Improving pre-release risk assessments for classical biological control agents by integrating behavioural, electrophysiological, and chemical-ecological methods for host specificity testing

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ABSTRACT

Classical biological control has proven to be a cost-effective method of suppressing pest populations in a variety of contexts, but it requires a thorough assessment of the potential non-target risks posed by a candidate agent in order to be environmentally safe. Pre-release host specificity testing is a cornerstone of safe and effective biological control programmes, and some kind of risk assessment is usually required by national regulators before a new organsim can be released. Decision makers often have to evaluate applications to release biological control agents (BCAs) based on physiological host range tests conducted in containment. For parasitoid agents, these usually manifest as no-choice oviposition tests, where a candidate BCA is confined in close proximity with a series of non-target species. These kinds of host range tests are a crucial first step in assessing host specificity because they offer unambiguous evidence of the ability of a BCA to recognise, attack, and develop in non-target species, thus confirming that species as a physiological host. However, the simplicity and artificiality of these tests are both an asset and a potential drawback. Physiological host range tests necessarily remove many important chemical cues from the host location process that parasitoids rely on for the natural expression of their ecological host ranges (the list of species they will actually attack in the field). The discrepancy between physiological and ecological host range has important implications for whether or not the candidate agent will be approved for release, and whether or not it will attack non-target species in the natural environment. The primary aim of my thesis was to apply chemical ecological methods to the study of host specificity, with a view toward integrating these methods into pre-release non-target risk assessments to provide more certainty to regulators about the risks a candidate agent may pose. My case study was the host-parasitoid complex of New Zealand stink bugs (Hemiptera: Pentatomidae) and three of their egg parasitoids (Hymenoptera: Scelionidae). New Zealand Pentatomidae taxa include: *Cermatulus nasalis hudsoni* Woodward, *Cermatulus nasalis nasalis* (Woodward), *Cermatulus nasalis turbotti* Woodward, *Cuspicona simplex* Walker, *Dictyotus caenosus* (Westwood), *Glaucias amyoti* (Dalla), *Hypsithocus hudsonae* Bergroth, *Monteithiella humeralis* Walker, *Nezara viridula* (L.), and *Oechalia schellenbergii* (Guérin). Egg parasitoids included: *Trissolcus japonicus* Ashmead, a BCA of brown marmorated stink bug (*Halyomorpha halys* Stål) conditionally approved for release in New Zealand in the event of the establishment of its target host; *Trissolcus basalis* (Wollaston), a BCA of green vegetable bug (*Nezara viridula*

[L.]) introduced into New Zealand in 1949; and *Trissolcus oenone* Johnson, a pentatomid parasitoid native to Australia and New Zealand.

Physiological host range testing of all three parasitoids revealed all were capable of attacking and developing in all pentatomid species tested, except *N. viridula* was not a host of *T. japonicus* and *T. oenone*, and *T. oenone* was unable to be tested with the endemic pentatomid *Hypsithocus hudsonae* (Bergroth). Development times were similar for the two resident New Zealand parasitoids on all pentatomid species. *Trissolcus japonicus* was shown to be capable of high parasitism rates against the endemic alpine shield bug, *H. hudsonae*.

The integration of electroantennography with open arena arrestment bioassays and competition tests helped to reveal the host preferences of *T. basalis* and *T. oenone* in relation to the exotic pentatomids *N. viridula* and *Cuspicona simplex* (Walker). Acetone extracts of *N. viridula* eggs elicited clear and consistent antennal responses in *T. basalis*, and these responses were stronger than those elicited by a hexane extract. Potential contact kairomones on the surfaces of eggs were tentatively identified to provide a foundation for future study in this area. Open arena arrestment bioassays were used to compare the retention time of the two parasitoids in arenas contaminated by one of the two pentatomid species. *Trissolcus basalis* spent four times longer searching in arenas for its primary host, *N. viridula*, than for *C. simplex*, while the reverse was true for *T. oenone*, which spent an even lower absolute length of time searching for *N. viridula*, a non-host. Parasitoids are therefore capable of distinguishing between these hosts based solely on adult footprint compounds left on substrates, and *T. oenone* is potentially capable of distinguishing between hosts and non-hosts. Competition tests between the two parasitoids on *C. simplex* eggs revealed *T. oenone* to be the superior competitor in both extrinsic and intrinsic contests. The native parasitoid successfully parasitized more eggs than *T. basalis*, and developed in over 90% of multiparasitised eggs. The combination of these approaches was useful for investigating the influence of chemical cues on the expression of host range. In particular, the results of arrestment studies clearly complement physiological host range tests and help to provide significant context especially when parasitism rates are similar.

The specific compounds associated with New Zealand species of stink bugs which elicit antennal responses in the three *Trissolcus* parasitoids were revealed through a combination of electrophysiological techniques and chemical analyses. Cuticular hydrocarbons and defensive compounds were extracted from adult stink bugs via immersion in hexane, and the resulting samples were analysed through GC-MS to identify the compounds present. Extracts were then exposed to the three species of parasitoids through gas chromatography coupled with electroantennographic detection (GC-EAD), which measures the change in voltage across an

insect antenna as compounds from an extract are fractionated and passed over its surface. After GC-EAD with extract, another round of recordings were made with synthetic standards. A final round of electroantennogram recordings were made by puffing individual compounds over the antennae and comparing responses to solvent controls. A total of eight compounds elicited responses, and seven of these were identified as follows: (*E*)-2-decenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, (*E*)-2-hexenal, (*E*)-2-decenyl acetate, *n*-tridecane, and *n*-dodecane. This work provides the foundation for future studies of the behavioural function of these compounds in stink bug egg parasitoids.

The work presented in this thesis shows the value of incorporating chemical ecological techniques into the study of host specificity, and for evaluating the non-target risks posed by classical BCAs. The results of olfactory and electrophysiological methods are complementary to physiological host range testing, and the combination of methods provides valuable insight into the chemical basis of host range. These kinds of studies provide results which are directly relevant for regulators to consider during the evaluation of applications to release new BCAs. A new non-target risk assessment framework incorporating these techniques is proposed.

Dedicated to Oliver Alan Saunders

The child thinks of growing old as an almost obscene calamity, which for some mysterious reason will never happen to itself. All who have passed the age of thirty are joyless grotesques, endlessly fussing about things of no importance and staying alive without, so far as the child can see, having anything to live for. Only child life is real life.

