposit when conditions are suitable in the spring and then die. The phenology of eriophyoid mites has been shown to be linked with temperature as demonstrated by Easterbrook (1978); the time of emergence of the pear rust mite *Epitrimerus pyri* (Nalepa), occurs at varying plant stages including the flowering, post blossom stage and green cluster stage. However, plant stage can also be a crucial factor for spring emergence as found for the hazelnut big bud mites *Phytoptus avellanae* Nalepa and *Cecidophyopsis vermiformis* (Nalepa), which emerge when daily temperatures are above 15°C (Webber *et al*, 2008). Although the 2018/19 winter in the UK was milder than average, there were some periods of intense snowfall and frost (Met Office, 2019). Whilst protected by insulation, the containers holding the plants experienced more extreme temperatures than would occur in a natural situation with containers being frozen for up to eight days in a row. These results demonstrate that the mites have the ability to survive cold temperatures and populations are not adversely affected by the frequent fluctuations in temperature that occur particularly during the change in season.



During the winter, plants continued to grow but mite presence had no effect on plant growth. This again indicated that mites were inactive during this time, as demonstrated by the lack of oviposition and lack of gall development. During the summer period, plant height was shorter where mites were present. In general, plant growth parameters did not differ greatly in the summer, however after several years of infestation and a greater starting population, differences could be greater in the field. This is supported by the fact that at the end of the experiment the average number of mites per plant was almost double that at the start, demonstrating that a higher starting population would be normal in a natural situation, particularly if winter conditions had been favourable to survival. Shoot number increased over time when mites were present although realistically, the mean number of shoots recorded across the whole experiment was low and varied between zero and three with increases in numbers being equally low.

### **Impact of shade**

In the shade there was more spread by adult mites between neighbouring plants within each replicate than in no-shade. Within the enclosure there was less air flow which is the main method of dispersal for *A. crassulae*, but also less disturbance from rainfall, which can be negatively associated with eriophyoid mite presence (Goolsby *et al.*, 2005a). As a result it was expected that there would be less dispersal between containers under these conditions; it has even been suggested that the dispersal of mites could be limited by the absence of wind (David *et al.*, 2019) although the results of this study contradict that theory for *A. crassulae*. The lack of airflow may have led mites to walk between neighbouring plants in order to reach new plants to colonise favouring short-distance spread. Increased spread under shade could also be attributed to the buffering effect of the shade from extreme heat during the summer. Off-host survival of eriophyoid mites can decline significantly under high temperatures and reduced humidity (Wosula *et al.*, 2015) and as such it is probable that *A. crassulae* would avoid dispersing under such desiccating conditions, but remain within the protective galls.

During the winter the shade equally provided some protection from extreme conditions and more mites were recorded there than outside of the shade enclosure. It is likely that the enclosure protected plants, and therefore mites, from exposure to wind and the most severe weather. Indeed, containers under the shade were frozen less regularly and for shorter durations than plants outside of the enclosure. The

recovery post-winter appeared to be faster under the shade as the number of eggs increased faster there than on plants outside of the shade; however the increase in mite numbers appeared to happen a few weeks earlier in the open treatment as mite development was more rapid under those conditions.

The number of mites and eggs dramatically declined in the shade at the last assessment. Plants in the shade enclosure were especially infected with powdery mildew with all containers hosting mildew-infested plants by the end of the experiment, although it was not possible to quantify the levels of infection per plant. This infection may have had an effect on mite populations. It is known that the presence of pathogens can affect herbivore performance, directly or indirectly by affecting plant chemistry (Hatcher, 1995) and in particular, there is evidence to show that mildew infection can affect the activity of herbivores (Tack *et al.*, 2012). It is possible that the mycelium on the surface of infected galls could be interfering with mite colonisation. Powdery mildew hyphae infect only the epidermal layer of plant cells and eriophyoid mites also use epidermal cells to feed so it could be speculated that the mites could be competing with the mildew for resources. As powdery mildew can enter plants via damaged epidermal cells (Lambertucci *et al.*, 2019) it could be feasible that damage caused by mite feeding allowed entry of the mildew into the plant and the mildew subsequently outcompeted the mites. Environmental conditions in the autumn are particularly suitable for mildew infection, especially in the cool, dark shade enclosure. Aside from mildew, there were more other invertebrates recorded on the plants in the shade enclosure than outside of it, including both predatory and plant-feeding species. It may be possible that there was more predation under the shade, although no predators were recorded within the galls.

In the shade treatment, plants grew taller than in the outside area. There was a greater difference between mite and control treatments in the shade enclosure than outside suggesting that the mites had a higher negative impact on plant growth when in shade. However, this was only evident for plant height but not shoot growth. This is contrary to what was found by Goolsby *et al.* (2004) reporting that the proportional differences between Old World climbing fern plants treated with the mite biological control agent, *F. perrepae*, and control plants was the same, whether they were in the shade or not. The leaves of plants grown in the shade may have a different chemical composition to leaves grown in full sunlight with lower levels of

defence chemicals and a reduced carbon to nitrogen ratio which could make them more attractive to herbivores (Herms & Mattson, 1992). Plants grown in the shade may also have thinner leaves with lower water content which also can lead to higher levels of herbivory (Guerra *et al.*, 2010). Chemical composition of the plants in and out of the shade was not assessed, however such chemical differences between the treatments could have resulted in an increased mite performance in the shade.

Overall these results can be reasonably extrapolated to represent a natural situation, however it should be noted that the water levels were controlled in this experiment and therefore have not fluctuated as regularly or as severely as would have occurred in a natural situation. Plants and mites may respond differently to changes in temperature and this must also be considered when interpreting the results.

## **6.7 Key conclusions**

These results provide important understanding of the mite – host system that will inform decision making for the release of *A. crassulae* as a biological control agent. The main points are summarised below:

- Initiation of mite activity occurs early in the spring and there is rapid population increase in adult mites in late spring. Therefore, mites can and should be released during this period. Mite populations continued to grow into early autumn and there was no significant decrease in populations as autumn approached suggesting that mites can still be released during this period.
- Reduced winter mortality under shade showed that shade provides mites with some protection from the harshest winter conditions. Shady sites may, therefore, be suitable as winter refuges at very exposed and windy sites, where winter survival may otherwise be reduced. Shade may also provide protection from high summer temperatures.
- The small impact of mite feeding on plant growth in this semi-natural field experiment was significant but not as great as in laboratory studies described in previous chapters. With a higher and more realistic starting population of mites colonising a greater number of plants, and a longer colonisation period before winter, a greater impact may have resulted.

- The mites demonstrated sufficient tolerance to conditions prevailing in the chosen experimental set-up suggesting that *A. crassulae* is likely to be able to survive under natural conditions at field sites in the UK. This study was conducted in individual insulated containers which clearly does not reflect a realistic situation in a waterbody with regard to temperature fluctuation. However, plants and mites in the containers would have experienced temperatures both higher and lower than found in a natural waterbody and therefore the container set-up represent a more extreme situation.

## 7 General Discussion

The aim of this PhD was to investigate the possibility of using biological control against the invasive, aquatic weed, *C. helmsii*. A summary of the objectives and key findings of each chapter is given in Table 7.1, below. The main findings and the wider relevance of the research presented in this thesis will be discussed here.

Table 7.1: The primary objectives and key findings from each research chapter.

Chapter	Objectives	Key findings
3	Assess the suitability of <i>C. helmsii</i> as a target for classical biological control	Surveys in Australia revealed a suite of natural enemies causing damage to <i>C. helmsii</i> . The stem-mining fly, <i>H. perplexa</i> and the fungal pathogens, <i>Colletotrichum</i> spp. and <i>Alternaria</i> sp. were found to be unsuitable candidates as biological control agents. The mite, <i>A. crassulae</i> was prioritised for further study with high potential as a biological control agent.
4	a) Investigate the establishment potential of <i>A. crassulae</i> b) Investigate the host range of <i>A. crassulae</i> c) Investigate the impact of <i>A. crassulae</i> on the growth of <i>C. helmsii</i>	The lower development threshold was calculated from the linear model to be 8.7°C below which complete development would not occur; however according to experimental data, the true lower developmental threshold is likely to be closer to 11°C. The degree day requirement for <i>A. crassulae</i> is 175 days above the threshold temperature. Mites are able to survive temperatures as low as -5°C for 48 hours which occur during UK winters. <i>Aculus crassulae</i> was confirmed to be host specific, only developing complete galls and sustainable colonies on <i>C. helmsii</i> . <i>Aculus crassulae</i> was also found to reduce growth in <i>C. helmsii</i> over time, and the negative impact of <i>A. crassulae</i> on plant fitness is greatest at the earlier stages of gall development.

Chapter	Objectives	Key findings
5	<p>a) Assess the suitability of the use of a sub-lethal dose of glyphosate for integration with the biological control agent, <i>A. crassulae</i>.</p> <p>b) Investigate the impact of integration of <i>A. crassulae</i> and glyphosate on <i>C. helmsii</i></p>	<p>A suitable sub-lethal dose of glyphosate for use on <i>C. helmsii</i> was identified as 0.6ml/l which resulted in reduced plant growth. With the addition of <i>A. crassulae</i>, plant height was reduced further. The acceleration of plant decay was also observed although this was not statistically significant. It was demonstrated that mites could survive treatment with a low dose of glyphosate.</p>
6	<p>a) Investigate the seasonal changes in <i>A. crassulae</i> abundance during the winter, spring and summer seasons and how this is affected by shade</p> <p>b) Investigate the associated changes in plants growth under both shade and non-shade.</p>	<p><i>Aculus crassulae</i> survives temperatures as low as -6°C in the winter and up to 43°C in the summer. Mites overwinter as adults. Mites move further into galls as the winter season progressed.</p> <p><i>Crassula helmsii</i> plants are still actively growing during the winter, though more slowly than in spring and summer; <i>A. crassulae</i> is inactive until the onset of spring. Winter mortality of mites is reduced in the shade, however mite populations increase more quickly in the spring in non-shaded areas. Under field conditions, mite-infested <i>C. helmsii</i> plants are shorter than those without mites akin to findings under laboratory conditions.</p>

In the last 20 years, awareness of the issues surrounding invasive non-native species has increased and there is more demand from both land managers and the general public for less environmentally damaging means of control, which currently include chemical and physical control. Most recently, significant financial support for the responsible management of invasive plants has come from the UK government through their obligation to align with the European Union's Water Framework Directive which came into force in 2000. A reduction in pesticide use in aquatic systems contributed to meeting the environmental objectives of this regulation and achieving a "good" status of health for waterbodies by 2015 (European Commission, 2019). The UK government launched two communication campaigns, *Be Plant Wise* and *Check, Clean, Dry*, in 2010 and 2011, respectively, aiming to raise awareness on invasive species and their spread. Additionally, novel methods of invasive species management were funded including biological control, and their ongoing support signifies a commitment by the UK government to alternative measures of control

Biological control is a technique that is already widely used and supported by conservation groups and other stakeholders in England and Wales for the control of another invasive aquatic plant, the waterfern *Azolla filiculoides* Lamarck, using the weevil, *Stenopelmus rufinasus* Gyllenhal (C. Pratt, pers. comm). The general public are also becoming more aware of the detrimental effects of invasive species and the benefits of biological control. Ill-fated biological control introductions of the past are slowly losing their association with modern day attempts through increased media coverage and the aforementioned support of the government. Still, such examples are not easily forgotten and occasionally recited. This is illustrated by the most infamous case of the cane toad which was released in Australia in 1935 to control sugarcane beetles, resulting in severe and wide-ranging impacts on native species, from invertebrate fauna to predators of the toads (Shine, 2010). In the context of the UK situation, current backing from all levels suggests that the acceptance and implementation of biological control of *C. helmsii* is likely. Indeed, experimental trials involving the release of the rust fungus, *P. komarovii* var. *glanduliferae* for the control of Himalayan balsam, has been accepted by many different types of stakeholder in Britain and demand for the rust remains high (Ellison *et al.*, 2020). The knowledge and acceptance of classical weed biological control in other parts of Europe is not as widespread, however this appears to be changing (Shaw *et al.*, 2018) and would not appear to impact on its use in the UK.

