The biology of Cassytha pubescens

An Australian native hemiparasitic vine

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Declaration

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Elizabeth C. Maciunas

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Persistence. Resilience. Tenacity.

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Prologue

Cassytha pubescens is an Australian native hemiparasitic vine with a relatively unknown life history. The majority of research on this species has focused on its potential use as a biological control agent for some invasive species, such as gorse (*Ulex europaeus*) and Scotch Broom (*Cytisus scoparius*), but little known about its propagation and seedling stages in particular. The most comprehensive study of the species to date was written in 1924, almost a century ago, and many of the observations and results contained within that paper are vague and difficult to replicate. The thesis presented here addresses many knowledge gaps in the lifecycle of this interesting plant and increases our understanding of an important Australian native species. Specifically, the work here examines dispersal, population genetics, germination triggers, seedling ecology and competitive effects on seedlings, and the effect of seedling infection on a novel host species. My work is presented as a series of manuscripts prepared for publication; since Chapter 2 has been published, for consistency all formatting of references follows the same style as presented in that journal. Chapter 5 is a manuscript presented in the style of a short communication, and Chapter 7 contains results that were limited by circumstance and are discussed as observations and trends in an essay-like style intended as a homage to McLuckie's original 1924 paper. Methods and results for that chapter are included as appendices.

Overall my research greatly advances our understanding of this native Australian parasite, and expands our knowledge of how it interacts with other species. I hope this information will be used to assist the application of *C. pubescens* as a biological control for invasive species, and more importantly to improve conservation and management outcomes for the parasite and the ecological communities it is found within.

Thesis abstract

This project improves our general understanding of the fundamental biology of one of our most interesting native plants, *Cassytha pubescens*. The implications of this research are far-reaching across many fields and have answered many diverse questions. To begin with, I collated all known dispersal vectors for all *Cassytha* species in a literature review, before examining scats of *Isoodon obesulus* and determining it may act as a dispersal vector for *C. pubescens* seeds. As the historical distribution of both species overlap significantly, it is likely that *I. obesulus* has played an important role in seed movement for the parasite over time.

C. pubescens forms patches seemingly growing clonally on different hosts, so I used genetic markers (SNPs) to understand the relatedness of individuals within and between sites at varying scales and to determine the relative importance of clonal reproduction for this species. Interestingly, my results show a high prevalence of clonal reproduction within sites, and diverging populations between national parks. This indicates seed dispersal may be limited, and that vegetative reproduction plays an important role in allowing populations to persist over long periods of time.

Fire is thought to stimulate germination in *C. pubescens*, based on laboratory testing. The field observations presented here are the first to confirm a strong fire response in this species. Importantly, significant time-delays were found between the burn and seedling emergence. Patterns of emergence in relation to the location of previous host plants, as well as observations of seed traps within the immediate landscape, also imply limited dispersal for this species. As seedlings emerged in high densities, I examined the effect of high seedling density at emergence, and assessed whether host species had any effect on their survivorship. Significant differences in survivorship were found between seedlings emerging at low and high density, indicating there is competition for resources between siblings.

I used a glasshouse experiment to examine the effect of host choice and density on establishment of seedlings. Contrary to expectations, greater host choice (and a correspondingly greater chance to find a host) did not correlate to greater establishment. It is proposed here that as seen in the field, competition plays a significant role in seedling success as greater host density increases competition and limits resource availability for emerging seedlings.

I also used *Rubus fruitcosus* in a glasshouse experiment to test whether *C. pubescens* seedlings could establish on a non-native host, and examined their effect on the performance of a host species with marked modular growth. I found that the parasite established well, induced strong resource partitioning effects in *R. fruticosus*, and that infection has a significant effect on overall biomass and thorniness. With these results, interesting parallels are drawn between the effects of parasitism and herbivory.

Finally, I present a compilation of information collected from the literature and from observations and short experiments. This includes a literature and herbarium data review to give perspective on the breadth of the host range for the parasite. Conditions required for seed germination, some results regarding seedling ecology, as well as an investigation into any potential mycorrhizal associations are also discussed.

Chapter 1

General introduction

Adult *Cassytha pubescens* wrapped around a woody stem. Note lateral shoots and haustoria.

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Introduction

The parasitic strategy is a successful one, with examples found across all kingdoms. Parasitic angiosperms have independently evolved at least 12 times, are spread across multiple families and genera, and parasitise a wide variety of hosts in many families (Nickrent, 2020; Watson et al., 2022). They have a variety of growth forms, host specialisation, vary in how and where they connect to their host (above or below ground), how much they depend on their host for survival, and which resources they acquire from their hosts. With such incredible diversity it is perhaps surprising that many groups are little studied, particularly considering our increasing understanding of the importance of parasitic species within plant communities (e.g. Chaudron et al., 2021). We now know that the effects of parasitism are not just restricted to those that the parasite directly has on its specific hosts; they can influence community dynamics on a much broader scale (Watson et al., 2022). For example, by influencing the vigour of their hosts parasitic plants have been known to suppress dominant species and allow for an increase in biodiversity and productivity through competitive release mechanisms (Musselman, 1996; Mutikainen et al., 2000). Parasitic plants also play a key role in processes such as nutrient cycling throughout a variety of habitats, particularly in semi-arid to arid zones (March & Watson, 2007; March & Watson, 2010; Quested, 2008; Quested et al., 2003). With such wide-reaching effects, it is unsurprising that they are increasingly seen as keystone species within ecosystems (Těšitel et al., 2021; Watson, 2009), not only for their influence on plant community structure but also by providing resources for fauna (Pincheira et al., 2023). Despite this, the vast majority of literature on parasitic plants still focuses on agriculturally problematic taxa, such as *Orobanchae*, *Striga* and *Cuscuta*, and the basic biology of many other genera remains largely unknown.

Study species: *Cassytha pubescens*

Cassytha L. (Lauraceae) is a genus of slender, twining hemiparasitic vines, and overall it has a wide-ranging distribution. Species are represented in Southern America, Southern Africa, Australia, New Zealand and the surrounding islands, with one pantropical species extending into the Northern hemisphere. However with 16 of the 23 species being endemic, the major centre of diversity is Australia (Weber, 1981). The genus is found throughout a wide variety of habitats, from sclerophyllous woodlands to tropical rainforest, with different species showing distinct preferences in terms of host structure. For example, smaller species such as *Cassytha glabella* typically parasitise low shrubs and groundcovers, whereas the large species such as *Cassytha melantha* typically parasitise trees (Weber, 1981).

C. pubescens is distributed throughout South-Eastern Australia; it occurs in South Australia, Victoria, New South Wales, Queensland and Tasmania (Maciunas et al., 2022; Weber, 1981). Throughout its extensive range, there is significant morphological variation (Weber, 1981), however, the species is characterised by its hairy mature stems and fruits (McLuckie, 1924). It typically parasitises shrubs (see Figure 1), and although it is assumed to have a broad host-range it is unknown whether there is a core host-range which the parasite preferentially grows on, or whether any host-preferences the species displays change throughout its life span.

Figure 1: *Cassytha pubescens* growing on *Bursaria spinosa*. The dense network of stems covering the outside of the shrub is typical of this species' growth form.

C. pubescens has previously been seen to damage and kill invasive species, whilst leaving natives relatively unscathed. In addition to negatively impacting on weedy species, a small scale study found that Scotch Broom is not just parasitised by *C. pubescens*, but is also a favoured host (Prider, 2009). In fact, the majority of the literature examining this species investigates the host-parasite relationship, and has an overarching theme of potentially utilising this differential impact on invasive hosts and growing the parasite as biological control agent. The most notable weedy species examined in such studies are two legumes: Scotch Broom (*Cytisus scoparius*) (Prider et al., 2008; Prider et al., 2011; Shen et al., 2010) and Gorse (*Ulex europaeus*) (Cirocco et al., 2016; Cirocco et al., 2017; Cirocco et al., 2018; Cirocco, et al., 2021; Tsang, 2010).

In order to consider using any species as a biological control agent a complete knowledge of that species' biology is needed, and *C. pubescens* is no exception. Even as a native itself, without in-depth knowledge of the biology of the organism to be used as the control agent the consequences of increasing the prevalence of the parasite could be dire. While some of the biology of *C. pubescens* is somewhat known from the literature (for example flowering and fruiting times, general habitat and approximate distribution), much of that information stems from a single study by McLuckie (1924) and focuses on specimens found only within the Sydney region of New South Wales. As the species is known to be variable across its range, and as much within that particular paper is largely based on observation alone, there are still many outstanding questions regarding this species' general biology. How and how far seeds are dispersed, what triggers germination, what environmental factors influence seedling survivorship, how prevalent vegetative spread of adults is, and the host range of both seedlings and adult plants are all important elements that need to be understood if this species is to see wide applications as a biological control agent for introduced species.

Knowledge gap: Dispersal

The mechanism a species uses to disperse its seeds is heavily dependent on the growth habit and abiotic factors that plant requires for survival. As such, dispersal mechanisms of different species can be predicted with a reasonable amount of certainty, even if the specific dispersal vector is unknown (Howe & Smallwood, 1982). In *C. pubescens,* the fruits are fleshy, about 0.5-1cm in diameter, contain a single, hard seed, and vary substantially in colour across its range. According to Weber (1981), the fruits may be "…grey, green-grey to green-red…. indumentum white and mixed red, often broadly streaked, alternately dull-green and dull-red-brown…" (Figure 2 provides an example). Overall, these traits are suggestive of biotic modes of dispersal,

Figure 2: Immature *C. pubescens* fruit. In the Mount Lofty ranges, fruit tends more grey-green with white indumentum.

although such variation in colour suggests that a suite of species may contribute to seed movement. *Cassytha* species are known to act as food and shelter sources for fauna (eg Bennett & Baxter, 1989; Hardman & Moro, 2006), and understanding the interactions at all levels of that community may improve conservation outcomes for all species. If dispersal is facilitated by birds and small mammals, the ongoing effects of fragmentation of our native forests and woodlands make understanding which species act as vectors even more important.

In *C. pubescens*, fruiting occurs during summer and the high water content of the fruit may act to attract a range of species. Based on field observations, a large amount of fruit falls to the ground around the parent plant and accumulates in the litter layer (Facelli and Maciunas pers obs.). It is certainly possible that birds and arboreal mammals (such as small possums) may consume fruit from nearer the canopy, but fallen fruit also provides an opportunity for other animals, notably fossorial mammals and lizards, to scavenge. It is unknown if such a large volume of fruit fall is part of this species historical ecology, or if a reduction in populations of small animals, particularly small mammals, since European settlement is now resulting in greater accumulation of fruits around the parent plants.

In order to determine whether or not an animal may disperse seed, two questions must be asked; firstly, does that animal consume the seed in the first place? And secondly, does that seed pass through the animal in a state that renders it undamaged and still viable? In the first instance, evidence can be collected from feeding experiments, or perhaps more reliably (in a natural setting) from examining the gut contents of captured animals. In feeding experiments, it can be difficult to differentiate seed consumption and dispersal from seed predation, as well as the effect of captivity on the preference for consuming fruits in the first place. However, in wildtrapped animals seeds can be recovered from scat samples, and differentiating damaged and undamaged seed can be the first step in identifying and discounting any seed predation that is occurring (Pufal, 2015). Recovering seeds whole allows viability to be tested, in most cases with germination or cut-tests, and dispersal vectors can be confirmed.

Knowledge gap: Population connectivity and prevalence of clonal reproduction

The distance that individual animals can disperse and the extent to which geneflow occurs between populations is increasingly used as a measure of the health, longevity and potential divergence of those populations (Kisel & Barraclough, 2010). In plant species, estimates of genetic similarities between populations can provide an insight into effective seed movement, pollen movement, or both, depending on the genome studied. By using chloroplast DNA, which is only maternally inherited, the contribution of seed movement specifically to genetic diversity can be studied. In turn, this can be used to determine the effective dispersal distances of seed (McCauley, 1995) and the likelihood that geneflow will continue to occur between populations. In *C. pubescens*, molecular studies are needed to determine the distance that seeds are dispersed, as well as to determine how closely related individuals with patches are. All *Cassytha* species can reproduce not only from seed but also vegetatively; this reproductive mode is in fact exploited in laboratory experiments in order to reproduce large numbers of mature vines relatively quickly. The extent to which clonal reproduction occurs in the field is unknown, however as this parasite can grow across multiple hosts and new clones in theory could form quickly, extensive clonal reproduction of individuals does have implications for the genetic diversity of populations.

Knowledge gap: Population recovery after fire and seedling ecology

In broad terms, fire response in plants can be divided into two main strategies; those that survive fire and recover by resprouting from protected cambial tissues, and those that are killed by fire and rely on the germination of seeds for population recovery (Bell, 2001). In the case of *C. pubescens,* adults lack root systems entirely and stems are herbaceous, therefore disturbances such as fire will remove all plants from an area. This species is therefore expected to rely on the soil seed bank, or require dispersal from other areas for patch regeneration. Laboratory tests have shown that *C. pubescens* seeds respond positively to high temperature (namely, boiling water), indicating that fire may be a strong germination cue (Tsang, 2010). Seedlings emerge within 6-8 weeks of the heat treatment in the lab and can survive for another 6-8 weeks while searching for a host. If no suitable host can be found in that time the seedling will then die (McLuckie, 1924; Prider *et al.,* 2009; Tsang, 2010). However, this is yet to be documented in the field, together with other aspects of the ecology of the species such as seed bank distribution, seedling survivorship, general seedling ecology (such as competitive and abiotic influences), and the influence of host species and availability are also still unknown.

In addition to specific germination cues, subsequent seedling survival is likely determined by complex ecological interactions. Parasitic plants require not only host species to be present, but they also have to be robust enough to support their growth and re-establishment. As a climbing vine, the growth habit of *C. pubescens* allows it a small amount of effective movement and access to different host species at any given time. It is possible that such movement may allow the opportunity for the use of intermediate, or nurse, hosts. Although this has been documented in a morphologically (although taxonomically unrelated) genus, *Cuscuta*, (Schoolmaster Jr, 2005), it is unknown whether this occurs in any *Cassytha* species. It is certainly possible that *C. pubescens* may make more use of different host species throughout different life stages, as it is a long-lived species and there is certainly post-fire successional change in community composition which would change host availability throughout the parasite's life. As re-establishment of patches after major disturbances can only occur through germination of seeds, understanding the ecology of the seedling stage is extremely important for managing populations.

Knowledge gap: Effects of seedlings on novel hosts

The effects of plant parasites on the host's health and general physiology vary depending on the parasite, how it parasitises the host, and whether or not the host species has developed any resistance to the parasite (Westwood *et al.,* 2010). However, all parasitic plants have, in theory, the chance of impacting the vigour of the host through creating an additional resource sink that reduces the resources that are otherwise available to that host (Marvier, 1996; Prider *et al.,* 2010). Other physiological responses to parasitism can include reduced photosynthetic activity due to shading by the parasite, water stresses due to the high transpiration rates of parasitic plants, photodamage, and a reduced reproductive output (Press and Phoenix, 2005). In addition to direct effects, parasitism can also have indirect effects on host health, including decreasing the resources available to the host plant to fight disease, infections, herbivory and to cope with abiotic stresses such as drought (Marvier, 1996; Press and Phoenix, 2005).

The vast majority of research considering the effect of *C. pubescens* on its host has focused on the natives *Leptospermum myrsinoides* and *Acacia paradoxa,* and the invasive species *Ulex europaes* and *Cytisus scoparius* (See the various works of Cirocco, et al., 2016-2022; Prider et al., 2008; Prider et al., 2009; Prider et al., 2011). For most studies the invasive species considered are leguminous hosts, and the effects of the parasite on non-leguminous hosts remains to be tested. All studies to date also examine the impact of mature parasites, with no data available on the effect of infection by seedlings. As this parasite is known to be a generalist at the adult stages, it is important to understand whether or not seedlings can also utilise non-native host species. In addition, whether the resource sink created by a seedlings is sufficient to promote a similar reduction in host growth to that seen under infection by adult parasites is important, as by understanding the suitability of invasive hosts for seedling establishment, the practical use of this parasite as a biological control agent may be significantly expanded.

Knowledge gap: Host range, germination cues and mycorrhizal associations

Host choice is a critical part of parasite life histories, as it can influence competition between parasitic species, and also resource availability for other organisms within that community (Pennings and Callaway, 2002). In many parasitic organisms host choice differs according to which stage of the lifecycle the parasite is at, so it is important to consider different life stages individually (Minchella, 2009).

As previously mentioned, there is some degree of host-specificity seen within the *Cassytha* genus. The size (stem diameter) of the parasite generally correlates with that of the hosts it is found on, with smaller species, such as *C. glabella*, parasitising mostly herbs and forbs, and larger species, such as *C. melantha*, parasitising trees (McLuckie, 1924). The degree of host-specificity for each species individually, however, is unknown. Whether host choice differs in different habitats is also unknown, as are the selection mechanisms for hosts. In more arid areas, available hosts may be a limiting resource, thereby forcing growth on to what would otherwise be less favoured species. In wetter ecosystems with higher diversity, such as *Eucalyptus* forests of the Mt Lofty Ranges, a complex understory and a wider range of potential host plants may allow for a greater host discrimination.

Initial field data indicates that adult *C. pubescens* plants may show some degree of host preference (Prider *et al.*, 2009). Although there is a lack of published material regarding the host choice of *Cassytha* species, they appear to be generalists and have been documented growing on a wide variety of species. However, there is also evidence that not all plants are susceptible to infection with *Acacia myrtifolia* has been shown to resist haustorial penetration (Facelli et al., 2020; Tsang, 2010). This indicates that not all species are not optimal hosts for this parasite, and further research into the use of different host species by *C. pubescens* is needed.

For many parasitic species, specific cues are needed to induce germination and seeds may remain dormant within the soil seed bank until germination is triggered by those cues (Mussleman et al., 1996). The timing of germination in obligate parasites is greatly important; if no host is available after germination then the seedling will die. To reduce the risk of germinating without suitable hosts, many parasitic species display physical and/or physiological dormancies of seeds, allowing for a more specific set of conditions that promote germination and less chance of germinating without the presence of suitable hosts (Mahadevan & Jayasuriya, 2013; Baskin et al., 2000). In *C. pubescens,* the hard seed coat and response in the laboratory to immersion in boiling water suggests there is some form of physical dormancy. However, the extent to which this interacts with other environmental conditions and any physiological dormancies is unknown.

Once dormancy is broken and seedlings emerge, access to resources will determine the survival of those seedlings. Community interactions such as competition, as well as mutualistic relationships such as mycorrhizal associations, can then affect establishment success. In *C. pubescens* this is especially important; the root of seedlings is short lived and must support the parasite while it searches for a host. Whether this species has the capacity to form mycorrhizal associations is unknown, however there are two possible mechanisms by which it could theoretically benefit from such a relationship. The traditional mycorrhizal relationship involves a mutualistic sharing of resources between fungi and the plant, but the benefit of increasing access to nutrients (and potentially water) for the seedling must outstrip the carbon cost of supporting the fungi. The other mechanism by which the parasite could utilise a mycorrhizal relationship is by effectively parasitising the mycorrhizal network of other species. Research has shown that establishment of seedlings in some species can be facilitated by connection to a common mycorrhizal network, whereby other plants in the community subsidise the carbon cost of the fungi and allow the seedling to hitch a ‘free ride’ (Bever et al., 2010; Booth & Hoeksema, 2010). Considering the transient nature of the root in *C. pubescens,* any benefit a fungal association could provide, however brief, could have lasting impacts on establishment and overall seedling success.

Overall project aims

This project will focus on the life cycle of *Cassytha pubescens*, to increase our understanding of its fundamental biology and interactions with other species. Specifically, this project encompasses:

1. Investigating a potential dispersal vector of *Cassytha pubescens*
2. Assessing population connectivity, and the prevalence of clonal reproduction in maintaining populations using genetic data
3. Examining fire response, intraspecific competition, survivorship, and potential host species selectivity of seedlings
4. Investigating the effect of host availability on seedling establishment success
5. Assessing the effects of infection on a new host species, *Rubus fruticosus*, when the parasite is grown from seed, and
6. Providing a variety of additional observations on host range, seedling ecology, germination biology, and an investigation into potential mycorrhizal associations in field and lab-germinated seedlings.

By answering these questions, substantial gaps in knowledge for this species can be filled. Ultimately it is expected that this will lead to enhanced management of populations of the parasite and its hosts, allowing this important species to continue to enhance ecosystem function within our increasingly fragmented natural landscapes. The knowledge gained here also has implications for this species' possible use as a biological control, particularly with regard to the spread of adult plants via vegetative (clonal) reproduction, as well as the ecology that governs the establishment of new populations after disturbances.

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Chapter 2

Seed traits and fate support probable primary dispersal of a native hemi-parasitic vine *Cassytha pubescens* (Lauraceae) by *Isoodon obesulus*, an endangered marsupial, in southern Australia

Adult southern brown bandicoot (*Isoodon obesulus*) and maturing fruit of *C. pubescens*. The historic range of *I. obesulus* overlaps significantly with that of *C. pubescens* and it is now thought that it disperses the parasite’s seeds.

Statement of Authorship

Title of Paper	Seed traits and fate support probable primary dispersal of a native hem-parasitic vine Cassytha pubescens (Lauraceae) by Isoodon obesulus, an endangered marsuipial, in southern Australia
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Conducted all lab work, analysis, literature review and wrote the manuscript. Prepared manuscript for publication, prepared figures.

Principal Author

Name of Principal Author (Candidate)	Elizabeth C Maciunas		
Contribution to the Paper	Conducted all lab work, analysis, literature review and wrote the manuscript. Prepared manuscript for publication, prepared figures.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/09/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 2 preamble

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Dispersal of *C. pubescens* seed by birds is almost certain, so in this chapter I focused on potential dispersal by bandicoots using scat samples of *Isoodon obesulus* that were collected in 2008 and 2009 by J. Packer during her studies of the marsupial's habitat use. These were examined under a dissecting microscope, and *C. pubescens* seeds were recovered. I also reviewed the literature to provide a comprehensive list of all known instances of all *Cassytha* species found within the gut or scat of any animal species, and compared the historic and current home ranges of *I. obesulus* to *C. pubescens* specifically to determine whether this association may have been wide-spread.

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Seed traits and fate support probable primary dispersal of a native hemi-parasitic vine *Cassytha pubescens* (Lauraceae) by *Isoodon obesulus*, an endangered marsupial, in southern Australia

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ABSTRACT

Consumption of seeds by fruit-eating animals is one of the most effective dispersal mechanisms for plants, yet little is known about dispersal by marsupial vectors. In the face of habitat fragmentation and the recent extinction or decline of many marsupial species in southern Australia, addressing knowledge gaps on vertebrate vectors is critical to determine deficiencies in seed dispersal. Here we examine one potential dispersal vector for the seeds of *Cassytha pubescens*, a native hemi-parasitic vine that contributes to vegetation complexity within many woodland habitats in southern Australia that are threatened by fragmentation. To determine the potential dispersal role of mammals for *Cassytha pubescens* within southern Australian woodlands, we collected and examined 46 scat samples from the omnivorous, nationally endangered southern brown bandicoot, *Isoodon obesulus*, over two fruiting (summer) seasons. Of the 46 scats, five had up to six seemingly undigested seeds of *C. pubescens*, indicating that *I. obesulus* consumes the fruits and may act as a disperser for this vine. Our findings improve understanding of the role of marsupials like bandicoots in dispersing native plant seed and may help to inform conservation management priorities to protect ecosystem function more effectively in fragmented woodlands threatened by loss of vertebrate seed vectors.

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Introduction

Seed dispersal is one of the most important factors determining the dynamics and spatial structure of plant populations (Nathan & Muller-Landau, 2000). The successful management of self-sustaining plant populations, therefore, relies on understanding how propagules are dispersed, as well as what influences their post-dispersal survival. Many factors change the ability of both plants and animals to disperse, with habitat fragmentation arguably one of the most important (K. F. Davies et al., 2000; McConkey et al., 2012). Different management strategies are required to maintain connectivity, depending on the

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dispersal syndrome and the types of dispersers present (Cramera et al., 2007). As such, knowledge of which extant species are important dispersers in degraded systems is critically important to understand the ecological consequences and further risks of fragmentation. For example, it is likely that decreasing faunal movement between habitat fragments reduces the effective dispersal distance for many plant species, particularly those with large seeds dependent on animal vectors (McConkey et al., 2012). Reduced dispersal distances have severe consequences for all species susceptible to inbreeding, genetic drift, and may also hinder recolonising patches after local extinctions (Farwig & Berens, 2012). Any species loss, particularly of digging Critical Weight Range (CWR) mammals (i.e. non-flying mammals in the weight range 35–4200 g (Burbidge & McKenzie, 1989), is known to be ecologically significant in many parts of Australia (Burbidge & Manly, 2002; Fleming et al., 2014). Remaining animal species are, therefore, increasingly important for the maintenance of various ecosystem functions, including the dispersal of seeds.

Small marsupials are known to be important contributors to a variety of ecosystem functions across a wide range of habitats, including acting as ecosystem engineers through bioturbation, dispersal of mycorrhizal fungi spores (e.g. Tay et al., 2018), and the dispersal of seeds (e.g. Cantor et al., 2010; Palmer et al., 2020; Vazquez et al., 2021). However, the extent to which marsupials act as seed predators, rather than seed dispersers, is a relatively unstudied but important distinction. It is known that in many cases, seeds are damaged after being ingested due to either the destruction of the seed while being consumed, or from whole seeds being damaged during passage through the gut (Beca et al., 2021; Junges et al., 2018; Lessa et al., 2013). Nevertheless, Beca et al. (2021) found that while seed predation does occur, marsupial woylies (*Bettongia penicillata ogilbyi*) and quenda (*Isoodon fusciventer*) are both able to disperse viable seed of some Australian native species. Given the diversity of CWR marsupials in Australia, understanding their role in dispersing native plant species will have high conservation value for protecting ecological communities across a wide variety of habitats.

Whilst the general mechanisms by which seeds are primarily dispersed can be inferred by fruit and seed properties (e.g. green, dull fruit are dispersed primarily by mammals; Valenta & Nevo, 2020), the specific vectors that are essential to move them to suitable germination habitats are often unknown (Amico et al., 2017; Jones et al., 2005; Wang & Smith, 2002). One plant species with a specialised life strategy, yet unknown dispersal ecology, is the Australian hemi-parasitic vine *Cassytha pubescens* R.Br. (Lauraceae). While the adult plants lack roots entirely, seedlings germinate in the soil and rely on a reduced root system until a host plant is found and an attachment is formed (McLuckie, 1924; Tsang, 2010). Most of the populations of *C. pubescens* now exist in highly fragmented habitats, such as dry forest fragments near Tomago (Atlas of Living Australia, 2022; Ross et al., 2002) and woodlands in the Mount Lofty Ranges (Atlas of Living Australia, 2022; Szabo et al., 2011), and whether effective connectivity occurs between patches is unknown. Understanding the dispersal ecology of *C. pubescens* is essential to determine whether, or how, to enhance connectivity, including enabling dispersal of seeds between fragmented habitat patches. Although adult plants may spread clonally from host to host, several factors can result in local extinction of the species. Fire, a common occurrence in systems where the species is found, completely kills *C. pubescens* because the plant lacks underground parts. Seed dispersal is therefore fundamental for maintaining populations in the landscape.