— George Orwell

ACKNOWLEDGEMENTS

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CHAPTER 1: A review of the application of chemical ecological methods to host-specificity testing

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1.1 Introduction

Classical biological control of emerging pests

Classical biological control operates on the idea that invasive species have lost their connection to natural enemies from their native ranges, and that by restoring this connection, pest densities and their associated damage can be reduced below an economic injury level (Ehler, 2006; Elton, 1958; Keane & Crawley, 2002; Stern, 1973). Importing and releasing a classical biological control agent (BCA) can be a cost-effective and environmentally safe method of pest control. However, newly introduced BCAs could also pose risks to non-target species (Caltagirone & Huffaker, 1980; Delfosse, 2005; Louda et al., 2003). Over the last 100 years, over 240 invasive pests have been successfully managed through the action of BCAs, with the vast majority of these through classical biological control (Van Driesche et al., 2008). When well planned and executed, successful biological control programmes can deliver improved crop yields, a reduction of chemical inputs, stronger ecosystem services, greater economic prosperity and stability, and considerable social benefits (Cock et al., 2015; De Clercq et al., 2011). The importation of vedalia beetle *Rodolia cardinalis* (Mulsant) from Australia to the United States is widely considered to be the first successful classical biological control program (Caltagirone & Doutt, 1989). It was introduced in 1889 against cottony cushion scale, *Icerya purchasi* Maskell, in order to rescue the United States citrus industry following a devastating outbreak of scale insects the previous year. The introduction of *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae) to French Polynesia in 2005 reduced populations of glassy-winged sharpshooter, *Homalodisca vitripennis* Germar (=*H. coagulata* Say) (Hemiptera: Cicadellidae) by more than 90% within the first year (Grandgirard et al., 2008). In another example, the introduction of ragwort flea beetle, *Longitarsus jacobaeae* (Waterhouse) into New Zealand in 1983 against ragwort, *Senecio jacobaea* L., is estimated to have saved the dairy sector in excess of NZD$1.1 billion, from a total project cost of NZD$468,000 (Cameron et al., 1987; Fowler et al., 2016). Past successes like these offer valuable lessons which can be applied to the control of emerging pests around the world.

Several recently emerging global pests highlight the importance of classical biological control as a useful component in a long-term management strategy. Spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is an Asian vinegar fly which has emerged as an important pest of soft-skinned fruit in North America and Europe since the late 2000s (Asplen et al., 2015). Unlike most vinegar flies which prefer spoilt fruit, *D. suzukii* is attracted to ripening or just ripe fruit, and it uses a serrated ovipositor to pierce the skin and lay eggs (J. C. Lee et al., 2011). This preference for ripening fruit means that by the time fruit are harvested, the damage from internal

larval feeding is already done. The three most dominant larval parasitoids of *D. suzukii* were found to be *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae), *Ganaspis brasiliensis* Ihering, and *Leptopilina japonica* Novković & Kimura (Hymenoptera: Figitidae). While laboratory tests show *A. japonica* tends to have higher parasitism efficiencies than the other two species, *G. brasiliensis* is more host-specific (Daane et al., 2021). Host-specificity testing work is ongoing in Europe and North America (Wang et al., 2021). Release of the most host-specific strain (G1) into North America has been approved by the North American Plant Protection Organisation, but the final regulatory decision by USDA-APHIS is still forthcoming.

Another high-risk pest is the spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), a highly invasive planthopper native to East Asia which damages a variety of shrubs and trees, including important crop plants such as apples, kiwifruit, and grapes (D.-H. Lee et al., 2019). The first specimens detected in the United States were found in September 2014, in Pennsylvania (Barringer et al., 2015). Parasitoid surveys in Northern China found *Anastatus orientalis* Yang & Choi (Hymenoptera: Eupelmidae) attacked around a third of spotted lanternfly egg masses, and achieved an average of 40% egg parasitism within masses (Yang et al., 2015). The authors also observed two generations of parasitoids emerging each year, whereas spotted lanternfly is restricted to one generation per year in Northern China. *Anastatus orientalis* cultures have been established in quarantine at USDA-APHIS labs in order to study the host-specificity and diapausing behaviour of the parasitoid as a candidate BCA (Hoelmer et al., 2018). Taken together, these three case studies of emerging pests show the importance of classical biological control as a critical component of a long-term IPM strategy to managing emerging invasive pests. Ideally, a suitable BCA should be screened and approved for release before the pest establishes to avoid any lag time in deploying the BCA, a strategy termed pre-emptive or pro-active biological control (Hoddle et al., 2018).

The brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), is a polyphagous horticultural pest native to East Asia, but invasive in North America since the mid-1990s, and in Europe since the mid-2000s (Haye et al., 2015; Hoebeke & Carter, 2003; D.-H. Lee et al., 2013). It has caused hundreds of millions of dollars in lost yields for growers in the United States and Europe (Bariselli et al., 2016; Leskey & Nielsen, 2018), led to a four-fold increase in pesticide application with a corresponding disruption to IPM-based strategies in some production systems (Blaauw et al., 2015), and has caused significant problems for residential and commercial property owners due to their habit of overwintering in large aggregations (Rice et al., 2014). *Trissolcus japonicus* Ashmead (Hymenoptera: Scelionidae) is a tiny egg parasitoid of pentatomoid bugs (Talamas et al., 2013), and because it has been identified as the most dominant natural enemy of *H. halys* in its native range (Buffington et al., 2018; Yang et al., 2009; Zhang et al., 2017), it is currently the subject of host-specificity testing in North America, Europe, and New Zealand (Charles et al., 2019; Haye et al., 2020; Rice et al., 2014; Saunders et al., 2021). Adventive populations of *T. japonicus* have recently been discovered in North America and Europe, but these have been

confirmed to be the result of self-introduced populations and not breaches of containment (Milnes et al., 2016; Stahl et al., 2019; Talamas et al., 2015).

Pre-release risk assessment

Non-target effects are a widely discussed issue within the classical biological control literature (Hinz et al., 2016; Suckling & Sforza, 2014; Van Driesche & Hoddle, 2016), but there has long been disagreement over how common they are and whether or not they have led to population-level impacts. Some classical biological control programmes in the past resulted in unintended non-target effects, particularly before the widespread adoption of scientific pre-release testing regimes in the early 2000s (Barratt, 2011; Follett & Duan, 2000; Howarth, 1983; Louda et al., 2003). For example, when the multi-coloured Asian ladybird beetle (*Harmonia axyridis*) was released into North America in 1916, it was effective in controlling infestations of its target aphid. However, while it is a predator of soft-bodied plant pests, it is now considered an invasive species owing to the fact that it is a generalist predator and will consume pests and other predatory arthropod species (Koch & Galvan, 2007). It is thought be responsible for the widespread decline of beneficial native coccinellids such as *Adalia bipunctata*, whose numbers have dropped in areas invaded by *H. axyridis* (Harmon et al., 2007). Another example is the tachinid fly *Compsilura concinnata* (Meigen), introduced in North America in 1906 against gypsy moth, *Lymantria dispar* (L.), a defoliator pest of deciduous trees (Fuester et al., 2014). Post-release work has linked it to severe declines in two native saturniid moths, and it has been shown to parasitise a rare endemic moth which has only been collected in limited numbers (Boettner et al., 2000). It is doubtful these kinds of agents would be approved for release today, and this is partly due to the development of improved testing methods for host-specificity (van Lenteren et al., 2006), in addition to more stringent regulations governing the process of releasing new organisms (Barratt et al., 2010; Hinz et al., 2014; Paynter et al., 2018).