It is well established that in the UK the invasive non-native species *C. helmsii* is extremely difficult to manage and complete eradication is unlikely (Dean, 2015); indeed the effectiveness of physical, chemical and mechanical eradication measures is low (van der Loop *et al.*, 2018). Although the full ecological impact of *C. helmsii* is not clear, substantial anecdotal evidence from conservationists and land managers suggest that the dominance of *C. helmsii* has led to the loss of native species (Crutchley & Wicks, 2001), and has stimulated the government and others, in particular the water industry, into taking action against this weed. Whether expensive and potentially aggressive management of *C. helmsii* using existing physical and chemical measures is worth the negative impact on non-target organisms is an additional issue currently under discussion (Diaz, 2012). The aim for conservationists and reserve managers should now be to reduce *C. helmsii* levels to below an ecologically damaging threshold where possible and reduce its spread to new sites. Current control measures are generally sporadic and taken in isolation thus using a more strategic integrated approach including a biological control agent would be more effective. These existing measures may be appropriate under certain circumstances but in general, fall short in managing *C. helmsii* long-term. A biological control agent would complement current practices and provide the long-term management of the invasive weed that is desired. The research presented in this thesis demonstrates the potential of using the mite, *A. crassulae* as an alternative additional control measure for an integrated management of *C. helmsii*.

*Aculus crassulae* was selected as the most suitable biological control agent following extensive surveys in Australia and subsequent laboratory studies. Native range surveys for natural enemies of *C. helmsii* in Australia revealed a similar suite of insect taxa as has been found during surveys for weed biological control agents on other aquatic plant targets including leaf and stem-mining Diptera, Coleoptera (mainly Curculionidae) and Lepidoptera (Spencer & Coulson, 1976; Bennett & Buckingham, 2000; Baars *et al.*, 2010). During the search for natural enemies of hydrillia, *H. verticillata*, in the Indo-Pacific region, over 25 herbivorous insects were found (Bennett & Buckingham, 2000) including six different *Hydrellia* spp. and eight different *Bagous* spp. weevils (Balciunas *et al.*, 2002). Despite a smaller geographic range targeted for surveys in the native range of *C. helmsii*, the type of natural enemies associated with *C. helmsii* in Australia was comparable. Finding fungal natural enemies suitable as classical biological control agents on completely submersed aquatic plants is not common, since the preferred groups of smuts, rusts



and mildews are not found on submerged parts (Shearer, 2008); what was found on *C. helmsii* is in line with this. Herbivorous mites have only been used once before as biological control agents of aquatic plants in the case of *Orthogalumna terebrantis* on water hyacinth, which was released in Zambia and subsequently found in other countries including South Africa (Marlin, 2010; Winston *et al.*, 2014). Ultimately, as chapter 3 and 4 describe, *A. crassulae* was chosen as the biological control agent for *C. helmsii* with the highest potential largely, but not exclusively, due to its high host specificity compared to that of the other initially prioritised species.

The release of *A. crassulae*, which only attacks emergent plant growth, would leave submerged plant growth unaffected. In other programmes additional agents were sought from the native range to attack those portions of the plant not affected by the agents currently in use. For example, *A. hygrophila*, the flea beetle which has been released in several countries for the biological control of alligator weed, *A. philoxeroides* and is an effective control agent for the emergent growth type of this plant. In Australia, the beetle does not control the terrestrial growth type nor plants in cooler environments and this has led to further exploratory work in the native range to search for new natural enemies which utilise the terrestrial plants (Julien *et al.*, 2012). The situation is different in China, however, where the beetle can thrive on the terrestrial growth form (Lu *et al.*, 2015, 2016). With respect to *C. helmsii* however, more surveys may not necessarily identify additional potential agents attacking other plant parts as Australian habitats generally do not support submerged growth of this species. As described in chapter 2, there were also few natural enemies found damaging the submerged portions of emergent *C. helmsii* plants in Australia. While the biotype of *C. helmsii* present in the UK is thought to originate from Australia, the species native range is broader and includes New Zealand. This country could host different potential agents that do attack the submerged part of the plants, since the amount of rainfall in New Zealand is more analogous to the UK and therefore plant growth type may also be similar. However, if this option were to be investigated further, it is important to acknowledge that *C. helmsii* in the UK is genetically more similar to plants from Australia than to those originating from New Zealand (G. Houliston, unpublished data) and as a result the susceptibility of UK *C. helmsii* plants to New Zealand natural enemies could be reduced.

Reserve managers and other affected stakeholders are expected to justifiably question what likely impact *A. crassulae* would have on *C. helmsii*. In chapter 4 it

was demonstrated that the main impact of the mite would be on the vegetative growth; colonisation by *A. crassulae* reduced growth in *C. helmsii* plants, in particular, plant height and lateral shoot growth. Sexual reproduction could also be affected indirectly by the reduction in vegetative growth. The creeping growth habit of *C. helmsii*, which was shown to be advantageous in invading new sites (Dean, 2015) was significantly reduced in mite-infested plants, also targeting its ability to spread within a site, particularly if sites have been recently invaded and plant population density is still lower. This may allow less competitive native plants to compete more successfully to occupy the bare ground preferred by *C. helmsii*. Native competitors were also shown to recolonise ground, left bare following removal of *C. helmsii*, much slower (Dean, 2015) and there are examples of this occurring in practice following conventional control (Gomes, 2005; Ewald, 2014). The establishment of *A. crassulae* at *Crassula*-infested sites could restrict the number of vegetative propagules available to spread. If otherwise unmanageable *C. helmsii* populations were infested with *A. crassulae*, other local sites at risk of invasion might receive less propagule pressure and the spread of the weed to new sites may be limited. Submerged plants could still provide propagules for new invasions as is currently the case, however, a reduction in the total density of *Crassula* from two of the three growth types is likely to lead to an overall reduction of propagules. Contribution of *A. crassulae* to the management of emergent *C. helmsii*, could thus result in a situation where some parts of a particular habitat are under natural control while other parts are not. Should *A. crassulae* maintain emergent *C. helmsii* populations lower than current levels and thereby contribute to partial control, this can be considered as a positive result for the overall habitat particularly in areas where no management is undertaken. A comparable scenario was observed with the flea beetle, *A. hygrophila* on alligator weed in parts of the USA, Australia and China (Buckingham, 1996; Ma & Wang, 2004; Julien *et al.*, 2012), and this has contributed considerably to its control in some areas despite the agent not attacking all growth forms (Sainty *et al.*, 1997). Population demographic modelling would be one way of understanding the effect of herbivory by *A. crassulae* on *C. helmsii* populations (Buckley *et al.*, 2003).

*Crassula helmsii* can actively grow in the autumn when native species in the same habitat are not doing so (Dean, 2015) and the studies in this thesis also confirmed winter growth at the temperatures experienced in winter 2018/19. Studies in chapter 6 demonstrated that *A. crassulae* was also still active in autumn. Mite activity at a

time when native plant species are inactive could help to prevent *C. helmsii* from gaining a foothold in sites where the weed is at an earlier stage of invasion by reducing its dominance at a time when other plants are least competitive.

Several biological features of successful biological control agents have been identified through analysing the outcome of past biological control agent releases. The most recent of these studies found that nearly 80% of all agent releases are from the orders Coleoptera, Diptera and Lepidoptera (Schwarzländer *et al.*, 2018) out of which members of the Coleoptera are the most widely used as biological control agents and the most effective at reducing plant size (Clewley *et al.*, 2012). There are some traits which successful agents are more likely to possess including a high intrinsic rate of increase, small body size and high voltinism (Crawley, 1989); these are traits also present in *A. crassulae*. The ability to reproduce quickly and produce several generations in one season indicates that there is potential for these mites to successfully contribute to the control of *C. helmsii*. Though of all guilds analysed, sap feeders, folivores and pathogen biological control agents were found to have a negative impact on plant fitness traits with pathogens having the greatest negative impact on plant biomass (Stiling & Cornelissen, 2005). Crawley (1989) also suggested that taxa with conspicuous eggs and larvae such as Lepidoptera may be more vulnerable to predation and disease which may affect their performance. Gall formers such as *A. crassulae* are also considered to have good potential as agents because galls, acting as sinks, can divert important plant resources from other parts of the plant and provide the respective gall forming agent with nutrients (Harris & Shorthouse, 1996), in addition to protection from predators. There are also several characteristics of weeds which may make them more challenging as biological control targets, including their ability to regrow following damage, or providing low quality food for herbivores. Another trait of difficult targets is wide genetic diversity in the host plant (Crawley, 1989), however, this would not appear to be an issue in the case of *C. helmsii* which grows mainly vegetatively. *Crassula helmsii* does have substantial ability to regrow following tissue damage which manifests itself in the production of new shoots and makes control of any type a challenge. However, *A. crassulae* mites are uniquely specialised to feed on meristematic tissue in growing shoots and as shown in chapter 4, infest these growing shoots and delay their growth. There are several issues that could limit the success of the mite, however. One important factor that can affect the success of aquatic weed biological control agents

is the changing water levels in the aquatic habitat (Cuda *et al.*, 2008). This has limited the establishment of several arthropod agents by affecting the availability of overwintering or pupation sites. For example, the weevils, *Bagous affinis* and *B. hydrillae* O'Brien released in the USA against hydrilla failed to establish due the absence of waterbody drawdowns required to complete their lifecycle (Godfrey *et al.*, 1994; Purcell *et al.*, 2019). Similarly affected is the weevil, *Euhrychiopsis lecontei* (Dietz) released in the USA against Eurasian water-milfoil, *Myriophyllum spicatum* L., which overwinters on the shoreline and is found to be more abundant there (Jester *et al.*, 2000). In Australia, mite infested *C. helmsii* plants were always found above the water line, although with *A. crassulae* evolving in an aquatic habitat, mites would be expected to survive temporary fluctuations in water level including submergence. However, the significant increase in water levels between autumn and spring in UK water bodies could prove a challenge for *A. crassulae* as when permanently submerged eriophyoid mites are unable to dispel waste and as a result not able to feed properly (pers. comm E. de Lillo). The predicted increase in the frequency and intensity of rainfall with climate change could affect the degree of change in water levels. In recent years the UK has seen more severe flooding and higher temperatures in the winter (Kendon *et al.*, 2019) and this could mean that habitats that were previously suitable for mite establishment could become unsuitable in the future. Further studies into the impact of long-term submergence should be undertaken to identify where the mites may have the greatest chance for establishment and how changing climate could affect this. In addition to changing water levels, the consistent availability of *C. helmsii* plants is essential for mite establishment. Low temperatures can affect the presence of *C. helmsii* and therefore the presence of *A. crassulae*. *Aculus crassulae* completes its lifecycle within the galls and only leaves these to disperse to new plants upon maturity. It is thus crucial that the galls on *C. helmsii* remain intact during cold periods, especially when the mites are inactive and their ability to transfer to live plants may be affected. In severe conditions, the top layer of *C. helmsii* plants can die back in the winter and this could affect overwintering mites, although this was not observed in the field studies described in chapter 6.

Another factor that could impact the development of sustainable mite populations is the effect of natural enemies of *A. crassulae*. Predatory mites were rarely observed in galls in Australia and particular efforts were made to exclude these from *A. crassulae* cultures. However, indigenous predatory mites in the UK could limit the potential

impact of *A. crassulae*, as found by Andres (1983) with predation of the Chondrilla mite, *A. chondrillae* by *Typhlodromus pyri* Scheuten in California. The activity of the gorse spider mite, *Tetranychus lintearius* Dufour was also found to be suppressed by phytoseiid mites used as biological control agents against agricultural pests in the USA (Pratt *et al.*, 2003). No predatory mites were observed within galls during field experimentation in the UK, however, as a factor that has the potential to affect mite establishment and activity, this should be monitored.

*Aculus crassulae* was granted permission by the UK government to be released from quarantine conditions for its use as a biological control agent against *C. helmsii* in June 2018. The first field releases took place in September of that year. The next stage would be to launch a nationwide release programme to establish *A. crassulae* across the UK. In the event that such a programme is agreed and funded, there are several factors that need to be considered. Critical to the successful establishment of a non-native species in a new environment is propagule pressure. This comprises the propagule size - the number of individuals arriving at a new environment - and propagule number - the number of introduction events; the higher the propagule pressure, the higher the likelihood of establishment. Some analyses have found that agent release size has no effect on establishment (Fauvergue *et al.*, 2012; Grevstad *et al.*, 2013) and it has been suggested that this is because weed biological control releases are generally sufficiently large to avoid these effects. It was also suggested that environmental variability could have a greater effect on establishment (Grevstad *et al.*, 2013). For the best chance of establishment, the mites should be released at as many sites as possible and where the environment is variable, more smaller releases would be beneficial (Grevstad, 1999). Investigations in chapter 4 showed that *A. crassulae* could complete more generations in the warmer regions of the south of the UK and fewer at higher altitudes and the north of the country. Release sites should be spread across regions and across different types of aquatic habitats where *C. helmsii* is present to allow the best chances of establishment. In South Africa, it was suspected that the full potential of the mite, *O. terebrantis* on water hyacinth was not realised because self-dispersal was low (Marlin, 2010). If this was found to be the case for *A. crassulae* human-led efforts to distribute the mites widely would also be necessary. The results of the glyphosate study in chapter 5 indicate that further studies into integrating both biological and chemical control should be pursued to further understand the effect of glyphosate on the mite, particularly since reserve

managers are likely to continue to use this herbicide to control *C. helmsii*, potentially until glyphosate is banned. It is currently approved for use in the EU until 2022.