Parasitic plants have been identified as keystone species, able to prevent over-abundance of dominant or invasive species (Prider et al., 2009; Těšitel et al., 2020), provide microhabitats that increase the diversity of multiple taxa (D. M. Davies et al., 1997; Pywell et al., 2004; Westbury et al., 2006), and create nutrient-rich patches (March & Watson, 2010; Phoenix & Press, 2005; Quested, 2008; Watson, 2016). The decline or disappearance of these keystone parasitic plants (including species of *Cassytha*) could, therefore, have severe effects on the integrity of ecosystems. It is known that fire is required for *C. pubescens* to germinate (Maciunas, unpublished data; Tsang, 2010); however, how seeds are dispersed and accumulate in the soil seed bank remains unclear.

Cassytha pubescens fruits vary from brown-red to green and contain a single, hard stone approximately 3.5 mm in diameter (Weber, 1981). There is some morphological variation in the size, shape, and colour of fruit of this species across its range, however the fleshy fruit consistently ripens during summer (November–January; McLuckie, 1924; Weber, 1981). The production of fleshy fruits in a dry season strongly suggests a vertebrate disperser, as many different plants provide fruits with specific water or caloric content during a particular season thereby catering to the needs of dispersers during those times (Herrera, 1982; Primack, 1987). The dull colouration of *C. pubescens* fruits suggests that they are unlikely to be dispersed primarily by birds (Weber, 1981). As a parasite, *C. pubescens* has access to the host plant's water supply, which allows it to produce fruit with a high water-content during dry times. The fleshy fruit is therefore likely to attract potential vertebrate vectors when other food and water sources are limited. Groups such as canids are known to disperse fruits of other hard-stoned species like Olives (Cuneo & Leishman, 2006; Spennemann, 2021), and omnivorous species are likely to play an important role in the dispersal of *C. pubescens*. Nevertheless, there is little evidence from the literature as to which species are responsible for the dispersal of the parasite, except for Taylor (1992) who indicated that the Tasmanian bettong (*Bettongia gaimardi*) is known to consume its seeds, and Bennett and Baxter (1989), whom recorded fruit consumption by the long-nosed potoroo (*Potorous tridactylus*) in Victoria. Both marsupials are absent from most of the large range of *C. pubescens*, including South Australia, raising questions as to what dispersal agents are active in those regions. Additionally, elsewhere in Australia and New Zealand seeds from similar sized species of *Cassytha* have been found in the gut and scats of small marsupials (such as bettongs; Taylor, 1992), small birds (Forde & Paton, 1986), emus (B. R. Quin, 1996) and even geckos (Whitaker, 1987). This range of reported vectors indicates a suite of vertebrates may play a role in dispersing *C. pubescens*.

The habitat of *C. pubescens* (sclerophyll forest, heathland and woodlands) is generally ecologically consistent across its range, but the frugivorous species assemblage may change substantially in different regions. It is therefore likely that various vectors disperse the seed of this species across its range. Considering that bettongs and potoroos ingest fruits and seeds in Tasmania and Victoria (Bennett & Baxter, 1989; Taylor, 1992), other

Figure 1. Distribution of *Cassytha pubescens* and *Isoodon obesulus*. Hatched areas represent current limits of *C. pubescens*. Light grey represents approximate historical limits, and dark grey current limits, of *I. obesulus* (including subspecies). Distribution envelopes of *C. pubescens* adapted from Australian Virtual Herbarium data, and *I. obesulus* adapted from Cooper et al. (2020), Pope et al. (2001), Rees and Paull (2000), Warburton and Travouillon (2016), and Zenger et al. (2005).

CWR marsupials that have a similar ecological role are likely to disperse seed in areas where these species are absent. For example, in South Australia, the foraging behaviour of the southern brown bandicoot (*Isoodon obesulus*) is similar to that of bettongs (Packer, 2014) and potoroos, and its diet includes fruits, arthropods, seeds and fungi (D. G. Quin, 1988). This bandicoot species had a historical distribution similar to the current distribution of *C. pubescens* (Figure 1), and although endangered (Commonwealth Government, 1999), small isolated populations still co-occur with *C. pubescens* throughout southeastern Australia (Atlas of Living Australia, 2022; Li et al., 2016). It is therefore possible that they can contribute to the dispersal of *C. pubescens* seed within this area.

To determine whether *I. obesulus* can be added to known CWR marsupial dispersers of *C. pubescens*, we combined literature review and field-based approaches to investigate this species in southern Australian woodlands. We aimed to determine whether *I. obesulus* is a probable disperser of *C. pubescens* within woodlands of the Mount Lofty Ranges. We first conducted a literature review on the ingestion of seeds of any species in the genus *Cassytha* to assess whether this marsupial species was a likely disperser. Second, we examined scats of *I. obesulus* collected from fragmented woodland patches in the Mount Lofty Ranges, South Australia, to determine if this marsupial ingested the fruit and seed of the parasitic plant and may therefore act as a disperser within the region.

Methods

A review of the literature was conducted to collate all known instances of ingestion of fruit of any species of *Cassytha* by vertebrates. Records were sourced through Google Scholar and Web of Science searches, using the terms "*Cassytha*" and any of the following: "mycophagy", "diet* OR frugiv", "dispersal", "bettongia" and "endozoochory". Reference lists of all papers found were also examined for additional sources. Data regarding the species of *Cassytha*, disperser species, condition of the seed and potential viability were extracted from each source.

Faecal samples (scats) were collected from individual bandicoots during January – March mark-recapture surveys in both 2008 and 2009, which season (summer) coincides with the peak fruiting of *C. pubescens*. Full details of the mark-recapture survey methods were provided in Packer et al. (2016), and all sites were within the distribution of both species. Scats (n = 46) were collected during trap checks (n = 2542), stored in 5 ml vials and frozen at ~5 degrees Celsius. The scats were then examined in petri dishes under a dissecting microscope. Scats were soft and dry when thawed and were disaggregated gently using blunt-end probes. Any seeds suspected to be *C. pubescens* were examined for damage before being removed from the scat samples and washed to remove debris, including the remains of the fruit. All recovered seeds were then compared to undigested seeds from a recent field collection. Seeds were classified as potentially viable if they were undamaged (i.e. visually undistinguishable from uneaten seeds with no apparent damage) after passing through the gut and being defaecated.

Results

The literature review revealed seven species of *Cassytha* reported as having seeds consumed by animals, including 12 bird species, 11 mammal species, and a single reptile (Table 1).

A total of 15 seeds of *C. pubescens* were found in five of the 46 scats examined, although seed abundance was extremely variable (Table 2). All seeds were recovered whole and undamaged. We, therefore, considered them to be potentially viable, although we acknowledge that due to being frozen their viability cannot be tested.

Discussion

Our scat analysis results showed that *I. obesulus* consumed and defaecated *C. pubescens* seeds in woodlands in the Mount Lofty Ranges. Based on seed trait (colour, size, and seasonal timing) and fate (whole, undamaged seeds), we therefore propose that *I. obesulus* is a probable disperser of *C. pubescens.*

Within the Mount Lofty Ranges, fruits of *C. pubescens* are produced during the dry, summer season. The fruits are dull grey, green-grey to green-red fleshy spheres up to 9 mm long (Weber, 1981), which are characteristics that indicate a larger, mammalian vector (Valenta & Nevo, 2020).

The presence of seeds of *C. pubescens* in the scats of *I. obesulus* confirms this bandicoot is one probable disperser for this vine species in South Australia. Endozoochory in the *Cassytha* genus has already been documented, and several bird and mammal species have

Table 1. Possible dispersal vectors reported for all *Cassytha* species globally (alphabetical order for mammals and birds). Records were based on fragments of vegetation and/or fruit within scat collections and were sourced through Google Scholar and Web of Science searches to February 2021. Seed condition and viability is stated where seed consumption was reported.

Cassytha Species	Vector species	Observations, seed condition and viability	Source
C. filiformis	Aders's duiker (*Cephalophus adersi*)	Vegetation and possible fruit consumption, only noted on one occasion	Fiske (2011)
	Allied Rock Wallaby (*Petrogale assimilis*)	Vegetation and possible fruit consumption	Horsup and Marsh (1992)
	Rufous hare-wallaby (*Lagorchestes hirsules*)	Vegetation and possible fruit consumption. Extensive fruit consumption with seasonal availability. Seed condition: unreported. Viability: unreported.	Lundie-Jenkins (1993) Jarman (1994)
	Spectacled hare-wallaby (*Lagorchestes conspicillatus*)	Seeds recovered. Seed condition: unreported. Viability: unreported.	Ingleby and Westoby (1992)
	Brown eared bulbul (*Hypsipetes amaurotis*)	One record of seed present. Seed condition: unreported. Viability: unreported.	Kawakami et al. (2009)
	Florida bobwhite (*Colinus virginianus floridanus*)	Seed consumption inferred. Seed condition: unreported. Viability: unreported.	Laessle and Frye (1956)
	Fruit Pigeons (Columbidae) and Yellow-Vented Bulbul (*Pycnonotus goiavier*)	Seed condition: whole seeds recovered. Viability: unreported	Whittaker and Jones (1994)
	Polynesian starling (*Aplonis tabuensis*)	Seed condition: whole seeds recovered. Viability: unreported.	Rinkie (1986)
	Seychelles black parrot (*Coracopsis barklyi*)	Fruit consumed. Seed condition: unreported. Viability: unreported.	Reuleaux et al. (2014)
C. glabella	Emu (*Dromaius novaehollandiae*)	Seed condition: whole seeds recovered. Viability: unreported.	B. R. Quin (1996)
	Ground parrot (*Pezoporus wallicus*)	Seed condition: fragments recovered. Viability: not viable Seed condition: fragments recovered. Viability: not viable.	Bryant (1994) McFarland (1991)
C. melantha	Ground parrot (*Pezoporus wallicus*)	Seed condition: fragments recovered. Viability: not viable.	Bryant (1994)
	Mallee fowl (*Leipoa ocellata*)	Seed condition: whole seeds recovered. Viability: unreported.	Booth (1986)
C. nodiflora	European rabbit (*Oryctolagus cuniculus*)	Vegetation and possible fruit consumption.	Martin et al. (2007)
C. paniculata	Northland green gecko (*Naultinus grayi*)	Fruit consumed. Seed condition: unreported. Viability: unreported.	Whitaker (1987)
C. pubescens	Long-nosed potoroo (*Potorous tridactylus*)	Fruit consumed. Seed condition: unreported. Viability: unreported.	Bennett and Baxter (1989)
	Southern brown bandicoot (*Isoodon obesulus*)	Fruit consumed. Seed condition: whole seeds recovered. Viability: unreported.	Maciunas et al. (2022 This study

(*Continued*)

Table 1. (Continued).

Cassytha Species	Vector species	Observations, seed condition and viability	Source
	Tasmanian bettong (*Bettongia gaimardi*)	Fruit consumed. Seed condition: unreported. Viability: unreported.	Taylor (1992)
	Rufus bristlebird (*Dasyornis broadbenti*), Spiny cheeked honeyeater (*Acanthagenys rufogularis*) & Singing honeyeater (*Lichenostomus virescens*)	Fruit consumed. Seed condition unreported. Viability: unreported.	Forde and Paton (1986)
C. racemosa	European rabbit (*Oryctolagus cuniculus*)	Vegetation and possible fruit consumption.	Martin et al. (2007)
Cassytha sp.	Mallee fowl (*Leipoa ocellata*)	Fruit and seed consumption. Seed condition: unreported. Viability: unreported.	Reichelt and Jones (2008)
	Yellow-vented bulbul *(Pycnonotus goiavier*)	Seed condition: whole seed recovered. Viability: unreported.	Zann and Male (1990)
	Black-gloved wallaby (*Macropus irma*) & Tammar wallaby (*Macropus eugenii*)	Vegetation and possible fruit consumption.	Shepherd et al. (1997)
	Bush rat (*Rattus fuscipes*)	Fruit consumed. Seed condition: unreported. Viability: unreported.	Cheal (1987)

Table 2. Mean and SD of *Cassytha pubescens* seeds/fruits in scats of *Isoodon obesulus*. Sample locations are longitudinal survey sites of *I. obesulus* populations within the Mount Lofty Ranges, South Australia.

Site	Number of scats	Seeds per scat
Ackland Hill Road	18	0.3 ± 1.34
Belair National Park	8	0
Mark Oliphant Conservation Park	8	0.75 ± 1.67
Mount Bold Reservoir Reserve	10	0.3 ± 0.65
Scott Creek Conservation Park	2	0

been reported to consume fruits of *C. pubescens* (Table 1). Although seed viability could not be confirmed here (because of post-sampling storage), the hard seed coat, moderate response to scarification and strong response to fire (Tsang, 2010), suggest that seeds of *C. pubescens* can survive gut passage and are likely adapted for vertebrate dispersal with later environmental germination triggers. Critically, in our study, all seeds were recovered whole rather than in a fragmented state, with no apparent changes to the aspect of the seed coat. In some cases, some remnants of fruit still surrounded the seed (pers. obs.), suggesting dispersal of viable seed by *I. obesulus* is likely. However, we acknowledge the ingestion of fruit and whole seeds does not in itself guarantee successful dispersal (Beca et al., 2021; Howe & Smallwood, 1982; Junges et al., 2018; Lessa et al., 2013). The effect of factors, such as the extent of seed damage (through intentional predation or incidental destruction of seed during fruit consumption), animal movement and gut retention times on successful dispersal events cannot be disregarded (Beca et al., 2021; Willson & Traveset, 2000). Gut retention times are typically shorter for larger seeded species, as smaller seeds are retained more easily in the gut than larger ones (Figuerola et al., 2010; Lessa et al., 2013), and therefore larger seed size may increase the chances of seeds remaining viable post-dispersal. In the case of *I. obesulus*, gut retention time is usually between 24 and 72 hours (McClelland et al., 1999), and the known viability of seeds from similarly sized plant species after ingestion by small mammals (for example, as presented in Lessa et al. (2013)) suggests that in this case viability is likely.

As it is likely that *C. pubescens* had or still has a wide variety of vertebrate dispersers, the role that other species play in seed dispersal should also be examined across its broad geographic range (Figure 1). Birds such as pigeons, honeyeaters and emu, as well as lizards such as blue-tongues (*Tiliqua* spp) may also play important roles in seed movements across southeastern Australia. Additionally, although smaller marsupials are proposed here to act as vectors for seed of *C. pubescens*, the role of larger mammals in seed movement in South Australia specifically remains to be tested. In northern Australia, seeds of *C. filiformis* have been identified in faecal pellets of small species of wallabies (See, Table 1; Horsup & Marsh, 1992; Ingleby & Westoby, 1992; Jarman, 1994; Lundie-Jenkins, 1993). Therefore, other dispersal vectors, such as small macropodids and other marsupials, may also be important in South Australia, and warrant further investigation.

Field observations of emerging seedlings suggest that a large number of *C. pubescens* fruits fall to the ground directly beneath the parent plant (pers. obs.), and these fruits could then be easily consumed by the ground-foraging *I. obesulus*. This species may therefore be an opportunistic consumer of *C. pubescens* fruit, rather than a reliable primary seed disperser. For plants with specialised life history strategies, such as parasitic plants, movement of viable seed may be especially important to facilitate dispersal into areas containing appropriate host species, which can be viewed as unique resources. Australia has lost many CWR mammal species since European settlement (Fleming et al., 2014; Fox, 1998), and this loss may lead to the surviving species playing an increasingly important role in the lifecycle of *C. pubescens* and other native plants. As such, increasing connectivity of remnant vegetation may assist the conservation of that vegetation, including *C. pubescens*, by also maintaining connectivity of marsupial populations and in turn improving effective dispersal distance of seeds. The potential importance of *C. pubescens* as a keystone species in sclerophyllous woodland systems cannot be overlooked, and landscape-scale management strategies to ensure persistence of this species may also maintain vegetation complexity and plant–frugivore mutualism within this system.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Chapter 3

Clonal reproduction and population isolation in an Australian hemiparasitic vine

Bank of adult *Cassytha pubescens* in native vegetation at site 5. Reproduction can be vegetative, or sexual.

Statement of Authorship

Title of Paper	Clonal reproduction and population isolation in an Australian hemiparasitic vine
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Contribution to the Paper	Conducted all field work, analysis, prepared figures. Wrote the manuscript. Note: labwork was completed by the Australian Genomics Research Foundation (AGRF)		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

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Chapter 3: Clonal reproduction and population isolation in an Australian hemiparasitic vine

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Abstract

The population genetics of parasitic plants are poorly understood despite their important ecosystem roles. *Cassytha pubescens* is one such species. A rootless, twining hemiparasitic vine with a broad distribution across the wetter parts of eastern Australia, it can be propagated vegetatively by cloning under glasshouse conditions. However, it is unknown how much sexual and asexual reproduction contribute to population spread in the field. Here we studied relatedness of *C. pubescens* individuals within five patches at small (>10km) spatial scales in South Australia's Mount Lofty Ranges by analysing SNP data. There was a high frequency of clonal reproduction in the largest site sampled, and inbreeding was high at all sites. There was little to no genetic connectivity between populations, which fall into one of just two genetic clusters. These results indicate that clonal reproduction is the prevalent mechanism that maintain populations of this species within the region, and that dispersal of seed may be limited. In an increasingly fragmented landscape where dispersal events are rare, this prevalence of clonal reproduction has important management implications for this species.

Introduction

The importance of parasitic plant species in natural ecosystems is often understated. The majority of research focuses on combating agriculturally problematic species (e.g. Cameron et al., 2008; Lanini & Kogan, 2005; Musselman, 1996; Nickrent, 2020; Sandler & Mason, 2010), however, in natural ecosystems parasitic plants may play important ecological roles and act as keystone species (Nickrent, 2020). They provide shelter and important sources of food for fauna, and contribute significantly to key processes, such as community dynamics and nutrient cycling (Bardgett et al., 2006; Grewell, 2008; Hatcher et al., 2012; Houston & Wolff, 2012; Press & Phoenix, 2005; Quested, 2008; Quested et al., 2003). Understanding the population dynamics of parasitic plants is therefore of critical importance for their management in natural communities. This may be particularly important when natural vegetation is fragmented, as disappearance of key parasitic plants in any plant community can change its structure and dynamics if recolonization does not happen readily. For example, it is important to distinguish the possibility of the parasitic species re-establishing from existing seed bank, or via dispersal from neighbouring patches. Molecular techniques for population genetics may be used to give a measure of the connectivity of populations in fragmented landscapes and can also provide insight into current and historical distributions (Fontúrbel et al., 2022; Sork et al., 1999).

One example of a wide-spread Australian parasitic plant with unknown population genetics is *Cassytha pubescens* (Lauraceae) R. Br. It has a wide distribution from south-eastern Queensland through to the Eyre Peninsula in South Australia, extending into Tasmania. It has a complex life history; it relies on fire to trigger germination in the soil seed bank (see Chapter 4), after which short-rooted seedlings emerge and are free-living for approximately 10 weeks while they seek a host plant (McLuckie, 1924; Tsang, 2010). Once seedlings attach to a host, the root senesces and the parasite remains a leafless, climbing vine with indeterminate growth. As *C. pubescens* is a generalist parasite with a large geographic range, the variety of species it can utilise as a host is broad (Facelli et al., 2020; McLuckie, 1924), and due to its twining growth habit a single individual parasite may cover multiple host plants resulting in large, dense patches. In *C. pubescens* the flowers are small (approximately 3 mm long) and white to yellowish, suggesting that this species is pollinated by small insects (Weber, 1981), however the specific species responsible for pollination are unknown. Each fruit contains a single, large seed, which is surrounded by thin moist flesh, however there is substantial morphological variation in the size, shape and colour of the fruit across its range (McLuckie, 1924; Weber, 1981). Dispersal vectors of *C. pubescens* are thought to be primarily birds and small mammals, with some fruit and/or seeds previously found in the scats of bettongs, bandicoots and emu (Bennett & Baxter, 1989; Maciunas et al., 2022; Taylor, 1992). Dispersal distance of this species is unknown, particularly as much of its large range is now heavily populated and has undergone significant fragmentation. Observations during a pilot study, and patterns of seedling emergence shown in Chapter 4, indicate that most fruits drop underneath the mother plant and suggest little direct dispersal. However, biotic secondary dispersal by scavenging species is certainly possible.

Unlike most mistletoes, arguably the most well known plant parasitic life form, reproduction in *C. pubescens* can be sexual via the production of seed, and/or clonal through vegetative reproduction. This plasticity presents a problem, whereby the two reproductive systems must be distinguished as they have different roles in the life cycle of the parasite, and therefore may influence community level population dynamics depending on which is more prevalent. It is known that the relative importance of each mode of reproduction can change with changing environments and disturbance regimes for many species (Dostál, 2023). In *C. pubescens* specifically, sexual reproduction is vital for the recovery of populations after fires, since the rootless, herbaceous individuals are killed even by slight fires (Pers. obs; See also Chapter 4). However, for periods between fires, clonal reproduction may dominate: the seed biology and observations during more than ten years of field work suggest seedling establishment is a very rare event in the absence of fires (Facelli and Maciunas Pers. obs). In addition, since fire regimes throughout Australia have changed substantially since European colonisation (Adeleye et al., 2022), clonal reproduction may now also increase the longevity of existing populations of the parasite and potentially alter host-parasite interactions in the area. Hence, examining population genetics will increase our understanding of the primary method of reproduction in *C. pubescens*. This knowledge of population genetics of parasitic plants may allow improved management of ecologically important parasitic species within native communities and the larger communities themselves.

In addition to community-wide effects, the ability to reproduce via vegetative clones will also influence the survival and overall fitness of the clones themselves. By reducing the reliance on suitable sexual partners in the immediate area, the resilience of individual clonal populations is increased (Dostál, 2023). However, a large population of clones may reduce pollen movement (Meloni et al., 2013), ultimately lowering genetic diversity within the patch and contributing to increased inbreeding. If this were the case, the ability to colonise new areas may also become limited due to the production of lower numbers of viable seed, and the importance of any remaining gene flow between populations would increase.

Because the effects of clonal reproduction on the population dynamics of *Cassytha pubescens* may be significant, this study aims to determine the extent of clonal reproduction in small populations, and to assess the extent of population connectivity at a small (less than 10 km) scale. Clonal reproduction is predicted to be high within patches, resulting in low genetic diversity and high inbreeding due to sexual reproduction between clones. If clonal spread is dominant, it is also expected that sexual reproduction may be localised, limiting population connectivity. Specifically, we address the following questions:

(1) Does clonal reproduction occur in the field?

(2) If it occurs in the field, how extensively does it occur? And

(3) How much connectivity (i.e., gene flow) is there between populations at a small (>10km) scale?

Methods

Sampling

Fresh stem material from *C. pubescens* (n=95), was collected from five sites in the Adelaide Hills, South Australia. Two sites were in Belair National Park, and three sites were in Mark Oliphant Conservation Park (Figure 1). Samples were collected from randomly chosen individuals at varying spatial scales, from within a meter of each other, to tens and hundreds of meters apart.

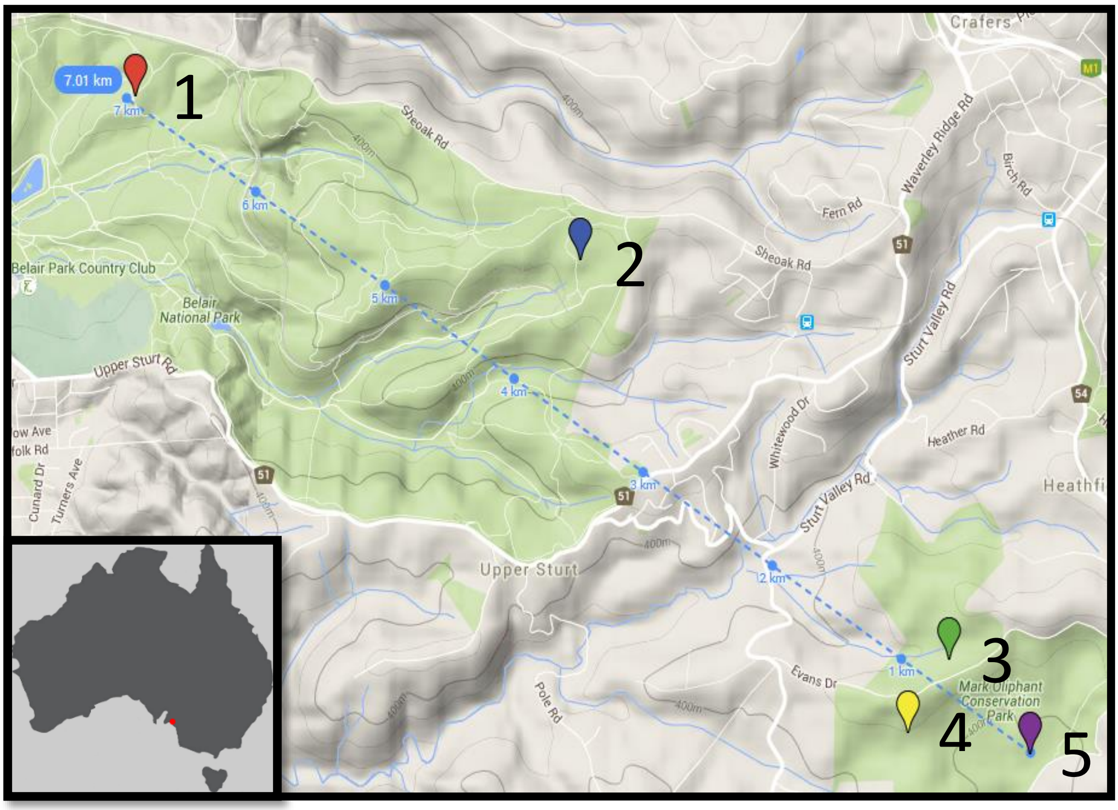

Figure 1: Map of sample area. Although *C. pubescens* is found throughout the Mount Lofty Ranges, two geographically close parks were sampled: Belair National Park, sites 1 (red) and 2 (blue), and Sir Mark Oliphant Conservation Park, sites 3 (green), 4 (yellow) and 5 (purple). Site 5 was more extensively sampled to test for clonality. Insert shows approximate location of sample sites (red circle) at a national scale.

Host plant species also varied across sampled plants. A high number of samples were also taken from a single site (Mark Oliphant, 5) to test for clonality, as extensive dense stands of the plant were well established (Figure 2). For all samples a geographic location (latitude/longitude) was recorded using a GPS (Magellan Meridian) (see Appendix A, Table 4 for sample locations).

Figure 2: General habit of *Cassytha pubescens* at site 5, Mark Oliphant Conservation Park. A dense shrub layer can enable extensive clonal reproduction for this parasitic vine.

DNA extraction

Fresh tissues were stored under dry conditions (with silica gel) until extraction for genomic DNA was undertaken at the Australian Genome Research Facility (AGRF) in Adelaide using a QIAGEN plant DNA kit.

Reduced representation genomic library preparation

Genotyping-by-sequencing (GBS) was performed at The University of Adelaide using a double digest with the restriction enzymes *Pst1* and *Msp1* and following the protocol of (Brown, 2011). Adapter titrations were performed as outlined in (Elshire et al., 2011). In brief, 200 ng of DNA from each individual sample was digested with the methylation sensitive restriction enzymes *Pst1* and *Msp1*. A water blank was included on the plate as a negative control. Individually barcoded adapters and common adapters were ligated onto the enzyme cut sites. The samples were then pooled equi-molar and PCR amplified using Illumina sequencing primers with ends complementary to the adapters. The final GBS library was sequenced by 100 bp single-end sequencing on a single lane of the Illumina HiSeq (AGRF in Melbourne).