Non-target effects are classified into one of two categories: non-target host use, where a BCA is observed attacking a non-target species; and non-target impacts, where non-target host use results in measurable population-level impacts on a non-target species (Van Driesche & Hoddle, 2016). Determining the thresholds at which non-target attack translates into impacts for a particular combination of BCA and non-target species remains an important research gap (Barratt et al., 2010; Van Driesche & Hoddle, 2017), as very few studies have convincingly attributed population-level declines of a non-target species to the action of an introduced BCA (Heimpel & Mills, 2017). Less than 1% of weed BCAs are reported to have caused significant non-target impacts, despite over 2,000 introductions of 550 agents over a period of many decades (Suckling & Sforza, 2014; Winston et al., 2014). Similarly, non-target host use has been documented in less than 2% of predator or parasitoid introductions worldwide, and of these, only one third are considered to have caused population-level impacts on a non-target species (Lynch & Thomas, 2000). Measuring rates of non-target use and uncovering evidence of non-target impacts is time consuming and resource intensive, due to the need

for thorough field monitoring, which likely means most instances of observed host use are never followed up (Lynch et al., 2001). Newer approaches to modelling, especially when combined with behavioural and molecular work, could make significant contributions to non-target risk assessment. For example, ecoclimatic modelling could be used to infer spatial and temporal overlap between agents and potential non-target species (Kriticos et al., 2021), while population modelling can be used to predict impacts on target and non-target pests based on traits associated with the agent (Mills & Kean, 2010). It's useful for regulators to be able to manipulate the inputs of models in order to visualise predicted outcomes under different scenarios.

There is no evidence to implicate BCAs in the extinction of a non-target species since the adoption of scientific host-range testing from the 1990s onward (Hoddle, 2016). While parasitoid BCAs have been blamed for declines in non-target native taxa, it can be difficult to separate the impact of a BCA from other factors such as loss of habitat. For example, in an influential and widely cited paper, Howarth (1991) blamed two classical BCAs—*Trichopoda pilipes* (F.) (Diptera: Tachinidae) and *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae)—for the decline of a native Hawaiian stink bug, *Coleotichus blackburniae* (White) (Hemiptera: Pentatomidae). However, Johnson et al. (2005) showed that up to 87% of egg mortality was instead due to accidentally introduced ants and spiders, that *T. basalis* parasitised the stink bug at low levels and only at lower elevations, and that *T. pilipes* only parasitised native stink bugs in large numbers at three out of twenty four study sites. While some species are likely to be very sensitive to parasitism, it's also possible to observe very high parasitism rates in the field without observing any measurable impacts on the species being attacked. For example, *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae) reaches parasitism rates of 50% against its target pest *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) in South Australia, apparently without causing any observable changes to the population densities of weevils (Hopkins, 1989). These kinds of results show how difficult it can be to attribute causality to BCAs for impacts against target or non-target taxa, even when these relationships appear simple.

One of the most effective ways to reduce the likelihood of non-target effects has been to improve the way potential BCAs are tested for their host-specificity before they are released (Barratt et al., 2010). Over the last three decades, researchers, biological control practitioners, and regulators, have contributed to defining and implementing stricter regulations around risk assessment and developing more robust frameworks for host range testing (Barratt, 2011; Cameron et al., 1987; Messing, 2008; Moeed et al., 2006; Van Driesche & Hoddle, 2016). At an international level, the importation of classical agents is regulated by the International Plant Protection Convention (IPPC, 2017), the UN Convention of Biological Diversity (Cock et al., 2010), and the FAO code of conduct for the import and release of exotic BCAs (FAO, 1995). These high-level agreements mandate pre-release risk assessments to ascertain a basic understanding of potential non-target risks related to new agents, and where possible, to avoid adverse effects on biological diversity as a result of biological

control programmes. The result is a set of guidelines which attempt to strike a balance between risk and economic viability of testing procedures (De Clercq et al., 2011). This shift towards a more scientific approach focussing on risk has precipitated a trend over the last couple of decades towards the release of classical agents with a genus or species-level host-specificity (Van Driesche & Hoddle, 2016).

Non-target risk assessments progress through a series of steps to identify and examine the chances of any potential adverse impacts that may eventuate from releasing a classical BCA (Barratt, 2011). As a first step, a thorough understanding of the taxonomy of both the target pest and proposed agent is critical to ensure correctly identified organisms are used in each step of the process. Natural enemies can show variation in host preferences and efficacy even within the same species, for example due to the presence of different biotypes (Phillips et al., 2008). A thorough review of scholarly literature, unpublished studies, specimen records, and climate records can be useful to establish the scope of a risk assessment (Sands & Van Driesche, 2004). Questions about the likelihood of natural enemy establishment, as well as potential non-target impacts, can be clarified at this stage to determine if it is worth proceeding (Hoddle, 2004). The next stage involves selecting non-target species to use in host range testing, which is based primarily on availability, phylogenetic relatedness, ecological similarity, and safeguard considerations such as targeting economically important species, or threatened taxa of high conservation value (Kuhlmann et al., 2006; Todd et al., 2015; van Lenteren et al., 2003; Wapshere, 1974). Non-target species are then exposed to the candidate BCA in a hierarchical procedure whereby a non-target can be removed from further consideration if no attacks are observed during the first step (van Lenteren et al., 2006). Subsequent steps increasingly try to replicate natural conditions to identify the point at which the proposed BCA loses interest in the offered non-target species, or prefers to attack another species, and these results are used to build an interpretation of the likely host range of the BCA if or when it is released (Bigler et al., 2006).

Host-specificity testing in quarantine is a common pre-requisite for assessing the environmental safety of biocontrol agents (Barratt, 2011) and results generated through this work are generally given considerable weight by regulators when deciding whether or not to approve an agent (Babendreier et al., 2006). The purpose of these experiments is to define the physiological (=fundamental) host range of the candidate BCA, which is the list of hosts the agent can attack and successfully develop in (van Lenteren et al., 2006). Typically, the agent is confined to a small space with a non-target species for a fixed period of time to observe whether it will attack the species offered, and to record any development or emergence from the host. Replicates containing non-target species without exposure to the agent provide important negative controls to measure host mortality and development in the absence of parasitism (Van Driesche & Murray, 2004). Confining a candidate BCA with non-target species provides robust evidence of the agent's physiological host range, but it also prevents a parasitoid from exploring the full repertoire of host location behaviours it will likely use in the field (Murray et al., 2010). Understanding why a biocontrol agent will attack some species

and not others, and how these host preferences are mediated by semiochemical cues, would be useful for designing host-specificity tests to more accurately characterise the risk of non-target attack (Avila et al., 2016b; Iacovone et al., 2016). Recent work is beginning to show how chemical ecology techniques can be applied to provide this kind of information to complement traditional approaches to non-target risk assessment (Avila et al., 2016a; Hedstrom et al., 2017; I. Park et al., 2018; Wheeler & Schaffner, 2013).