Other factors under consideration during a biological control programme are the cost and potential benefits that could result from the implementation of a such a programme against *C. helmsii*. Biological control programmes can be expensive, particularly if the target weed has not been under investigation elsewhere before. It was estimated that the cost of a new programme in New Zealand would cost NZ\$1.9 million (approximately £1 million) (Paynter *et al.*, 2015) and current estimates for this programme against *C. helmsii* are around two thirds of that amount (unpublished data). The cost of releasing an agent like *A. crassulae* can be substantial, especially the costs of mass production of biological control agents possibly under controlled conditions and extensive post-release monitoring across many sites. The ultimate aim of classical biological control is that the agent develops self-sustained populations which regulate the pest population. Occasionally, a biological control agent may not be able to develop large enough populations, either short or long-term, to impact on the target pest and can be released as an augmentative agent. This is the case for *S. rufinasus*, a control agent for *A. filiculoides* in the UK, where the weevil is now mass-produced to provide widespread control (Reeder *et al.*, 2018). Current methods of mass-rearing *A. crassulae* are labour intensive and expensive and should *A. crassulae* have similar issues, it is unlikely that such a strategy could be employed long-term using the current methodology.

Weed control measures need to be cost effective or land managers will simply not use them. Benefit:cost analyses have documented how successful weed biological control can result in huge financial savings in the long-term. In Australia, the control of rubber vine, *Cryptostegia grandiflora* (Roxb.) R.Br. using the rust fungus, *Maravalia cryptostegiae* (Cummins) Ono and the moth, *Euclasta whalleyi* Popescu-Gorj & Constantinescu, resulted in significant control of the weed and post-release analyses calculated a benefit-cost ratio of 108.8:1 (Page & Lacey, 2006) and these benefits increase with time as the cost of the programme decrease over time.

Calculating the benefit-cost ratio for environmental weeds can be difficult since the ecological impacts and the level of control needed to alleviate these impacts are required parameters and this information may not be available (Morin *et al.*, 2009) as is the case with *C. helmsii*. Analyses suggest that between £1.6 and £2.2 million is spent per annum on three aquatic weeds; floating pennywort, *H. ranunculoides*,

parrot's feather, *M. aquaticum* and *C. helmsii* in Great Britain (Oreska & Aldridge, 2011). Another study puts estimations at £3 million per annum (Williams *et al.*, 2010). Many volunteer hours are spent managing affected habitats which are often unaccounted for, and if the cost is too high, minimal management is undertaken. It is difficult to assign a cost to the value of biodiversity to the general public, however the UK Office for National Statistics (ONS) attempted this with its UK Freshwater Ecosystem Assets and Services Accounts by estimating outdoor recreation as a cultural service provided by the natural environment. It was calculated that the monetary asset value of recreational ecosystem services provided by UK freshwater ecosystems in 2008 and 2012 was around £13.5 billion and £13.4 billion in 2012 prices, respectively (Office for National Statistics, 2015). A reduction in the abundance of *C. helmsii* could lead to a more biodiverse environment which would be more enjoyable for the general public.

In conclusion, the studies in this thesis have described the process of investigating the use and potential success of biological control as an appropriate option for the long-term and sustainable management of *C. helmsii*. These experiments found that the biological control agent, *A. crassulae* has the potential to contribute to the control of *C. helmsii*, and could be an important option where few options exist. There could be many benefits of successful management of *C. helmsii*; ecological, financial and social. Future work should be focussed on the implementation of a nationwide release programme for the mite and subsequently, the integrated use of *A. crassulae* with currently practiced control measures should be trialled among land managers.

## 8 References

- Ainsworth, N. (2003) Integration of herbicides with arthropod biocontrol agents for weed control. *Biocontrol Science and Technology*, **13**, 547–570.
- Andres, L.A. (1983) *Considerations in the use of phytophagous mites for the biological control of weeds. Biological control of pests by mites* (ed. by M.A. Hoy, G.L. Cunningham, and L. Knutson), pp. 53–60. University of California Division of Agriculture and Natural Resources.
- Asadi, G., Ghorbani, R. & Cristofaro, M. (2014) The impact of the flower mite *Aceria acroptiloni* on the invasive plant Russian knapweed, *Rhaponticum repens*, in its native range. *BioControl*, **59**, 367–375.
- Australian Government Bureau of Meteorology (2019) Climate statistics for Australian locations.  
[http://www.bom.gov.au/climate/averages/tables/cw\\_090147.shtml](http://www.bom.gov.au/climate/averages/tables/cw_090147.shtml). (Accessed 29th November 2019)
- Baars, J.R., Coetzee, J.A., Martin, G., Hill, M.P. & Caffrey, J.M. (2010) Natural enemies from South Africa for biological control of *Lagarosiphon major* (Ridl.) Moss ex Wager (Hydrocharitaceae) in Europe. *Hydrobiologia*, **656**, 149–158.
- Bailey, K.L. (2014) *The bioherbicide approach to weed control using plant pathogens. Integrated Pest Management: Current Concepts and Ecological Perspective* (ed. by D.P. Abrol), pp. 245–266. Elsevier Inc., San Diego, CA.
- Baker, C.R.B., Blackman, R.L. & Claridge, M.F. (1972) Studies on *Haltica carduorum* Guerin (Coleoptera: Chrysomelidae) an alien beetle released in Britain as a contribution to the biological control of creeping thistle, *Cirsium arvense* (L.) Scop. *Journal of Applied Ecology*, **9**, 819–830.
- Balbuena, M.S., Tison, L., Hahn, M.L., Greggers, U., Menzel, R. & Farina, W.M. (2015) Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, **218**, 2799–2805.



- Balciunas, J.K., Grodowitz, M.J., Cofrancesco, A.F. & Shearer, J.F. (2002) Hydrilla. Biological control of invasive plants in the eastern United States (ed. by R. van Driesche, S. Lyon, B. Blossey, M. Hoddle, and R. Reardon), pp. 91–114. USDA Forest Service Publication FHTET-2002-04.
- Bale, J.S. & Hayward, S.A.L. (2010) Insect overwintering in a changing climate. *Journal of Experimental Botany*, **213**, 980–994.
- Barratt, B.I.P., Moran, V.C., Bigler, F. & van Lenteren, J.C. (2018) The status of biological control and recommendations for improving uptake for the future. *BioControl*, **63**, 155–167.
- Barton, J. (2004) How good are we at predicting the field host-range of fungal pathogens used for classical biological control of weeds? *Biological Control*, **31**, 99–122.
- Barton, J. (2012) Predictability of pathogen host range in classical biological control of weeds: an update. *BioControl*, **57**, 289–305.
- Baudoin, A.B.A.M., Abad, R.G., Kok, L.T. & Bruckhart, W.L. (1993) Field evaluation of *Puccinia carduorum* for biological control of musk thistle. *Biological Control*, **3**, 53–60.
- Bennett, C.A. & Buckingham, G.R. (2000) *The herbivorous insect fauna of a submersed weed, Hydrilla verticillata (Alismatales: Hydrocharitaceae). Proceedings of the X International Symposium on Biological Control of Weeds, 4-14 July, 1999* (ed. by N.R. Spencer), pp. 307–313. Montana State University, Bozeman, Montana, USA.
- Biological Records Centre (2020) Online atlas of the British and Irish flora. [www.brc.ac.uk/plantatlas/plant/crassula-aquatica](http://www.brc.ac.uk/plantatlas/plant/crassula-aquatica). (Accessed 25th January 2020)
- Blossey, B. & Nötzold, R. (1995) Evolution of increased competitive ability in invasive nonindigenous plants: a hypothesis. *Journal of Ecology*, **83**, 887–889.
- Blumenthal, D. (2005) Interrelated causes of plant invasion. *Science*, **310**, 243–244.
- Bock, I.R. (1990) The Australian species of *Hydrellia* Robineau-Desvoidy (Diptera: Ephydriidae). *Invertebrate Taxonomy*, **3**, 965–93.

- Boldt, P.E., Rosenthal, S.S. & Srinivasan, R. (1998) Integrated pest management distribution of field bindweed and hedge bindweed in the USA. *Journal of Production Agriculture*, **11**, 377–381.
- Bond, N.R., Lake, P.S. & Arthington, A.H. (2008) The impacts of drought on freshwater ecosystems: an Australian perspective. *Hydrobiologia*, **600**, 3–16.
- Boughton, A.J. & Pemberton, R.W. (2011) Limited field establishment of a weed biocontrol agent, *Floracarus perrepae* (Acariformes: Eriophyidae), against Old World climbing fern in Florida - a possible role of mite resistant plant genotypes. *Environmental Entomology*, **40**, 1448–1457.
- Boulton, A.J. (2003) Parallels and contrasts in the effects of drought on stream macroinvertebrate assemblages. *Freshwater Biology*, **48**, 1173–1185.
- Bowers, R.C. (1986) Commercialization of Collego<sup>TM</sup>— an industrialist’s view. *Weed Science*, **34**, 24–25.
- Bownes, A. (2014) Suitability of a leaf-mining fly, *Hydrellia* sp., for biological control of the invasive aquatic weed, *Hydrilla verticillata* in South Africa. *BioControl*, **59**, 771–780.
- Boydston, R.A. & Williams, M.M. (2004) Combined effects of *Aceria malherbae* and herbicides on field bindweed (*Convolvulus arvensis*) growth. *Weed Science*, **52**, 297–301.
- Briese, D.T. (2005) Translating host-specificity test results into the real world: the need to harmonize the yin and yang of current testing procedures. *Biological Control*, **35**, 208–214.
- Briese, D.T. & Walker, A. (2008) Choosing the right plants to test: The host-specificity of *Longitarsus* sp. (Coleoptera: Chrysomelidae) a potential biological control agent of *Heliotropium amplexicaule*. *Biological Control*, **44**, 271–285.
- Brock, M.A., Nielsen, D.L., Shiel, R.J., Green, J.D. & Langley, J.D. (2003) Drought and aquatic community resilience: the role of eggs and seeds in sediments of temporary wetlands. *Freshwater Biology*, **48**, 1207–1218.
- Bruzzese, E. & Hasan, S. (1983) A whole leaf clearing and staining technique for host specificity studies of rust fungi. *Plant Pathology*, **32**, 335–338.

- Buckingham, G.R. (1996) Biological control of alligatorweed, *Alternanthera philoxeroides*, the world's first aquatic weed success story. *Castanea*, **61**, 232–243.
- Buckingham, G.R., Okrah, E.A. & Christian-Meier, M. (1991) Laboratory biology and host range of *Hydrellia balciunasi* [Diptera: Ephydriidae]. *Entomophaga*, **36**, 575–586.
- Buckingham, G.R., Okrah, E.A. & Thomas, M.C. (1989) Laboratory host range tests with *Hydrellia pakistanae* (Diptera: Ephydriidae), an agent for biological control of *Hydrilla verticillata* (Hydrocharitaceae). *Environmental Entomology*, **18**, 164–171.
- Buckley, Y.M., Briese, D.T. & Rees, M. (2003) Demography and management of the invasive plant species *Hypericum perforatum* II. Construction and use of an individual-based model to predict population dynamics and the effects of management strategies. *Journal of Applied Ecology*, **40**, 494–507.
- CABI (2016) *Weed Biological Control Progress Report September 2016*, CABI Switzerland. <https://www.cabi.org/Uploads/CABI/about-us/4.4-global-locations/Switzerland/Weeds%20progress%20report%202016.pdf>
- Cabrera Walsh, G., Maestro, M., Dalto, Y.M., Shaw, R., Seier, M., Cortat, G. & Djeddour, D. (2013) Persistence of floating pennywort patches (*Hydrocotyle ranunculoides*, Araliaceae) in a canal in its native temperate range: effect of its natural enemies. *Aquatic Botany*, **110**, 78–83.
- Callaway, R.M. & Ridenour, W.M. (2004) Novel weapons: invasive success and the evolution of increased competitive ability. *Frontiers in Ecology and the Environment*, **2**, 436–443.
- Campbell, A., Frazer, B.D., Gilbert, N., Gutierrez, A.P. & Mackauer, M. (1974) Temperature requirements of some aphids and their parasites. *Journal of Applied Ecology*, **11**, 431–438.
- Cannon, P.F., Damm, U., Johnston, P.R. & Weir, B.S. (2012) *Colletotrichum* - current status and future directions. *Studies in Mycology*, **73**, 181–213.
- Caresche, L.A. & Wapshere, A.J. (1974) Biology and host specificity of the Chondrilla gall mite *Aceria chondrillae* (G. Can.) (Acarina, Eriophyidae). *Bulletin of Entomological Research*, **64**, 183–192.