Bioinformatics for SNP calling (genotyping)

Single Nucleotide Polymorphism (SNP) calling on the Illumina data was carried out using the Universal Network Enabled Analysis Kit (UNEAK) pipeline (Lu et al., 2013). The UNEAK pipeline filters the reads for sequences containing the barcode and enzyme cut-site, trims the reads to 64 bp and removes the barcodes. Identical reads (>10) were collapsed into tags, with tag pairs differing by 1 bp identified for SNP calling using a network filter. The error tolerance rate in the network filter was set to 0.03 (default) and a ‘tags by taxa’ file was generated, giving the number of reads of each tag per individual sample. Genotypes were then assigned for each individual, with SNPs filtered based on a minimum minor allele frequency of 0.05, a maximum minor allele frequency of 0.5 (defaults), and a minimum call rate of 0.8. The SNPs were further filtered based on read coverage and heterozygous ratio.

This was to remove low coverage data that may contribute to the under-calling of heterozygotes.

Data Analyses

Data file conversion

The HapMap output file from the UNEAK pipeline was converted to the format for STRUCTURE (Falush et al., 2003) using the recode function from the 'car' package in R (Fox & Weisberg, 2011; R Core Team, 2014). The STRUCTURE file was then converted to a BAYESCAN input file using PGDSpider version 2.0.5.2 (Lischer & Excoffier, 2012).

Detection of outlier loci

In order to obtain a neutral marker dataset free from potential bias (by loci under selection or outlier loci), detection of genetic markers under selection was carried out in BAYESCAN version 2.1 (Foll & Gaggiotti, 2008). The program examines allele frequency differences between sampling sites in order to detect any outlier loci, calculating a posterior probability for each locus for the model including selection. Data from the five sites were analysed as five different putative populations. BAYESCAN was run to identify outlier loci which were statistically significant at a False Discovery Rate of 5% and these loci were then removed from further analysis. The statistical significance of the multiple testing was calculated as a q-value.

Testing for clonal reproduction

Testing for clonal reproduction and population genetic data analyses were carried out in GenAlEx (Peakall & Smouse, 2006). A pairwise matrix of genetic distance was made between individuals, inputted as an Allelic Distance Matrix for

Wrights F-Statistics (number of permutations = 999), and the partitioning of the variance within and among populations was estimated by Analysis of Molecular Variance (AMOVA). Samples that had a similarity greater than 0.95 were considered vegetative clones, and all but one representative sample (for each set) was removed from the dataset for all remaining analyses (Table 5, Appendix A). Individuals retained were chosen from samples with the least missing data. Geographic distance between clones was calculated using the Haversine equation, based on the latitude-longitude of all clonal pairs.

Basic population genetic data analyses

The number of polymorphic loci per population was calculated, and observed heterozygosity was compared with that expected for a single randomly mating population. The multi-locus, multi-sample dataset was visualized using Principle Coordinates Analysis (PCoA). Pairwise F_{ST} was then calculated for each population. Spatial genetic analyses were carried out including the Mantel test to test matrix correspondence between genetic and geographic distance, and a Multivariate Spatial Autocorrelation Analysis with putative populations set as each distance class.

Population genetic structuring

The program STRUCTURE version 2.3 (Pritchard et al., 2000) was used to explore population genetic structuring in the SNP dataset. The admixture ancestry model was used to determine the likelihood of the data being partitioned into different genetic clusters, both with and without sampling location included as a prior, and using correlated allele frequency models (Falush et al., 2003). Initial runs with a burn-in time of 10,000 and 10,000 runtime steps for the parameter estimation (individual population membership) were carried out for 1 through to 6 populations (*k*). The least negative values for estimation of the likelihood came from the correlated allele frequency model without location set as a prior. The runs were repeated five times, implementing the admixture ancestry model with correlated allele frequencies and using a burn-in time of 10,000 and 100,000 runtime steps for *k*=1 to *k*=6.

The output from the five STRUCTURE runs was inputted into STRUCTURE HARVESTER (Earl & VonHoldt, 2012) and the number of genetic clusters was estimated using the method of (Evanno et al., 2005). Alignment of cluster assignment across replicates was carried out in CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) and the final aligned cluster assignment was visualized using DISTRUCT version 1.1 (Rosenberg, 2004).

Results

SNP calling

The UNEAK pipeline identified 3039 SNPs. The blank sample and one other (sample 93; Appendix A) were negative and removed from the dataset. The data was filtered to 10 reads coverage, a ratio of alleles of 0.2 and a 90% call-rate, leaving 542 SNPs (24% of the original dataset). Individuals with a large number of missing loci were also removed (sample numbers 41, 42, 44, 45; Table 4, Appendix A) leaving 90 individuals.

Detection of outlier loci

Eight SNPs (118, 127, 137, 349, 398, 487, 509 and 516) were identified as outliers and removed with positive values of the estimated alpha coefficient (the locus-specific component of selection), indicating a locus under diversifying selection. All outlier SNPs could be translated to amino acids without any stop codons, indicating that they are most likely from coding regions of the genome.

Testing clonal reproduction

Fifty-one clonal samples were identified, with all samples then plotted against their location coordinates and clonal groups colour coded for easy visual identification (Figure 3).

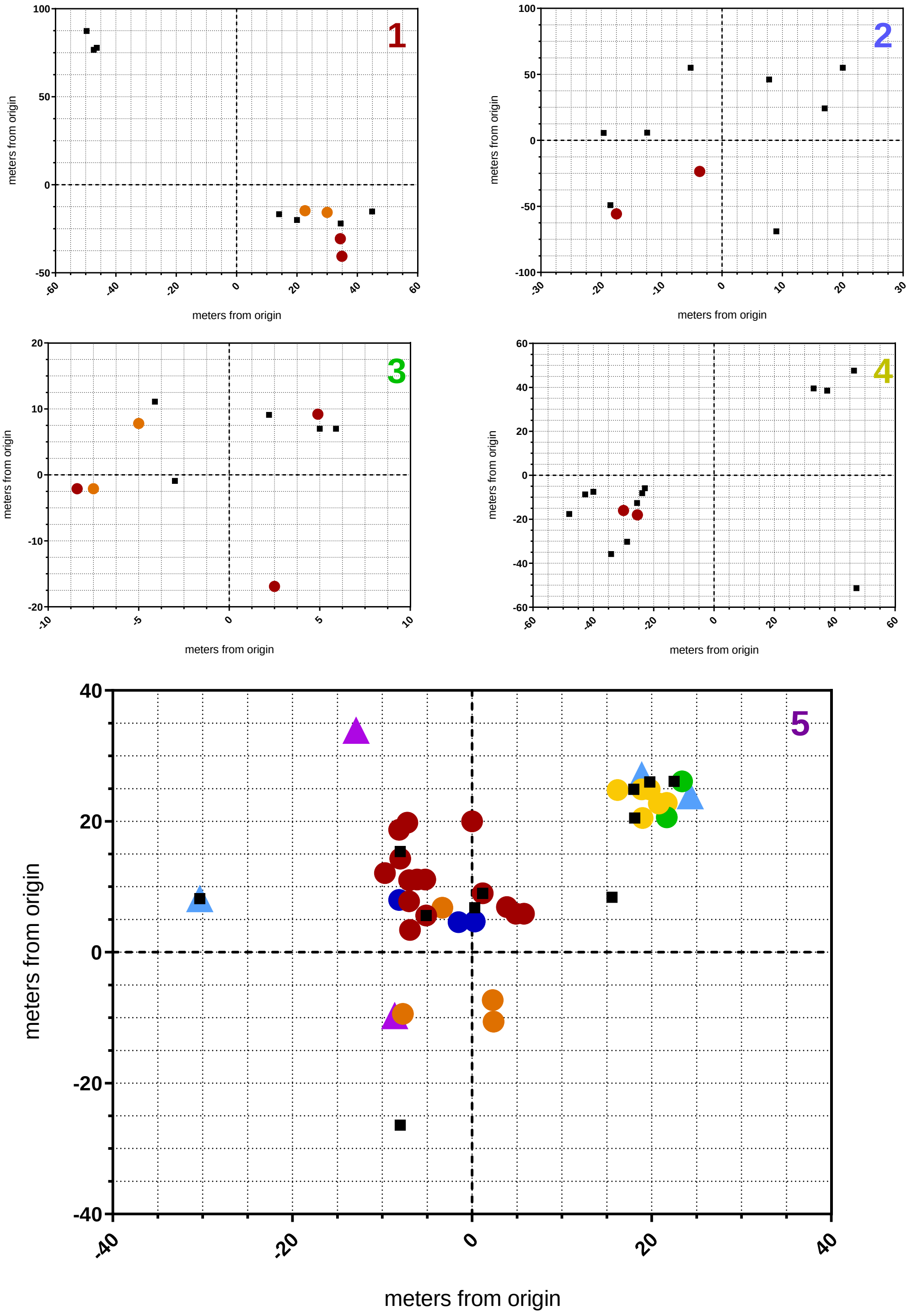

Figure 3: Location of all samples, scatter plotted with the center of the site taken as the origin. Sites 1 through 5 placed from top left to bottom right. Coloured circles and triangles represent clonal samples, warm colours through cool represent largest to smallest groups of clones sampled. Black squares are non-clonal samples. Triangular points indicate clonal groups with at least 2 samples more than 20 meters apart. Note that clonal groups are unique to each site, and the graphic for site 5 is enlarged to emphasise the large number of clones and show greater detail than other sites.

Most clonal individuals were geographically grouped closely, with the average distance between clonal samples 9.2 meters. However, the largest distance found between individual clones was, remarkably, 62.4 meters (Figure 3). Overall the frequency of clonal samples decreased substantially after 15 meters (Figure 4).

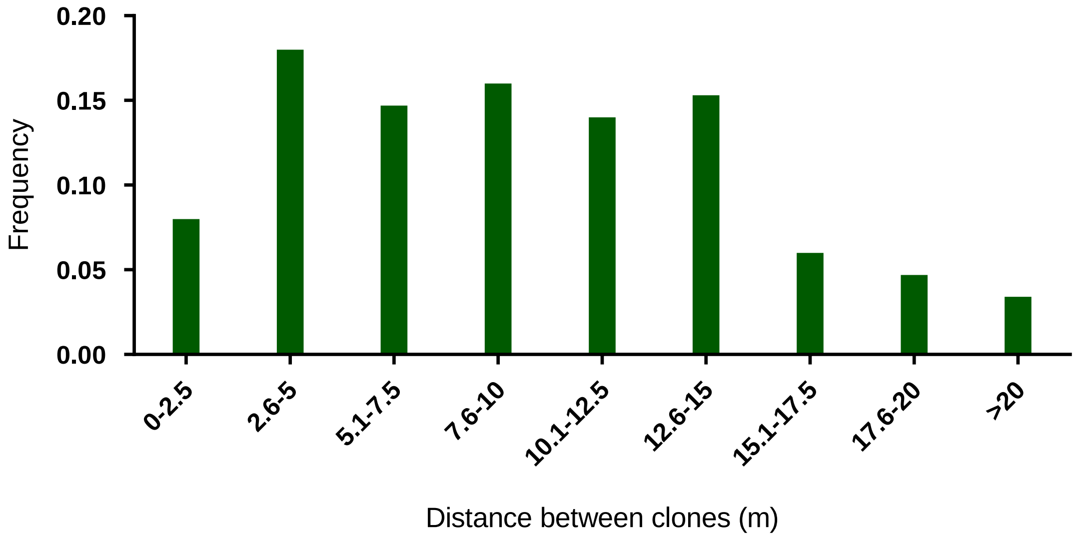

Figure 4: Frequency distribution of distances between clonal pairs. Distance was calculated between all possible pairs of clones within all clonal groups.

Population genetic analyses

Amongst non-clonal samples the percentage of polymorphic loci at each site varied. Observed heterozygosity was slightly greater than that expected in a randomly mating population, however this was not significant and there was no evidence of excess heterozygosity (Table 1).

Table 1: Mean and SE of heterozygosity of loci for each population and for the total population. Ho= Observed heterozygosity; He=Expected heterozygosity (1-$\sum\pi^2$); uHe = Unbiased expected heterozygosity (2N/(2N-1)*He; F= Fixation index ((He-Ho)/He=1-(Ho/He). Π is the frequency of the ith allele for the population and $\sum\pi^2$ is the sum of the squared population allele frequencies.

Pop		Ho	He	uHe	F
1	**Mean**	0.314	0.273	0.287	-0.129
	SE	0.010	0.007	0.008	0.014
2	**Mean**	0.293	0.223	0.239	-0.227
	SE	0.014	0.009	0.009	0.022
3	**Mean**	0.291	0.267	0.281	-0.068
	SE	0.011	0.008	0.008	0.017
4	**Mean**	0.279	0.276	0.286	0.034
	SE	0.010	0.007	0.007	0.018
5	**Mean**	0.285	0.309	0.313	0.067
	SE	0.009	0.006	0.006	0.017
Total	**Mean**	0.292	0.270	0.281	-0.052
	SE	0.005	0.003	0.003	0.008

PCoA of the genetic distance matrix comparing individual samples showed two clusters, with 28% of the variation explained by axis 1 of the PCoA and 33% by axis 2 (Figure 5).

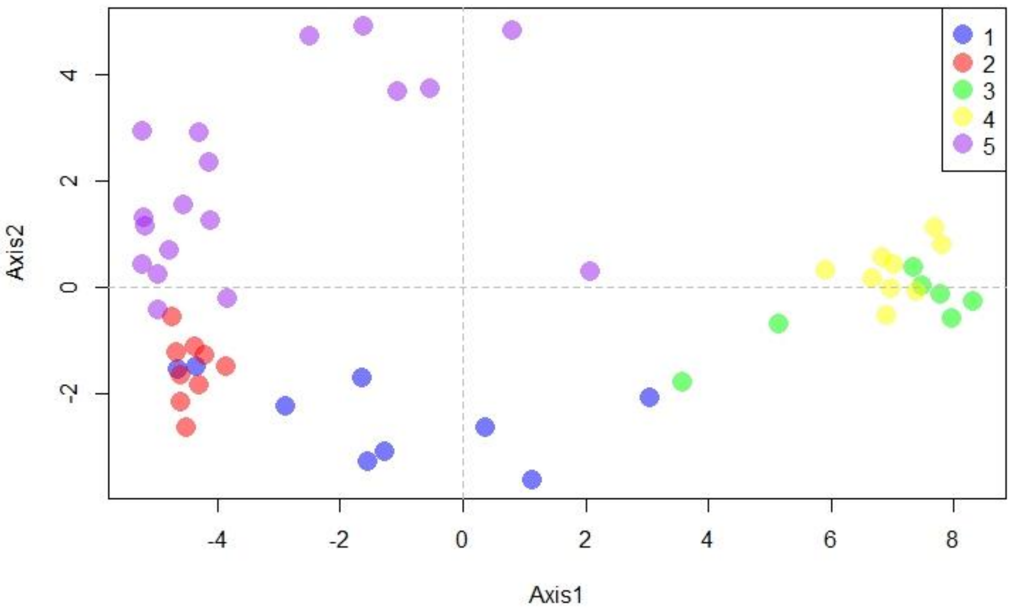

Figure 5: PCoA ordination of individual genetic sample distance matrix. First and second axes of variation are represented. As in Figure 1, sites 1 through 5 represented by different colours, with sites 1 and 2 geographically furthest from site 5.

Results of AMOVA showed that the majority of genetic variation was within individuals (Table 2). An estimated overall F_{ST} value of 0.0195 (p=0.001) is indicative of low level of differentiation between sites, and the F_{IS} value (-0.049; p=0.945) implied that there was a reduction of heterozygosity within populations suggestive of inbreeding.

Table 2: AMOVA tables showing source of genetic difference within and between sites.

Source	df	SS	MS	Est. var.	%
Among Sites	4	1649.989	412.497	17.189	19%
Among Indiv	47	3167.300	67.389	0.000	0%
Within Indiv	52	3864.829	74.324	74.324	81%
Total	103	8682.117		91.513	100%

Pairwise comparison of the populations showed the greatest differentiation between populations 2 and 4 (Table 3). The mantel test for isolation of populations also suggests there is evidence for isolation by distance at this spatial scale.

Table 3: Pairwise population Fst Values (below diagonal). Probability, P (rand>=data) based on 999 permutations is shown above the diagonal (shaded light grey). Greatest differentiation is between sites 2 and 4 (highlighted in dark grey).

	Site1	Site 2	Site 3	Site 4	Site 5	
Site1	0.000	0.013	0.001	0.001	0.005	**Site1**
Site 2	0.071	0.000	0.001	0.001	0.019	**Site 2**
Site 3	0.207	0.325	0.000	0.089	0.001	**Site 3**
Site 4	0.247	0.366	0.028	0.000	0.001	**Site 4**
Site 5	0.062	0.045	0.261	0.290	0.000	**Site 5**
	Site1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	

Population genetic structuring

Two genetic clusters were identified from the STRUCTURE output. Each site contained admixed individuals from the two genetic clusters, although many individuals were assigned exclusively to either cluster.

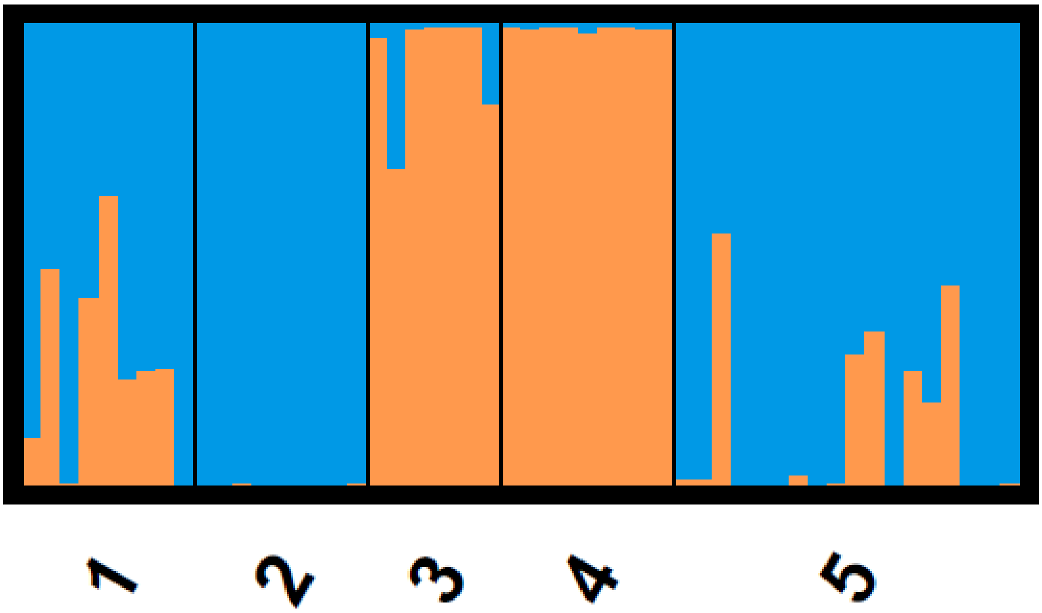

Figure 6: Visual representation of genetic cluster assignment (mean of 5 aligned replicates) for individual *C. pubescens* samples in the neutral SNP dataset. Labels are of sites 1 through 5. Each coloured vertical bar represents the probability of assignment to the orange or blue genetic cluster.

Individuals from sites 1 and 2 were mainly from a single genetic cluster (Figure 6, blue), although site 1 individuals showed greater admixture compared to site 2 individuals. Individuals from sites 3 and 4 formed a second genetic cluster (orange) with greater admixture observed at site 3 compared to site 4. In contrast, individuals from site 5 mainly clustered together in the same group as the Belair individuals (sites

1 and 2, blue) rather than the other Mark Oliphant individuals (site 3 and 4, orange), although admixture was still observed at this site.

Discussion

We found that a large proportion of samples (51 out of 90) could be identified as clones of one or more other samples and that some of the members of a clone were separated by up to 62 meters (Figure 3). This study provides the first evidence that *C. pubescens* undergoes extensive vegetative reproduction in the field, and that one genetic individual may extend beyond identifiably continuous patches. The growth of new vegetative clones is generally considered a lower-risk strategy than undergoing extensive sexual reproduction, particularly under favourable environmental conditions, and can lead to a rapid increase in the number of individuals in the population (Honnay & Bossuyt, 2005; Meloni et al., 2013). As such, clonal reproduction is important for many species as it increases the longevity of patches (Doyle et al., 2023), and for *C. pubescens* this is especially important as the interval between disturbances required for seed germination, namely fire, may be many decades. In plants that are extensively clonal, an increase in the size of the population may also lead to a greater risk of sexual reproduction, as inbreeding and pollen discounting is increased significantly (Meloni et al., 2013). In *C. pubescens*, the low genetic diversity within patches may be partially explained by these effects, as although sexual reproduction may still occur, the likelihood of selfing will be proportionate with the numbers of clones regardless of the effectiveness of pollen movement within the patch. Excessive selfing across clones may also explain the high inbreeding coefficient, as even if pollination occurs, pollen is likely to originate from a genetically similar (or identical) individual. However, the pollination biology of this species is yet to be examined, and the extent to which this species naturally outcrosses or selfs remains unknown.

The larger distances (those in excess of 30 meters) between clones were unexpected, but not unreasonable as the area with the greatest distance between clones had not experienced a fire for 20 years. Considering that distance between the two furthest points in this study may represent the two furthest points of spread (as the ‘starting point’ is unknown), *C. pubescens* would theoretically spread at a minimum rate of half the distance between clones divided by years of growth, in this case a minimum rate of 0.62 meters per year. Studies by Weber (1981) and McLuckie (1924) report that under good growing conditions, tendrils of some *Cassytha* species can grow up to 5 cm in a single day, indicating that our estimate may be very conservative. Regardless, spread of parasites such as these are often limited by the availability of suitable hosts in a given area, as well as by the disturbance regime of their environment. Understanding the extent that this parasite in particular can spread is increasingly important, as research indicates it may be a suitable biological control agent for multiple introduced species (Cirocco et al., 2016a; Cirocco et al., 2016b; Cirocco et al., 2020; Facelli et al., 2020; Prider et al., 2009; Těšitel et al., 2020).

Genetic isolation within the study area is significant, with low genetic diversity seen within sites and two distinct genetic clusters seen across the study area as a whole (Figures 5 & 6). Historically, gene flow may have been greater than it is today, as site 5 was more closely grouped with sites 1 and 2 than with 3 and 4, which were geographically the closest. Why this is the case we do not know; a reasonable hypothesis is this result reflects a population with historic connectivity, and subsequent fragmentation into more disconnected populations. However, the historical distribution of this species at this scale is unknown, and patchy populations with low numbers of individuals may be the naturally occurring distribution pattern. A consequence of a lack of site connectivity appears to be high inbreeding, which may be indicative of either longer-term isolation, resulting in increasingly low outcrossing and an overall drop in the genetic diversity of patches, or may represent a founder effect. In either case, purging of deleterious genes from populations may allow individuals to survive with higher levels of inbreeding (Bataillon & Kirkpatrick, 2000; Byers & Waller, 1999).

The reason for patch isolation, particularly of sites 3 and 4, is uncertain. Fragmentation of natural vegetation may prevent effective pollen and/or seed dispersal via the removal or disruption of the dispersal vectors. For example, small mammals such as bandicoots are known to disperse *C. pubescens* seeds (see Bennett & Baxter, 1989; Maciunas et al., 2022; Taylor, 1992), however, of the seven species of bandicoot found in South Australia at the time of European settlement only one remains in the Mount Lofty Ranges. Woodland birds are seeing serious declines within the same region (Ford et al., 2001; Szabo et al. 2011; Watson et al. 2002) and are also almost certainly critical dispersers of *C. pubescens* seeds. The loss of large numbers of small mammal and bird species may explain much of the lack of population connectivity in *C. pubescens*. Although fragmentation of native vegetation began more than 100 years ago and the resulting species loss is considerable, the broader implications for the ecology of many species, including *C. pubescens*, may only just be recognised. It is known that regeneration of patches of this perennial species from seed occurs primarily after fire (Tsang, 2010; see also Chapter 4), and morphology strongly suggests that seeds survive for long periods of time in the soil seed bank. As such, given that there have been substantial changes to the Australian fire regime since European settlement (Mariani et al. 2022), it may take many decades for changes to the genetic makeup of seeds of all species held within the seed bank to change, and even longer for those changes to become apparent through post-fire regeneration of patches (Broadhurst et al., 2017). In short, establishment of new populations may be limited due to decreased suitable habitat and decreased dispersal distances and frequency (through fragmentation), as well as changed frequency of the environmental conditions required for germination to occur (in this case fire). When dispersal events do occur, dependence on fire for germination will still cause a time-lag in changes to the overall genetic structure of the population, as those germination events may occur only once every 20-30 years. Additionally, a decrease in fire events may contribute to the high proportion of clones present in local sites, with decreasing germination events increasing the importance of vegetative reproduction in the long-term persistence of populations. In areas where fire events have increased, shorter inter-fire intervals will reduce the overall quantity of seeds entering the seed bank potentially limiting recovery of patches. In addition, the effect of changing fire intensity on the viability of seeds within the seed bank is still unknown. What is clear from this study is that genetic diversity within sites may change substantially over time, largely depending on the age of the patch and hence the inter-fire interval. Therefore, understanding changes to fire regimes and managing fire events where *C. pubescens* is present will be important for maintaining genetic diversity of populations. More work is needed to understand the long-term implications of altered fire regimes on the genetic diversity of populations for this species.

As the distribution of *C. pubescens* is so broad, covering much of eastern Australia, the lack of population connectivity across such small distances raises further questions regarding the relatedness of individuals from a much greater geographic range. The scale at which this study was undertaken was small (maximum range of approximately 10 kilometres) for such a wide-spread species. If the pattern of genetic isolation is similar across this species' entire range, for example comparing South Australian populations to those found in New South Wales, it would have profound evolutionary implications. From these results it is reasonable to hypothesise that if this lack of connectivity predates European changes to the landscape, rather than one species with a very large range *C. pubescens* may in fact be a complex of cryptic species, or at least show multiple, diverging sub-species. If the lack of connectivity is due to recent fragmentation, it is reasonable to suggest there is great potential for increasing divergence between populations as time goes on. The hypothesis of a species complex or the potential emergence of subspecies is supported at least anecdotally by McLuckie (1924) and Weber (1981) who describe significant regional morphological variation within *C. pubescens* across its range, in the flowers, fruits and inflorescence structure. However, there is no molecular phylogeny of the genus to date and the sampling used in this study is inadequate for species-level taxonomy. Ideally any future study incorporating variability in habitat (as outlined previously) could also explore multiple populations from different states, in order to clarify the taxonomic status of this species.

As demonstrated above, it is important to consider the species ecology when considering its population dynamics. For *C. pubescens*, factors such as host species and when the last fire disturbed the site will all affect successful founding events, and hence may influence the results of studies of this species' population genetics. In the present study, aspect, rainfall, time since last fire and host species were recorded for all samples, however the small number of sites and large number of clonal samples confounded all attempted analyses. Ideally, such ecological parameters should be built into any future sampling of this species, with sample sites being drawn from a larger and more variable area, and a larger sample size used. It is suggested that sampling be undertaken at a slightly larger scale (tens to hundreds of meters to several kilometres) in order to avoid a high proportion of clones reducing the effective sample size for analysis. A variety of sites should also be used to encompass a larger variety of fire histories and host species, as sampling populations both before and after fire events would help build an understanding of the effect of sexual versus clonal reproduction, as well as the relative contributions made to the seed bank by plants in that area versus dispersal event from elsewhere. Overall, such approaches may help build a comprehensive picture of the dynamics of population genetics and how they change over time in this wide-ranging species.

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Appendix A

Sample identification and clonal sample information

Table 4: Sample location and individual identification information. *denotes sample removed from SNP dataset due to large number of missing loci. ** was removed prior to SNP calling due to negative sample.