Parasitoid wasps are a megadiverse group of Hymenoptera united by their parasitic mode of reproduction which kills their host (Godfray, 1994; LaSalle & Gauld, 1993). They are the most commonly used class of BCA because they are often highly effective at locating and attacking hosts (Van Driesche et al., 2008). Vinson (1976, 1998) proposed a general framework illustrating how parasitoids find their hosts by exploiting different kinds of cues as they get closer to their hosts. Female parasitoids first seek out a potential community of hosts by relying on long range visual or volatile chemical cues associated with their hosts' food plants, which are highly detectable, but relatively unreliable in confirming the presence or suitability of hosts (Vet & Dicke, 1992). Next a female enters a potential host patch and transitions from flying to ambulatory movement as she searches the substrate for cues produced by the host as it feeds, defecates, builds shelter, or releases pheromones or defensive compounds (Group II cues *sensu* Vinson, 1998). Once a female parasitoid finds a potential host, she must recognise the host is suitable, and to do this she uses Group III cues, which are chemical or tactile cues associated with the life stage attacked (Bin et al., 1993). A final set of cues (Group IV) are detected by the female through her ovipositor to confirm correct mechanical orientation of her ovipositor, and to confirm attractive physiological attributes of the host during the act of oviposition (Vinson, 1998). Following oviposition, the development of parasitoid offspring depends largely on the suitability of the host (nutritional adequacy, the presence of immune responses or toxins, competition with other parasitoids) (Vinson & Iwantsch, 1980b), and the ability of the parasitoid to regulate the host's movement, development, physiological state, or nutritional profile (Vinson & Iwantsch, 1980a). A greater understanding of how and why parasitoids locate and decide to attack certain species and not others, based on chemical cues, could be applied to designing biological control programmes that maximise the efficacy of BCAs on their target pests, while also minimising the risks to non-target species.

This review aims to summarise literature on the subject of applying chemical ecological methods to host-specificity testing of classical BCAs, and in particular, parasitoid wasps. Parasitoids rely on their ability to detect and exploit a range of volatile semiochemicals emitted or produced by their hosts in order to reproduce (Blomquist & Ginzel, 2021; Colazza & Wajnberg, 2013). Experiments which demonstrate the attraction of parasitoids to different hosts provide important information relevant to measuring the strength of attraction to non-target species, and for assessing the efficacy of the agent on its target host (Avila et al., 2016a; Conti et al., 2004; I. Park et al., 2018). In particular I focus on: 1. moving air bioassays involving olfactometers to demonstrate directed

movement towards or away from an odour stimulus; 2. substrate-borne arrestment studies involving the use of behavioural arenas to measure the intensity of searching effort in relation to host associated compounds; and 3. electrophysiological techniques such as gas-chromatography coupled with electroantennographic detection, and single sensillum recording where the response profile of an insect to host-associated compounds is characterised in relation to the types of olfactory sensilla present on the antennae. I believe these kinds of experiments could be applied more widely to host-specificity testing, in order to improve my understanding of the risks posed by classical BCAs to non-target species.

1.2 Olfactometer odour-specificity bioassays

Olfactometer bioassays measure the rate and direction of movement of an insect in response to odour stimuli in an airstream (Hare, 1998). Olfactometers are generally made out of glass tubes or channelled plastic bases with clear acrylic fixed tightly over the top, in order to form a conduit for the merging of multiple odour sources into a single stream, at the base of which an insect is released (Hare, 1998). Odour sources are usually contained within a chamber which may be separated from the rest of the device with fine mesh to prevent the insect walking inside it. Filtered, humidified air is pumped or sucked through the device, and an air flow meter is used to ensure consistent and reproducible test conditions (Avila et al., 2017). It is important to minimise outside visual distractions for the insect being tested, and the device must be uniformly lit (or used in the dark) in order to prevent a biased result due to orientation towards a light source. In between each trial, it is best practise to clean the device with a solvent or combination of solvents (e.g. hexane and ethanol) before rinsing with distilled water, and baking in an oven, as traces of test odour or insect residues could remain inside the device and bias the results (I. Park et al., 2019). The allocation of odours to testing arms should also be rotated with each replicate in order to avoid any potential directional biases induced by the experimental set up.

       Researchers use a wide variety of variables and metrics to define when an insect has made a choice (Avila et al., 2017; Ballhorn & Kautz, 2013; Turlings et al., 2004). Common variables include walking a certain distance inside one of the arms, reaching the end of an arm, the arm with the greater residence time, a combination of orthokinetic variables to show searching motivation, or the analysis of video recorded walking trails with software to quantify spatial preferences (Colazza, McElfresh, et al., 2004). Insects are typically given between five and ten minutes to make a choice, or to show their preference through residence time, before the next bioassay is conducted. The simplest kinds of comparative tests involve measuring a behavioural choice in a Y-tube olfactometer between an arm containing a semiochemical source, and a blank control arm, in order to determine the attractiveness of the semiochemical under consideration (Hare, 1998). Attraction to a single odour can be measured with a blank control arm, or the preference between two odours can be measured at the same time. The insect is released at the base and walks upstream before making a choice at the

junction of the two arms. The advantages of Y-tube olfactometers include a relatively cheap and simple design which is able to show a preference for an odour over a blank arm, and then a preference for one odour over another, but disadvantages include turbulence at odour junctions which can confuse insects, and the need to conduct a high number of replicates to demonstrate non-random movement when only two choices are available for the insect (Hare, 1998; Vet et al., 1983).

Multi-arm olfactometers were first used by Pettersson (1970), and subsequently improved by Vet et al. (1983), to employ a greater number of odour source arms which flow into a central arena and are exhausted through a central port, which can also be used to introduce the insect. Advantages over Y-tube designs include the ability to test a greater number of different odour sources for greater statistical power, and a lesser degree of odour plume mixing to confuse the test insect, but disadvantages include the need for highly accurate construction and monitoring of airflow to ensure all arms offer an identical medium for test odours to flow through (Hare, 1998). Turlings et al. (2004) developed an olfactometer system designed to test six insects for their responses to six odours at a time, while simultaneously trapping part of the odour stream in filters for chemical analyses using GC-FID and GC-MS. Odour chamber choices were similar when wasps were released one at a time or when released in groups of six, demonstrating how group releases of parasitoids into such a device can save a significant amount of time over conventional single insect bioassays.