- Castagnoli, M. & Simoni, S. (2000) Observations on intraplant distribution and life history of eriophyoid mites (Acari: Eriophyidae, Phytoptidae) inhabiting evergreen cypress, *Cupressus sempervirens* L. *International Journal of Acarology*, **26**, 93–99.
- Centre of Aquatic Plant Management (2004) *Centre of Ecology and Hydrology Information Sheet 12: Australian swamp stonecrop*, Wallingford, UK.
- Charlton, P.E., Gurney, M. & Lyons, G. (2010) Large-scale eradication of New Zealand pygmy weed *Crassula helmsii* from grazing marsh by inundation with seawater, Old Hall Marshes RSPB reserve, Essex, England. *Conservation Evidence*, **7**, 130–133.
- Cilliers, C.J. (1991) Biological control of water hyacinth, *Eichhornia crassipes* (Pontederiaceae), in South Africa. *Agriculture, Ecosystems and Environment*, **37**, 207–217.
- Clement, S.L. & Cristofaro, M. (1995) Open-field tests in host-specificity determination of insects for biological control of weeds. *Biocontrol Science and Technology*, **5**, 395–406.
- Clewley, G.D., Eschen, R., Shaw, R.H. & Wright, D.J. (2012) The effectiveness of classical biological control of invasive plants. *Journal of Applied Ecology*, **49**, 1287–1295.
- Coetzee, J.A., Centre, T.D., Byrne, M.J. & Hill, M.P. (2005) Impact of the biocontrol agent *Eccritotarsus catarinensis*, a sap-feeding mirid, on the competitive performance of waterhyacinth, *Eichhornia crassipes*. *Biological Control*, **32**, 90–96.
- Compere, H. (1961) The red scale and its insect enemies. *Hilgardia*, **31**, 173–278.
- Coon, B.R., Harms, N.E., Cuda, J.P. & Grodowitz, M.J. (2014) Laboratory biology and field population dynamics of *Trichopria columbiana* (Hymenoptera: Diapriidae), an acquired parasitoid of two hydrilla biological control agents. *Biocontrol Science and Technology*, **24**, 1243–1264.
- Cordeau, S., Triolet, M., Wayman, S., Steinberg, C. & Guillemin, J.P. (2016) Bioherbicides: dead in the water? A review of the existing products for integrated weed management. *Crop Protection*, **87**, 44–49.

- Cortat, G., Grosskopf-Lachat, G., Hinz, H.L., Thuis, A. & Tateno, A. (2012) Annual Report 2011. Biological control of field bindweed, *Convolvulus arvensis*, CABI, Wallingford, UK.
- Crawley, M.J. (1989) The successes and failures of weed biocontrol. *Biocontrol News and Information*, **10**, 213–223.
- Crutchley, S. & Wicks, D. (2001) Australian swamp stonecrop (*Crassula helmsii*) in the New Forest: an assessment of current distribution and potential for eradication, Hampshire Wildlife Trust, UK.
- Cuda, J.P., Charudattan, R., Grodowitz, M.J., Newman, R.M., Shearer, J.F., Tamayo, M.L. & Villegas, B. (2008) Recent advances in biological control of submersed aquatic weeds. *Journal of Aquatic Plant Management*, **46**, 15–32.
- Cullen, J.M. (2012) *Chondrilla juncea* L.–skeleton weed. Biological control of weeds in Australia, pp. 150–161. CSIRO Publishing, Collingwood, Victoria.
- Cullen, J.M. (1989) *Current problems in host specificity screening. Proceedings of the VII International Symposium on Biological Control of Weeds* (ed. by E.S. Delfosse), pp. 27–36. Istituto Sperimentale per la Patologia Vegetale, Ministero dell'Agricoltura e delle Foreste, Rome, Italy.
- Cullen, J.M. (1995) *Predicting effectiveness: fact and fantasy. Proceedings of the VIII International Symposium on Biological Control of Weeds, Lincoln University, Canterbury, New Zealand, 2-7 February 1992* (ed. by E.S. Delfosse) and R.R. Scott), pp. 103–109. CSIRO Publishing, Melbourne, Australia.
- Cullen, J.M., Groves, R.H. & Alex, J.F. (1982) The influence of *Aceria chondrillae* on the growth and reproductive capacity of *Chondrilla juncea*. *Journal of Applied Ecology*, **19**, 529–537.
- D'hont, B., Denys, L., Jambon, W., De Wilde, R., Adrians, T., Packet, J. & van Valkenburg, J. (2016) Reproduction of *Crassula helmsii* by seed in western Europe. *Aquatic Invasions*, **11**, 125–130.
- Dalle Fratte, M., Bolpagni, R., Brusa, G., Caccianiga, M., Pierce, S., Zanzottera, M. & Cerabolini, B.E.L. (2019) Alien plant species invade by occupying similar functional spaces to native species. *Flora: Morphology, Distribution, Functional Ecology of Plants*, **257**, 151419.

- Damos, P. & Savopoulou-Soultani, M. (2012) Temperature-driven models for insect development and vital thermal requirements. *Psyche: A Journal of Entomology*, **12**, 1–13.
- David, A.S., Jones, I.M. & Lake, E.C. (2019) Wind speed predicts population dynamics of the eriophyid mite *Floracarus perrepae* on invasive Old World climbing fern (*Lygodium microphyllum*) in a shade house colony. *Experimental and Applied Acarology*, **78**, 263–272.
- Dawson, F.H. (1989) *Natural habitat and population control mechanisms of Crassula helmsii (Australian swamp stonecrop) in Australia. Report to the Winston Churchill Memorial Trust.*
- Dawson, F.H. (1994) *Spread of Crassula helmsii in Britain. Ecology and Management of Invasive Riverside Plants* (ed. by L.C. de Waal, L.E. Child, P.M. Wade and J.H. Brock), pp. 1–13. John Wiley and Sons Ltd, Chichester.
- Dawson, F.H. & Warman, E.A. (1987) *Crassula helmsii* (T. Kirk) Cockayne: is it an aggressive alien aquatic plant in Britain? *Biological Conservation*, **42**, 247–272.
- Dean, C., Day, J., Gozlan, R.E., Green, I., Yates, B., Diaz, A. & Lodge, T. (2013) Estimating the minimum salinity level for the control of New Zealand Pygmyweed *Crassula helmsii* in brackish water habitats. *Conservation Evidence*, **10**, 89–92.
- Dean, C.E. (2015) The ecology, impacts and control of *Crassula helmsii*. PhD Thesis. Bournemouth University, UK.
- DeBach, P. (1964) *Biological control of insect pests and weeds*, Chapman and Hall, London, UK.
- Denys, L., Packet, J., Jambon, W. & Scheers, K. (2014) Dispersal of the non-native invasive species *Crassula helmsii* (Crassulaceae) may involve seeds and endozoochorous transport by birds. *New Journal of Botany*, **4**, 104–106.
- Dhileepan, K., Senaratne, K.A.D.W. & Raghu, S. (2006) A systematic approach to biological control agent exploration and prioritisation for prickly acacia (*Acacia nilotica* ssp. *indica*). *Australian Journal of Entomology*, **45**, 303–307.
- Diaz, A. (2012) *Crassula helmsii* (T. Kirk) Cockayne (New Zealand pygmyweed). *A handbook of global freshwater invasive species* (ed. by R.A. Francis), pp. 37–46. Routledge, Oxford, UK.

- DiTomaso, J.M. (2008) *Integration of biological control into weed management strategies. Proceedings of the XII International Symposium on Biological Control of Weeds, La Grande Motte, France, 22-27 April, 2007* (ed. by M.H. Julien, R. Sforza, M.C. Bon, H.C. Evans, P.E. Hatcher, H.L. Hinz and B.G. Rector), pp. 649–654. CAB International, Wallingford, UK.
- Dodd, A.P. (1959) *The biological control of prickly pear in Australia. Biogeography and Ecology in Australia* (ed. by A. Keast, R.L. Crocker and C.S. Christian), pp. 565–577. Springer Netherlands, Dordrecht, the Netherlands.
- Doyle, R.D., Grodowitz, M., Smart, R.M. & Owens, C. (2002) Impact of herbivory by *Hydrellia pakistanae* (Diptera: Ephydriidae) on growth and photosynthetic potential of *Hydrilla verticillata*. *Biological Control*, **24**, 221–229.
- Easterbrook, M.A. (1979) Life history of *Aculus schlechtendali* on apple in England. *Annals of Applied Biology*, **91**, 287–296.
- Easterbrook, M.A. (1978) The life history and bionomics of *Epitrimerus piri* on pear. *Annals of Applied Biology*, **88**, 13–22.
- Ellison, C.A., Evans, H.C. & Ineson, J. (2004) *The significance of intraspecies pathogenicity in the selection of a rust pathotype for the classical biological control of Mikania micrantha (mile-a-minute weed) in Southeast Asia. Proceedings of the XI International Symposium on Biological Control of Weeds, Canberra, Australia, 27 April - 2 May 2003* (ed. by J.M. Cullen, D.T. Briese, D.J. Kriticos, W.M. Lonsdale, L. Morin and J.K. Scott), pp. 102–107. CSIRO Entomology, Canberra, Australia.
- Ellison, C.A., Pollard, K.M. & Varia, S. (2020) Potential of a coevolved rust fungus for the management of Himalayan balsam in the British Isles: first field releases. *Weed Research*, **60**, 37–49.
- English, K., Hoffmann, J.H., Phiri, E. & Lyons, C. (2019) *Identifying factors influencing establishment discrepancies of a bud-feeding weevil, Dicomada rufa: a case study from South Africa. Proceedings of the XV International Symposium on Biological Control of Weeds, Engleberg, Switzerland, 26-31 August 2018* (ed. by H.L. Hinz, M.C. Bon, G. Bourdôt, M. Cristofaro, G. Desurmont, D. Kurose, H. Müller-Schärer, M. Rafter, U. Schaffner, M. Seier, R.F.H. Sforza, L. Smith, S. Stutz, S. Thomas, P. Weyl and R. Winston), p. 249. Engelberg, Switzerland.

- Environment Agency (2010) Managing Invasive Non-native Plants. *Managing invasive non-native plants webpage*. [http://a0768b4a8a31e106d8b0-50dc802554eb38a24458b98ff72d550b.r19.cf3.rackcdn.com/LIT\\_5001\\_d24b8c.PDF%5Cnwww.environment-agency.gov.uk](http://a0768b4a8a31e106d8b0-50dc802554eb38a24458b98ff72d550b.r19.cf3.rackcdn.com/LIT_5001_d24b8c.PDF%5Cnwww.environment-agency.gov.uk), 16, (Accessed 15th September 2019).
- EPPO (2007) Data sheets on quarantine pests *Crassula helmsii*. *EPPO Bulletin*, **35**, 225–229.
- EPPO (2016) EPPO global database. <https://gd.eppo.int/taxon/CSBHE/distribution>
- European Commission (2019) WDF: Timetable for implementation. [https://ec.europa.eu/environment/water/water-framework/info/timetable\\_en.htm](https://ec.europa.eu/environment/water/water-framework/info/timetable_en.htm). (Accessed 21st March 2020)
- Evans, H.C. (2000) Evaluating plant pathogens for biological control of weeds: An alternative view of pest risk assessment. *Australasian Plant Pathology*, **29**, 1–14.
- Evans, H.C. (1995) *Pathogen-weed relationships the practice and problems of host range screening. Proceedings of the VIII International Symposium on Biological Control of Weeds, Lincoln University, Canterbury, New Zealand, 2-7 February 1992* (ed. by E.S. Delfosse and R.R. Scott), pp. 539–551. CSIRO Publishing, Melbourne, Australia.
- Ewald, N.C. (2014) *Crassula helmsii* in the New Forest – final report on the status, spread and impact of this non-native invasive plant, and the efficacy of control techniques following a 3-year trial. Prepared on behalf of the New Forest Non-Native Plants Project, Freshwater Habitats Trust, Oxford, UK.
- Farr, D.F. & Rossman, A.Y. (2020) Fungal Databases, U.S. National Fungus Collections, ARS, USDA. <https://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm>.
- Fauvergue, X., Vercken, E., Malausa, T. & Hufbauer, R.A. (2012) The biology of small, introduced populations, with special reference to biological control. *Evolutionary Applications*, **5**, 424–443.
- Ferrarese, E.R. & Garono, R.J. (2011) Purple loosestrife biocontrol agent aggregation and overwintering are influenced by tidal inundation. *Wetlands*, **31**, 113–123.