Sample number	Park	Population number	Latitude	Longitude
1	Belair	1	-35.007471	138.661518
2	Belair	1	-35.007322	138.661312
3	Belair	1	-35.007327	138.661412
4	Belair	1	-35.007326	138.661498
5	Belair	1	-35.007371	138.661561
6	Belair	1	-35.007444	138.661571
7	Belair	1	-35.007530	138.661551
8	Belair	1	-35.007222	138.661566
9	Belair	1	-35.008168	138.642592
10	Belair	1	-35.008134	138.662496
11	Belair	1	-35.008144	138.662485
12	Belair	2	-35.003270	138.641800
13	Belair	2	-35.003033	138.642585
14	Belair	2	-35.003408	138.641478
15	Belair	2	-35.003418	138.641544
16	Belair	2	-35.003143	138.640346

17	Belair	2	-35.003429	138.642091
18	Belair	2	-35.003357	138.642093
19	Belair	2	-35.003063	138.642277
20	Belair	2	-35.003155	138.642496
21	Belair	2	-35.003285	138.642585
22	Mark Oliphant	3	-35.033748	138.695257
23	Mark Oliphant	3	-35.033848	138.695265
24	Mark Oliphant	3	-35.033873	138.695166
25	Mark Oliphant	3	-35.033882	138.695166
26	Mark Oliphant	3	-35.033828	138.695178
27	Mark Oliphant	3	-35.033739	138.695257
28	Mark Oliphant	3	-35.033776	138.695278
29	Mark Oliphant	3	-35.033839	138.695298
30	Mark Oliphant	3	-35.033773	138.695218
31	Mark Oliphant	3	-35.033749	138.695279
32	Mark Oliphant	4	-35.031826	138.698492
33	Mark Oliphant	4	-35.031814	138.698350
34	Mark Oliphant	4	-35.031780	138.698472
35	Mark Oliphant	4	-35.031953	138.698565
36	Mark Oliphant	4	-35.031062	138.699128
37	Mark Oliphant	4	-35.031926	138.698577
38	Mark Oliphant	4	-35.031764	138.698571

39	Mark Oliphant	4	-35.031755	138.698593
40	Mark Oliphant	4	-35.032006	138.698476
***41**	Mark Oliphant	4	-35.031781	138.698526
***42**	Mark Oliphant	4	-35.031054	138.698139
43	Mark Oliphant	4	-35.031196	138.699047
***44**	Mark Oliphant	4	-35.031151	138.699037
***45**	Mark Oliphant	4	-35.031867	138.698294
46	Mark Oliphant	5	-35.034131	138.708713
47	Mark Oliphant	5	-35.034201	138.708591
48	Mark Oliphant	5	-35.034200	138.708547
49	Mark Oliphant	5	-35.034182	138.708569
50	Mark Oliphant	5	-35.034201	138.708623
51	Mark Oliphant	5	-35.034183	138.708624
52	Mark Oliphant	5	-35.034211	138.708667
53	Mark Oliphant	5	-35.034212	138.708700
54	Mark Oliphant	5	-35.034203	138.708711
55	Mark Oliphant	5	-35.034260	138.708852
56	Mark Oliphant	5	-35.034182	138.708569
57	Mark Oliphant	5	-35.034146	138.708559
58	Mark Oliphant	5	-35.034164	138.708581
59	Mark Oliphant	5	-35.034228	138.708634
60	Mark Oliphant	5	-35.034211	138.708656

61	Mark Oliphant	5	-35.034108	138.708440
62	Mark Oliphant	5	-35.034107	138.708407
63	Mark Oliphant	5	-35.034192	138.708624
64	Mark Oliphant	5	-35.034128	138.708560
65	Mark Oliphant	5	-35.034211	138.708249
66	Mark Oliphant	5	-35.034217	138.708416
67	Mark Oliphant	5	-35.034212	138.708593
68	Mark Oliphant	5	-35.034208	138.708419
69	Mark Oliphant	5	-35.034119	138.708603
70	Mark Oliphant	5	-35.034128	138.708581
71	Mark Oliphant	5	-35.034092	138.708582
72	Mark Oliphant	5	-35.034082	138.708572
73	Mark Oliphant	5	-35.034073	138.708572
74	Mark Oliphant	5	-35.034119	138.708603
75	Mark Oliphant	5	-35.033906	138.708774
76	Mark Oliphant	5	-35.033950	138.708718
77	Mark Oliphant	5	-35.033975	138.708597
78	Mark Oliphant	5	-35.034434	138.708595
79	Mark Oliphant	5	-35.034434	138.708595
80	Mark Oliphant	5	-35.033942	138.708784
81	Mark Oliphant	5	-35.033933	138.708773
82	Mark Oliphant	5	-35.033951	138.708762

83	Mark Oliphant	5	-35.033914	138.708741
84	Mark Oliphant	5	-35.033888	138.708752
85	Mark Oliphant	5	-35.033897	138.708774
86	Mark Oliphant	5	-35.033914	138.708719
87	Mark Oliphant	5	-35.033923	138.708740
88	Mark Oliphant	5	-35.033915	138.708752
89	Mark Oliphant	5	-35.033933	138.708762
90	Mark Oliphant	5	-35.033941	138.708718
91	Mark Oliphant	5	-35.033969	138.708761
92	Mark Oliphant	5	-35.033942	138.708762
****93**	Mark Oliphant	5	-35.033924	138.708773

Table 5: Sample numbers for the 54 clonal samples in dataset, with middle column showing genetic clones of (retailed) samples in left-hand column. Sample chosen for retention was based on the least missing information.

Sample retained	Clone(s) removed from dataset	Sample Site
3	2	1
6	5	2
12	14	2
23	24	3
30	31, 25	3
32	34	4
57	67, 64	5
58	61, 62, 68	5
66	55	5
70	46, 47, 48, 49, 50, 51, 53, 54, 59, 60, 63, 69, 71, 72, 73	5
78	80, 84	5
83	85, 86	5
89	87, 90, 91, 92	5
94	95	5

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Chapter 4

Post-fire population recovery and seedling survivorship in *Cassytha pubescens*

Seedling of *Cassytha pubescens*, approximately 2 weeks old. Seeds germinate in the spring, following a fire, and establish on resprouting vegetation.

Statement of Authorship

Title of Paper	Post-fire population recovery and seedling survivorship in Cassytha pubescens	
Publication Status	☐ Published ☐ Submitted for Publication	☐ Accepted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Conducted all fieldwork, analysis, prepared figures and wrote the manuscript.	

Principal Author

Name of Principal Author (Candidate)	Elizabeth C Maciunas		
Contribution to the Paper	Conducted all field work and sampling, analysis, prepared figures. Wrote the manuscript.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/09/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Jose M Facelli		
Contribution to the Paper	Assisted with experimental design, advised statistical analyses, manuscript drafting		
Signature		Date	6-09-23

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Please cut and paste additional co-author panels here as required.

Chapter 4: Post-fire population recovery and seedling survivorship in *Cassytha pubescens*

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Abstract

Fire is a common disturbance for much of the native Australian vegetation, and how species respond to it is an important component of their life history strategies. Re-sprouting and rapid re-colonisation from the seed bank are frequently observed and well-studied strategies. In contrast, the re-appearance of parasitic plants in areas recovering from fire is generally poorly understood, despite the importance of this group in modulating the structure and function of the plant community. The species studied here, *Cassytha pubescens*, being rootless is wholly dependent on the seed bank for recovery after fires, however little is known about its seedling stages. We followed patch recovery after fire and found that seedlings emerged 11 months after the event. This could reflect a strategy of delaying germination to allow sufficient time for potential hosts plants to have grown enough to sustain a population of the parasites. Density of emerged seedlings was high but concentrated around former hosts destroyed by the fire. Seedling survivorship was approximately 38%, however, mortality was much higher in plots where seedling density was high. This suggests that much of the mortality was density dependent. We recorded host use, but little host-discrimination was found, indicating the generalist nature of this parasitic species can be extended to its seedling stages.

Introduction

Plant parasites have evolved strategies that fine-tune germination to maximise the chances of overcoming the critical independent seedling stage and attach to a host before seed reserves are exhausted (Hatcher & Dunn, 2011; Těšitel, 2016; Watson, 2009; Zagorchev et al., 2021). The parasitic *Cassytha pubescens* R.Br. (Lauraceae), a rootless vine, establishes from seeds in the soil. Its germination requirements suggest that germination may be triggered by fires (Tsang, 2010). If this is the case, there could be a peculiar delayed germination dynamic since rapid germination after fires can be risky as suitable hosts may not yet be present. Some mechanisms may exist that delay emergence until such time when suitable hosts can be easily reached by the seedling. Until now there is surprisingly little information about the biology of this native Australian plant, despite the well known important effects that parasitic plants have on community structure and dynamics (Grewell, 2008; Hatcher & Dunn, 2011; March & Watson, 2007; Press & Phoenix, 2005; Těšitel et al., 2020; Watson, 2022).

The distribution of *C. pubescens* at the local scale is highly aggregated and may form extensive patches of clonal adults concentrated around suitable hosts (Pers obs., see Chapter 3). A previous, unpublished, study found that most fruits dropped from the plant and accumulated under the host shrub (Facelli, unpublished) which agrees with observations presented in McLuckie (1924). However, small fossorial mammals and some birds also consume the fruit and disperse the seeds (Maciunas et al., 2022). The growth habit of the parasite, a scrambling, dense vine, together with habitat preferences for small marsupials such as bandicoots, undoubtedly results in higher seed densities around small shrubs that act as both seed traps for fallen fruit, as well as cover for small mammals. This aggregation of propagules needs to be considered to understand the establishment patterns after fires. In many cases, the shape and size of seed shadows can have important repercussions for germination and establishment success, since more favourable conditions found within nucleation sites often facilitate establishment (Dalotto et al., 2018; Franks, 2003; Fujita, 2016; Janzen, 1971; Willson & Traveset, 2000). However, although nucleation sites (such as the seed traps created by small plants) may exhibit favourable conditions for germination, the density of propagules may then lead to intraspecific competition amongst germinating seedlings (Callaway & Walker, 1997; Holmgren et al., 1997). In species with complex life-histories, such as parasitic plants, this sibling rivalry may be further compounded by additional interspecific competition with surrounding neighbours despite the clear need for germination in close proximity to potential hosts. Therefore, for parasitic plants showing patchy distribution and complex ecology, understanding how both inter- and intraspecific competition affects the survivorship of highly aggregated seedlings is of critical importance.

Cassytha pubescens is a rootless perennial hemiparasitic vine, with a widespread distribution across much of south-eastern Australia (Maciunas et al., 2022; McLuckie, 1924). As adults lack underground parts or any other fire-resistant organ, fire events result in the complete destruction of all plants in the affected area, and the species solely recovers from the soil seed bank. *Cassytha* species are all rootless as adults, and the seedlings have only a short-lived, shallow root that senesces after the seedling attaches to a host, or when seed reserves are exhausted. *C. pubescens* reportedly has only 8-10 weeks in which to find a host and attach to it before the seedling dies (McLuckie, 1924). The combination of an inability to survive long periods without a host and the rootless adult life history stage make a study of the seedling ecology of this species fundamental to understand its distribution and the long-term persistence of populations.

From germination trials carried out by Tsang (2010) it is known that seeds respond to heat treatments in a way suggesting that fire could break the hard seed's dormancy. However, a field study is needed to confirm post-fire emergence for this species. In laboratory and glasshouse studies, seeds of *C. pubescens* have shown a high germination rate but poor establishment success, with the seedlings suffering a high mortality prior to establishment on hosts (see Chapters 5 & 7), however, it is unknown if this mortality is reflected in field.

This study aims to examine seedling distribution and to document the recovery of this species after fire, including emergence and establishment on new host individuals. Specifically, we asked:

(1) Does this species show high emergence as a response to fire?

(2) How are seedlings distributed in relation to pre-fire adult plants?

(3) Does the seedling density influence survivorship?

(4) Does the first host species a seedling reaches influence survivorship? And;

(5) Is there host selection present for this species at seedling stage?

Methodology

Study system

The first field study was conducted at a site within Mark Oliphant Conservation Park (S 35°01.196; E 138°41.840) within the Mount Lofty Ranges. This region is characterised by a Mediterranean-type climate, with a variable rainfall ranging from 600 to 900mm per year. Topography is varied, and soil and vegetation characteristics can be changeable within relatively small areas. The site for the first study examining seedling distribution was selected as a location of a planned spring burn by the

Department for Environment and Water. While this allowed pre- and post- fire measurements to be made, it limited the number and location of the site studied. The vegetation before the fire was a Eucalypt woodland, with an understory dominated by *Leptospermum myrsinoides.* Importantly, the site was sloped. Many individual *L. myrsinoides* acted as a host for *C. pubescens* before the fire, however the distribution of the parasite was heterogeneous, as is typical of the species.

The second study also utilised a patch of open Eucalypt woodland, in an area subjected to a controlled fire in spring by the Country Fire Service. This site was level, and the open understory was dominated by *Pultanea daphnoides* and *Dodonaea viscosa*. As with the first site, the distribution of *C. pubescens* was heterogenous, and after the fire parts of the parasite's burned stem could still be seen on some remains of shrubs in the area, indicating the controlled burn was of low intensity.

Site 1: Seedling distribution

To determine the location of emerged seedlings in relation to the presence of plants infected by *C. pubescens* before the fire, a single 10x10 meter quadrat was established. The quadrat was divided into 1x1 meter cells, and all existing infected hosts within in the cells before the fire were noted. This was done to test the hypothesis that emergence from seeds in the soil bank would be clustered around host plants, since seeds dispersal may be limited. Follow-up post-fire surveys were conducted starting shortly after the burn (in October 2010) until late 2012. Patch recovery took longer than expected, with the site monitored every 2 weeks for 12 weeks, and then monthly for the rest of the year. Seedling emergence was not observed until 11 months after the burns (September 2011). At this time much of the vegetation was regrowing and some new seedlings of some species had established.

Site 2: Seedling survivorship

To examine survivorship of individual seedlings, in November 2013 four smaller scale 50x50 cm quadrats with 10x10 cm cells were established at Ironbank in the Mount Lofty Ranges (S 35° 02.548; E 138° 41.146) in an area subjected to a spring burn the year before by the Country Fire Service. Seventy-four newly emerged *C. pubescens* seedlings were individually numbered and measured (this time was considered here as week 0 for survivorship purposes), and their subsequent establishment or death was assessed every 2 weeks over the following 18 weeks. Establishment was recorded as successful if the seedling survived for at least two weeks after roots had visually senesced. All other plant species within the quadrats were recorded, as was the distance from the base of the nearest potential host plant to the point of emergence of all *C. pubescens* seedlings.

Seedlings were further divided for analyses based on the density of seedlings within that cell (1, or 2 or more seedlings per cell), and into pre- and post-coiling data sets for the purposes of analysis of potential competitive effects. Analysis was undertaken using the inbuilt tools in Graphpad Prism 9.0 (GraphPad Software, San Diego California USA), with the significance of competitive effects on survivorship determined using a survival curve (Kaplan-Meier), where seedling death was treated as the event (1) and survivorship was censored (0). The resulting curves were compared using a log-rank (Mantel-Cox) test. Survivorship in relation to distance to the nearest host was analysed using a simple logistical regression. It is important to note that when analysing the effect of density and survivorship, for some data sets n is greatly reduced (due to low survivorship). Therefore for density-dependent effects, we consider p-values of <0.1 to be meaningful.

Results

Seedling distribution

No emergence occurred until 11 months after the burns. The majority of seedlings emerged in areas of lower cover and grew rapidly (Pers obs.). Within the quadrats seedling densities were sometimes high (>50 per square meter in some cells), with most seedlings emerging within two meters of pre-fire host plants (Figures 1 and 2).

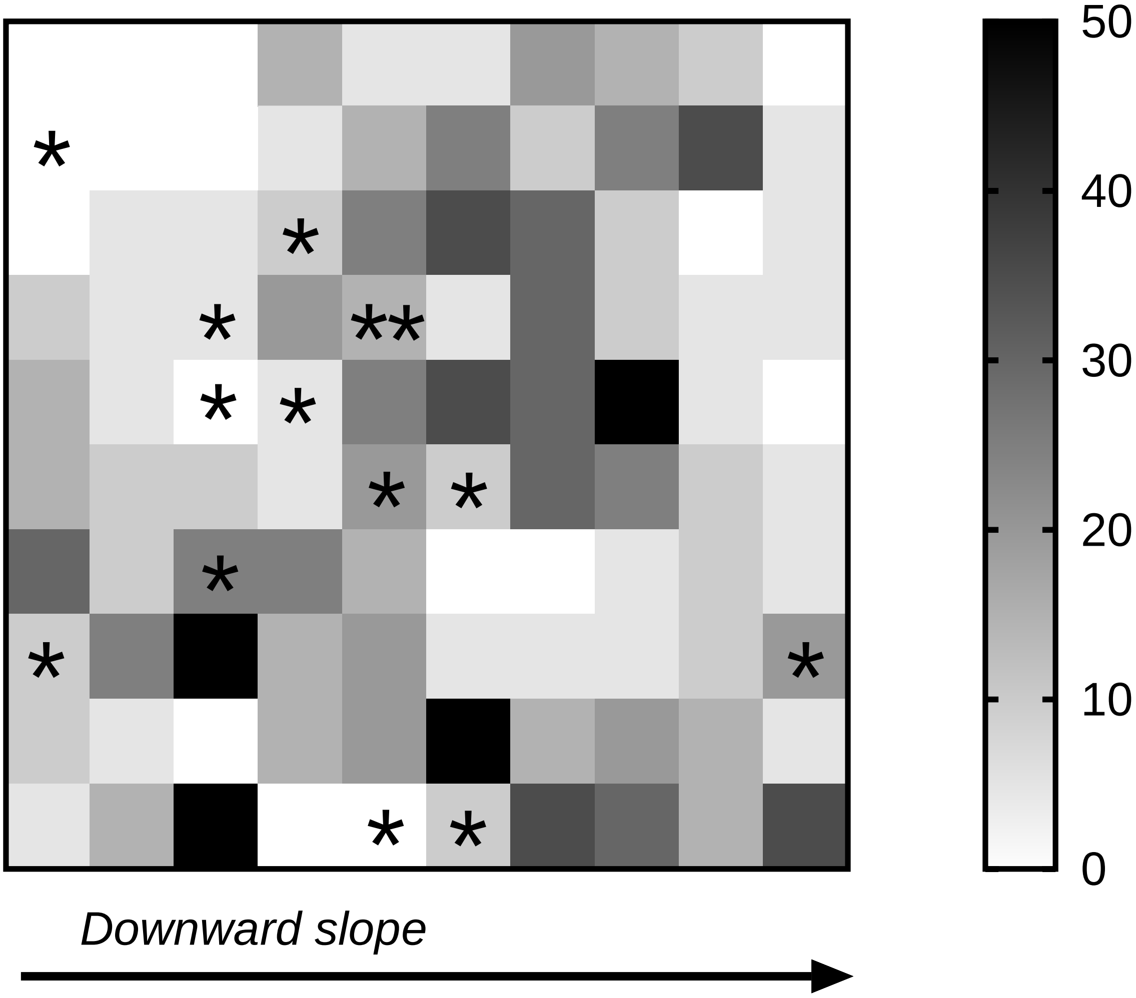

Figure 1: Seedling distribution at MO 11 months post-fire, with density/m^2 key on right. Asterisks denote locations of pre-fire adult parasites on host plants. Accumulation of seeds corresponds to downward slope, however dispersal vectors in this species are largely unknown.

Interestingly, seedling densities were overall greater in cells 1-2 meters from host plants, showing a strong bias towards the down-slope side (Figure 1). More cells with no seedlings present were also found closer than expected to pre-fire host plants (Figure 2).

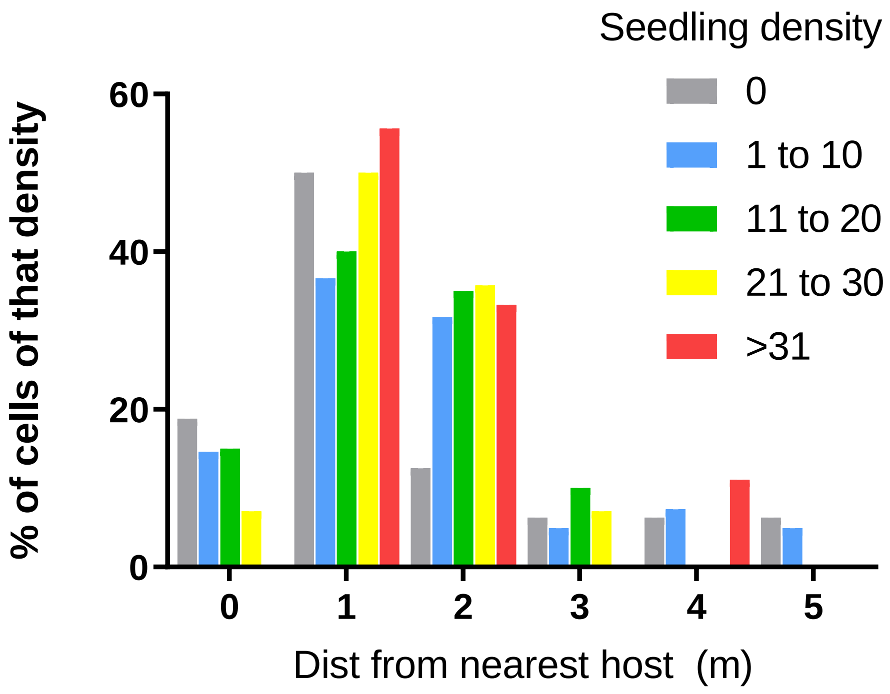

Figure 2: Seedling density and distance from pre-fire host plants. A high proportion of seedlings found at all densities were within 2 meters of the pre-fire host plants, with more than 50% of all seeds at a density of 31 seedlings/m^2 or higher found within 1 meter of the pre-fire host plant.

Survivorship

The survivorship study at Ironbank demonstrates that total seedling establishment was 36% (Figure 3). At week 8, all but three seedlings were coiled on a host, and by week 10 every seedling had either coiled around a host or died. Of the 27 surviving seedlings, only 3 had not coiled around a host by week 6, agreeing with McLuckie (1924) that seedlings which do not find a host in the first few weeks struggle to establish, even if they reach one in later weeks.

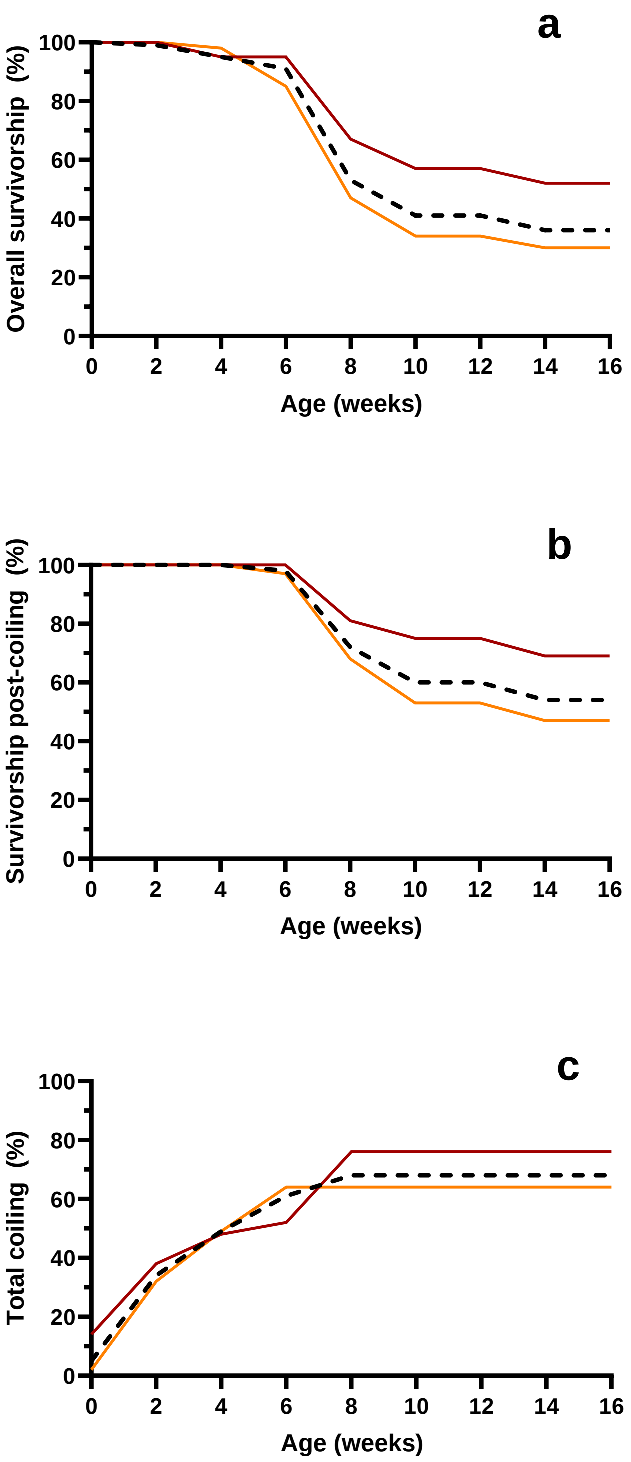

Figure 3: Survivorship curves *for C. pubescens* seedlings from emergence to establishment, showing overall survivorship (a), survivorship post-coiling (b) and total coiling activity by seedlings (c). Black (dashed) series indicates overall survivorship or coiling across all cells, red series indicates survivorship or coiling of seedlings in cells with only one seedling, and orange series indicates survivorship or coiling in cells with 2 or more seedlings present.

Some density-dependent effects can be seen, with seedlings in cells (10 x 10 cm) with one sibling showing lower survivorship (p=0.073) than those in cells by themselves (Figure 3a). Although not statistically significant (p=0.157), this trend is also observed when survivorship of only those seedlings that found a host is considered (Figure 3 b). Interestingly, the ability to coil on a host does not appear to show density-dependent effects (Figure 3c).

Distance from the point of emergence to the nearest host species significantly influences the survivorship of seedlings, with closer emergence increasing success (p=0.0338; Figure 4a). However, when host species are analysed separately, *C. pubescens* seedlings have increased survivorship with increasing proximity to the host, but only for the host species *Pultanea daphnoides* (p= 0.0305; Figure 4b).