Parasitoids have evolved the ability to discriminate between different hosts based on their odours, or differences in the odours their hosts induce in plants, and this ability facilitates the selection of high quality or developmentally suitable hosts (Avila et al., 2016a; Chiappini et al., 2012; Salerno et al., 2009). For example, Fors et al. (2018) used Y-tube olfactometers to measure attraction of the eulophid *Asecodes parviclava* to larvae of two closely related hosts: *Galerucella calmariensis* and *G. pusilla* (Coleoptera: Chrysomelidae). Parasitoids reared on *G. calmariensi* showed a clear preference for that host in olfactometers while those reared on *G. pusilla* showed no preference. Parasitoids were attracted by a blend of two terpenes produced at elevated levels by plants infested with *G. calmariensi*. These results showed that the parasitoid responds to a specific blend of compounds from plants infested with *G. calmariensi* , and interestingly, previous work has shown parasitoids achieve far higher reproductive success in this host compared to *G. pusilla*, as a result of a weaker immune response against developing parasitoid offspring (Fors et al., 2014). Olfactometer bioassays have also revealed the ability of stink bug egg parasitoids to perceive VOCs offering information relating to the sex and mating status of their hosts (Colazza et al., 2007; Colazza, Fucarino, et al., 2004). For example, Salerno et al. (2019) demonstrated how spermathecal extracts from female *N. viridula* induced the emission of volatiles in green beans which were attractive to the egg parasitoid *T. basalis* in olfactometer experiments, but only when stink bugs were mated. This suggests female *T. basalis* are able to prioritise movement toward plants which are likely to harbour female stink bugs ready to lay eggs, and this is a very reliable cue to the presence of exploitable hosts. Nurkomar et al. (2017) tested the attractiveness of cucumber plants infested with *Diaphania indica*

Saunders (Lepidoptera: Crambidae) to the potential BCA *Apanteles taragamae* Viereck (Hymenoptera: Braconidae) in four-arm olfactometers. Female parasitoids were attracted to uninfested plants and mechanically damaged plants over clean air, and showed a clear preference to host infested plants over uninfested plants. Demonstrating parasitoid attraction to target species is an important first step in testing the relative attraction of non-target species.

Results from olfactometer bioassays are valuable for pre-release risk assessments of classical BCAs because they can show the relative search motivation for a parasitoid in response to a variety of host-associated or plant-associated cues (Conti et al., 2004; Salerno et al., 2006). The odour-specificity or odour preferences exhibited by a parasitoid offer indirect signals for the risk it poses to non-target species, based on attraction to volatile compounds associated with potential hosts or their habitats. For example, Avila et al. (2016b) tested the introduced BCA *Cotesia urabae* Austin and Allen (Hymenoptera: Braconidae) in odour-specificity assays to measure attraction of parasitoids to non-target New Zealand lepidoptera and their host plants in a retrospective risk assessment study. They showed that while non-target host-plant complexes were attractive to parasitoids in Y-tube bioassays, the target host-plant complex (*Uraba lugens* Walker on *Eucalyptus*) was most attractive to parasitoids in four-arm olfactometer bioassays. In addition, parasitoids with previous oviposition experience in non-target species were no more likely to be attracted to non-target host odours, suggesting that parasitoids were unable to associate novel non-target odours with oviposition opportunities, or that these opportunities were not valued by the parasitoid (Avila et al., 2016b). Hedstrom et al. (2017) used Y-tube bioassays to show that *Trissolcus japonicus* favoured its target host *Halyomorpha halys* over one non-target pentatomid, but displayed no preference for two of the other non-target species tested. One of these, *Banasa dimidiata*, was parasitized as frequently as *H. halys* in no-choice tests, and the combination of oviposition and olfactory results suggests it may be at risk of non-target attack in the field. Combining olfactory and oviposition experiments provides a more holistic view of the risk posed by a candidate BCA than either test alone.

Thanikkul et al. (2017) used four-arm olfactometers to compare the attractiveness of odours from maize plants infested with target and non-target species to *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae) a biocontrol agent of *Mythimna separata* Walker (Lepidoptera: Noctuidae). Parasitoids were attracted to plants infested with hosts, and not to healthy plants, but only when plants were infested with three or five larvae, and not just a single one, and parasitoids were attracted to non-host volatiles more than uninfested control plants. The characteristics of plant odour plumes are complex, but parasitoids are likely able to switch between plumes when they perceive more reliable cues indicating the presence of their hosts, or cues indicating a more preferred host is nearby (Beyaert & Hilker, 2014). Ferracini et al. (2015) used olfactometer bioassays to assess the non-target risks of *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), which was introduced in Japan, the United States, Italy, and France, as a BCA of the chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu. Three parasitoids were found to emerge from wild-collected chestnut galls from one non-

target species, *Biorhiza pallida*, and this was the first record of this happening. However, *T. sinensis* never showed a preference in Y-tube tests for any non-target galls compared to its target, so the emergence of parasitoids from field-collected non-target galls is likely to be very rare.

Other factors that may influence the ability of parasitoids to locate their hosts include the host they were reared on, toxins in the environment, and contact kairomones on their hosts. Belda and Riudavets (2012) investigated the host preferences of *Venturia canescens* (Gravenhorst), a generalist ichneumonid parasitoid which also attacks stored product pests, to determine if rearing host influenced odour preferences in Y-tube olfactometers. Parasitoids reared on *Ephestia kuehniella* preferred their rearing host in olfactometer assays, but those reared on *Plodia interpunctella* still preferred *E. kuehniella*. This may be explained by the fact that parasitoids emerging from *E. kuehniella* were larger than those emerging from *P. interpunctella*, and parasitoid size (as measured by hind tibia length) is often used as a proxy for fitness (Sagarra et al., 2001). It is possible that *V. canescens* is able to distinguish between the hosts based on the presence or absence of a specific compound, and despite being a generalist, it may have an innate preference for hosts emitting this particular compound in order to prioritise higher quality hosts. Bayram et al. (2010) exposed *Telenomus busseolae* Gahan to sublethal concentrations of deltamethrin and cyfluthrin, in order to determine the impact on its ability to locate its target host *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae). Parasitoids treated with cyfluthrin failed to orient toward the sex pheromones of their target host in Y-tube olfactometers, suggesting the use of this pyrethroid in the field may impair the ability of *T. busseolae* to locate its target host. Sublethal exposure of pesticides to natural enemies is known to impair the neuroethology, reduce the longevity, and diminish the fecundity of biocontrol agents (Desneux et al., 2006). Tognon et al. (2020) investigated the egg chemistry of the soybean pest *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae) in order to identify compounds eliciting a kairomonal effect in the egg parasitoid *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae). Hexane extracts contained 31 compounds, and synthetic mixtures of limonene, camphene, benzaldehyde, β-myrcene and with or without β-pinene, elicited parasitoid attraction in Y-tube olfactometers relative to hexane control arms. This result is somewhat unusual when compared with recent work by Michereff et al. (2016) testing hexane and acetone rinses of *E. heros* eggs in relation to *T. podisi* attraction who showed parasitoids only responded in Y-tube olfactometers to egg masses or acetone egg extracts, and not hexane extracts. Pioneering work by Bin et al. (1993) showed kairomonal activity was elicited in *T. basalis* only in acetone extracts of *N. viridula* when applied to glass beads, suggesting kairomones could only be extracted from stink bug eggs with a partially polar solvent such as acetone, and not hexane.