- Gallardo, B., Clavero, M., Sánchez, M.I. & Vilà, M. (2016) Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biology*, **22**, 151–163.
- Gaskin, J.F., Bon, M.C., Cock, M.J.W., Cristofaro, M., De Biase, A., De Clerck-Floate, R., Ellison, C.A., Hinz, H.L., Hufbauer, R.A., Julien, M.H. & Sforza, R. (2011) Applying molecular-based approaches to classical biological control of weeds. *Biological Control*, **58**, 1–21.
- Gassmann, A., Cock, M.J.W., Shaw, R. & Evans, H.C. (2006) The potential for biological control of invasive alien aquatic weeds in Europe: a review. *Hydrobiologia*, **570**, 217–222.
- Gaupp-Berghausen, M., Hofer, M., Rewald, B. & Zaller, J.G. (2015) Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. *Scientific Reports*, **5**, 1–9.
- Genovesi, P., Carboneras, C., Vilà, M. & Walton, P. (2015) EU adopts innovative legislation on invasive species: a step towards a global response to biological invasions? *Biological Invasions*, **17**, 1307–1311.
- Gill, J.P.K., Sethi, N., Mohan, A., Datta, S. & Girdhar, M. (2018) Glyphosate toxicity for animals. *Environmental Chemistry Letters*, **16**, 401–426.
- Godfrey, K.E., Anderson, L.W.J., Perry, S.D. & Dechoretz, N. (1994) Overwintering and establishment potential of *Bagous affinis* (Coleoptera: Curculionidae) on *Hydrilla verticillata* (Hydrocharitaceae) in Northern California. *Florida Entomologist*, **77**, 221–230.
- Goeden, R.D. (1983) Critique and revision of Harris' scoring system for selection of insect agents in biological control of weeds. *Protection Ecology*, **5**, 287–301.
- Goeden, R.D. & Louda, S.M. (1976) Biotic interference with insects imported for weed control. *Annual Review of Entomology*, **21**, 325–342.
- Gomes, B. (2005) Controlling New Zealand pygmyweed *Crassula helmsii* in field ditches and a gravel pit by herbicide spraying at Dungeness RSPB reserve Kent, England. *Conservation Evidence*, **2**, 62.

- Goolsby, J.A., Alexander Jesudasan, R.W., Jourdan, H., Muthuraj, B., Bourne, A.S. & Pemberton, R.W. (2005a) Continental comparisons of the interaction between climate and the herbivorous mite, *Floracarus perrepae*. *Florida Entomologist*, **88**, 129–134.
- Goolsby, J.A., Zonneveld, R. & Bourne, A. (2004) Prerelease assessment of impact on biomass production of an invasive weed, *Lygodium microphyllum* (Lygodiaceae: Pteridophyta), by a potential biological control agent, *Floracarus perrepae* (Acariformes: Eriophyidae). *Environmental Entomology*, **33**, 997–1002.
- Goolsby, J.A., Zonneveld, R., Makinson, J.R. & Pemberton, R.W. (2005b) Host-range and cold temperature tolerance of *Floracarus perrepae* Knihinicki & Boczek (Acari: Eriophyidae), a potential biological-control agent of *Lygodium microphyllum* (Pteridophyta: Lygodiaceae). *Australian Journal of Entomology*, **44**, 321–330.
- Government of Canada (2015) Import and release of biological control agents into Canada. <https://www.inspection.gc.ca/plant-health/plant-pests-invasive-species/biological-control-agents/eng/1514956211166/1514956212112>. (Accessed 2nd April 2020)
- Great Britain non-native species secretariat (2015) *The Great Britain Invasive Non-native Species Strategy*, Sand Hutton, York, UK.  
[www.nationalarchives.gov.uk/doc/open-government-licence/version/3/](http://www.nationalarchives.gov.uk/doc/open-government-licence/version/3/)  
[www.nationalarchives.gov.uk/doc/open-government-licence/version/3/](http://www.nationalarchives.gov.uk/doc/open-government-licence/version/3/)
- Grevstad, F.S. (1999) Factors influencing the chance of population establishment: implications for release strategies in biocontrol. *Ecological Applications*, **9**, 1439–1447.
- Grevstad, F.S., Coombs, E.M. & McEvoy, P.B. (2013) *Revisiting release strategies in biological control of weeds: are we using enough releases? Proceedings of the XIII International Symposium on Biological Control of Weeds, Waikoloa, Hawaii, USA, 11-16 September 2011* (ed. by Y. Wu), T. Johnson), S. Sing), S. Raghu), G. Wheeler), P. Pratt), K. Warner), T. Center), J. Goolsby), and R. Reardon), pp. 368–376. U.S. Forest Service, Forest Health Technology Enterprise Team, Morgantown, West Virginia, USA.

- Grodowitz, M.J., Owens, C.S., Smart, R.M. & Nachtrieb, J.G. (2007) *Impact of herbivory and plant competition on the growth of Hydrilla in small ponds*, U.S. Army Engineer Research and Development Center. ERDC/TN APCRP-BC-08 Vicksburg, MS, USA.
- Guerra, P.C., Becerra, J. & Gianoli, E. (2010) Explaining differential herbivory in sun and shade: the case of *Aristotelia chilensis* saplings. *Arthropod-Plant Interactions*, **4**, 229–235.
- Gurevitch, J., Fox, G.A., Wardle, G.M., Inderjit & Taub, D. (2011) Emergent insights from the synthesis of conceptual frameworks for biological invasions. *Ecology Letters*, **14**, 407–418.
- Harms, N.E., Cronin, J.T., Diaz, R. & Winston, R.L. (2020) A review of the causes and consequences of geographical variability in weed biological control successes. *Biological Control*, **151**.
- Harris, P. (1991) Classical biocontrol of weeds: its definitions, selection of effective agents, and administrative-political problems. *The Canadian Entomologist*, **123**, 827–849.
- Harris, P. (1973) The selection of effective agents for the biological control of weeds. *Canadian Entomologist*, **105**, 1495–1593.
- Harris, P. & Shorthouse, J.D. (1996) Effectiveness of gall inducers in weed biological control. *The Canadian Entomologist*, **128**, 1021–1055.
- Hart, A.J., Tullett, A.G., Bale, J.S. & Walters, K.F.A. (2002) Effects of temperature on the establishment potential in the U.K. of the non-native glasshouse biocontrol agent *Macrolophus caliginosus*. *Physiological Entomology*, **27**, 112–123.
- Hasan, S. (1974) Recent advances in the use of plant pathogens as biocontrol agents of weeds. *PANS Pest Articles & News Summaries*, **20**, 437–443.
- Hatcher, P.E. (1995) Three-Way interactions between plant pathogenic fungi, herbivorous insects and their host plants. *Biological Reviews*, **70**, 639–694.
- Hatherly, I.S., Bale, J.S., Walters, K.F.A. & Worland, M.R. (2004) Thermal biology of *Typhlodromips montdorensis*: implications for its introduction as a glasshouse biological control agent in the UK. *Entomologia Experimentalis et Applicata*, **111**, 97–109.

- Hatherly, I.S., Hart, A.J., Tullett, A.G. & Bale, J.S. (2005) Use of thermal data as a screen for the establishment potential of non-native biological control agents in the UK. *BioControl*, **50**, 687–698.
- Heimpel, G.E. & Mills, N.J. (2017) *Biological control: ecology and applications*, Cambridge University Press, Cambridge, UK.
- Herbert, H.J. (1979) Population trends and behavior of the pear rust mite, *Epitrimervs pyri* (Prostigmata: Eriophyoidea), on pears in Nova Scotia. *The Canadian Entomologist*, **111**, 955–957.
- Herbert, L.T., Vázquez, D.E., Arenas, A. & Farina, W.M. (2014) Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *Journal of Experimental Biology*, **217**, 3457–3464.
- Herms, D.A. & Mattson, W.J. (1992) The dilemma of plants: to grow or defend. *The Quarterly Review of Biology*, **67**, 283–335.
- Hill, M.P., Coetzee, J.A. & Ueckermann, C. (2012) Toxic effect of herbicides used for water hyacinth control on two insects released for its biological control in South Africa. *Biocontrol Science and Technology*, **22**, 1321–1333.
- Hill, R.L. (1999) *Minimising uncertainty - in support of no-choice tests. Host specificity testing in Australasia: towards improved assays for biological control* (ed. by T.M. Withers, L. Barton Browne and J.N. Stanley), pp. 1–10. Scientific publishing, Brisbane, Australia.
- Hinz, H.L., Schwarzländer, M., Gassmann, A. & Bouchier, R. (2014) Successes we may not have had: a retrospective analysis of selected weed biological control agents in the United States. *Invasive Plant Science and Management*, **7**, 565–579.
- Hislop, R.G. & Prokopy, R.J. (1981) Integrated management of phytophagous mites in Massachusetts (USA) apple orchards. 2. Influence of pesticides on the predator *Amblyseius fallacis* (Acarina: Phytoseiidae) under laboratory and field conditions. *Protection Ecology*, **3**, 157–172.
- Hoelmer, K.A. & Kirk, A.A. (2005) Selecting arthropod biological control agents against arthropod pests: Can the science be improved to decrease the risk of releasing ineffective agents? *Biological Control*, **34**, 255–264.

- Hoffman, J.H. (1995) *Biological control of weeds: the way forward, a South African perspective. Weeds in a changing world. British Crop Protection Council Symposium Proceedings No. 64* (ed. by J.K. Waage), pp. 77–98. British Crop Protection Council.
- Howe, R.W. (1967) Temperature effects on embryonic development in insects. *Annual Review of Entomology*, **12**, 15–42.
- Hulme, P.E. (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. *Journal of Applied Ecology*, **46**, 10–18.
- Hussner, A. (2009) Growth and photosynthesis of four invasive aquatic plant species in Europe. *Weed Research*, **49**, 506–515.
- Igrc, J., Deloach, C.J. & Zlof, V. (1995) Release and establishment of *Zygogramma suturalis* F. (coleoptera: Chrysomelidae) in Croatia for control of common ragweed (*Ambrosia artemisiifolia* L.). *Biological Control*, **5**, 203–208.
- IUCN (2017) Invasive species. <https://www.iucn.org/theme/species/our-work/invasive-species>. (Accessed 7th January 2017)
- Jadhav, A., Hill, M.P. & Byrne, M. (2008) Identification of a retardant dose of glyphosate with potential for integrated control of water hyacinth, *Eichhornia crassipes* (Mart.) Solms-Laubach. *Biological Control*, **47**, 154–158.
- Jadhav, A.M. (2011) Integrated control of water hyacinth using a retardant dose of glyphosate herbicide. PhD Thesis. University of Witwatersrand, Johannesburg.
- Jester, L.L., Bozek, M.A., Helsel, D.R. & Sheldon, S.P. (2000) *Euhrychiopsis lecontei* distribution, abundance, and experimental augmentations for Eurasian watermilfoil control in Wisconsin lakes. *Journal of Aquatic Plant Management*, **38**, 88–97.
- Jian, S., Soltis, P.S., Gitzendanner, M.A., Moore, M.J., Li, R., Hendry, T.A., Qiu, Y.L., Dhingra, A., Bell, C.D. & Soltis, D.E. (2008) Resolving an ancient, rapid radiation in Saxifragales. *Systematic Biology*, **57**, 38–57.
- Jones, C.G., Lawton, J.H. & Shachak, M. (1994) Organisms as ecosystem engineers. *Oikos*, **69**, 373–386.
- Julien, M., Sosa, A., Chan, R., Schooler, S. & Traversa, G. (2012) *Alternanthera philoxeroides* (Martius) Grisebach - alligator weed. *Biological control of weeds in Australia*, pp. 43–51. CSIRO Publishing, Melbourne, Australia.

- Julien, M.H. & White, G. (1997) *Biological control of weeds: theory and practical application*, Australian Centre for International Agricultural Research, Canberra, Australia.
- Jupp, P.W., Briese, D.T., Cullen, J.M. & Entomology, C. (1997) Evidence for resistance in *Hypericum perforatum* to a biological control agent, the eriophyid mite *Aculus hyperici*. *Plant Protection Quarterly*, **12**, 67–70.
- Katembo, N., Hill, M.P. & Byrne, M.J. (2013) Impacts of a sub-lethal dose of glyphosate on water hyacinth nutrients and its indirect effects on *Neochetina* weevils. *Biocontrol Science and Technology*, **23**, 1412–1426.
- Keane, R. & Crawley, M. (2002) Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology & Evolution*, **17**, 164–170.
- Kendon, M., McCarthy, M. Jevrejeva, S. Matthews, A. & Legg, T. (2019) State of the UK climate 2018. *International Journal of Climatology*, **39**, 1–55.
- Kenney, D.S. (1986) DeVine ® - the way it was developed - an industrialist's view. *Weed Science*, **34**, 15–16.
- Kew Herb. IMI. <http://www.herbimi.info/herbimi/home.htm>. (Accessed 2nd October 2011)
- Klavsén, S.K. & Maberly, S.C. (2009) Crassulacean acid metabolism contributes significantly to the in situ carbon budget in a population of the invasive aquatic macrophyte *Crassula helmsii*. *Freshwater Biology*, **54**, 105–118.
- Knihinicki, D.K., Petanović, R., Cvrković, T. & Varia, S. (2018) A new species of *Aculus* mite (Acari: Eriophyidae), a potential biocontrol agent for Australian swamp stonecrop, *Crassula helmsii* (Crassulaceae). *Zootaxa*, **4497**, 573–585.
- Konigsberg, E.R. (2014) Factors involved in the success and establishment of the field bindweed gall mite *Aceria malherbae* Nuzzaci (Acari: Eriophyidae), MSc Thesis, Montana State University, Bozeman, Montana, USA.
- Kontodimas, D.C., Eliopoulos, P.A., Stathas, G.J. & Economou, L.P. (2004) Comparative temperature-dependent development of *Nephus includens* (Kirsch) and *Nephus bisignatus* (Boheman) (Coleoptera: Coccinellidae) preying on *Planococcus citri* (Risso) (Homoptera: Pseudococcidae): evaluation of a linear and variou. *Physiological Ecology*, **33**, 1–11.