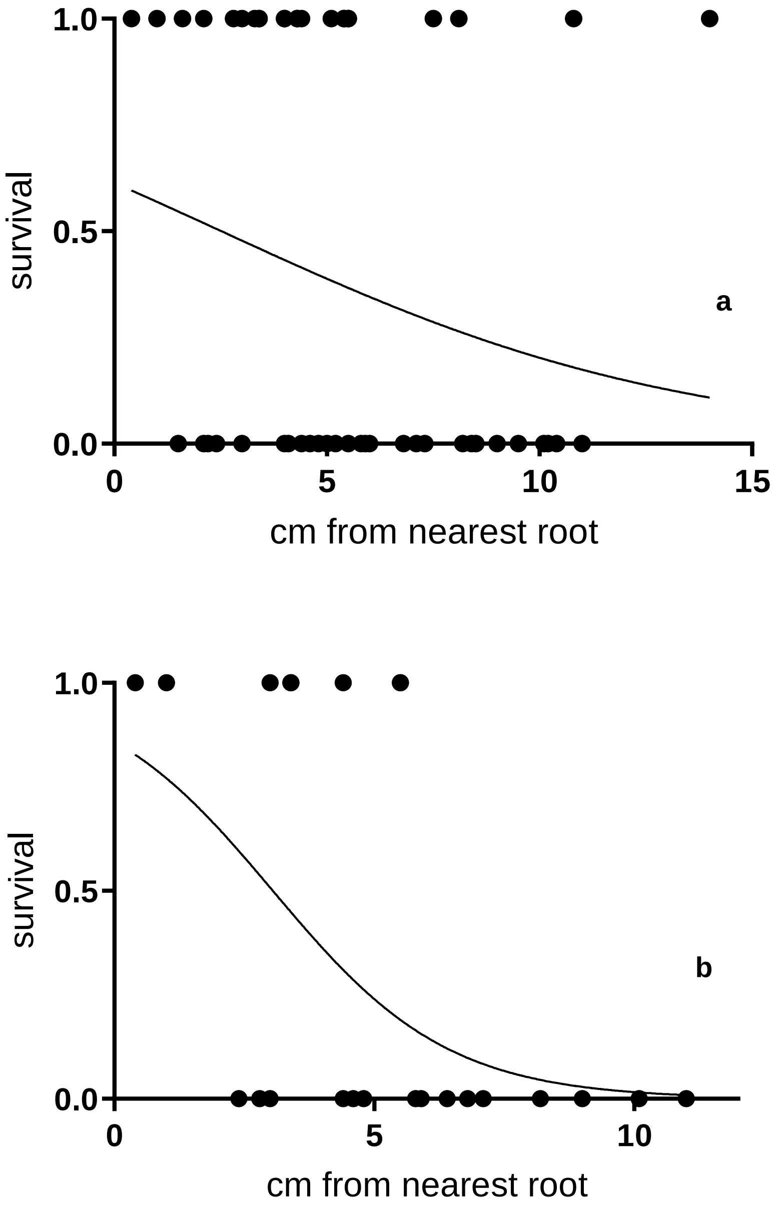

Figure 4: Survivorship (1= success, 0=death) of *C. pubescens* seedlings in relation to distance from the nearest host plant. Distances were taken from the point of seedling emergence to the nearest point of the host. Graph A: relationship for all species sampled (P=0.0338), and Graph B: survivorship when grown on *Pultanea daphnoides* (P=0.0075), the only individual species to show any significant effect.

Observations of coiling and foraging behaviour of seedlings indicate there is no selection of host species, rather coiling will occur around any material the seedling comes into contact with. However, although there is no apparent host-selection, survival post-establishment is influenced by the type of host encountered. Shrubby species resulted in the best survival, and ephemeral species, such as annuals and spring-flowering ground orchids, the worst (Figure 5). The short-lived hosts are, however, often associated with subsequent coiling on other host individuals, with survivorship of seedlings coiled on grasses reduced to zero when seedlings with second hosts are removed from the dataset. It is also important to note that no seedlings died within the period of this study once coiling had occurred around a second host, even after root senescence.

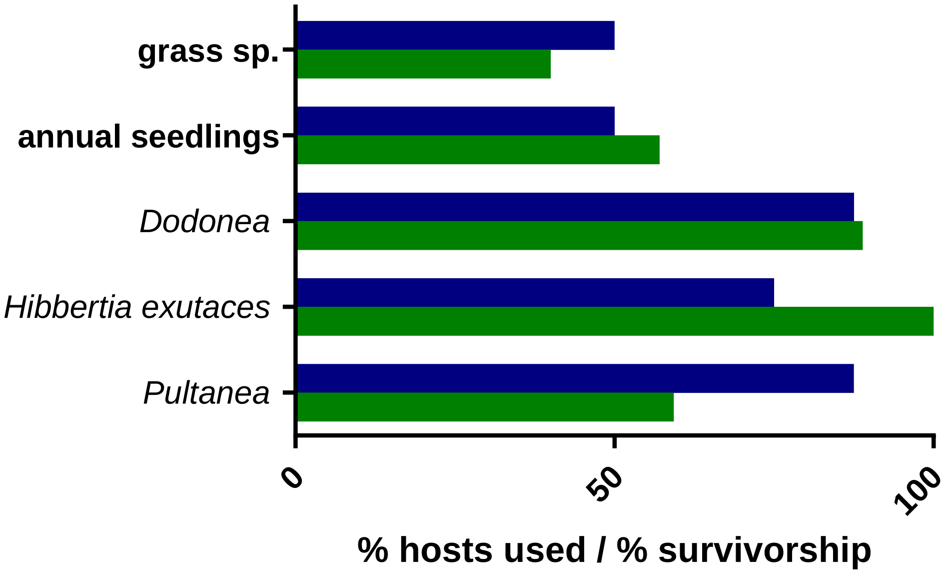

Figure 5: Host availability and survivorship for *C. pubescens* seedlings, group by host species. Blue bars indicate survivorship as a percentage of seedlings utilising that host species, and green bars the percentage of available hosts used within quadrats. Note that grasses and annual seedlings only show greater than 0 survivorship due to *C. pubescens* seedlings coiling onto secondary hosts. All seedlings that remained attached only to grasses and annuals did not survive.

Discussion

The re-establishment of *C. pubescens* after a fire is a complex process, and it is clear the response of this species to fire is strong, albeit delayed. The limited dispersal suggested by the emergence patterns (supported by population genetics, see Chapter 3) and resulting high seedling density lead to competitive effects in seedlings during their most vulnerable life-stage. However, a lack of strong host selectivity may help mitigate these competitive effects.

Seedling distribution

Given the life history strategy of this species, some delay between the disturbance (in this case fire) and emergence makes sense, as the parasite needs to allow time for the recovery of the host's population in order to have enough resources (i.e. robust enough host plants) for their own recovery. However, such a long delay was unexpected in an environment containing many species that respond to fire by resprouting, and indicates that this species does not just rely on physical dormancy to delay germination. Whilst the timing of the field study does not allow a full examination of the germination phenology (on one monthly site visit no seedlings were present, and the next they were), glasshouse experiments indicate that seedling germination occurs over a period of approximately 6 weeks. A long delay in germination followed by a staggered emergence is a good bet-hedging strategy for a species which relies on the recovery of the surrounding vegetation, as infecting hosts very early after their emergence may result in low resource availability. However, by the time most parasite emergence occurred the resprouting and growth of most of the perennial species in the area was quite advanced, thus most host plants were able to support the parasite.

While the distribution of seedlings close to the where infected hosts were located before the fire was not completely unexpected, the degree of clustering and the high densities observed were surprisingly high (Figure 1). Because the seedling stage of this parasite is sensitive to environmental extremes, the consequences of low dispersal rates are important, as the resulting distributions do influence seedling survivorship. For a parasitic species such as *C. pubescens,* seed nucleation around potential host plants with the capacity to re-sprout after fires is clearly advantageous for future establishment of seedlings. In this case, behaviour of dispersal vectors (for example bandicoots; see Maciunas et al., 2022), particularly with regard to their use of low vegetation as cover, may be important for the distribution of seed into areas where potential host species are present. An example of this is seen in mistletoes, where the successful establishment of seedlings is entirely dependent on dispersal onto a suitable host, and therefore on targeting of those host species by the parasite's dispersal vectors (García et al., 2009; Luo et al., 2016). However, in the case of the generalist parasite *C. pubescens,* the high density of seeds in very close proximity to adult plants suggests that either dispersal distance is often limited, or seed accumulation around adult plants with secondary movement of seed occurring over short distances (primarily about 1-2 meters, Figure 2). To what extent this is a result of a reduction of abundance of potential seed dispersers need to be ascertained. It must be noted, however, that low dispersal may ensure seed presence around a host that can re-establish after a fire that would destroy the mother plant. It is known that the different resources available at different nucleation sites of seeds can influence subsequent seedling survivorship (Verdu & García-Fayos, 1996), and in the instance of a parasitic species, those resources must include the type and distribution of host species.

Factors such as the length of time over which seed accumulates and seed longevity within the seed bank are known to influence post-fire recovery in Australian native species (Auld et al., 2000), and the requirement for *C. pubescens* seedlings to find a suitable host plant quickly after germinating adds more complexity to the post-disturbance recovery of this species. As density of seed within the seed bank may increase substantially over time, longer intervals between burns may have a strong influence on the regenerative capacity of those patches, particularly as seedlings are seen to be influenced by intraspecific competition (Figure 3).

Survivorship

Whilst accumulation of seed near a resprouter host plant can be important for the subsequent survival and establishment of parasitic seedlings, the cost of interspecific competition with the host plant as well as intraspecific competition within those high density patches of seedlings may affect the overall establishment success of new patches after fires. Some intraspecific competition does occur at low densities, however, it appears to affect primarily the success of the seedlings at the coiling stage, with effects disappearing when only post-coiling survivorship is considered (Figure 3). This is not surprising, given that once a seedling has formed a functional connection to its host plant any root competition with other seedlings becomes irrelevant. In this species, the root system is significantly reduced (McLuckie, 1924), and the ability of that root system to support the seedling for extended periods of time is limited (See Chapter 7). Glasshouse observations indicate seedlings in this species are sensitive to dry conditions (Pers. obs.), although the functionality of the reduced root system is unknown. Small changes in abiotic factors, such as moisture availability, may therefore have significant effects on the establishment and survivorship of individual seedlings, accounting for differing survivorship seen under intraspecific competition in the field.

We also found some evidence of interspecific competition, as there was some indication that distance from the host may affect survival for one host species (Figure 4b). These effects should be explored in more detail in future studies, as the limited sample size for all hosts but *P. daphnoides* may explain the non-significant results of those other species. As this is a parasitic species which needs to attach to a host promptly after emergence, there must be some trade-off between the seedling growing close enough to a host plant to ensure successful establishment and suffering from interspecific competition from the prospected host. Due to the reduced root system of the seedlings, any negative interaction between the host plant and the parasite whilst it is free-living may be limited spatially.

The survivorship of the parasite on broad types of host was variable, and smaller seedlings and annual plants appeared to be less likely to sustain survivorship of the parasite (Figure 5). Although sample size is low (and hence statistical analyses not possible), these host plants are commonly found in the field and therefore this element of the parasite’s ecology requires further investigation (McLuckie, 1924; Pers. obs.). The exception to this lowered survivorship was when a secondary host was reached soon after coiling around the first, since then these smaller hosts acted more as a ‘stepping stone’ to more robust, longer term ones rather than a sole host themselves. The phenomenon of stepping stone hosts is known from other parasitic taxa (Schoolmaster Jr, 2005), and here may be important for the survivorship of seedlings even if the seedlings do not draw resources from them directly. Even if they act just as a physical support, it would allow those seedlings to reach high quality hosts that can support them in the long-term. However, if a second host is not found, survivorship of the parasite seems to be unlikely (Figure 5). It is also important to note that although there is no host selectivity shown at the seedling stage, the one nitrogen-fixing host studied here, *Pultanea daphnoides*, did result in a higher survivorship than other host species.

This study demonstrates that *Cassytha pubescens* shows a strong response to fire, with seedlings emerging *en masse.* The significant time delay between the fire event and emergence reflects the high degree of attunement to fire events, while the clustering of emerging seedlings near adult plants suggests that dispersal may be limited. While this might be a result of scarcity of dispersers, it may also reflect adaptation to exploit a patch of proven quality (in this case potential presence of a suitable host after a fire). Importantly, the results presented here suggest that there may be significant competitive effects when seedling density is high, therefore the limited dispersal and any clustering of seedlings around adult plants is an important consideration when examining the population dynamics of this species.

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Chapter 5

Competition affects establishment of seedlings in a rootless hemiparasitic vine

Cassytha pubescens seedling establishing on *Acacia paradoxa* in a glasshouse experiment. As the functional haustoria form, the short root dies back and the parasite becomes fully dependent on its new host.

Statement of Authorship

Title of Paper	Competition affects establishment of seedlings in a rootless hemiparasitic vine
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Conducted all fieldwork, analysis, prepared figures and wrote the manuscript.

Principal Author

Name of Principal Author (Candidate)	Elizabeth C Maciunas
Contribution to the Paper	Conducted all field work and sampling, analysis, prepared figures. Wrote the manuscript.
Overall percentage (%)	90
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Signature		Date	07/09/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Assisted with experimental design, advised statistical analyses, manuscript drafting

Signature		Date	6-09-23

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Contribution to the Paper	

Signature		Date	

Please cut and paste additional co-author panels here as required.

Chapter 5: Competition affects establishment of seedlings in a rootless hemiparasitic vine

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Abstract

Complex interactions between multiple species can have a large influence on the maintenance of populations, particularly for species with unusual life-history strategies. Here we examined the effect of host diversity and density on the seedling establishment in the hemiparasitic *Cassytha pubescens*. This parasite is rootless as an adult but relies on a reduced and short-lived root in the seedling stage. We introduced seedlings of the parasite to pots with individual host plants, or a multi-species assemblage. Surprisingly we found that competitive effects outweighed the benefits of host diversity: successful establishment was significantly greater on individually planted hosts. We interpret this as evidence that strong competition for resources, particularly water, has a strong effect on establishment and survivorship during this vulnerable life stage.

Introduction

The life history strategies of parasitic species are arguably some of the most complex amongst plants. Both host specificity as well as a sensitivity to changing abiotic conditions may influence establishment and seedling survivorship, however this may also be dependent on the life history strategy of the parasitic species in question. For example, in mistletoes seed establishment is already limited by dispersal on to a suitable host, and the availability of both space and light can influence establishment success (Mellado & Zamora, 2014). On the other hand, seedlings of generalist root parasites such as *Santalum* spp. may be more sensitive to water availability until they attach to the root systems of a range of available hosts (Binu et al., 2015; Woodall & Robinson, 2002). As different host species may provide different benefits to the parasite once attached (Pennings & Callaway, 2002), a diversity of potential host species may be important for maximising establishment success for these parasites. This raises questions of how host plants and parasitic seedlings interact in terms of competitive effects, and whether there may be trade-offs for parasitic seedlings that grow in close proximity to their host.

The obligate hemiparasitic vine *Cassytha pubescens* is an herbaceous, perennial rootless parasite and presents an interesting and special case. Although adult *C. pubescens* plants are generalists, some potential host species are known to resist infection by this parasite (Facelli et al., 2020) and the range of species that seedlings can infect is unknown. Germinating in the soil, the parasite develops a short, fleshy root to support it until a suitable host can be found and a functional connection established (McLuckie, 1924). Eventually, when the host is acquired (or after the seed reserves are exhausted) the root senesces, and the shoot-parasite relies solely on its host plants for nutrients and water. Being obligate parasites, establishment on a suitable host plant quickly after germination is essential for long-term survival, making the seedling stage particularly vulnerable to environmental stressors. Interestingly, seed germination occurs in springtime following a disturbance from fire in the previous year (Chapter 4), which make rapid growth and establishment on a host plant before the hot dry summer begins important. The reduced and short-lived root may leave the parasite seedling vulnerable to low water availability, raising questions regarding possible trade-offs between the proximity of the seedling to the host (allowing fast infection), and competition with the roots of the potential host plants for resources such as water.

A morphologically similar, although physiologically different (i.e., holoparasitic) species, *Cuscuta gronovii*, is known to require particular host species in order for seedlings to successfully establish on, before they can move on to other hosts (Schoolmaster Jr, 2005). This phenomenon is comparable to 'nurse plant' effects that are well known from a wide variety of ecosystems (Callaway & Pennings, 1998). Both *Cuscuta gronovii* and *Cassytha pubescens* are twining vines that can spread from an initial host plant and infect several hosts simultaneously, and the significant similarities in the life cycle of these two parasites raise further questions of the effects of host species on *Cassytha pubescens* establishment. Therefore, understanding initial establishment patterns can help to understand their potential spread in populations recovering after, e.g. bushfires.

This study examines whether host species affects establishment of *C. pubescens* seedlings, as well as whether the presence of multiple small hosts in close proximity influences survivorship. Three common understory species were selected as nurse hosts based on seedling availability, survivability under cultivation, and whether that species has been observed acting as a host for this parasite in the field. Additionally, as *C. pubescens* seedlings establish during recovery from fires, one grass, and seedlings of one small shrub and one tree species were selected as nurse hosts to attempt to simulate the diversity of growth forms (bushy, resprouting vegetation and newly growing seedlings) seen in the field during fire recovery. Our primary research questions were 1) does a greater host choice increase establishment success? 2) does host species affect establishment success? And 3) does competition for resources influence seedling establishment?

Methods

Study species

Cassytha pubescens is an Australian native, herbaceous hemiparasitic vine. As an adult it is rootless and coils and attaches to a variety of perennial species growing clonally between hosts. It has a wide-ranging distribution across south-eastern Australia and is a generalist parasite that can be found on a multitude of different species (Facelli et al., 2020). The root in the seedlings of this species is short-lived, senescing after establishment on a host and reported in the literature to last no longer than 8-10 weeks (McLuckie, 1924), although it was observed in both the field and glasshouse to survive up to 16 weeks (Maciunas Pers. obs). Recovery from disturbances such as fire is crucial for this species, as they are common in its habitat, and the adults, lacking underground organs, are killed by fires (McLuckie, 1924; Tsang, 2010). Therefore, the interactions with potential hosts at the sensitive seedling stage have potentially large consequences for patch dynamics (See particularly Chapter 4).

Five different host species were used in this study. Two are considered 'main' hosts: *Leptospermum myrsinoides* (Australian native) and *Rubus fruticosus* (invasive) and were selected for their physical structure and because they frequently act as host in the field. Three 'understory' species were also used: *Rytidosperma caespitosa* to represent the grass growth form, *Goodenia ovata* to represent shrubby low growth form, particularly as seen in resprouting species, and *Acacia paradoxa* to represent larger shrub and tree species' seedling form. All five species have previously been observed supporting *C. pubescens* in the field (i.e. the parasite was observed growing and coiling on them – see Chapter 7) and were used together to provide a complex selection of growth forms for new seedlings to establish within.

Experimental methodology

C. pubescens fruits were collected in the field in December/January 2010-11 and left to dry over a period of five days. Once the fruits were semi-dried the seeds were removed and thoroughly cleaned and dried, and then stored for three months under dark conditions at ambient temperature prior to treatment for germination.

Host plants were established in 180mm diameter, 4.2 litre pots in the glasshouse, using commercially available sandy-loam soil and a slow-release Australian native plant fertiliser (Scotts Osmocote, NPK : 21.8 : 0.7 : 7.2) mixed according to the manufacturer's instructions. Five treatments, each with 20 replicates, were established. The five treatments were: *Leptospermum myrsinoides* grown individually, *L. myrsinoides* grown with the three understory species, *Rubus fruticosus* grown individually, *R. fruticosus* grown with the three understory species, and one treatment with the 3 understory species only. *R. fruticosus* were removed from the field as crowns, transplanted, allowed to establish for 4 weeks before being pruned back to ground level. At this point understory species were also added to the pots for the relevant treatments. All Australian native species, including *L. myrsinoides,* were purchased as tube-stock, and all treatments were maintained for four months before *C. pubescens* seedlings being planted. At the time of infection, *L. myrsinoidies* hosts were well established and approximately 30 centimetres tall.

To break dormancy, *C. pubescens* seeds were boiled in RO water for 1 minute (following Tsang 2010), and then placed in petri dishes containing a thin layer of sterilised sandy-loam soil. These were kept moist under controlled conditions in a germination cabinet (model treil-200-1-SD; Thermoline scientific, Australia) with a day length of 8 hrs at 22^{o}C and a night length of 16 hrs at 18^{o}C until germination occurred.

C. pubescens seedlings were introduced to the pots approximately 3 cm from the base of each main host plant. For the treatment where no main host was included, seedlings were placed equidistant from all understory species. According to McLuckie (1924), the lifespan of *C. pubescens* seedlings can be as short as 8-10 weeks unless they attach to a suitable host. Therefore, germinating seeds were moved into the pots with hosts (planted at 3 cm depth) every 2 weeks over a period of 8 weeks, with each cohort of seedlings spread evenly across all treatments, until all pots in each treatment had been planted out with 3 living seedlings.

Establishment of *C. pubescens* seedlings was recorded as successful if the seedling survived for at least two weeks after the root had senesced, once the base of the stem and root had dried and visually separated from the rest of the parasite. Mortality of seedlings that had coiled around hosts during that time was also recorded. It is important to note that although many seedlings were growing vigorously, by the conclusion of the experiment none had grown across multiple host plants. Therefore all species can be considered as separate, individual hosts for the following data analysis.

Results were analysed using the chi square analysis in Graphpad Prism 9.0 (GraphPad Software, San Diego California USA).

Results

Seedlings in pots with understory plants only, had a significantly lower establishment (5-10%) than those in pots with only a single host plant (43% for both *L. myrsinoides* and *R. fruticosus*) (Figure 1; Table 1; p=<0.0001).

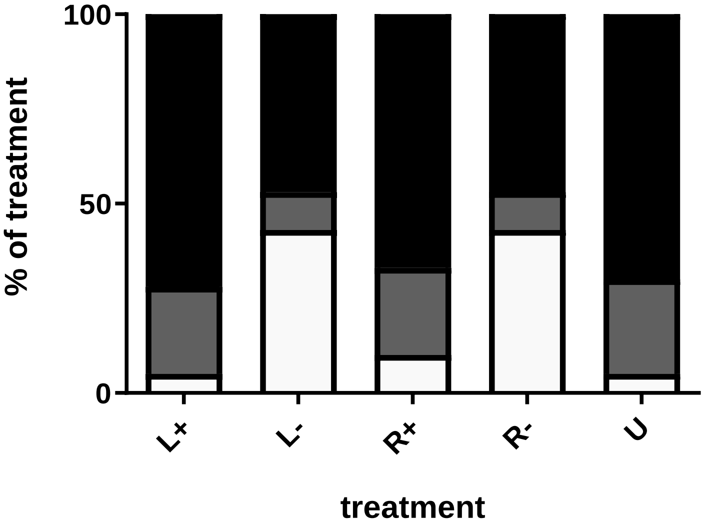

Figure 1: *C. pubescens* seedling survivorship, also considers coiling and successful/unsuccessful establishment on host. White sections indicate successful establishment on host, grey sections indicate coiling on host but subsequent seedling death before establishment, and black sections indicate seedlings showed no coiling before death. Main host species are *Leptospermum myrsinoides* (L) and *Rubus fruticosus*. (R), understory species are *Goodenia ovata*, *Rytidosperma caespitosa* and *Acacia paradoxa* seedlings. Negative values on host species labels indicate no understory present, positive values indicate understory presence. U indicates only understory species present, with no main host species.

Table 1: Chi square analyses of all 5 treatments.

P value and statistical significance	
Test	Chi-square
Chi-square, df	59.80, 8
P value	<0.0001
P value summary	****
Statistically significant (P < 0.05)?	Yes
Data analyzed	
Number of rows	5
Number of columns	3

Survivorship of seedlings that did coil around host plants was also much lower in the understory treatments. Of the three understory species used, coiling was highest in *R. caespitosa*, however there was no survivorship on this host species (Table 2).

Table 2: Survivorship of parasitic seedlings on understory species. Treatments L+, R+ and U all pooled to give totals for all host species**.**

Host species	Total coiled	Total died	Survivorship (%)
A. paradoxa	14	10	29
G. ovata	8	6	25
R. caespitosa	21	21	0

Discussion

We predicted that a greater diversity of potential host species would allow a greater opportunity for establishment to occur, however, we found that the risks of competitive effects far outweighed any benefit of having a greater host choice. Overall, seedlings performed far better in pots without multiple host species, and all species except *R. caespitosa* allowed successful establishment (Table 1). Nevertheless, *R. caespitosa* still supported coiling of the parasite; indeed the highest number of seedlings coiled on this understory species, but no seedlings produced viable haustoria on *R. caespitosa* and all died. Whether this is due to the internal anatomy of this species or another factor requires further investigation, as *R. caespitosa* was seen in the field to support adult *C. pubescens.* It is important to note that adult *C. pubescens* in the field may coil around and parasitise multiple hosts simultaneously (Cirocco et al., 2022; McLuckie, 1924), so while it may be unable to draw resources from *R. caespitosa* it may use some plants more as a physical support that allow the stems to reach a functional host.

In addition to successful establishment, differences in seedling activity between treatments was also observed (coiling vs non-coiling). Treatments with a better developed understory showed less seedling establishment and reduced seedling activity. Excepting those coiling on *R. caespitosa*, host species and type (shrub, seedling, grass) appears to have little effect on the success of establishment in *C. pubescens* seedlings, however multiple hosts in a small area did affect establishment. While this result was unexpected, it does agree with the field observations of emergence slightly away from existing vegetation (Pers. obs; see Chapters 4 and 7).

As the roots of the parasitic seedlings are greatly reduced, it is likely that they are susceptible to even low levels of competition for resources. Competition for water is perhaps the most likely factor influencing survivorship here and is worthy of further research. In this experiment multiple plants per pot may have decreased water availability in the top level of soil, the root-zone for the seedlings, particularly as many understory species have shallow and dense roots. Field observations suggest that competition between siblings and the host plants may play a role in establishment success (see Chapter 4), however field and glasshouse conditions may vary substantially. It is likely that competition for light and nutrients also influenced survivorship, although the nutrition from seed reserves for initial growth of the parasite is likely adequate for establishment. Light is known to affect performance of adult *C. pubescens* (Cirocco et al., 2016), and it is reasonable to assume shading from more densely growing host plants does have similar effects on seedlings.

Conclusions

The conditions governing successful seedling establishment in this species are undeniably complex, with host species and host density affecting both establishment and survival. Seedlings were more likely to actively coil on hosts (and therefore more likely to survive) when presented with hosts at lower density, however, the understory species results indicate further research is needed into which host species can support the parasite seedlings. Responses to infection by mature *C. pubescens* range from tolerance to immunity (Facelli et al., 2020; Tsang, 2010), and these results suggest such variability also occurs at the seedling stages. Understanding how the conditions in a complex understory affects the recovery of *C. pubescens* after disturbance is an important consideration for the management of this parasite.

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Chapter 6

Rubus fruticosus agg. responds to infection by *Cassytha pubescens* by partitioning effects and raising physical defences.

Close up of *Cassytha pubescens* haustoria on *Rubus fruticosus* (top left), seedling *C. pubescens* establishing in a glasshouse experiment (top right) and an example of the high densities adult *C. pubescens* can reach when growing on *R. fruticosus* in the field (bottom).

Statement of Authorship

Title of Paper	Rubus anglocandicans responds to infection by a hemiparasitic plant by compartmentalising effects and raising physical defences
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Conducted all fieldwork, lab work, sampling, analysis, prepared figures and wrote the manuscript.

Principal Author

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Contribution to the Paper	Conducted all fieldwork, lab work, sampling, analysis, prepared figures and wrote the manuscript.		
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Chapter 6: *Rubus fruticosus* agg. responds to infection by *Cassytha pubescens* by partitioning effects and raising physical defences.

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Abstract

Here we report the modular response of a host (*Rubus fruticosus* agg) to infection by a hemiparasitic plant (*Cassytha pubescens*) and the induction of anti-herbivore defences in response to infection. We infected *R. fruticosus* with the hemiparasitic vine *Cassytha pubescens* and measured the impact on host physiology, final biomass and thorn production. We measured uninfected hosts, and infected and uninfected branches in infected hosts, and physiological indicators of performance were generally lower under infection; however no physiological measurements were significant. In contrast, infected stems had a significantly lower biomass than uninfected stems from the same individuals, and the number of thorns on infected branches was also significantly higher than on uninfected branches in infected individuals. Overall the results suggest that this host responds to infection by a parasite by partitioning resources differentially: more resources were seemingly allocated to uninfected branches while infected branches suffered reduced growth. The increase in thorniness in infected plants suggests a response akin to that to herbivory, with inducible defences expressed in response to parasitism.