1.3 Arrestment to substrate-borne semiochemicals

Substrate-borne semiochemicals are deposited by hosts when they walk, feed, defecate, build shelter, or emit pheromones, and these kinds of cues are used by parasitoids for short range host finding

(Group II cues *sensu* Vinson 1998). Plants may also produce substrate-borne contact synomones in response to egg deposition by herbivores (Conti et al., 2010). Parasitoids detect semiochemicals through smell (olfaction) and taste (contact chemoreception), but in order to exploit these cues, they must first orient towards their source by using a search strategy (Bell et al., 1995). Substrate-borne arrestment studies are normally conducted through the use of arena bioassays, and they aim to identify bioactive compounds by observing whether or not a parasitoid engages in searching behaviour when exposed to a particular host-associated blend or extract, and to measure the strength of attraction to that substance by measuring variables associated with parasitoid searching behaviour (Colazza et al., 2014). Commonly measured variables include the retention time of the parasitoid on the substrate, as well as locomotory variables such as walking velocity, walking distance, angular velocity, and these variables are compared with a control treatment where the substrate was left uncontaminated (Malek et al., 2019, 2021; Peri et al., 2006). Arrestment occurs when a parasitoid is introduced to a substrate which has been contaminated by a host or host extract containing a kairomone, and in these cases, parasitoids often display characteristic behaviour (Colazza et al., 2014): freezing for a short time, slower walking speed and increased turning, drumming of the antennae on the substrate, and initiation of a search pattern whereby the parasitoid may leave a contaminated area only to be pulled back in as it searches for the source of the odour.

Open arena bioassays measure how motivated a parasitoid is to search for a given host without physically confining it, and such tests are useful for assessing the responses of parasitoids to kairomones from potential hosts (Colazza et al., 2014). If a parasitoid shows a very short retention time, or is actively repelled by the odour of a host, it is reasonable to infer the likelihood of the parasitoid finding the host in a natural environment is low (Conti et al., 2004). Similar to olfactometer odour-specificity bioassays, arrestment studies have great potential to inform pre-release risk assessments of classical BCAs by providing complementary information alongside oviposition tests. This is especially the case when oviposition results are similar. For example, in chapter three I demonstrated that the introduced BCA *T. basalis* and the native pentatomid parasitoid *T. oenone* are both highly efficient in their discovery (>95%) and parasitism (>90%) of *Cuspicona simplex* eggs. I also found their development times on this host to be almost identical. This confirms *C. simplex* is highly suitable as a physiological host for both parasitoids, but in light of additional results, it would be incorrect to assume that both parasitoids are equally likely to find and attack this host in the field. In chapter five I showed that *T. basalis* was highly motivated to search for *N. viridula* in open arenas contaminated with footprint compounds, whereas it spent only a fifth of the time searching in arenas contaminated by *C. simplex*. The reverse was true for *T. oenone*: it spent at least twice as long searching for *C. simplex* as it did for *N. viridula*, and it spent even less time searching for *N. viridula* than *T. basalis* spent searching for *C. simplex*. These results show that, like other scelionid egg parasitoids, *T. oenone* is capable of distinguishing adult hosts based solely on the footprint kairomones they leave behind on a substrate (Colazza et al., 2007; Conti & Colazza, 2012). It also

suggests *T. oenone* may be able to distinguish between physiological hosts and non-hosts, based on the lower amount of time it spent searching for *N. viridula*, relative to the amount of time *T. basalis* spent searching for *C. simplex*. As far as I know, these are the first arrestment results reported for a scelionid parasitoid in relation to a physiological host versus a non-host, and the possibility that these parasitoids are able to distinguish host from non-host based solely on substrate-borne semiochemicals is certainly worth exploring further.

Parasitoids often exploit multiple cues to find their hosts, including semiochemicals, vibratory signals, taste, colour, shape, and host morphology (Bin et al., 1993; Colazza et al., 2014; Iacovone et al., 2016). Ohsaki et al. (2020) elegantly demonstrated how physical cues and substrate-borne chemical cues can act synergistically to mediate arrestment and searching behaviour. *Chelonus inanitus* L. (Hymenoptera: Braconidae), an egg-larval parasitoid of Lepidoptera, antennated and probed glass beads when they were similar in size to real-life hosts (0.4-1mm in diameter) but not to eggs measuring 0.2mm in diameter. Parasitoids also searched filter paper arenas contaminated with egg extracts of the host *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). When Ohsaki et al. (2020) applied host extract to 0.2mm beads, all parasitoids antennated and probed them, and parasitoids searched for much longer than in treatments containing untreated glass beads or extract on filter paper alone. These experiments demonstrate how parasitoids use multiple sensory modalities when making decisions about whether or not to attack a host, and these kinds of studies are directly relevant for assessing the likelihood of a BCA attacking a non-target in the field.

Plants can react to the presence of herbivores by producing volatile synomones attractive to parasitoids in response to feeding (herbivore induced plant volatiles; HIPVs) or oviposition (oviposition-induced plant volatiles; OIPVs) (Colazza, Fucarino, et al., 2004; Turlings & Erb, 2018). These volatile compounds can be used by parasitoids to locate plants infested with hosts over long ranges (Thanikkul et al., 2017), or they can be used as contact cues on the surfaces of plants. Contact choice assays are commonly used to test responses of parasitoids to substrate-borne herbivore-induced plant compounds (Hilker & Meiners, 2006). Conti et al. (2010) showed that *Brassica oleracea* L. plants were capable of producing their own substrate-borne compounds in response to feeding by the pentatomid pest *Murgantia histrionica* (Hahn), and that these cues were detected and exploited by the egg parasitoid *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae). Similarly, oviposition by *Pieris brassicae* L. (Lepidoptera: Pieridae) on Brussels sprouts appears to induce plants to produce compounds which arrest *Trichogramma brassicae* Bezdeko (Hymenoptera: Trichogrammatidae) on the surfaces of leaves, but these compounds are only produced 72 hours following oviposition (Fatouros et al., 2005). The tritrophic interactions between plants, herbivores, and parasitoids add an additional layer of complexity to the chemical basis of host-location, but more research into these systems would contribute significantly to a better understanding of the ecology underlying host-parasitoid relationships.