- Kudsk, P. & Mathiassen, S.K. (2020) Pesticide regulation in the European Union and the glyphosate controversy. *Weed Science*, **68**, 214–222.
- Kyung, S., Seong, J., Kook, J., Lee, H. & Lee, J. (2018) Predicting early spring emergence and late season overwintering movement of *Calepitrimerus vitis* (Nalepa) (Acari: Eriophyidae) in grapevine. *Journal of Asia-Pacific Entomology*, **21**, 1182–1185.
- Lactin, D.J., Holliday, N.J., Johnson, D.L. & Craigen, R. (1995) Improved rate model of temperature-dependent development by arthropods. *Environmental Entomology*, **24**, 68–75.
- Lake, E.C. & Minter, C.R. (2018) A review of the integration of classical biological control with other techniques to manage invasive weeds in natural areas and rangelands. *BioControl*, **63**, 71–86.
- Lambertucci, S., Orman, K.M., Das Gupta, S., Fisher, J.P., Gazal, S., Williamson, R.J., Cramer, R. & Bindschedler, L.V. (2019) Analysis of barley leaf epidermis and extrahaustorial proteomes during powdery mildew infection reveals that the PR5 thaumatin-like protein TLP5 is required for susceptibility towards *Blumeria graminis* f. sp. *hordei*. *Frontiers in Plant Science*, **10**.
- Langdon, S.J., Marrs, R.H., Hosie, C.A., MaAllister, H.A., Norris, K.M. & Potter, J. (2004) *Crassula helmsii* in U.K. ponds: effects on plant biodiversity and implications for newt conservation. *Weed Technology*, **18**, 1249–1352.
- Lansdown, R. V. (2015) New Zealand Pigmyweed, *Crassula helmsii* factsheet. 2–4.
- Laundon, J.R. (1961) An Australian species of *Crassula* introduced into Britain. *Watsonia*, **5**, 59–63.
- Leach, J. & Dawson, H. (1999) *Crassula helmsii* in the British Isles - an unwelcome invader. *British Wildlife*, **10**, 234–239.
- Levine, J.M., Adler, P.B. & Yelenik, S.G. (2004) A meta-analysis of biotic resistance to exotic plant invasions. *Ecology Letters*, **7**, 975–989.
- Lindquist, E.E. (1996) *External Anatomy and Notation of Structures. Eriophyoid Mites. Their Biology, Natural Enemies and Control* (ed. by E.E. Lindquist, M.W. Sabelis and J. Bruin), pp. 3–29. Elsevier Science Publishing, Amsterdam, the Netherlands.

- Lindquist, E.E. & Oldfield, G.N. (1996) *Evolution of eriophyoid mites in relation to their host plants. Eriophyoid Mites. Their Biology, Natural Enemies and Control* (ed. by E.E. Lindquist, M.W. Sabelis and J. Bruin), pp. 277–300. Elsevier Science Publishing, Amsterdam, the Netherlands.
- Lockton, A.J. (2010) Species account: *Crassula helmsii*. Botanical Society of the British Isles. <http://sppaccounts.bsbi.org/content/crassula-helmsii-2.html>. (Accessed 16th October 2020)
- Logan, J.A., Wollkind, D.J., Hoyt, S.C. & Tanigoshi, L.K. (1976) An analytic model for description of temperature dependent rate phenomena in arthropods. *Environmental Entomology*, **5**, 1133–1140.
- van der Loop, J.M.M., de Hoop, L., van Kleef, H.H. & Leuven, R.S.E.W. (2018) Effectiveness of eradication measures for the invasive Australian swamp stonecrop *Crassula helmsii*. *Management of Biological invasions*, **9**, 343–355.
- Lu, X., Siemann, E., He, M., Wei, H., Shao, X. & Ding, J. (2015) Climate warming increases biological control agent impact on a non-target species. *Ecology Letters*, **18**, 48–56.
- Lu, X., Siemann, E., He, M., Wei, H., Shao, X. & Ding, J. (2016) Warming benefits a native species competing with an invasive congener in the presence of a biocontrol beetle. *New Phytologist*, **211**, 1371–1381.
- Lym, R.G. & Nelson, J.A. (2002) Integration of *Aphthona* spp. flea beetles and herbicides for leafy spurge (*Euphorbia esula*) control. *Weed Science*, **50**, 812–819.
- Ma, R.Y. & Wang, R. (2004) Effect of morphological and physiological variations in the ecotypes of alligator weed, *Alternanthera philoxeroides* on the pupation rate of its biocontrol agent *Agasicles hygrophila*. *Acta Phytoecological Sinica*, **28**, 24–30.
- Mangan, R. & Baars, J.R. (2016) Can leaf-mining flies generate damage with significant impact on the submerged weed *Lagarosiphon major*? *BioControl*, **61**, 803–813.



- Manson, D.C.M. & Oldfield, G.N. (1996) *Life forms, deuterogyny, diapause and seasonal development. Eriophyoid Mites. Their Biology, Natural Enemies and Control* (ed. by E.E. Lindquist, M.W. Sabelis and J. Bruin), pp. 173–183. Elsevier Science Publishing, Amsterdam, the Netherlands.
- Marchante, H., López-Núñez, F.A., Freitas, H., Hoffmann, J.H., Impson, F. & Marchante, E. (2017) First report of the establishment of the biocontrol agent *Trichilogaster acaciaelongifoliae* for control of invasive *Acacia longifolia* in Portugal. *EPPO Bulletin*, **47**, 274–278.
- Marlin, D. (2010) The role of the mite *Orthogalumna terebrantis* in the biological control programme for water hyacinth, *Eichhornia crassipes*, in South Africa. PhD Thesis. Rhodes University, South Africa.
- Marohasy, J. (1998) The design and interpretation of host-specificity tests for weed biological control with particular reference to insect behaviour. *Biocontrol News and Information*, **19**, 13–21.
- Marsden, J.S., Martin, G.E., Parham, D.J., Smith, T.J.R. & Johnston, B.G. (1980) *Returns on Australian agricultural research. The joint Industries Assistance Commission-CSIRO benefit-cost study of the CSIRO Division of Entomology.*, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia.
- Marshall, V.G. & Clayton, M.R. (2004) Biology and phenology of *Cecidophyopsis psilaspis* (Acari: Eriophyidae) on Pacific yew (Taxaceae). *Canadian Entomologist*, **136**, 695–710.
- Martínez Jiménez, M. & Gómez Balandra, M.A. (2007) Integrated control of *Eichhornia crassipes* by using insects and plant pathogens in Mexico. *Crop Protection*, **26**, 1234–1238.
- McClay, A.S. & Balciunas, J.K. (2005) The role of pre-release efficacy assessment in selecting classical biological control agents for weeds - applying the Anna Karenina principle. *Biological Control*, **35**, 197–207.
- McFadyen, R.E.C. (1998) Biological control of weeds. *Annual Review of Entomology*, **43**, 369–93.

- McFadyen, R.E.C. (2002) *Does ecology help in the selection of biocontrol agents? Improving the Selection, Testing and Evaluation of Weed Biological Control Agents. CRC for Weed Management Systems Technical Series: No.7* (ed. by H. Spafford Jacob and D.T. Briese), pp. 5–9. Glen Osmond, Australia.
- McFadyen, R.E.C. (2000) *Successes in biological control of weeds. Proceedings of the X International Symposium of Biological Control of Weeds, 4-14 July, 1999* (ed. by N.R. Spencer), pp. 3–14. Montana State University, Bozeman, Montana, USA.
- McFadyen, R.E.C. & Spafford Jacob, H. (2004) *Insects for the biocontrol of weeds: predicting parasitism levels in the new country. Proceedings of the XI International Symposium on Biological Control of Weeds, Canberra, Australia, 27 April - 2 May 2003* (ed. by J.M. Cullen, D.T. Briese, D.J. Kriticos, W.M. Lonsdale, L. Morin and J.K. Scott), CSIRO Entomology, Canberra, Australia.
- Messersmith, C.G. & Adkins, S.W. (1995) Integrating weed-feeding insects and herbicides for weed control. *Weed Technology*, **9**, 199–208.
- Messing, R. & Brodeur, J. (2018) Current challenges to the implementation of classical biological control. *BioControl*, **63**, 1–9.
- Met Office (2019) Seasonal summary: Winter 2018/2019.  
[https://www.metoffice.gov.uk/binaries/content/assets/metofficegovuk/pdf/weather/learn-about/uk-past-events/summaries/uk\\_monthly\\_climate\\_summary\\_winter\\_2019.pdf](https://www.metoffice.gov.uk/binaries/content/assets/metofficegovuk/pdf/weather/learn-about/uk-past-events/summaries/uk_monthly_climate_summary_winter_2019.pdf). (Accessed 20th December 2019)
- Meyer, J.Y., Duploux, A. & Taputuarai, R. (2007) Dynamique des populations de l'arbre endémique *Myrsine longifolia* (Myrsinacées) dans les forêts de Tahiti (Polynésie française) envahies par *Miconia calvescens* (Mélastomatacées) après introduction d'un champignon pathogène de lutte biologique. *Revue d'Ecologie (Terre et Vie)*, **62**, 17–33.
- Meyer, J.Y. & Fourdrigniez, M. (2011) Conservation benefits of biological control: the recovery of a threatened plant subsequent to the introduction of a pathogen to contain an invasive tree species. *Biological Conservation*, **144**, 106–113.
- Michalska, K., Skoracka, A., Navia, D. & Amrine, J.W. (2010) Behavioural studies on eriophyoid mites: an overview. *Experimental and Applied Acarology*, **51**, 31–59.

- Milan, J.D., Harmon, B.L., Prather, T.S. & Schwarzländer, M. (2006) Winter mortality of *Aceria chondrillae*, a biological control agent released to control rush skeletonweed (*Chondrilla juncea*) in the western United States. *Journal of Applied Entomology*, **130**, 473–479.
- Moore, J.L. & Remais, J. V (2014) Developmental models for estimating ecological responses to environmental variability: structural, parametric and experimental issues. *Acta biotheoretica*, **62**, 69–90.
- Moran, V.C. & Hoffmann, J.H. (2015) The fourteen International Symposia on Biological Control of Weeds, 1969-2014: delegates, demographics and inferences from the debate on non-target effects. *Biological Control*, **87**, 23–31.
- Morin, L. (2020) Progress in biological control of weeds with plant pathogens. *Annual Review of Phytopathology*, **58**, 1–23.
- Morin, L. & Edwards, P.B. (2006) Selection of biological control agents for bridal creeper: a retrospective review. *Australian Journal of Entomology*, **45**, 287–291.
- Morin, L., Evans, K.J. & Sheppard, A.W. (2006) Selection of pathogen agents in weed biological control: critical issues and peculiarities in relation to arthropod agents. *Australian Journal of Entomology*, **45**, 349–365.
- Morin, L., Reid, A.M., Sims-Chilton, N.M., Buckley, Y.M., Dhileepan, K., Hastwell, G.T., Nordblom, T.L. & Raghu, S. (2009) Review of approaches to evaluate the effectiveness of weed biological control agents. *Biological Control*, **51**, 1–15.
- Motta, E.V.S., Raymann, K. & Moran, N.A. (2018) Glyphosate perturbs the gut microbiota of honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, **115**, 10305–10310.
- Müller-Schärer, H., Lewinsohn, T.M. & Lawton, J.H. (1991) Searching for weed biocontrol agents – when to move on? *Biocontrol Science and Technology*, **1**, 271–280.
- Nowell, D. & Maynard, G. V. (2005) *International guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms (ISPM No. 3). Second International Symposium on Biological Control of Arthropods* (ed. by M. Hoddle), pp. 726–734. USDA Forest Service, Davos, Switzerland.