Introduction

There is ample evidence of the importance of parasitic plants in natural systems where they can have significant ecological effects, such as affecting the abundance of host species (Press & Phoenix, 2005), altering structure and diversity of communities (Callaway & Pennings, 1998; Chaudron et al., 2021; Grewell, 2008; Hatcher & Dunn, 2011; Marvier, 1998; Pennings & Callaway, 2002; Watson, 2009) and modulating nutrient cycling (Grewell, 2008; March & Watson, 2007; March & Watson, 2010; Quested, 2008).

The effects of parasitic plants have been widely studied, particularly in crops due to the high impact parasites can have on yields (Lanini & Kogan, 2005; Musselman, 1996). These studies show remarkable variation in the effects of parasitic plants on their hosts (Cameron et al., 2006; Zagorchev et al., 2021). The direct effects of parasites are variable because the tolerance of hosts to infection by different parasitic plants ranges from immunity (Tsang, 2010), to limited physiological and growth effects of infection due to high resistance (Cirocco et al., 2016a; Cirocco et al., 2015; Prider et al., 2009; Tjiurutue et al., 2016; Zagorchev et al., 2021), to the death of the host (Marvier, 1996; Zagorchev et al., 2021). In hosts that fall in the middle of this continuum, more moderate responses to infection may be seen in both physiology and growth. These include as a general reduction in physiological performance (both photosynthesis and water relations) and partitioning of growth (Aflakpui et al., 2002), or a reduction in fecundity of the infected individual (Aflakpui et al., 2002; Graves, 1995; Prider et al., 2011). Interestingly, somehow similar responses are common in plants under pressure from herbivory, and may be the result of either resource removal or physical injury. In some cases, comparisons between the two processes are justified (e.g. Pennings & Callaway, 2002).

How species respond to injury and resource removal by both parasites and herbivores can vary with season, environment and host characteristics. Additionally, abiotic conditions and ecological interactions such as herbivory or parasitism rarely occur uniformly across a plant's canopy, and it is known that responses to such effects can be highly localised to the region, or 'module', of the individual on which they occur (Haukioja et al., 1990; Honkanen & Haukioja, 1998; Kawamura, 2010). For example, in studies on South American pepper, Marquis (1992) found that in a plant with a modular structure herbivory triggered resource partitioning effects, and factors such as season heavily influenced the effect on the plant. Similar results are reported in Honkanen & Haukioja (1994) and Haukioja et al. (1990), indicating these effects extend to a multitude of different species. While these studies examined herbivory rather than parasitism, it may be expected that resource removal by a parasite could induce a somehow similar response. If individual stems function as semi-autonomous units and resource flow between them is limited, it can be hypothesised that parasitism may also lead to differential effects on plants with these kinds of modular structures.

Many plants defend themselves against herbivores through the production of volatile chemicals, resource partitioning, or physical defences such as spines or thorns (Herms & Mattson, 1992; Howe & Schaller, 2008; Moreira et al., 2015; Züst & Agrawal, 2017). Anti-herbivore defences are known to be plastic traits in most species, and are often inducible via hormonal pathways after damage to tissues ('wounding') or infection by pathogens (Baron & Zambryski, 1995; Davies, 1987; Gibson et al., 1993). Similar to herbivore damage and pathogen attack, infection by plant parasites involves wounding (through haustorial penetration), and resource removal, thus there may be similarities in the defence responses produced by hosts. In some host species, inducible defences to infection by stem parasites comparable to both herbivory and pathogen attack have been detected (Runyon et al., 2010; Smith et al., 2009; Tjiurutue et al., 2016). Whether physical defences may also be promoted by parasitism is an interesting question, and remains to be investigated.

This paper tests the effects of localised infection by *Cassytha pubescens* on *Rubus fruticosus*. Using this host species allowed us to test the effect of infection with *C. pubescens* on a plant with a modular structure, and to observe any resource partitioning that may occur in response to that removal. *R. fruticosus* also has prominent physical defences (thorns) that are known to be plastic in response to herbivory, as Gibson et al. (1993) found thorn production changed under different nutrient and browsing regimes. Therefore, *R. fruticosus* provides a good model to study the effects of infection with a plant parasite on the production of antiherbivore defences. We measured the effect of infection with *C. pubescens* on physiology, biomass and thorn production of *R. fruticosus*.

Specifically, we asked:

(1) Does infection by *C. pubescens* change the growth and biomass of *R. fruticosus*?

Due to observations made as biomass data was collected, two further questions were then posed:

(a) Does the modular structure of the host result in differential growth effects between infected and uninfected branches? And

(b) Does infection prompt changes in physical inducible defences, hence can comparisons be made to herbivory?

Methods

Study species

Cassytha pubescens R. Br. (Lauraceae) is a hemiparasitic vine native to South-Eastern Australia. Reproduction may be either clonal, through the spread of the parasite over multiple host plants, or through establishment of new seedlings. Seedlings are rooted and free-living for approximately 6-8 weeks, after which either the seedling establishes successfully on a new host plant, or it dies (McLuckie, 1924).

Rubus fruticosus L. aggregate (Rosaceae) is an aggregate genus of blackberries of European origin in Australia (Evans et al., 2007). It is a weed of national significance and has a wide distribution throughout most moderate to high rainfall areas (Evans & Weber, 2003). It is semi-deciduous, losing many but not all leaves during the colder months, and grows rapidly in warmer weather (Evans & Bruzzese, 2003). Both sexual and clonal reproduction occurs, with even small patches able to reproduce rapidly via the growth of runners. The branching, modular pattern of runners raises questions about possible partitioning effects of herbivory and parasitism in this species (Gibson et al., 1993).

Experimental methods

R. fruticosus crowns of similar size were removed from an infested open Eucalypt woodland in the Mount Lofty Ranges, South Australia (S 35°02’815”; E 138°41’553”), in summer (January) 2011 and potted into 2L containers filled with sandy loam. The growth habit of *R. fruticosus* consists of a central crown at ground level, from which single long stems emerge. First year stems are typically non-branching, and in this experiment some stems on infected plants remained completely uninfected. This allowed individual stems to be harvested and analysed separately after classifying them into infected and uninfected stems. To begin the experiment, stems were cut back to 10cm long at transplant, and allowed to grow for 4 weeks until all plants had recovered, before being cut back to ground level. Plants were then grown in a glasshouse for 4 months prior to infection with *C. pubescens*. During this time they were kept well watered and supplied with liquid fertiliser according to the manufacturer's recommended directions (Amgrow Nitrosol N 10.5; K 6.5) every 2 months. After 4 months, plants were randomly assigned to either infected or uninfected treatments, with n=9 for each.

C. pubescens seeds were treated in June according to Tsang (2010) and kept in a controlled environment cabinet (day length 8 h at 22°C; night length 16 h at 18°C) for eight weeks until germination began. As germination occurs over a period of several weeks, seedlings were re-planted (one per pot) into host pots over a period of 4 weeks, at a distance of 5 cm from the main host stem. Once seedlings were placed in pots with the host plants, they were allowed to coil naturally on them. *C. pubescens* seedlings were grown on the host plants for a further 6 months before physiological and growth measurements were taken in March 2012.

All of the following physiological measurements were taken on the youngest fully expanded leaf, which, for infected plants, was on an infected stem. All data was taken on days that were clear, and at midday when it was approximately 30 degrees Celsius, unless otherwise stated.

Water potential of hosts was measured using a Scholander-type pressure chamber (PMS Instrument Company, Albany, OR, USA) at pre-dawn and midday. Photosynthesis and stomatal conductance were measured at midday using a Ciras-2 Gas Exchange System (PP systems, Amesburg MA, USA), and rapid light response curves were measured using a Mini-PAM chlorophyll flurometer (Walz, Effelrich) immediately following the Ciras-2 measurements. ETRmax was calculated by measuring yield and following the standard method as shown in (White & Critchley, 1999). It is used here to compare photosynthetic efficiency between treatments.

After 8 months of infection the experiment was concluded and all plants were destructively harvested (May 2012) to measure biomass. Firstly, stems were removed from the roots and roots were washed and weighed. Stems from each plant were separated and weighed individually, noting which were infected and which were uninfected. Because of a failure in the environment control system just before the harvest, leaves were heat-damaged, and some had dropped. Therefore, all remaining leaves were removed from all stems before weights were taken, to ensure consistency. Once stems were weighed, they were measured and the number of thorns were counted. These were counted from the base at the crown to the last fully expanded leaf of every stem (on average this excluded the last 5 centimetres). Thorn density was calculated as the number of thorns divided by stem length.

All results were analysed and graphed in Graphpad Prism 9.0 (GraphPad Software, San Diego California USA), with one-way ANOVA used to test for differences in physiological measurements as well as stem biomass and number of thorns. A simple linear regression was used to examine the relationship between total host biomass and *Cassytha* biomass.

Results

There was no significant infection effect in any physiological measurements taken (Table 1).

Table 1: Results of t-tests for the effects of infection on physiology of *R. fruticosus*. Numbers in bold indicate significant values.

	t	*p-value*	*df*
Waterpotential	0.9869	0.3384	16
Stomatal Conductance	0.5409	0.5961	16
Photosynthesis	1.607	0.1276	16
ETR_{max}	1.877	0.0789	16

For stem growth data, the biomass of individual infected stems was significantly lower than uninfected stems on the same plant (P = 0.0097; Figure 1a), but the length of individual stems was greater for uninfected stems on infected plants compared to both the control as well as infected stems (P=0.0196; Figure 1b).

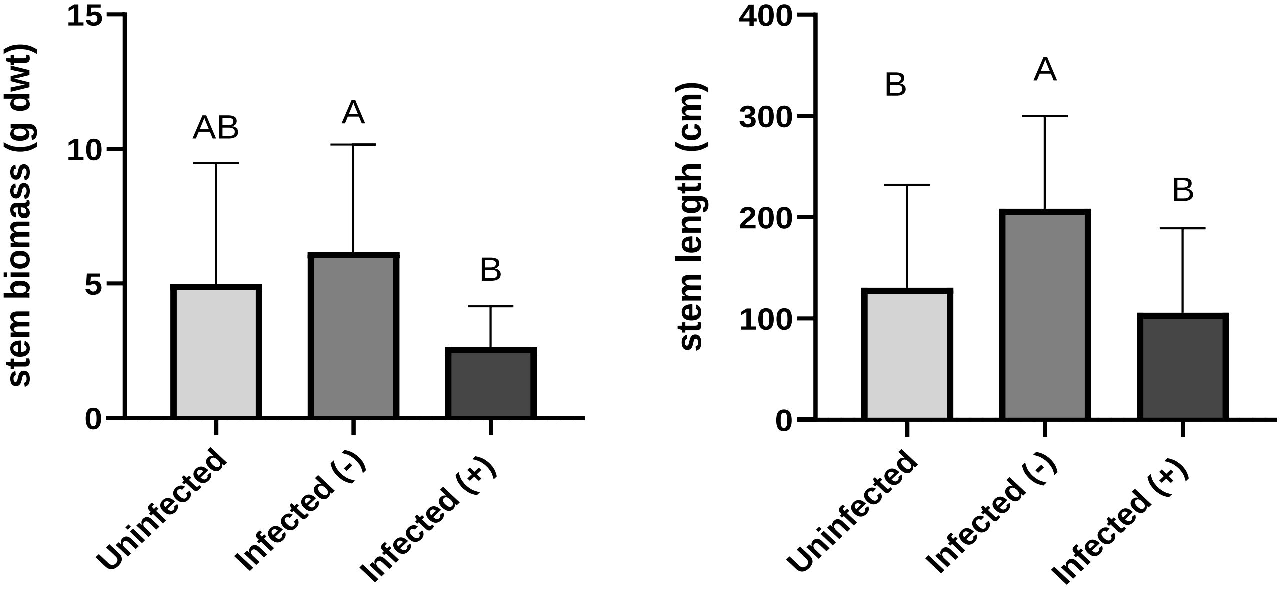

Figure 1: Biomass of individual stems (Left) and stem length (Right), for uninfected *R. fruticosus* plants (light grey bar), uninfected stems on infected plants (medium grey bar, -), and infected stems (dark grey bar, +). Data are means ±SD, n= 37, 11, and 19 respectively, whiskers represent 95% confidence intervals with capital letters indicating significant differences.

There was a significant negative relationship between *C. pubescens* biomass and total stem biomass of *R. fruticosus* (infected and uninfected pooled; *P*= 0.0043; Figure 2), and thorn density was significantly greater on both infected than uninfected stems on infected plants, as well as stems on totally uninfected plants (*P*= 0.0011; Figure 3).

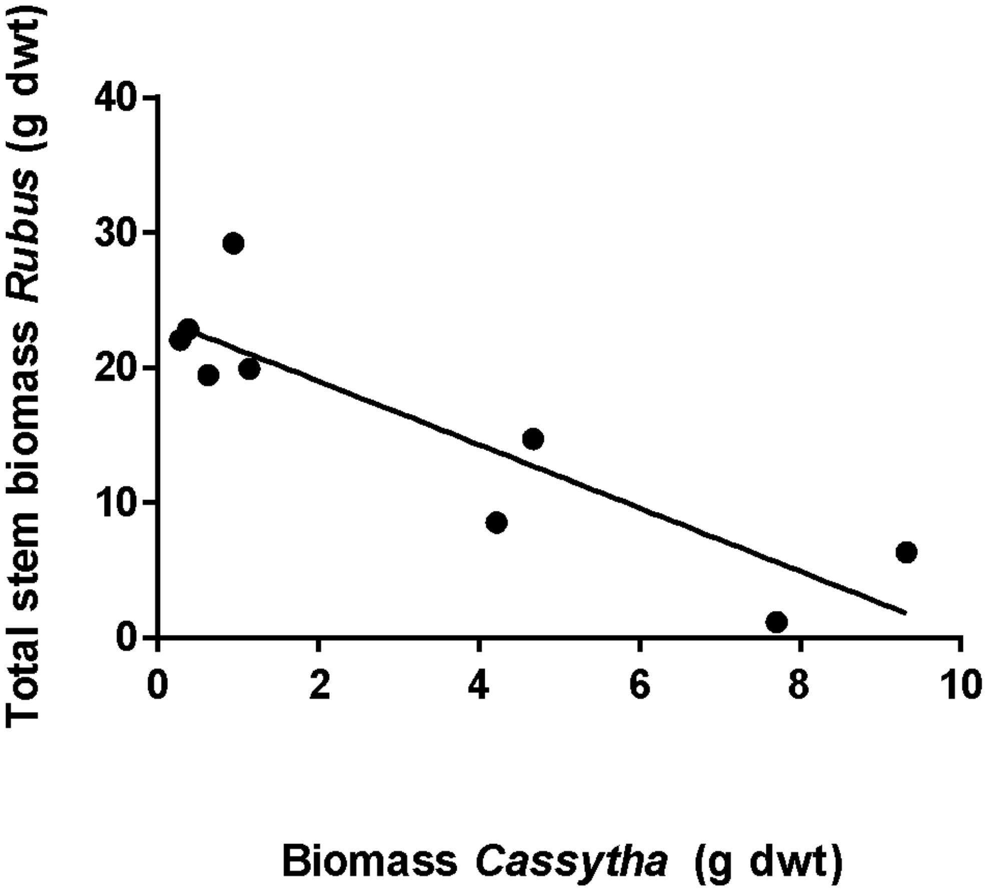

Figure 2: Relationship between biomass of *C. pubescens* and stem biomass of infected *R. fruticosus,* with infected and uninfected branches pooled for infected plants. n=9, R^2=0.7114.

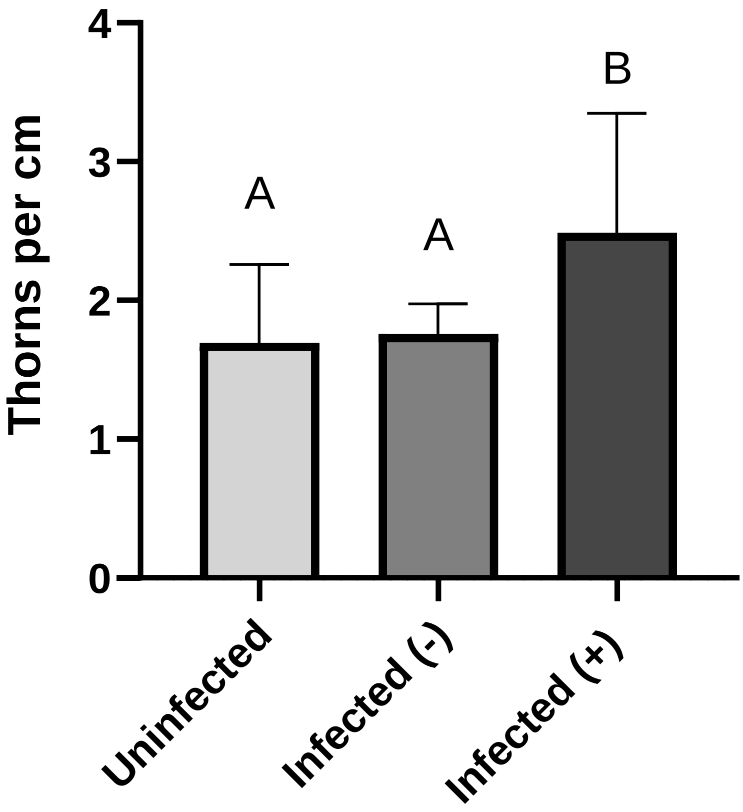

Figure 3: Thorn density (thorns cm^{-1}) for *R. fruticosus* stems on uninfected plants (light grey), and uninfected (medium grey, -) and infected stems (dark grey, +) on infected plants. Data are means ±SD, n= 37, 11, and 19 respectively, whiskers represent 95% confidence intervals with letters indicating significant differences.

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Discussion

Our results indicate that the effects of *C. pubescens* on this host species appear to be localised to directly infected stems, and anti-herbivore responses may be triggered in response to infection. Although most biomass effects are indicative of reduced performance under infection, the physiological measurements were not significant. There is evidence to suggest that branches in some plants may function as semi-autonomous units, i.e. those that are adversely affected by certain environmental conditions may not pass on the effects of those conditions to other branches (Marquis, 1992). If each branch were to function semi-independently in *R. fruticosus*, the effects of parasitism could be contained to only the branch that was directly infected. Such a partitioning response was seen in the biomass results and supports the argument for infection effects to be stronger in those branches which are directly infected than those which are not.

Although it was not significant, physiological measurements taken on uninfected branches showed a trend for higher photosynthetic activity than the control group, indicating the potential for compensatory mechanisms in uninfected stems. This is supported by the stem length data, where uninfected stems in infected treatments were significantly longer than the control group. A similar effect has been found in plants subject to resource removal by sap-sucking insects (for example, Retuerto et al., 2004), and as physiological data was collected only on uninfected plants and infected stems on infected plants before the modular difference between infected and uninfected stems within the same plant was evident in this study, further research on this response to similar resource removal by a parasite is recommended. In addition to this, more regular measurements of all non-destructive physiological measurements (particularly gas exchange and chlorophyll fluorescence) is recommended, as small differences must be cumulative to result in differing biomass. Attempting to capture subtle but ongoing differences over time between infected and uninfected stems will aid our understanding of the potential movement and allocation of mobile resources (photosynthates and nutrients) within this host species.

In this experiment, branches that were infected grew very little and although not significantly different from the control, uninfected branches did grow significantly longer than infected ones from the same plant (Figure 1b). Interestingly, the slight difference between stem length and biomass results indicate that even if within the same plant uninfected stems can elongate more than infected ones, it does not necessarily translate into greater biomass. In addition to this, some heavily infected plants never actually produced new stems (Pers. obs.) supporting the idea that despite being relatively low in biomass, the rapidly growing parasite was acting as a strong resource sink. The modular structure of *R. fruticosus* may explain such partitioning effects, as a strategy to combat parasitism seen in plants with such modular structures is to produce more, independent clones, or to completely sacrifice infected branches or clones and instead allocate more resources to uninfected modules (Fischer & Van Kleunen, 2001).

In many species the removal of above ground biomass elicits a wounding response which stimulates growth and also prompts the production of anti-herbivore defences (eg Howe & Schaller, 2008; Maschinski & Whitham, 1989; McNaughton, 1983; Strauss & Agrawal, 1999; Thomson et al., 2003). In some host plants, there is also evidence that infection by a parasite may induce a similar chemical response to that of wounding (Westwood et al., 1998), and in this case these responses may explain the slightly higher ETRmax in infected *R. fruticosus.* If in this case the removal of water and nutrients rather than biomass itself is considered, parallels may be drawn between the processes of parasitism and herbivory. Growth compensation effects can be seen in many plants under stress from herbivory (McNaughton, 1983), and infected stems in this study did have significantly more thorns when compared to both the uninfected stems from the same plant, as well as the control (Figure 3). Whether this is a response to wounding or resource removal and subsequent protection of valuable tissues would be an interesting avenue for further study, particularly considering these results contrast those of (Cirocco et al., 2022a) who found a small but significant decrease in thorniness of infected stems in *R. anglocandicans*, one of the species that makes up this aggregate. It is important to note that *R. fruticosus* is highly morphologically variable and has somewhat plastic traits, which may allow for rapid changes to morphology depending on the conditions the individual plant is subjected to (Caplan & Yeakley, 2013). There have been several studies focusing on the response of *Rubus* species to browsing by herbivores, with effects varying according to both abiotic and biotic stressors (Bazely et al., 1991; Björkman & Anderson, 1990; Gibson et al., 1993; Thexton & Bajcz, 2021). Members of this genus are typically heavily defended with sharp thorns that deter most browsers, however Bazely et al. (1991) and Gibson et al. (1993) found that the density of the thorns can be a response to both browsing intensity and nutrient limitation. It is worth noting that whilst this study was undertaken under glasshouse conditions and using seedlings of the parasite, the study by Cirocco et al. 2022 was carried out under field conditions and using adult *C. pubescens.* Small changes such as these may account for differing results, and is an interesting comparison to draw for future studies. Overall, the interactions between nutrient and water limitation, herbivory and parasitism are complex, and further experimentation is recommended for this species particularly when comparing field-collected data to in-lab experimental results.

Adaptations to reduce herbivory, or in this case parasitism, are especially important when a plant is grown under low nutrient conditions, as it may be more costly to a plant to replace photosynthetic tissues when critical nutrients are limited (Coley et al., 1985). However, the cost of producing different tissue types is not necessarily easily measured (Björkman & Anderson, 1990), as the tissue produced most efficiently may differ depending on which resources are limiting (Herms & Mattson, 1992). When considering parasitism, it is likely that differences would be seen between the nutrient stress induced by photosynthetic and non-photosynthetic parasites, as these remove different resources (for example water and nitrogen versus photosynthates) from their host plants. It is also known that the more herbivory (or parasitism) is localised to one part of the plant, the greater the impact on that part of the plant (Herms & Mattson, 1992). In this case, *C. pubescens* is a hemiparasite which removes water and nitrogen from its host. As the home range of the parasite is naturally seasonally dry and with low-nutrient availability, common environmental conditions may work to increase the negative effects on this invasive host species. Further investigation of infection of *Rubus* by this parasite under field conditions is warranted, particularly regarding its growth and the production of antiherbivore defences.

The overall results of this study agree with previous work on *C. pubescens* (e.g. Cirocco et al., 2016a; Cirocco et al., 2016b; Cirocco et al., 2015; Cirocco et al., 2022a; Cirocco et al., 2022b; Prider et al., 2009; Tsang, 2010), which demonstrated that non-native hosts are adversely affected by this parasitic species. Additionally, this study indicates that such results are not restricted to nitrogen-fixing host species, and can probably be applied more generally to a larger spectrum of invasive species. Caution should be used when directly comparing these results to other studies, as this is the first to examine the effects of this parasite when grown from seedlings, rather than through clonal infection from adult plants. However it is likely that if adult clones had been used rather than seedlings, the impact on the host may have been even larger, as a smaller overall biomass of the seedlings would act as a smaller resource sink than that of adult clones.

In this study *R. fruticosus* branches that were heavily parasitised put minimal resources into growth while instead favouring thorn production to protect the photosynthetic tissues already present, theoretically allowing the plant to allocate more resources to the growth of new, uninfected branches. The combination of lower biomass and increased thorns on infected branches indicates that *R. fruticosus* may use a resource partitioning strategy to cope with infection by this parasite. Overall infection by *C. pubescens* limits the vigour of *R. fruticosus*, however only for those branches that are directly infected. An important aspect of this study was the use of young *C. pubescens*, with a very small biomass (infecting from seedlings). Repeating the above study using adult parasites to infect clonally may provide a better understanding of the partitioning ability of *R. fruticosus* as a response to parasitism.

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Chapter 7

Natural history observations on the germination and seedling stage of *Cassytha pubescens*

Cassytha pubescens seedling germinated in the lab.

Statement of Authorship

Title of Paper	Natural history observations on the germination and seedling stage of Cassytha pubescens
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Principal Author

Name of Principal Author (Candidate)	Elizabeth C Maciunas		
Contribution to the Paper	Conducted all experimental work, analysis, prepared figures. Wrote the manuscript.		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	07/09/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	

Please cut and paste additional co-author panels here as required.

A note to the examiners

This chapter is presented in an unusual format; a short narrative text with extensive supporting methodologies and limited results following as an appendix. I have chosen to present it in this style as an homage to John McLuckie, who's 1924 paper was, until this thesis, the only overview of the ecology of this species. The following chapter includes data from experiments that were limited by circumstance (for example limited seed germination and later seedling survivorship) and field observations that still contribute greatly to our knowledge of this species, but are unfortunately limited in statistical power. The information here is most certainly useful to anyone who intends to examine the ecology of this species in the future, and is hence worthy of inclusion in this thesis.

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Chapter 7: Natural history observations on the germination and seedling stage of *Cassytha pubescens*

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Introduction

Parasitic plants are a diverse group in terms of physiology, morphology and taxonomy, and as a consequence of adaptations to their unique strategy, many show patterns of growth and development very different from those seen in non-parasite plants (Musselman et al., 1995). Germination and seedling ecology is one such area, with many parasites requiring very specific environmental conditions for germination, and subsequent establishment, to occur (Boone et al., 1995). In some root parasites such as *Orobanche* and *Striga*, root exudates of particular species induce germination (Logan & Stewart, 1992; Mohamed et al., 1998; Musselman et al., 1995; Musselman, 1980; Worsham, 1987). The strength of this trigger is enough that this can even be used to induce suicidal germination in agricultural pests (Jamil et al., 2021; Mwakha et al., 2020). In contrast, in other groups, such as mistletoes, germination occurs readily post-dispersal but the parasite is restricted by its seedling ecology; whereby establishment only occurs on a compatible host-species (Van, 1993).

In obligate parasites there are often extended periods of seed dormancy in addition to the requirement for specific germination triggers (Logan & Stewart, 1992). For example, an after-ripening period is a common form of physiological dormancy (PD), whereby the embryo must undergo a further period of maturation after dispersal before it can respond to germination stimuli (Finch-Savage & Leubner-Metzger, 2006). PD is common in root parasites and is known to occur in Orobancaeae, primarily in the genera *Striga* and *Orobanche* (Logan & Stewart, 1992; Mohamed et al., 1998; Musselman et al., 1995; Musselman, 1980; Worsham, 1987). Another strategy to delay germination is Physical Dormancy (PY), whereby the cellular anatomy of the seed coat prevents germination triggers such as water from penetrating the seed coat, and therefore delays development of the embryo (Baskin et al., 2000; Finch-Savage & Leubner-Metzger, 2006). PY is known from parasitic genera, including both *Cuscuta* (Convolvulaceae) (Baskin et al., 2000) and *Cassytha* (Lauraceae) (Mahadevan & Jayasuriya, 2013) and has been recorded in at least 16 other non-parasitic families (Baskin et al., 2000; Jaganathan et al., 2016). The combination of these two strategies can lead to complex germination requirements for some species, and for plants that require strict conditions for seedling success this may provide an evolutionary advantage. Parasitic plants are one such group, and how germination cues from both hosts and the environment combine with breaking of any physiological or physical dormancy is little-known.