Stink bugs (Hemiptera: Pentatomidae) and their hymenopteran parasitoids in the family Scelionidae have been a model system for substrate-borne arrestment studies for decades, but there is still much to learn about how chemical ecology influences the ecological host ranges of egg parasitoids (Austin et al., 2005; Conti & Colazza, 2012). Stink bugs leave footprint kairomones in epicuticular waxes on leaf surfaces which are then exploited by egg parasitoids as cues for host location (Colazza et al., 2009; Conti et al., 2003; Frati et al., 2013). Female scelionid parasitoids are capable of discriminating between adult hosts, not only based on their sex, but also based on their reproductive stage (Colazza et al., 1999). Colazza et al. (2007) compared blends of hydrocarbons from different sexes of *N. viridula* using solvent extracts and direct-contact solid phase microextraction of their cuticles, and footprint compounds left on glass plates. They used open arena bioassays to show how female *T. basalis* use *n*-nonadecane to discriminate between male and female *N. viridula*, as female bugs do not leave this compound behind. Building on this work, Salerno et al. (2009) showed that *T. brochymenae* are able to rank hosts based not only on their sex, but also on their reproductive state. Female parasitoids spent longer searching in arenas for mated female stink bugs in pre-ovipositional states, and remarkably, this preference was directly related to the successful transfer of sperm between pentatomids. These kinds of studies show the importance of well-designed behavioural experiments in the process to identify compounds eliciting important behaviours in parasitoids, and arrestment experiments provide such a method.

Stink bugs and their egg parasitoids are also an excellent system for demonstrating the utility of comparing results from no-choice oviposition tests with results from arrestment experiments to improve risk assessments for parasitoids (Conti et al., 2004). For example, *Telenomus podisi* Ashmead and *Trissolcus urichi* (Crawford) both parasitise the eggs of *Piezodorus guildinii* (Westood) at high rates in no-choice oviposition tests (Cingolani et al., 2014). But subsequent work showed these two parasitoids differed in their retention times in arenas contaminated with the footprints of this same host (Cingolani et al., 2019). Another comparison between the generalist egg parasitoid *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) and *Trissolcus basalis*, showed that *O. telenomicida* spent the same amount of time searching in open arenas contaminated by different *N. viridula* treatments as it did in uncontaminated control arenas (Peri et al., 2011). This suggests *T. basalis* would be more successful in locating *N. viridula* under field conditions as it is able to perceive footprint kairomones left behind by *N. viridula* and able to discriminate between different sexes and reproductive stages of its hosts. This ability would allow it to rank the hierarchical value of hosts based on the reliability of finding egg masses to exploit (Peri et al., 2006). The superior host-finding ability of *T. basalis* over *O. telenomicida* was confirmed experimentally in a two year field and semi-field study examining intraguild interactions and parasitism rates against *N. viridula* for these two parasitoids in Western Sicily (Peri et al., 2014). If a parasitoid is unable to perceive substrate-borne kairomones left by a potential host, or if it shows little interest in searching for these cues, then it is reasonable to infer the parasitoid will spend less time and effort searching for that host in the field.

Comparing different parasitoids based on how strongly they are arrested by different hosts can indicate their relative host finding abilities, and offer clues as to their likely performance against those hosts in the field.

Hedstrom et al. (2017) combined no-choice oviposition tests with arrestment experiments to examine the suitability and attractiveness of native North American Pentatomidae to *T. japonicus*, compared to its target host *H. halys*. Parasitoids were retained in open arenas at similar times to *H. halys* for three non-target species: *Chinavia hilaris* (Say), *Chlorochoa ligata* (Say), and *Banasa dimidiate* (Say). However, parasitoids discovered less than 30% of *Chi. hilaris* and *Chl. ligata* egg masses, and parasitoids developed in less than 6% of eggs for these species, suggesting the eggs are both unattractive and unsuitable for development. On the other hand, fifty nine percent of *B. dimidiate* egg masses were discovered by parasitoids, and parasitoids developed in 48% of eggs, which suggest the parasitoid may be more likely to find and exploit this host in the field. Lagôa et al. (2020) demonstrated how a thorough understanding of searching motivation can provide highly relevant information on host preferences, which could then be applied to pre-release risk assessments. They conducted a series of open arena arrestment experiments with *Tr. basalis* and *Te. podisi* to natural footprints and footprint extracts from *Euchistus heros*, *Dichelops melacanthus*, and *N. viridula*. *Trissolcus basalis* spent longer searching in arenas contaminated by *N. viridula* or their extracts, while *Te. podisi* spent longer searching for the other two species, with higher average residence times for *E. heros*. These experiments demonstrated the innate ability for each parasitoid to discriminate between three different pentatomid hosts. The methodology developed by Lagôa et al. (2020) could easily be applied to examine searching motivation in parasitoids which are being evaluated for non-target risks as part of pre-release host-specificity testing. No-choice and choice arrestment studies would be a valuable complement to no-choice and choice oviposition tests, and would contribute relevant information about the hierarchical value of hosts for each parasitoid.

Arrestment to substrate-borne host compounds could also be used to examine the strength of host-parasitoid relationships, or the risk of native parasitoids falling into 'evolutionary traps' through their attraction to and oviposition in novel hosts, which may be unsuitable for parasitoid development (Abram et al., 2014). *Coptera occidentalis* Muesebeck (Hymenoptera: Diapriidae), a pupal parasitoid of fruit flies (Diptera: Tephritidae), was arrested by larval trails of four fruit fly species, but showed the greatest retention time on its natural host (Granchietti et al., 2012). This is despite parasitoids having been reared on a factitious host (one of the other three fruit fly species tested) for over 80 generations, which shows the link between the parasitoid and its natural host is very strong. González et al. (2011) used open arena bioassays to test hexane extracts made with the cocoons, meconia, and prepupae of the mud dauber wasp *Trypoxylon politum* Say (Hymenoptera: Crabronidae) to identify kairomonal activity mediating behavioural responses by its ectoparasitoid *Melittobia digitata* Dahms (Hymenoptera: Eulophidae). Parasitoids responded strongly to cocoon and meconium extracts, alone and when mixed together, but not to the prepupa, which is the stage these parasitoids attack. A

reconstructed blend of the major fatty acids detected in meconium and cocoon extracts elicited a weak response, suggesting minor or undetected compounds may play an important role in arresting the parasitoid. Peri et al. (2021) showed how *T. basalis* exhibited typical arrestment behaviour in the presence of volatiles from the invasive *H. halys*, but that in oviposition tests, *T. basalis* emerged at very low rates (6%) indicating low developmental compatibility between host and parasitoid. This suggests *T. basalis* could fall into an evolutionary trap by pursuing BMSB but then failing to develop or emerge in sufficiently high numbers, which could then have an impact on the efficacy of this parasitoid as a biocontrol agent against *N. viridula*.