- Office for National Statistics (2015) *UK Natural Capital – Freshwater Ecosystem Assets and Services Accounts*,  
<https://www.wavespartnership.org/en/knowledge-center/uk-natural-capital—freshwater-ecosystem-assets-and-services-accounts>.
- Oldfield, G.N. (1996) *Toxemias and other non-distortive feeding effects. Eriophyoid Mites. Their Biology, Natural Enemies and Control* (ed. by E.E. Lindquist, M.W. Sabelis, and J. Bruin), pp. 243–249. Elsevier Science Publishing, Amsterdam, the Netherlands.
- Oldfield, G.N. & Proeseler, G. (1996) *Eriophyoid mites as vectors of plant pathogens. Eriophyoid Mites. Their Biology, Natural Enemies and Control* (ed. by E.E. Lindquist, M.W. Sabelis and J. Bruin), pp. 259–275. Elsevier Science Publishing, Amsterdam, the Netherlands.
- Oreska, M.P.J. & Aldridge, D.C. (2011) Estimating the financial costs of freshwater invasive species in Great Britain: a standardized approach to invasive species costing. *Biological Invasions*, **13**, 305–319.
- Page, A.R. & Lacey, K.L. (2006) *Economic impact assessment of Australian weed biological control. CRC for Weed Management Systems Technical Series: No.10*, pp. iii–160.
- Pakyari, H., Fathipour, Y. & Enkegaard, A. (2011) Estimating development and temperature thresholds of *Scolothrips longicornis* (Thysanoptera: Thripidae) on eggs of two-spotted spider mite using linear and nonlinear models. *Journal of Pest Science*, **84**, 153–163.
- Palmer, W.A. & Haseler, W.H. (1992) The host specificity and biology of *Trirhabda bacharidis* (Weber) (Coleoptera: Chrysomelidae), a species introduced into Australia for the biological control of *Baccharis halimifolia* L. *The Coleopterists Bulletin*, **46**, 61–66.
- Palmer, W.A., McLaren, D. & Sheppard, A.W. (2014) *Australia's present scientific capacity to progress the biological control of weeds. Proceedings of the XIV International Symposium on Biological Control of Weeds, Kruger National Park, South Africa, 2-7 March 2014* (ed. by F.A.C. Impson, C.A. Kleinjan and J.H. Hoffmann), pp. 183–186. University of Cape Town, South Africa.

- Paynter, Q. (2003) Integrated weed management: effect of herbicide choice and timing of application on the survival of a biological control agent of the tropical wetland weed, *Mimosa pigra*. *Biological Control*, **26**, 162–167.
- Paynter, Q., Fowler, S. V., Hayes, L. & Hill, R.L. (2015) Factors affecting the cost of weed biocontrol programs in New Zealand. *Biological Control*, **80**, 119–127.
- Perkins, D. (1974) Arthropods that stress waterhyacinth. *PANS Pest Articles & News Summaries*, **20**, 304–314.
- Petanović, R. & Kielkiewicz, M. (2010a) Plant-eriophyoid mite interactions: Cellular biochemistry and metabolic responses induced in mite-injured plants. Part I. *Experimental and Applied Acarology*, **51**, 61–80.
- Petanović, R. & Kielkiewicz, M. (2010b) Plant-eriophyoid mite interactions: specific and unspecific morphological alterations. Part II. *Experimental and Applied Acarology*, **51**, 81–91.
- Pimentel, D., McNair, S., Janecka, J., Wightman, J., Simmonds, C., O'Connell, C., Wong, E., Russel, L., Zern, J., Aquino, T. & Tsomondo, T. (2001) Economic and environmental threats of alien plant, animal, and microbe invasions. *Agriculture, Ecosystems and Environment*, **84**, 1–20.
- Plant Health Australia (2001) Australian Plant Pest Database. <https://appd.ala.org.au>. (Accessed 20th January 2011)
- Pratt, P.D., Coombs, E.M. & Croft, B.A. (2003) Predation by phytoseiid mites on *Tetranychus lintearius* (Acari: Tetranychidae), an established weed biological control agent of gorse (*Ulex europaeus*). *Biological Control*, **26**, 40–47.
- Pratt, P.D., Herr, J.C., Carruthers, R.I. & Cabrera Walsh, G. (2019) Complete development on *Elodea canadensis* (Hydrocharitaceae) eliminates *Hydrellia egeriae* (Diptera, Ephydriidae) as a candidate biological control agent of *Egeria densa* (Hydrocharitaceae) in the USA. *Biocontrol Science and Technology*, **29**, 405–409.
- Purcell, M., Harms, N., Grodowitz, M., Zhang, J., Ding, J., Wheeler, G., Zonneveld, R. & de Chenon, R.D. (2019) Exploration for candidate biological control agents of the submerged aquatic weed *Hydrilla verticillata*, in Asia and Australia 1996–2013. *BioControl*, **64**, 233–247.

- QGIS.org (2020) QGIS Geographic Information System. Open Source Geospatial Foundation Project.
- Quinn, B. (2017) A critical review of the use and performance of different function types for modeling temperature-dependent development of arthropod larvae. *Journal of Thermal Biology*, **63**, 65–77.
- R Core Team (2019) R: A language and environment for statistical computing. R Foundation for statistical computing, Vienna, Austria. <https://www.r-project.org/>.
- Rebaudo, F. (2018) devRate: quantify the relationship between development rate and temperature in ectotherms.
- Rebaudo, F., Struelens, Q. & Dangles, O. (2017) Modelling temperature-dependent development rate and phenology in arthropods: The devRate package for R. *Methods in Ecological Evolution*, **9**, 1144–1150.
- Rebaudo, J.-F. & Rabhi, V.-B. (2018) Modeling temperature-dependent development rate and phenology in insects: review of major developments, challenges, and future directions. *Entomologia Experimentalis et Applicata*, **166**, 607–617.
- Reeder, R.H., Bacon, E.T.G., Caiden, M.J., Bullock, R.J. & González-Moreno, P. (2018) Effect of population density of the Azolla weevil (*Stenopelmus rufinasus*) on the surface cover of the water fern (*Azolla filiculoides*) in the UK. *BioControl*, **63**, 185–192.
- Relyea, R.A. (2005) The lethal impact of roundup on aquatic and terrestrial amphibians. *Ecological Applications*, **15**, 1118–1124.
- Reznik, S.Y., Spasskaya, I.A., Dolgovskaya, M.Y., Volkovitsh, M.G. & Zaitzev, V.F. (2008) *The ragweed leaf beetle Zygommatia suturalis F. (Coleoptera: Chrysomelidae) in Russia: current distribution, abundance and implication for biological control of common ragweed, Ambrosia artemisiifolia L. Proceedings of the XII International Symposium on Biological Control of Weeds, La Grande Motte, France, 22-27 April, 2007* (ed. by M.H. Julien), R. Sforza), M.C. Bon), H.C. Evans), P.E. Hatcher), H.L. Hinz), and B.G. Rector), pp. 614–619. CAB International, Wallingford, UK.

- Robertson, M.P., Kriticos, D.J. & Zachariades, C. (2008) Climate matching techniques to narrow the search for biological control agents. *Biological Control*, **46**, 442–452.
- Sainty, G., McCorkelle, G. & Julien, M. (1997) Control and spread of alligator weed *Alternanthera philoxeroides* (Mart.) Griseb., in Australia: lessons for other regions. *Wetlands Ecology and Management*, **5**, 195–201.
- Salgado-Luarte, C. & Gianoli, E. (2010) Herbivory on temperate rainforest seedlings in sun and shade: resistance, tolerance and habitat distribution. *PLoS ONE*, **5**, 1–7.
- de Saraiva, A.S., Sarmiento, R.A., Pedro-Neto, M., Teodoro, A.V., Erasmo, E.A.L., Belchior, D.C.V. & de Azevedo, E.B. (2016) Glyphosate sub-lethal toxicity to non-target organisms occurring in *Jatropha curcas* plantations in Brazil. *Experimental and Applied Acarology*, **70**, 179–187.
- Schaffner, U., Dingle, K., Swart, C. & Cristofaro, M. (2012) *Biological control of Russian knapweed, Acroptilon repens, annual report 2011*, CABI Switzerland.
- Schaffner, U., Smith, L. & Cristofaro, M. (2018) A review of open-field host range testing to evaluate non- target use by herbivorous biological control candidates. *BioControl*, **63**, 405–416.
- Schroeder, D. & Goeden, R.D. (1986) The search for arthropod natural enemies of introduced weeds for biological control - in theory and practice. *Biocontrol News and Information*, **7**, 147–155.
- Schwarzländer, M., Hinz, H.L., Winston, R.L. & Day, M.D. (2018) Biological control of weeds: an analysis of introductions, rates of establishment and estimates of success, worldwide. *BioControl*, **63**, 319–331.
- Seebens, H. (2019) Invasion ecology: expanding trade and the dispersal of alien species. *Current Biology*, **29**, R120–R122.
- Seebens, H., Essl, F., Dawson, W., Fuentes, N., Moser, D., Pergl, J., Pyšek, P., van Kleunen, M., Weber, E., Winter, M. & Blasius, B. (2015) Global trade will accelerate plant invasions in emerging economies under climate change. *Global Change Biology*, **21**, 4128–4140.
- Sharon, A., Amsellem, Z. & Gressel, J. (1992) Glyphosate suppression of an elicited defense response. *Plant Physiology*, **98**, 654–659.

- Shaw, R., Schaffner, U. & Marchante, E. (2016) The regulation of biological control of weeds in Europe - an evolving landscape. *EPPO Bulletin*, **46**, 254–258.
- Shaw, R.H., Ellison, C.A., Marchante, H., Pratt, C.F., Schaffner, U., Sforza, R.F.H. & Deltoro, V. (2018) Weed biological control in the European Union: from serendipity to strategy. *BioControl*, **63**, 333–347.
- Shaw, R.H., Tanner, R., Djeddour, D. & Cortat, G. (2011) Classical biological control of *Fallopia japonica* in the United Kingdom - lessons for Europe. *Weed Research*, **51**, 552–558.
- Shearer, J.F. (2008) Is classical biocontrol using fungi a viable option for submersed aquatic plant management? *Journal of Aquatic Plant Management*, **46**, 202–205.
- Shearer, J.F. & Nelson, L.S. (2002) Integrated use of endothall and a fungal pathogen for management of the submersed aquatic macrophyte *Hydrilla verticillata*. *Weed Technology*, **16**, 224–230.
- Sheppard, A.W. (2002) *Prioritising agents based on predicted efficacy: Beyond the lottery approach. Improving the selection, testing and evaluation of weed biological control agents. CRC for Weed Management Systems Technical Series: #7* (ed. by H. Spafford Jacob and D.T. Briese), pp. 11–22.
- Sheppard, A.W., Klinken, R.D. Van & Heard, T.A. (2005) Scientific advances in the analysis of direct risks of weed biological control agents to nontarget plants. *Biological Control*, **35**, 215–226.
- Sheppard, A.W., Shaw, R.H. & Sforza, R. (2006) Top 20 environmental weeds for classical biological control in Europe: a review of opportunities, regulations and other barriers to adoption. *Weed Research*, **46**, 93–117.
- Shine, R. (2010) The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *The Quarterly Review of Biology*, **85**, 253–291.
- Silvestri, L., Sosa, A., Mc Kay, F., Vitorino, M.D., Hill, M., Zachariades, C., Hight, S., Weyl, P., Smith, D., Djeddour, D. & Mason, P.G. (2020) Implementation of access and benefit-sharing measures has consequences for classical biological control of weeds. *BioControl*, **65**, 125–141.



- Skoracka, A., Kuczyński, L. & Rector, B. (2007) Divergent host acceptance behavior suggests host specialization in populations of the polyphagous mite *Abacarus hystrix* (Acari: Prostigmata: Eriophyidae). *Environmental Entomology*, **36**, 899–909.
- Skoracka, A., Smith, L., Oldfield, G., Cristofaro, M. & Amrine, J.W. (2010) Host-plant specificity and specialization in eriophyid mites and their importance for the use of eriophyid mites as biocontrol agents of weeds. *Experimental and Applied Acarology*, **51**, 93–113.
- Smith, L. (2005) Host plant specificity and potential impact of *Aceria salsolae* (Acari: Eriophyidae), an agent proposed for biological control of Russian thistle (*Salsola tragus*). *Biological Control*, **34**, 83–92.
- Smith, L. (2019) *Influence of host plant on wind dispersal by an eriophyid mite, Aceria salsola. Proceedings of the XV International Symposium on Biological Control of Weeds, Engleberg, Switzerland, 26-31 August 2018* (ed. by H.L. Hinz), M.C. Bon), G. Bourdôt), M. Cristofaro, G. Desurmont, D. Kurose, H. Müller-Schärer, M. Rafter, U. Schaffner, M. Seier, R.F.H. Sforza, L. Smith, S. Stutz, S. Thomas, P. Weyl and R. Winston), p. 135. Engelberg, Switzerland.
- Smith, L., de Lillo, E. & Amrine, J.W. (2010) Effectiveness of eriophyid mites for biological control of weedy plants and challenges for future research. *Experimental and Applied Acarology*, **51**, 115–49.
- Smith, R., Mangan, R. & Coetzee, J.A. (2019) Risk assessment to interpret the physiological host range of *Hydrellia egeriae*, a biocontrol agent for *Egeria densa*. *BioControl*, **64**, 447–456.
- Smith, T. & Buckley, P. (2015) The growth of the non-native *Crassula helmsii* (Crassulaceae) increases the rarity scores of aquatic macrophyte assemblages in south-eastern England. *New Journal of Botany*, **5**, 192–199.
- Spencer, N.R. & Coulson, J.R. (1976) The biological control of alligatorweed, *Alternanthera philoxeroides*, in the United States of America. *Aquatic Botany*, **2**, 177–190.
- Sternlicht, M. (1969) Effect of different wave lengths of light on the behaviour of an eriophyid bud mite. *Entomologia Experimentalis et Applicata*, **12**, 377–382.