Cassytha (Lauraceae) is a genus of twining hemiparasitic vines, with a center of diversity in Australia. Of the 23 species, 16 are endemic to Australia and all show similar growth form; that is elongation and coiling of the main and lateral stems (rather than tendrils). There is significant variation in the ecology of species within the genus, with species with a larger stem diameter parasitising generally larger host species (Weber, 1981). *C. pubescens* is a medium-sized species, parasitising primarily shrubs, and has a wide distribution throughout south eastern Australia (Maciunas et al., 2022). Its distribution throughout a wide variety of habitats and climates make it an interesting species to study, particularly as its complex life-history it relies on climatic, habitat and host conditions that must vary substantially throughout its range. Much can no doubt be learned from observations of its habit, and by comparison to other parasitic groups. However, with the exception of McLuckie (1924) and some of the work of Tsang (2010), little is known about the germination and seedling biology of this species. This information is critical as this species has been proposed, and successfully trialled, as a biological control agent for ecological weeds (Cirocco et al., 2018; Cirocco, et al., 2021; Cirocco et al., 2022a).

Ecology and parasitic habit of *C. pubescens*

The mechanisms by which parasitic plants forage for a host vary, with some showing no foraging behaviour. An example of this are most mistletoes, which germinate shortly after dispersal, regardless of the host species they have come into contact with. Survivorship is then determined by the suitability of that host species, whereas many root parasites such as *Orobanchae* (Orobanchaceae) germinate only in the presence of root exudates that indicate a suitable host is present nearby (Kuijt, 1969). The first strategy may be advantageous in terms of ease of germination, but is highly limiting for survival of seedlings due to chance factors involved in seed dispersal. However, although the latter increases the chance of a seedling to find a suitable host, it may also cause those seedlings to undergo competition from not only the host plant's root system (prior to establishment) but also from other seedlings germinating in close proximity. The benefits and trade-offs of different strategies for many other parasitic species are largely unknown (Kuijt, 1969), and are an interesting avenue for future research.

The first step in understanding potential foraging behaviours in any parasitic species, is to understand the breadth of the range of host species that the parasite can utilise. To determine this for *C. pubescens*, a combination of literature searches and reviewing herbarium records were used (Tables 1 and 2). The generalist nature of this parasite allows it to grow on host species both native to, and introduced to, its range, and with a minimum of 107 host species from 60 genera recorded overall there is clearly considerable amplitude in what species can be used as a host. Indeed, such is the diversity of taxa that make up the host pool of this parasite it is almost easier to assume that any species may act as a host, until shown otherwise. *Acacia myrtifolia* is one such host, with a study by Facelli et al. (2020) indicating that although *C. pubescens* may form haustoria upon it they are not functional. This, and studies of performance on native and introduced hosts (see particularly the work of Cirocco, 2016-2022; Facelli et al., 2020; Prider et al., 2008; Prider et al., 2009; Tsang, 2010), suggests that there may be variation in how conducive various hosts are to parasite performance.

Figure 1: Examples of diverse hosts for *C. pubescens*. Top *Rubus fruticosus* (left), *Acacia pycnantha* (right), bottom *Acacia paradoxa* (left) and *Goodenia ovata* (right).

The generalist nature of adult *C. pubescens* seems to apply to the seedling stage also, however, some results do indicate that vigour and growth of seedlings on some types of host structure (for example graminoids) can be limited (see Chapters 4 and 5). In natural vegetation, such limitation is easily overcome by the scrambling habit of the parasite, with multiple hosts able to be infected by a single individual. In the case of *A. myrtifolia,* this may explain why *C. pubescens* can be forming haustoria on it that have been found to be non-functional, yet the parasite is still seemingly healthy. Connections to other hosts may be enough to sustain adult parasites, but the long-term effects of poorer-quality hosts at the point of seedling establishment is still yet to be determined.

Germination and seed dormancy

As *C. pubescens* is a vine with no underground parts from which to regenerate, fire is a major disturbance for this species (Tsang, 2010; Weber, 1981; see also Chapter 4). Fire events will result in the complete removal of not only all adult plants in the area, but also a delay before this species can recover from the seed bank. As an obligate parasite, no new individuals can establish in a burned area until the surrounding vegetation has recovered to a point at which it can host the parasite. From the germination trials carried out by Tsang (2010), it is known that seeds respond to heat treatments, however, in the laboratory *C. pubescens* has shown a high germination rate but poor establishment success. In many cases, seedlings suffer a high mortality prior to establishment on hosts in the laboratory (see Chapter 5).

The variability in germination from seeds of the same individual is seen as a bet-hedging strategy in many species (Clauss & Venable, 2000; Silvertown, 1984), and in *C. pubescens* this may have an important influence on establishment success.

In contrast to non-parasites, obligate parasitic plants require an additional resource, which is a suitable host plant, in order to successfully establish. Not only is the host species important, the individual must also be robust enough to support the new parasite. As such, a delay in germination or a wide temporal spread of germination may therefore provide the best chance for a parasitic seedling to encounter such a host, particularly if that host is also recovering from a disturbance such as fire. To test this, a series of treatments were used on *C. pubescens* seed (for methods and results, see Appendix B; unless stated otherwise seed dormancy was broken by immersing seed in boiling water for 1 minute). We found that there was substantial intraspecific variation in seed response to germination stimuli, with the total overall germination fraction 51.6% for one collection, yet only 11.6% for the other. For both collections, germination was staggered across a period of about ten weeks. However, there was also a substantial difference between collections in terms of the time taken from treatment to germination, with the 2011 collection taking just 7 weeks to begin germinating once treated and the 2012 cohort taking 16. Both collections were taken from different source plants and were stored for different lengths of time before treatment, leading to questions regarding potential physiological dormancy (particularly after-ripening dormancy). The presence of physiological constraints on germination makes adaptive sense in a species with such specific resource requirements, and physiological dormancy (PD) has been known from a wide variety of plants for a long time (eg Shull, 1923). After such variable germination in the first experiment, a second study was undertaken with a single collection (2013) and used to investigate PD. We found here that the freshest treated seed did show a greater lag in time from treatment to first germination than did older seed, even with an increase in daytime temperature. The fresher seed also took longer to reach peak germination, showing a wider temporal spread of germination overall. However, there was no significant effect on total overall germination percentage (Figure 2, Appendix B). As seedlings were assessed for germination alone, it is unknown if this delay would pose any advantage to fresher seeds regarding establishment, with the presence of more established hosts for seedlings that emerge later. The presence of Physical Dormancy (PY) has also been confirmed within the *Cassytha* genus (Mahadevan & Jayasuriya, 2013) and it is probable that the heat treatment developed by Tsang (2010) allows germination by breaking that physical dormancy and allowing water imbibition. This, combined with the results from experiments on seasonal variability and PD show that germination cues are highly complex, and further investigation into the dormancy of *C. pubescens* is needed.

In addition to a slightly longer delay in germination for fresher seed, the results from the PD experiment indicate that colder temperatures and winter day lengths may be required to stimulate seeds and assist with breaking dormancy. Considering that seeds germinated 6 weeks after the day length and temperature were increased, an overwintering period may be the missing factor for this experiment. Hence, it would be reasonable to re-examine the results of Tsang (2010) with regard to cold stratification, as the temperatures used in this experiment were milder that those used in the previous study. Using a very cold and dark treatment (where seeds were incubated at 5^{o}C in complete darkness for 6 weeks) resulted in a reduction in germination for heat-treated seeds in this species. It is possible, however, that using a milder temperature (such as the one used in this study) is a more realistic model for seeds overwintering within a soil seed bank throughout the range of this species.

Due to the failure of germination in Experiment 3 (A)-(E), (Appendix B) further work is also required to isolate the effects of various environmental factors on the germination of *C. pubescens*. The effects of smoke, drought, light and temperature need to be re-examined, as field observations demonstrate that *C. pubescens* does emerge seasonally (in spring) after fire and the importance of each of these factors in breaking dormancy is still unknown.

Seedling ecology, and the effect of light, nutrients and water limitation

A study into the effects of light and parasitism by *C. pubescens* on *Leptospermum myrsinoides* (Cirocco et al., 2015) found that *C. pubescens* had less growth and a lower photosynthetic capacity under low-light conditions. Any strong trend shown in adult plants is worth comparing to seedlings responses, as in addition to the effect of light, the inability to survive long periods without a host makes the seedlings particularly vulnerable to other environmental stresses such as drought and nutrient limitation. The root in this species is short lived, greatly reduced, and anatomically simple (McLuckie, 1924), raising questions regarding its functionality. A limited ability to explore the soil have strong effects on the seedling's growth, as soil resources may become quickly exhausted in proximity to that root. How interactions with roots of other individuals (either siblings or the host plants themselves) may influence the acquisition of these critical resources by seedlings is another avenue most certainly worth exploring, with intriguing results from a small scale survivorship study indicating competitive effects do exist in this species (see Chapter 4).

To examine the effects of abiotic stressors the seedlings may encounter in the field, 3 short experiments were conducted in the glasshouse with newly germinated seedlings (see Appendix C). Although too few seedlings established and survived in the light experiment to determine any significant light effects, there was a trend towards better seedling establishment under higher light conditions. This agrees with experiments conducted on adult *C. pubescens*, where light was found to have a significant effect on growth and photosynthesis of this parasite (Cirocco & Waterman 2015). It would be expected that the effect seen in adult plants would also be seen in seedlings, and that light levels would therefore have a large influence on successful seedling establishment. It is worth noting that after fires seedlings appear to emerge *en masse* close to the previous location of adult plants, often directly under the canopy of the new host (see Chapter 4), and therefore light may be limited by direct shading from the host. This suggests a complex balance between sufficient proximity to the host to reach it and attach to it, and the ability to acquire other critical resources during a sensitive life stage.

Nutrient availability can strongly influence seedling success across many species. In *C. pubescens*, it is unknown how functional the root may be, with the short root system having a limited lifespan. The growth of this species on nitrogen-fixing hosts is well documented (eg Cirocco et al., 2017; Prider et al., 2008; Prider et al., 2009; Shen et al., 2010), and in many cases these hosts have been shown to be highly beneficial for the parasite. As nutrition at a seedling stage may affect development and growth of the seedling for years to come, and the results of this experiment were inconclusive, the functionality of the root still needs to be assessed. When the seedling attaches itself securely to a host plant the short fleshy root senesces, and the parasite becomes dependent on that host for survival. However, the root can only support the seedling for a limited time and the seedling seems unable to continue seeking for a host after about 16 weeks, resulting in eventual death after approximately 20 weeks (Pers. obs). It should be noted that this is double the lifetime of free living seedlings reported by McLuckie (1924).

Most stem parasites (e.g. mistletoes) do not grow roots in the soil before infecting their host. However due to this life history strategy, *C. pubescens* may be subjected to below-ground interactions with other species through processes such as root competition. In the root competition pot experiment, seedlings not subjected to root competition were more likely to establish on a host plant than those that did encounter root competition from the host. Whether this competition is primarily for soil nutrients or water, or whether the host roots exude enzymes or any other compounds, or harbour microbes that may hinder parasite development is unknown. Additionally, the strength of the effects of root competition may explain the results from the light and nutrient experiments, with the interaction of the host root and that of the parasite probably more important in small pots with large host plants.

The effect of root competition in the glasshouse in this case is no doubt greater than that seen in the field, with potted plants showing rapid top soil drying (despite regular watering), possibly due to a lack of litter layer and shading from the overstory. In the field seedlings typically emerge close to existing plants, or where extensive vegetation was present pre-fire, since they often emerge close to resprouting and newly germinated host plants. Adult plants have been observed to suffer from water stress more quickly than their hosts (Cirocco et al., 2022a), and are also known to show better growth under higher light conditions {Cirocco et al., 2016). Based on these results it is reasonable to suggest that seedlings show similar sensitivities, and that there is an optimum distance from host plants that may give them the best chance of establishment. This occurs somewhere between the trade-off of over-shading and root-competitive positions underneath the new host plant, and an open space far from hosts with available light and no root competition.

Observations of roots and a search for mycorrhizae

Resource limitation can have immediate impacts on establishment patterns in seedlings, as well as lasting effects on population structure within plant communities. In many plant species, a mutualistic relationship with mycorrhizal fungi can provide a competitive advantage and allow faster and more successful establishment (Bennett & Groten, 2022). This is particularly true for nutrient-poor environments, such as the dry sclerophyll forests of south eastern Australia. However, the benefits gained from mycorrhizal associations, from an evolutionary perspective, must outstrip the costs of maintaining them (Smith et al., 2009). In many species this benefit is clear-cut, but in species with complex life histories, such as parasitic plants, it is less so. Indeed, most parasitic plants are assumed to be non-mycorrhizal (Brundrett, 2009), particularly those that are holoparasitic. Although *C. pubescens* is a hemiparasite, that is it has substantial photosynthetic capabilities and relies on its host for water and associated nutrients, it also has a limited capacity to photosynthesise and needs to attach to a host plant as quickly as possible. The root system in this species is greatly reduced, appears to have limited functionality, and once the plant establishes on a host it dies completely (McLuckie, 1924). For such a critical life stage, it appears that the root has a very limited capacity to support the parasite on its own for any extended period. In addition to this, seedlings of *C. pubescens* appear to be affected strongly by both inter- and intraspecific competition (see above, and Chapters 4 and 5).

The limitations of the root in *C. pubescens* and the strong effects of competition on seedling survivorship present an interesting question; would the potential benefit from a mycorrhizal association outweigh the cost of spending the limited resources the seedling has in order to secure a host quickly? Research has shown that ectomycorrhizal associations may allow seedlings to tap into resources that other plants are accessing, and this association can be particularly important for the distribution of water (Bingham & Simard, 2011). The use of a common mycelial network has the potential to counter competitive effects and facilitate establishment in some species (Booth & Hoeksema, 2010), and in *C. pubescens*, it may reduce effective competition from their hosts. In a species where the root is a transient organ, a true mycorrhizal association may not be worth the investment. However, if the plant can effectively parasitise the relationship between other species and their mycorrhizae, a substantial advantage may be gained. Parasitism of mycorrhizae is known in other plants, namely orchids (Rasmussen & Rasmussen, 2014), and given the parasitic nature of adult *C. pubescens* this is an interesting relationship to explore.

In order to test for any mycorrhizal relationship, samples from the glasshouse and field collected seedlings were examined for the presence of fungal hyphae. Interestingly, no seedlings from any sample showed any indication of being infected with mycorrhizae, regardless of the conditions under which they were grown. It should be noted that Lauraceae are known to be mycorrhizal (eg Chen et al., 2021; Song et al., 2020; Songachan et al., 2011), therefore the root alone must be sufficient to provide the nutritional resources the seedling needs for rapid growth over short periods. It is interesting that even with potential benefits from connection to a common mycelial network no infection was found, indicating that at some point the parasitic seedlings have sacrificed the ability to take advantage of any mycorrhizal association.

Overall conclusions

The host range, germination requirements and ecology of *C. pubescens* seedlings are complex and diverse. As this species is found across such a broad geographic area, such diversity is not unexpected as the habitats available across that range differ substantially. How different germination factors combine, for example different climate and fire regimes, are important questions that remain to be tested. However, the results presented here indicate there may be complex interactions between factors that do ultimately affect seedling establishment (for example the success in germination for the after ripening physiological dormancy experiment only occurring post-cold stratification). We now understand the importance of the after-ripening period for this species, and other types of dormancy in *C. pubescens* should also be investigated. A lack of assistance from mycorrhizal associations implies the seedling has very limited resources with which to establish on a new host plant, increasing the importance of understanding the competitive effects that have been observed in the field. Overall this fascinating species shows great complexity in its ecology, much of which remains to be unravelled.

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Appendix A: Host range study

To create a comprehensive host-species list for *C. pubescens* (Table 1), Herbarium records were searched and a review of published literature was carried out in February/March 2023. Australian Virtual Herbarium records (1067) were manually checked for host information, as this information is recorded in different fields depending on the record. Overall 165 host-entries were found, indicating that for parasitic plants a systematic way of recording host-species information is lacking and would be a useful addition to collections.

A literature review of all known *Cassytha pubescens* publications was also undertaken to include hosts both used in glasshouse experiments, as well as observed in the field.

Table 1: Host species for *C. pubescens.* Herbarium collections contained within the Australian Virtual Herbarium database. Minimum species is a conservative estimate, not including any records listed as sp., as that may be a duplicate of a listed species**.**

Family	Genus	Min. species	Number of records
Apocynaceae	*Alyxia*	1	1
Asphodelaceae	*Xanthorrhoea*	1	1
Asteraceae	*Cassinia*	1	1
	Chrysocephalum	1	1
	Olearia	1	1
Casuarinaceae	*Allocasuarina*	4	8
	Casuarina	9	13
Cupressaceae	*Callitris*	1	1
Cyperaceae	*Lepidosperma*	1	1

Dilleniaceae	*Hibbertia*	1	1
Ericaceae	*Acrotriche*	2	2
	Brachyloma	3	4
	Epacris	1	1
	Leucopogon	1	2
	Monotoca	1	1
Euphorbiaceae	*Ricinocarpos*	1	1
Fabaceae	*Acacia*	12	25
	Daviesia	3	3
	Dillwynia	1	1
	Hovea	1	1
	Indigofera	1	1
	Phyllota	1	1
	Platylobium	1	2
	Pultenaea	2	2
	Genista	1	1
	Ulex	1	7
Lamiaceae	*Westringia*	1	1
Lauraceae	*Cryptocarya*	1	1
Loranthaceae	*Amyema*	1	1
Myrtaceae	*Baeckea*	1	1
	Callistemon	1	1
	Eucalyptus	4	9
	Kunzea	2	3

	Leptospermum	7	25
	Lysicarpus	1	2
	Melaleuca	6	18
Picrodendraceae	*Petalostigma*	1	2
Pittosporaceae	*Bursaria*	1	1
	Pittosporum	1	2
Poaceae	*Spinifex*	1	2
Proteaceae	*Banksia*	5	5
	Grevillea	1	4
	Hakea	1	2
	Lambertia	1	1
Ranunculaceae	*Clematis*	1	1
Rhamnaceae	*Pomaderris*	1	1
	Spyridium	1	1
Rosaceae	*Cotoneaster*	1	1
	Rubus	1	4
Rubiaceae	*Coprosma*	1	1
Rutaceae	*Boronia*	1	1
	Correa	1	1
Santalaceae	*Exocarpus*	1	2
Sapindaceae	*Dodonaea*	1	4
Thymelaeaceae	*Pimelea*	1	1

Table 2: All host species for *C. pubescens* known from published literature. Note some studies include field observations, however the majority cover hosts used for glasshouse experiments.

Family	Species	Citations
Fabaceae	*Acacia myrtifolia*	Facelli et al., 2020; Tsang, 2010
	Acacia paradoxa	Cirocco, 2016; Cirocco et al., 2021; Cirocco et al., 2017; O'Connor d'Arlach Espinoza, 2021; Těšitel et al., 2020
	Acacia pycnantha	Cirocco et al., 2022b; O'Connor d'Arlach Espinoza, 2021
	Cytisus scoparius	Facelli et al., 2020; O'Connor d'Arlach Espinoza, 2021; Prider et al., 2009; Prider et al., 2011; Těšitel et al., 2020
	Ulex europaeus	Britton, 2002; Cirocco, 2016; Cirocco et al., 2016; Cirocco et al., 2017, 2018, 2020; Cirocco et al., 2021; Facelli et al., 2020; Těšitel et al., 2020
Malvaceae	*Pavonia praemorsa*	Heide-Jørgensen, 2013
Myrtaceae	*Leptospermum myrsinoides*	Cirocco, 2016; Cirocco et al., 2016; Cirocco et al., 2015; Facelli et al., 2020; Prider et al., 2009; Těšitel et al., 2020
Pittosporaceae	*Bursaria spinosa*	O'Connor d'Arlach Espinoza, 2021; Cirocco, 2022b
Rosaceae	*Rubus anglocandicans*	Cirocco et al., 2022a, 2022b
Sapindaceae	*Dodonaea viscosa*	O'Connor d'Arlach Espinoza, 2021

Appendix B: Seed germination trials

Methodology

Fruits were collected from several localities throughout the Mount Lofty Ranges in various years (Table 3), with fruit from multiple individuals pooled together . For all following experiments, seeds were maintained in a germination cabinet (model treil-200-1-SD; Thermoline scientific, Australia) and kept moist until either germination occurred, or 20 weeks had elapsed, whichever occurred sooner. If germination occurred, the experiment was concluded when there were 4 consecutive weeks with no further germination. If no germination occurred, in some cases seeds were re-treated and left for up to another 20 weeks. Each treatment consisted of 3 petri dishes, each with 20 seeds taken as a sub-sample from the relevant collection (unless stated otherwise).

Table 3: Collection locations and collection sizes for *Cassytha pubescens* seeds**.**

Collection	Location	Date	Total
MO-11	Mark Oliphant Conservation Park	18/01/2011	406
IB-11	Ironbank Road	27/01/2011	662
MO-12	Mark Oliphant Conservation Park	29/12/2011	449
IB-12	Ironbank Road	30/12/2011	701
MO-13	Mark Oliphant Conservation Park	23/12/2012	405
LW-13	Longwood Road	12/01/2013	500

For all collections, whole fruits were removed from the field and were semi-dried by leaving them in the sun for several days. Seeds were then extracted, and stored in a dark, cool environment until pre-treatment. All seeds were pre-treated by boiling them in water for 1 minute, following Tsang (2010), unless stated otherwise. Seeds were incubated in a germination cabinet on a bed of sandy loam, checked every 2-3 days and watered with 4mL RO water. Germination was counted as successful if the radicle emerged from the seed coat.

As there was no difference in any experiment in germination between sites, collections are from hereon combined into total 2011, 2012 and 2013 collections.

Assessment of germination success across two years

Following pre-treatment as described above, seeds from the 2011 and the 2012 collections were incubated at 16^{o}C/22^{o}C, 16/8 hrs darkness/light beginning in mid-February 2012. All seeds were stored under the same cool, dry conditions between collection and treatment, with the 2011 collection stored for 1 year longer than the 2012 collection.

Testing for after-ripening physiological dormancy

Seeds from 2013 were pre-treated in batches at 3-days, 2, 4, or 6 weeks post-collection, to test for potential post-ripening dormancy (PRD). Seeds were incubated for a total of 18 weeks at 16^{o}C /22^{o}C, 16/8 hrs darkness/light.

As no germination occurred within those 18 weeks, the temp/day length was then increased to 18^{o}C /24^{o}C, 12/12 hrs darkness/light for a period of 6 more weeks.

Five short experiments testing the influence of abiotic, biotic and seasonal factors on germination success

Seed from the 2013 collection was used to assess the effects of several abiotic, biotic and seasonal factors on germination success. With the exception of experiment A, the standard boiling water pre-treatment of Tsang (2010) was used on all seeds. All seeds were maintained on a bed of sandy loam, checked every 2-3 days and watered with 4mL RO water, except for droughting treatments. With the exception of experiment E, all seeds were incubated at 18^{o}C /24^{o}C, 12/12 hrs darkness/light.

A. Comparison of the effects of heat and smoke water on germination

A total of seven treatments were used, all smoke water treatments were a concentration of 5% and seeds were kept submerged for 24 hours post-treatment. The first treatment (control for established method) was included to determine a baseline germination fraction, rather than as a direct comparison to treatments.

(1) control for the established pre-treatment method (boiling water for 1 minute, cooling to room temperature on moist paper towel),

(2) dry heat then steeping in room temperature water for one day,

(3) dry heat then steeping in room temperature smoke water for 1 day.

(4) boiling smoke water for one minute, then steeping in smoke water for 1 day,

(5) boiling smoke water for one minute, then steeping in room temperature water for one day,

(6) steeping seed in room temperature smoke water for 1 day,

(7) steeping seed in room temperature water for 1 day

B. Effect of droughting on germination

As germination occurs in late spring in the field, three treatments were used to test the effect of dry conditions on germination. (1) A control, where seeds were kept under wet conditions at all times, (2) One wet/dry cycle where seeds were kept wet for 1 week, allowed to dry out completely and maintained dry for a week, then wet again and kept wet until any germination occurred, and (3) two wet/dry cycles, treated as for 1 cycle, but with two drying cycles, keeping seeds wet for 2 weeks between dry cycles.

C. Seed age

A total of seven treatments were used to examine the effect of the age of seeds on germination. Four lots of ‘aged’ seeds with various times of storage were used, collected by H.T. Tsang on or around 01/12/04, 07/01/07, 08/11/07, 29/12/11, at unknown locations. One treatment of seeds from the 2013 collection was also included, as well as one treatment where seeds from the 2012 had been treated to break dormancy but had failed to germinate. One specific control for the re-treatment was also used, whereby previously pre-treated seeds were kept wet for the duration of the experiment but not subjected to another boiling water pre-treatment. All “aged” seeds had been stored in a cool, dry environment since collection.

Due to limited seed availability, 3 replicates with 10 seeds each were used for this experiment.

D. Seed size

The diameters of seeds from the 2012 collection were measured longitudinally, and the collection divided into 6 size classes. Due to limited seed, 3 replicates of 10 seeds were used for each size class in this experiment.

The six size classes were defined as follows: <3mm; 3-3.2; 3.2-3.4; 3.4-3.6; 3.6-3.8; >3.8mm

E. Testing the effect of seasonal conditions on germination

Pre-treated and not pre-treated seeds from the 2012 collection were subjected to one of 2 sets of conditions; 2 or 4 weeks of winter day length and temperatures (16°C /22°C, 16/8 hrs darkness/light) then warming to spring/summer day length & temperatures (18°C /24°C, 12/12 hrs darkness/light).

Results

Assessment of germination success across two years

Seeds from the 2011 collection germinated after 7 weeks, with an overall germination success of 51.6%. However, seeds from the subsequent 2012 collection germinated after 16 weeks, and had an overall germination success of only 11.6%.

Testing for post-ripening dormancy

No seeds germinated within the expected 8 weeks, however seeds were left in the germination cabinet under the same light and heat for approximately 10 more weeks before the day length and temperature were increased to 12 hrs/24°C night 12hrs 18°C. Six weeks after this, germination occurred in seeds across all 4 treatments.

There was no difference in the overall number of seeds germinating between fresh and stored seed, however treatments using fresh seeds took on average 4 weeks longer to show significant germination success than seeds that had been stored for short periods of time (Figure 2).