- Sternlicht, M. & Goldenberg, S. (1971) Fertilisation, sex ratio and postembryonic stages of the citrus bud mite *Aceria sheldoni* (Ewing) (Acarina, Eriophyidae). *Bulletin of Entomological Research*, **60**, 391–397.
- Stevens, P.F. (2019) Angiosperm Phylogeny Website. Version 14.  
<http://www.mobot.org/MOBOT/research/APweb/>. (Accessed 15<sup>th</sup> February 2019)
- Stiling, P. & Cornelissen, T. (2005) What makes a successful biocontrol agent? A meta-analysis of biological control agent performance. *Biological Control*, **34**, 236–246.
- Stoeva, A., Harizanova, V., de Lillo, E., Cristofaro, M. & Smith, L. (2012) Laboratory and field experimental evaluation of host plant specificity of *Aceria solstitialis*, a prospective biological control agent of yellow starthistle. *Experimental and Applied Acarology*, **56**, 43–55.
- Strayer, D.L. (2010) Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. *Freshwater Biology*, **55**, 152–174.
- Sukno, S.A., García, V.M., Shaw, B.D. & Thon, M.R. (2008) Root infection and systemic colonization of maize by *Colletotrichum graminicola*. *Applied and Environmental Microbiology*, **74**, 823–832.
- Swale, E. & Belcher, H. (1982) *Crassula helmsii*, the swamp stonecrop, near Cambridge., *Nature in Cambridgeshire*, **25**, 59–62
- Tack, A.J.M., Gripenberg, S. & Roslin, T. (2012) Cross-kingdom interactions matter: fungal-mediated interactions structure an insect community on oak. *Ecology Letters*, **15**, 177–185.
- Tate, T.M., Spurlock, J.O. & Christian, F.A. (1997) Effect of glyphosate on the development of *Pseudosuccinea columella* snails. *Archives of Environmental Contamination and Toxicology*, **33**, 286–289.
- Thiede, J. & Eggli, U. (2007) *Crassulaceae. The Families and Genera of Vascular Plants. Volume IX Flowering plants. Eudicots* (ed. by K. Kubitzki), pp. 83–118. Berlin-Heidelberg, Germany.
- Thomma, B.P.H.J. (2003) *Alternaria* spp.: From general saprophyte to specific parasite. *Molecular Plant Pathology*, **4**, 225–236.

- Tullett, A.G., Hart, A.J., Worland, M.R. & Bale, J.S. (2004) Assessing the effects of low temperature on the establishment potential in Britain of the non-native biological control agent *Eretmocerus eremicus*. *Physiological Entomology*, **29**, 363–371.
- USDA (2017) Technical Advisory Group for Biological Control Agents of Weeds. [https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/permits/regulated-organism-and-soil-permits/biological-control-organism-permits/sa\\_tag/ct\\_technical\\_advisory\\_group\\_biological\\_control\\_agents\\_weeds](https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/permits/regulated-organism-and-soil-permits/biological-control-organism-permits/sa_tag/ct_technical_advisory_group_biological_control_agents_weeds) (Accessed 2nd April 2020)
- Vacante, V. (2015) *The Handbook of Mites of Economic Plants: Identification, bio-ecology and control*, CABI, Wallingford, UK.
- Valenzano, D., Bari, G., Valeria, M. & Lillo, E. De (2019) Off-host survival of Eriophyoidea and remarks on their dispersal modes. *Experimental and Applied Acarology*, **79**, 21–33.
- Varia, S. & Shaw, R. (2013) *Potential for the biological control of Crassula helmsii in the U.K. Proceedings of the XIII International Symposium on Biological Control of Weeds, Waikoloa, Hawaii, USA, 11-16 September 2011* (ed. by Y. Wu, T. Johnson, S. Sing, S. Raghu, G. Wheeler, P. Pratt, K. Warner, T. Center, J. Goolsby and R. Reardon), p. 179. USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown, West Virginia, USA.
- Varia, S., Wood, S., Pratt, C., Adair, R. & Murphy, S. (2019) *A mitey solution to Australian swamp stonecrop. Proceedings of the XV International Symposium on Biological Control of Weeds, Engleberg, Switzerland, 26-31 August 2018* (ed. by H.L. Hinz, M.C. Bon, G. Bourdôt, M. Cristofaro, G. Desurmont, D. Kurose, H. Müller-Schärer, M. Rafter, U. Schaffner, M. Seier, R.F.H. Sforza, L. Smith, S. Stutz, S. Thomas, P. Weyl and R. Winston), pp. 242–243. Engelberg, Switzerland.
- Vilà, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarošík, V., Maron, J.L., Pergl, J., Schaffner, U., Sun, Y. & Pyšek, P. (2011) Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. *Ecology Letters*, **14**, 702–708.

- Villamagna, A.M. & Murphy, B.R. (2010) Ecological and socio-economic impacts of invasive water hyacinth (*Eichhornia crassipes*): a review. *Freshwater Biology*, **55**, 282–298.
- Walton, C. (2005) *Reclaiming lost provinces - a century of weed biological control in Queensland*, Queensland Department of Natural Resources and Mines, Brisbane, Australia.
- Wapshere, A.J. (1974a) A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology*, **77**, 201–211.
- Wapshere, A.J. (1974b) Host specificity of phytophagous organisms and the evolutionary centres of plant genera or sub-genera. *Entomophaga*, **19**, 301–309.
- Warren, R.J., Candeias, M., Labatore, A., Olejniczak, M. & Yang, L. (2018) Multiple mechanisms in woodland plant species invasion. *Journal of Plant Ecology*, **12**, 201–209.
- Watson, W. (2001) An unwelcome invader! *Worcestershire Record*, **10**.
- Webb, C.J., Sykes, W.R. & Garnock-Jones, P.J. (1988) *Flora of New Zealand. Volume IV. Naturalised pteridophytes, gymnosperms, dicotyledons.*, Botany Division, DSIR Christchurch, New Zealand.
- Webber, J., Chapman, R.B. & Worner, S.P. (2008) Forecasting emergence and movement of overwintering hazelnut big bud mites from big buds. *Experimental and Applied Acarology*, **45**, 39–51.
- Wei, H., Lu, X. & Ding, J. (2014) Direct and indirect impacts of different water regimes on the invasive plant, alligator weed (*Alternanthera philoxeroides*), and its biological control agent, *Agasicles hygrophila*. *Weed Biology and Management*, **15**, 1–10.
- Westphal, E. & Manson, D.C.M. (1996) *Feeding effects on host plants: gall formation and other distortions. Eriophyoid Mites. Their Biology, Natural Enemies and Control* (ed. by E.E. Lindquist, M.W. Sabelis and J. Bruin), pp. 231–242. Elsevier Science Publishing, Amsterdam, the Netherlands.

- Weyl, P., Cristofaro, M., Smith, L., Vidovic, B., Petanović, R., Marini, F., Asadi, G.A. & Stutz, S. (2019) *Eriophyid mites and weed biological control: does every silver lining have a cloud? Proceedings of the XV International Symposium on Biological Control of Weeds, Engleberg, Switzerland, 26-31 August 2018* (ed. by H.L. Hinz, M.C. Bon, G. Bourdôt, M. Cristofaro, G. Desurmont, D. Kurose, H. Müller-Schärer, M. Rafter, U. Schaffner, M. Seier, R.F.H. Sforza, L. Smith, S. Stutz, S. Thomas, P. Weyl and R. Winston), pp. 9–11. Engelberg, Switzerland.
- Williams, F., Eschen, R., Harris, A., Djeddour, D., Pratt, C., Shaw, R.H., Varia, S., Lamontagne-Godwin, J., Thomas, S.E. & Murphy, S.T. (2010) *The economic cost of invasive non-native species on Great Britain*, Project number VM10066, p199, CABI UK.
- Willis, A.J., Berentson, P.R. & Ash, J.E. (2003) Impacts of a weed biocontrol agent on recovery from water stress in a target and a non-target *Hypericum* species. *Journal of Applied Ecology*, **40**, 320–333.
- Winston, R.L., Schwarzländer, M., Hinz, H.L., Day, M.D., Cock, M.J.W. & Julien, M.H. (2014) *Biological control of weeds: a world catalogue of agents and their target weeds*, 5th edition, FHTET-2014-04. USDA Forest Service, pp 838, Forest Health Technology Enterprise Team, Morgantown, West Virginia, USA.
- Wosula, E.N., McMechan, A.J. & Hein, G.L. (2015) The effect of temperature, relative humidity, and virus infection status on off-host survival of the wheat curl mite (Acari: Eriophyidae). *Journal of Economic Entomology*, **108**, 1545–1552.

**Appendix 1: Sites surveyed for natural enemies of *Crassula helmsii* in Australia between 2011 and 2013.**

<b>Site ID</b>	<b>Identifying landmark</b>	<b>State</b>	<b>Type of water body</b>	<b>Latitude</b>	<b>Longitude</b>
1	Kangaroo Lake	Victoria	Lake	-35.5982	143.7683
2	Cohuna	Victoria	Lake	-35.798212	144.233318
3	Shepparton	Victoria	Artificial lake	-36.390817	145.396133
4	Horsham	Victoria	Creek	-36.760433	142.391767
5	Heathcote	Victoria	Artificial pond	-36.928833	144.68075
6	Mansfield	Victoria	Wetland	-37.051733	146.076883
7	Amphitheatre	Victoria	Creek	-37.197733	143.398133
8	Blampied	Victoria	Pond	-37.358633	144.055067
9	Blampied 2	Victoria	Pond	-37.3649	143.990467
10	Waterloo	Victoria	Drainage ditch	-37.3695	143.417283
11	Eden Park	Victoria	Creek	-37.4893	145.02215
12	Penshurst	Victoria	Creek	-37.856533	142.2767
13	Penshurst botanical garden	Victoria	Artificial pond	-37.872967	142.29105
14	Paynesville	Victoria	River edge	-37.889017	147.6595
15	Dandenong	Victoria	Wetland	-37.970557	145.231209
16	Cranbourne	Victoria	Drainage ditch	-38.121383	145.31255
17	Geelong	Victoria	Wetland	-38.176171	144.353821
18	Moriac	Victoria	Pond	-38.233383	144.203933
19	Winslow	Victoria	Lake	-38.244683	142.45625
20	Hazelwood	Victoria	Pond	-38.255417	146.486417
21	Lake Purrumbete	Victoria	Lake	-38.284067	143.21595
22	Stonyford	Victoria	Wetland	-38.300286	143.302369
23	Lake Colac	Victoria	Lake	-38.330867	143.596933
24	Warrnambool	Victoria	Wetland	-38.37425	142.43883
25	Port Fairy	Victoria	Pond	-38.364967	142.1989
26	Whoorel	Victoria	Lake	-38.396233	143.834033
27	Palana	Tasmania	Creek	-39.764633	147.892133

Site ID	Identifying landmark	State	Type of water body	Latitude	Longitude
28	Lackrana	Tasmania	Drainage stream	-40.10144	148.18842
29	Coast Road	Tasmania	Creek	-40.195433	148.172517
30	Big Waterhouse Lake	Tasmania	Lake	-40.896183	147.614633
31	Lake Dulverton	Tasmania	Lake	-42.299033	147.377617
32	Kamona	Tasmania	Meadow	-41.15593	147.66249
33	Newnham	Tasmania	Wetland	-41.38214	147.10272
34	Tamar Island	Tasmania	Wetland	-41.390367	147.073317
35	Victoria Valley	Tasmania	Creek	-42.309417	146.7064
36	Lake Tiberias	Tasmania	Lake	-42.422674	147.381543