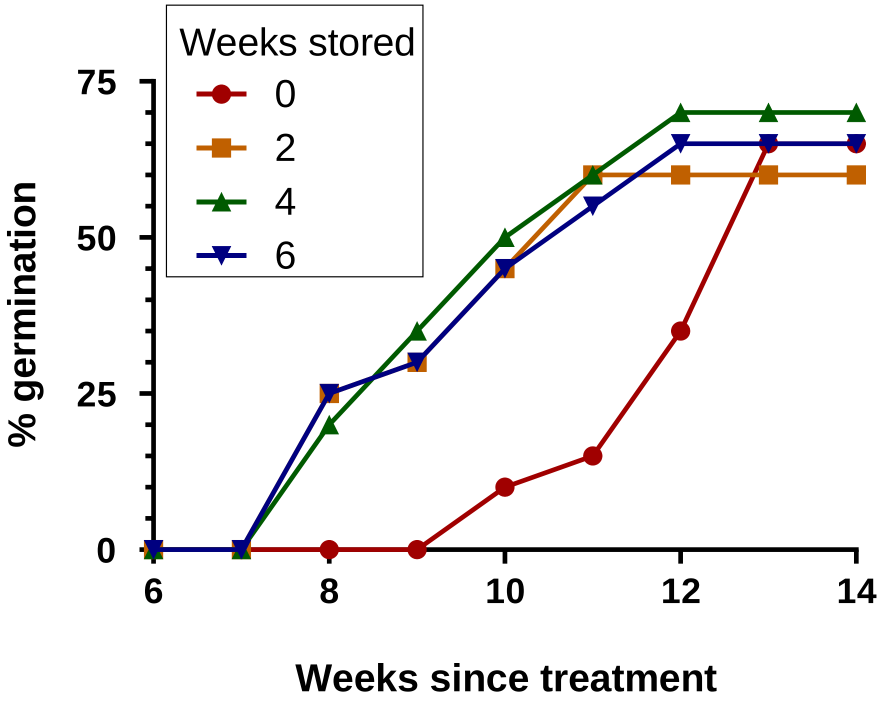

Figure 2: The effect of storage time (in weeks) on cumulative germination of *Cassytha pubescens* seeds. Seeds in the 0 week treatment were pre-treated (see Methods for description) within 3 days of collection, remaining seeds were kept in cool dark conditions until pre-treated at 2, 4, and 6 weeks post-collection**.**

Influence of abiotic, biotic and seasonal factors on germination success

For unknown reasons, no seeds from experiments A to E germinated.

Appendix C: Seedling ecology

Methodology

To achieve germination, seed pre-treatment and germination conditions were as described in Appendix B. All experiments used seed from the 2012 collection.

For all the following experiments, except where stated otherwise, once host plants were established the *C. pubescens* seedlings were introduced to the pots approximately 2-3cm from the base of each host plant. According to McLuckie (1924), the lifespan of *C. pubescens* seedlings can be as short as 8-10 weeks unless they attach to a suitable host. Therefore, germinating seeds were moved into the pots with hosts (planted at 3 cm depth) every 2 weeks over a period of 8 weeks until all pots in each treatment had seedlings present.

Establishment of *C. pubescens* seedlings was recorded as successful if the seedling survived for at least two weeks after root senescence. Mortality of seedlings that had coiled around hosts during that time was also recorded.

Light availability

Twenty *Acacia paradoxa* seedlings were grown in 2L pots as host plants. Ten were placed in full sun, and ten kept under a shade cloth structure (35% light penetration) to impose a shade treatment. Successful establishment, survival and photosynthetic ability (using a minipam flurometer) were recorded and compared between high-and low-light treatments.

Nutrient availability

Twenty *Acacia paradoxa* seedlings were grown in 2L pots as host plants. Ten were watered only with RO water, and ten were watered weekly with a standard liquid fertilizer (Nitrosol, Rural Research Ltd, Auckland, NZ; N : P : K 8 : 3 : 6). Successful establishment, survival and photosynthetic ability (using a minipam flurometer) were recorded and compared between high-and low-N treatments

Root competition

Forty rectangular 1L pots were paired and joined together. Ten pairs had a 2.5x6 cm hole cut in one side, and these were paired to create a gap to allow movement of roots from one side to the other. Twenty *Acacia* were grown as host plants, ten in pots that were joined, ten were maintained separately. All *C. pubescens* seedlings used in this experiment were transplanted into the pot without the host, in order to maintain a ‘no root competition’ control. Establishment and survival were recorded for all *C. pubescens* seedlings.

Results

Light & Nutrient availability, Root Competition

For unknown reasons, the light and nutrient availability experiments showed little seedling survival or establishment. Of the 40 seedlings used across the two experiments, only 6 established successfully. 3 of these were from the high light treatment, one from low light and one from the low nutrient treatment. The root competition experiment had higher establishment, with 4 of the 10 seedlings in the ‘no root competition’ treatment establishing, and 2 in the ‘root competition’ treatment successfully establishing. Additional observations of seedlings within these experiments are as follows:

(1) While under low light conditions, seedlings appeared to elongate substantially more than under high light, however even when presented with a host fail then to coil on it.

(2) Nutrient availability did not have any effect on either in survival or elongation. Host plant health did also not appear to differ between treatments.

(3) Conjoined pots in the root competition experiment were much drier than those kept separate, and *C. pubescens* seedlings appeared to show water stress well before any sign could be detected in host plants.

Appendix D: Assessment of mycorrhizal associations

Methodology

The roots of 15 lab-germinated seedlings (2010 collection) and of 15 seedlings collected from the field (S 35^o 01.196; E 138^o 41.840) were measured with digital callipers. All lab-grown seedlings used for root studies had been growing for a minimum of 14 weeks, to ensure maximum root-length had been achieved prior to harvesting.

In order to examine the surface-features, such as root-hairs, and to look for any mycorrhizal associations, roots were carefully cut from the shoots using a razor blade. They were then cleared and stained using acidified Schaffer black ink, following the protocol of (Vierheilig et al., 1998).

In addition, some roots from a native host, *Leptospermum myrsinoides*, were collected from the same field location and laboratory material, and cleared and examined for the presence of mycorrhizae.

Results

The root system of seedlings of *C. pubescens* is very small with a short primary root (length 0.5mm ±0.1mm), and two lateral roots (length of 6.8-8.1mm ±1.1mm), although some seedlings showed additional aborted lateral roots. As reported in McLuckie (1924), large unicellular hairs develop from the base of the primary root along the lateral roots to the growing tips, becoming progressively shorter and wider towards the apex.

There were no mycorrhizae found in association with *C. pubescens* roots from either field or lab-germinated samples. However *Leptospermum myrsinoides* did show mycorrhizal associations, indicating the protocol used was effective.

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Chapter 8

General discussion

Adult *C. pubescens* attempting to parasitise *Acacia*. This is one genus that has been shown to have resistance to infection

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Introduction

Until this research was conducted, the only study outlining any elements of the biology of *Cassytha pubescens* was McLuckie (1924). With this project, I aimed to create a much more in-depth knowledge and updated information regarding many elements of the ecology of the parasite, from most parts of its life cycle. The new knowledge presented here has implications not only for the management of this species, but also for those plants and animals that interact with it at a broader ecosystem level. At times, choosing a structure for this thesis has been difficult; the concepts underlying all chapters are interconnected and together form a jigsaw puzzle that, although not quite complete, is certainly starting to outline the full picture. In this discussion I generally present ideas in the order in which they have appeared previously, however there is certainly some crossover between earlier and later chapters that helps demonstrate the complexities of the ecology of this species as a whole.

Dispersal

New knowledge

To begin with, an investigation of seed dispersal vectors helped me generate an understanding of this species at broader ecological level. From Chapter 2 we now know that in the Mount Lofty Ranges, *C. pubescens* acts as a food source for at least one of Australia's endangered mammals. The Southern Brown Bandicoot (*Isoodon obesulus*) is a fossorial marsupial, and is known to interact with low, dense habitat types such as those created by high densities of *C. pubescens*. Other studies have demonstrated that *Cassytha* species may be important for providing not only a food source, but also physical habitat structure for small marsupials (Hardman & Moro, 2006; Lundie-Jenkins, 1993). Interestingly, the results in Chapter 6 indicate *C.*

pubescens also grows well on *Rubus fruticosus* (blackberry). This is important, as previous studies have also shown that *R. fruticosus* can form a critical, if novel, habitat type for *I. obesulus* (Bruce et al., 2022; Packer, 2014; Packer et al., 2016). As *C. pubescens* can gradually remove the invasive species from the landscape, it can maintain habitat structure and provide food sources for animals that depend on that habitat whilst controlling and removing the weed. This contrasts current management techniques such as slashing, which completely and suddenly change the habitat type and foods sources available, to the disadvantage of bandicoots. Therefore new knowledge that *C. pubescens* can both impact the growth of the invasive, together with it providing important habitat and food for the marsupial population, gives us more holistic management approaches that can be of benefit to all elements of the native ecosystem.

Future directions

In addition to the benefit *C. pubescens* provides *I. obesulus*, we must also consider the relationship from the other perspective. In order to disperse seed, the parasitic vine is likely reliant on a suite of species, however, with declines seen in woodland bird populations (Bennett & Watson, 2011; Ford et al., 2001) and extensive extinctions of small mammals since European settlement (Woinarski et al., 2015), the continued dispersal by *I. obesulus* may be of more importance now than a single species historically was. If there is indeed a decreasing number of seed dispersing species available to the plant, in an increasingly fragmentated landscape we would have to expect that dispersal is, over time, becoming more limited. Overall, it is important to consider other species in the ecosystem that may play a role in dispersing *C. pubescens*. In particular, species that are more common along with those with larger home ranges or more migratory habits (for example Emu) should be examined as potential dispersers. Species with wider ranges may be particularly important for longer-distance dispersal, and the maintenance of genetic diversity across landscapes. The other element needed to complete this picture are viability tests of seeds once they have passed through the gut of an animal. Captive populations of bandicoots were unavailable for feeding trials, and the scat samples were taken before this study was designed; all samples had been frozen or preserved in ethanol, hence viability tests were not possible. However, for future work on other potential dispersal species, germination experiments or simple cut tests would provide additional, valuable information regarding viability of seeds.

Population connectivity

New knowledge

Species such as *I. obesulus* have relatively small home ranges, and as such we would expect their effective dispersal distances for *C. pubescens* seeds to be small. The genetic data analysed in Chapter 3 supports this and has two important implications for dispersal and management of populations. Firstly, distinctive genetic clusters are seen within parks, which indicates that although there is seed movement within intact vegetation, mixing of populations through fragmented vegetation between national parks is limited. Secondly, within populations there can be extensive clonal reproduction occurring and whether this affects the long-term genetic diversity of those populations is unknown. Assuming the genetic diversity of the soil seed bank is greater than the genetic diversity in adult plants surviving in older populations, a logical model of population dynamics is that over time, some individuals within that population die and are replaced by clones of surviving individuals. The maintenance of populations occurs primarily by clonal reproduction; although this may be at the cost of genetic diversity. As time goes on, an increased numbers of clones within the population would increase the likelihood that selfing occurs, and subsequent input into the seed bank may reflect this decrease in genetic diversity. If dispersal is not particularly limited, transfer of seed between populations may help substantially in maintaining a diversity within the seed bank. However, we hypothesise that dispersal is now somewhat limited by habitat fragmentation, small home ranges of extant dispersal vectors and a reduced suite of species for dispersal of seeds (see Chapters 2 and 3). In addition, since European settlement fire regimes have changed substantially throughout the range of *C. pubescens* (Adeleye et al., 2022), and with longer time intervals between recruitment events as well as limited dispersal, it may now take many decades for changes that are currently occurring to the genetic structures of seed banks to become apparent in adult populations.

Future directions

Although *C. pubescens* can be seemingly locally common, it has a patchy distribution throughout its extensive range. Considering the differentiation between the populations studied here, questions should be raised regarding the taxonomy of the species as a whole. If populations within a 10 kilometre scale are showing clear genetic signatures, it stands to reason that populations from, for example, the Sydney region where McLuckie conducted his original research in 1924 and the populations studied here may in fact be reproductively isolated and hence could potentially be in fact separate species. This is supported anecdotally by the wide diversity seen in fruit colour, size and shape across its range (Weber, 1981), and although these could be plastic traits and naturally highly variable, a future study examining the species-level taxonomy of *C. pubescens* is still very desirable. It would be expected, based on these results, that the species is at the very least a group of diverging populations, if not a complex of cryptic species or diverging subspecies.

The other element of this aspect of my research that would benefit from further study are genetic diversity across a larger scale and the prevalence of clonal reproduction, particularly paired with an association with fire history. We now know that extensive clonal reproduction does occur naturally, and we would expect older populations to contain mainly clones and less genetic diversity. Unfortunately, all populations sampled within my study had the same fire history, but examining populations with different age structures would provide better insight into these dynamics. In addition, sampling populations both before and after fires and comparing the diversity of populations across time would help with our understanding of how the genetic structures of those populations change, as well as our understanding of the implications of these changes for future diversity.

Recovery from fire and seedling ecology

New knowledge

The results in Chapters 2 and 3 suggest dispersal may be increasingly limited for this species, and therefore we would expect to see higher seedling density in close proximity to the location of mother plants. In the long-term study monitoring seedling emergence after fire in Chapter 4, I found there were indeed high densities of seedlings emerging within 2 meters of where adult plants had occurred before the fire. However, while the results of this study are interesting they are unfortunately limited. Initial study sites were limited to just 2 places that both had *C. pubescens* present and were going to be subject to a controlled burn that spring. I had planned to repeat this experiment with both autumn and spring burns the following year, however, due to time constraints and the lack of results before autumn, this was abandoned in favour of the more targeted and smaller scale survivorship study. One of the 2 initial sites (Belair National Park) had only two *C. pubescens* individuals present before the fire, and follow-up surveys failed to find any seedlings emerging following the fire. For that site in particular, the results were not surprising; overall density of *C. pubescens* in the area is low and both plants present before the fire were small, suggesting they may not have been established long enough to contribute substantially to the soil seed bank. The second site in Mark Oliphant Conservation Park had significant quantities of *C. pubescens* both within the quadrat and the surrounding area, and that site therefore provided the data for the seedling density observations. Interestingly, there is a strong slope at this site, and the seed shadow of parent plants can be clearly seen to bias along that gradient. This is suggestive of small amounts of fruit and seed redistribution abiotically, however as discussed above with Chapter 3, the timeframes over which this might occur could be many decades. It is also further evidence of high quantities of fruit fall, and may explain how ground-foraging bandicoots can find fruits easily in the litter layer (see Chapter 2).

The hypothesis that seeds germinate in response to fire is a logical one, considering the complete removal of adult plants from areas that are burned. Nevertheless, until now no study has documented the recovery of *C. pubescens* specifically after a fire. As seeds subjected to heat treatments in the lab germinate after approximately 8 weeks, it was expected that after the fire seedlings should emerge in about the same timeframe. This was not the case, however, and I found a significant time-lag for the emergence of seedlings, leading to more questions regarding the conditions required to stimulate germination.

The technique used to germinate seeds in the laboratory applies both heat and water (submerging seeds in boiling water for 1 minute). This is an important difference from germination triggers in the field; fire provides dry heat, smoke and most often occurs in summer months when rainfall is also low. From my results, it appears seeds need a complex suite of conditions to promote germination in the field, with an 11-month delay in germination suggesting both water availability and temperature stratification may play a role in breaking dormancy. If we consider the complex ecology of parasitic plants, a delay in germination is a sound strategy. For most plants, seedling establishment is easily affected by the favourability of environmental conditions, and for *C. pubescens,* not only will climatic variation influence success, but also the availability of new host plants to infect. The Mount Lofty Ranges are characterised by wetter winters and dry summers, and most plants in the region recover from fire relatively quickly. Higher growth rates should correlate with higher water availability throughout winter and spring, so a delay in germination of the parasite until this boom in growth occurs in its hosts does make sense.

One draw-back of a delay in germination until the potential host plants are robust enough (and present in great enough density) to allow for more successful establishment, is potential competition with host plants for resources. From the study in Mark Oliphant Conservation Park, we now know seedlings emerge in high density, and from the more targeted survivorship study at Ironbank we know they also show greater survivorship when emerging nearer to a host. The strength of competition between siblings does not appear to affect their ability to find a host, but does affect survivorship. On top of this, although seedlings do not appear to exhibit any host selectivity, one species in particular (*Pultenea daphnoides*) did allow for the greatest establishment success. Based on observations in the field, as well as the literature review conducted in Chapter 7, it is clear that nitrogen-fixing legumes are well represented as hosts for this parasite, and it is possible that greater establishment success may be found on those species that provide high nutrient availability.

Whether the establishment of seedlings is being influenced by competition for water, nutrients or light is inconclusive, as is the extent to which competition with their hosts can affect survival. To examine the effect of host availability and diversity on establishment, a small glasshouse study was run concurrently with the field observations conducted at Ironbank (see Chapter 5). This experiment aimed to assess the effect of the presence of a more complex understory on establishment, however, I found that density of hosts and resulting competition was a larger driver of seedling success than what the individual host species were. This was initially surprising, although had this experiment been undertaken after the survivorship study at Ironbank it would perhaps have been designed to more clearly test host density and water availability, rather than host abundance and species. Overall, the results for this experiment clearly support the suggestion that competition for resources at the seedling stage strongly influences establishment success.

Future directions

The recovery of *C. pubescens* after a fire is a long process. Whilst seedlings may establish within 10 weeks after emergence, the length of time and seasonal conditions required for emergence makes any future studies logistically difficult. However, the results here suggest a much greater scope for exploring the complex interactions both between siblings and between seedlings and their hosts. The effect of water and nutrient limitations in particular should be further examined. In addition, although patterns of survivorship and factors that affect seedlings growth is now better known, long term development of populations and recovery post-fire is yet to be understood. For example, how long surviving seedlings need to mature, fruit and contribute back to the seed bank is important for understanding the minimal inter-fire interval this species requires. During the past decade I have had the opportunity to revisit the same site (not used for this PhD project) each year with an undergraduate class to collect data on fire recovery in native vegetation. Observations there suggest that it takes at least ten years for *C. pubescens* to produce any quantity of fruit after it recovers from fire (Pers obs.). It is likely that the timeframe required depends heavily on host availability and quality; the propensity of *C. pubescens* to grow on leguminous hosts, as well as the increased survivorship of seedlings on such hosts indicates this is an area worth exploring further.

Effects of seedlings on novel hosts

New knowledge

In addition to establishing whether the novel host, *Rubus fruticosus* was suitable for seedling establishment, I also asked what effect growing the parasite may have on that host species. At the time, no study had yet examined the effects of the parasite on any invasive species other than Gorse (*Ulex europaeus*) or Scotch Broom (*Cytisus scoparius*). We now know that the seedlings of *C. pubescens* can indeed utilise novel hosts for establishment, and that the resource sink they create can affect the growth of that host.

Rubus fruticosus is an interesting host to consider, as it has a substantially modular structure; long branches grow from a central point, and such structure lends it to having an infection along some individual stems, but not others. This allowed the differential impact of direct infection to be compared across infected branches on infected plants, uninfected branches on infected plants, and completely uninfected plants. Further work by Cirrocco (2022a; 2022b) since the completion of data collection for this study has shown the effects on *R. fruticosus* extend to field plants, however, the resource removal by the parasite resulted in unique responses here. In contrast to that study, I found no significant results for any physiological measurements, although host biomass was substantially impacted by infection. The physiological changes may have been small, incremental and over a long period of time, resulting in a cumulative effect that only became visible in the biomass data. The contrast between this study and the results of Cirocco et al., (2022b) may well be due to both the size of the parasite and its host, as this study used both small hosts and seedling parasites. Evidence from a study Gorse of (*Ulex europaeus)* showed smaller plants may be more impacted by infection (Cirocco et al., 2020), and it is reasonable to suggest that this may extend to *R. fruticosus,* explaining the differences seen between these two data sets. The modular, partitioning response in *R. fruticosus* was unexpected, however this is supported by the field study, particularly in the fruiting data (Cirocco et al., 2022a). The extent to which *R. fruticosus* responds to resource withdrawal by sacrificing affected branches was a new insight and may be an important consideration for its future management.

Future directions

Perhaps the most interesting result here is the interaction between thorn production and infection. Again, this contrasts (Cirocco et al., 2022a) who found that infection resulted in fewer thorns. It is worth considering that field conditions vary substantially, and the removal of resources from isolated plants in a glasshouse may result in very different stressors to those field plants are subjected to. To understand the effects more fully, comparing infections from both adult (clonally reproduced) parasites and seedlings that are newly established would be ideal. Ongoing physiological measurements, ongoing biomass (leaf) sampling to track foliar nitrogen changes over time, growth rate measurements and final biomass data could be collected to clarify the mechanisms underlying the structural changes seen in *R. fruticosus* in response to the parasite. Understanding whether seedlings can act to suppress growth as effectively as adult plants would be useful knowledge if this parasite were to be implemented as a biological control for this weedy host species.

In addition to understanding the effect of parasitism on thorn production, the extent to which the growth and fecundity of *R. fruticosus* is impacted by the parasite is worthy of additional study. From Chapter 2 and the literature, we know that *Isoodon obesulus* interacts with *C. pubescens* and *R. anglocandicans* and also uses both species as a food source (see Chapter 2 as well as Maciunas et al., 2022; Packer, 2014). As the growth rate and fecundity of the invasive species is affected by infection (see Chapter 6 here and Cirocco et al., 2022a), the effect of infection on the overall habitat quality for *I. obesulus* should be assessed. How much the fruit of *C. pubescens* may make up for a reduction in the availability of *R. fruticosus* fruit due to infection, as well as the importance of the cover *C. pubescens* provides when the growth of the invasive is limited by the parasite is important to understand for the management of all three species. If the reduction in growth of the host is countered by the increase in biomass of the parasite, as seen in this experiment, there may be an opportunity to both reduce the population of the invasive species while simultaneously preserving, and potentially enriching, the habitat of the endangered marsupial.

Germination cues and host range

New knowledge

Chapter 7 is an unusual collection of observations, and although many of the results presented there are very limited, all are important and useful elements for understanding the ecology of *C. pubescens*, particularly its seedling stages. There are several major conclusions to draw from this chapter; firstly, the host range of this parasite is extremely broad. Considering the previous work undertaken on invasive species, which demonstrates the ability of the parasite to use novel hosts, this is not surprising. However, the importance of a large host-range cannot be overstated. In real terms, a highly generalist nature may make this parasite easier to establish in new areas, on new hosts, and enable reintroduction to any areas in which it has been lost. Its potential use as a biological control agent is both a reflection of this broad host range, but may also be limited by it; as a generalist it will be important to fully consider the potential impact on other species present in systems it is introduced to as here we can say it is very likely to spread to other hosts within that community.

Secondly, the seed germination and seedling ecology is incredibly complex in this species, with limited results here supporting the tentative conclusions drawn from Chapters 4 and 5. The presence of both physical and physiological dormancies makes sense when the time-delay for emergence of seedlings seen in Chapter 4 is considered; fire may break physical dormancy, but seasonal conditions are still required to induce germination. Whether these conditions are simply the correct temperature regime remains to be tested, as do the abiotic conditions that affect seedling establishment. The trends displayed in these small studies provide a tantalising glimpse into some of the complexities of the ecology of this parasite, for example increased survival in higher light environments, however there are still many questions to be answered here.

Finally, how effective the root is for supporting this sensitive life stage is now better known. Most angiosperms form interactions with mycorrhizal fungi, and *C. pubescens* presented an interesting case; could the short-term cost of such an association be outweighed by the long-term potential gain? We know from previous chapters (notably 4 and 5) that competition between both siblings and the host species can be strong, and it was thought that any mycorrhizal relationship may provide seedlings with the advantage they needed to establish more successfully. This relationship could structured in one of 3 ways; by paying the carbon cost directly, by parasitising the fungi itself (similarly to the relationship between orchid seedlings and mycorrhizal fungi) or by getting a ‘free ride’ from an established communal hyphal network (Bever et al., 2010). No seedlings collected either from the field nor grown in the laboratory showed any associations with fungi, so we can conclude that in this case the extra carbon sink any mycorrhizal association would create is more detrimental than acquisition of other resources. It is interesting that parasitic relationships were also not found here, indicating the short-lived root is sufficient to sustain the seedling. For seedlings that quickly reach a host, not spending resources to invest in any kind of mycorrhizal relationship does make sense, as once attached to that host they have access to as many mineral nutrients as the host does. In that case, the parasite is still indirectly benefiting from mycorrhizal associations in the host, without having to pay the cost of establishing an association itself.

Future directions

Although this chapter has uncovered a wealth of small fragments of knowledge, there is much that can be expanded, particularly with regards to germination biology and seedling ecology. From a germination biology perspective, several collections made over several seasons and treated as a fully blocked design is recommended. Understanding why the standard boiling water treatment worked on some collections and not on others would go a long way to understanding also how the seeds held within natural soil seed banks respond, or not, to germination stimuli. This is of particular importance for management of this species, as population recovery must occur from the soil seed bank. It is worth noting, however, that germination requirements may vary substantially across this species' range. Populations in New Zealand, for example, are unlikely to be subjected to regular fire events. Therefore it is likely that there are a range of factors that may promote germination, and this is certainly an area of research that should be pursued.

From several studies by Cirrocco (in particular see Cirocco et al., 2017 and Cirocco et al., 2021), it is clear that the ability of adult parasites to acquire more nitrogen from its host can influence their success. Whether this has any implication for seedling success is an important question. When examining host choice, the high level of seedling success on *P. daphnoides* reported in Chapter 4 indicates that high nitrogen hosts may similarly influence seedling growth. This is supported somewhat by the limited data here; results from my attempts to tease apart the competitive effects seen in the field indicate that nutrient levels can affect seedling survivorship. However, unfortunately due to limited success both with germination in that cohort as well as an unusually hot summer period, survival within those experiments was too limited to draw any strong conclusions. Ideally this experiment should be repeated with a larger quantity of seeds, as well as comparisons made between growth of seedlings when presented with both nitrogen fixing and non-leguminous hosts.

Similarly, the effects of simple shading and droughting cannot be ignored in this study, with seedlings showing substantial water stress well before water levels within the pots appeared to lower. Whether this was due simply to the limited volume of the pots, a lack of litter layer or a lack of interactions with other species as occurs in the field is unknown. However, the availability of water potentially limiting the establishment of seedlings is an important consideration here. I found that *C. pubescens* seedlings emerge in late spring (see Chapter 4); a time of year where water is still available in the soil but the environment is, overall, drying. For seedlings that emerge late, or compete strongly with their siblings or hosts for a rapidly dwindling resource, a sensitivity to water loss may be a substantial detriment. I suggest that another experiment to test the effects of abiotic factors including nutrient, water and light limitation is needed.

Overall significance & concluding remarks

Our knowledge of the fundamental biology of this parasitic species has been very limited, and what we historically knew was in great need of an update. My research has greatly improved our understanding of this native parasitic plant and filled substantial gaps in knowledge. Not only has this greatly broadened our understanding of *C. pubescens* itself, but also how it interacts with some of the plant and animal species around it. We now know that the fruits form a part of the diet of an endangered marsupial, and that at least one species is, most likely, still functioning as an active dispersal vector within the Mount Lofty Ranges. My examination of genetic data, combined with this knowledge of dispersal, informs us of the connectivity of populations, and we now have an important insight into the role clonal reproduction plays in maintaining populations in the long-term. Further research is required to understand how the population structures change genetically over time, with extensive clonal reproduction and a reliance on fire for recruitment from seed complicating sampling across not only space, but also time. Another important consideration not examined here is the movement of pollen and geneflow on a smaller scale within populations. Together this should build a comprehensive picture of the dynamics of population genetics in this species across its range.

My study has also contributed greatly to understanding the seedling dynamics of this parasite, including confirming its fire response and its ability to establish from seed on a novel host. Although these results are clear cut, more research is also needed in this area to uncover other potential germination triggers in populations not frequently subjected to fire, as well as to establish more clearly how competition and abiotic conditions can influence seedling establishment. The seedling ecology component of this work is an important step in understanding seedling establishment, and is of critical importance for conserving *C. pubescens* for it's own sake, for conserving other species that depend on it, and for its use as a biological control for noxious weeds.

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