1.4 Electrophysiological identification of kairomones

Insect antennae are complex sensory structures specialised to detect chemical stimuli important for mating, avoiding predators, dispersing, feeding, or finding hosts (Hansson, 1999). Parasitoids rely heavily on olfaction in most stages of the host location process (Meiners & Peri, 2013; Vinson, 1998), and because their capacity to locate hosts is directly tied to their reproductive success, their sensory abilities are under strong selective pressure to distinguish between reliable and unreliable cues indicating the presence of their hosts (Hansson & Stensmyr, 2011). Parasitoids have often evolved to detect and exploit a set of innately attractive cues associated with their hosts, and some of these associations are plastic enough to be modified through learning (Giunti et al., 2015; Vet & Groenewold, 1990). Cuticular hydrocarbons, defensive secretions, and pheromones are commonly used by parasitoids as reliable medium to short-range kairomones (Blomquist & Ginzel, 2021; Fatouros et al., 2008). Plant volatiles function as longer range cues, and these can include compounds emitted as part of an inducible defence to feeding or oviposition by herbivores (i.e., herbivore-induced plant volatiles; HIPVs) (Hilker & Meiners, 2006; McCormick et al., 2012; Mumm & Dicke, 2010; Turlings & Erb, 2018). The study of chemical ecological interactions between parasitoid BCAs and their potential hosts can provide important insights into host-specificity, and can contribute to a greater understanding of how motivated a parasitoid is to search for a particular host (Ngumbi et al., 2009, 2010). This type of information is extremely valuable for non-target risk assessments of classical BCAs, particularly when no-choice oviposition results are often the only kind of evidence available (Cingolani et al., 2019; Conti et al., 2004). Unravelling the chemical identity of key compounds mediating these behaviours requires the ability to separate and characterise the components of host extracts containing complex blends of volatile organic compounds (VOCs) (Blomquist & Ginzel, 2021; Fatouros et al., 2008; Moraes et al., 2008). The process of identifying olfactory-active compounds in host extracts broadly follows a three step process (Barbosa-Cornelio et al., 2019): extraction of analytes, chemical analysis, and recording antennal detection.

The first step for identifying olfactory-active compounds is to extract, purify, and pre-concentrate analytes of interest from host insects or plants (Reyes-Garcés et al., 2018). First a biological sample is exposed to an extractant material which adsorbs analytes. The most common

VOC extraction formats are solvent rinses, headspace collection, and direct contact between extractants and biological samples. For solvent extraction, whole insects, specific body parts, or tissues are immersed in a non-polar solvent (hexane, pentane, dichloromethane) or a partially polar solvent (acetone), times varying between five minutes and several days (Barbosa-Cornelio et al., 2019; Jones & Oldham, 1999). Analytes extracted in solvent may then be concentrated to ensure there are sufficient amounts for detection during chemical analyses (Barbosa-Cornelio et al., 2019; Pawliszyn, 2012), and this usually involves the partial or total evaporation of solvent under a gentle nitrogen stream, followed by resuspension of analytes in fresh solvent. For headspace extraction, analytes can be collected statically/passively, where insects are placed into a bag and compounds collect on an adsorbent material, or they may be collected dynamically/actively, where insects are placed in a chamber connected to a vacuum pump which draws the headspace through a filter which traps compounds of interest (Blight, 1990). Solid phase microextraction (SPME) is a popular technique for extracting analytes in conjunction with headspace collection, which involves the use of small volumes of coated fibers to extract small quantities of analytes from a sample. SPME is considered to be faster and more versatile than other techniques as it requires no solvent, works with liquid or gaseous samples, and is highly sensitive (Pawliszyn, 2012). Multiple treatments can be extracted in this way simultaneously. For example, an insect, an insect on a plant, a plant, and a blank control treatment can all be extracted simultaneously through parallel air flow circuits. Important variables in dynamic extraction include the volume of enclosures, air flow rate, sampling duration, and the amount of adsorbent material used to capture analytes (Jones & Oldham, 1999). Once analytes have been extracted and concentrated, the resulting mixture is analysed to identify compounds in the extract (and to ensure extraction procedures have been successful).

Analytes in a biological sample can be separated with gas chromatography, and then identified through the coupling of flame ionization detection and mass spectrometry to the GC (Barbosa-Cornelio et al., 2019). Gas chromatography works on the basis that each compound in a sample has a slightly different affinity for the special coating applied to the inner surface of the column the sample moves through. The sample is heated by a programmed series of temperature ramps, until each component in the sample has eluted out of the GC and across a detector (Barbosa-Cornelio et al., 2019). Flame ionization detectors are commonly used to detect eluting compounds, and the resulting retention indices can be compared with a homologous series (for example n-alkanes) to tentatively identify compounds. These detectors can also be used to quantify the amounts of compounds in a sample when internal standards are added before analysis. Gas chromatography coupled with mass spectrometry is a more reliable identification method, as the mass spectral 'fingerprint' of each compound can be calculated and compared with large databases holding spectral values for a wide range of compounds (Jones & Oldham, 1999). Once the compounds in a biological extract have been identified and/or quantified, the insect neurophysiological system itself can be used to screen extracts for kairomones and other semiochemicals (Pickett et al., 2012).

Schneider (1957) pioneered electroantennography by recording the olfactory receptor potential in relation to different VOCs across two glass capillary microelectrodes positioned on either side of a bumblebee antenna. Electroantennograms display the difference in electrical potential when olfactory receptor neurons depolarise following detection of a responsive stimulus (Roelofs, 1984). The magnitude of responses usually rise with the concentration of the stimulus up to a saturation point, although certain ecologically relevant compounds can show very high responses at low doses (Morawo et al., 2017). The electrodes consist of silver wires sheathed with glass capillaries pulled to fine points, and these capillaries are filled with a saline-based solution such as Ringers or Kaisslings solution (Kaissling, 1995). The reference electrode is positioned into contact with the insect body or excised portion of the head, while the recording electrode is positioned into contact with the distal tip of one of the insect's antennae (and sometimes it is useful to excise half of the distal flagellomere). This type of insect preparation can be used with GC-EAD, where a sample is fractionated in the GC and delivered over the antenna one compound at a time (Arn et al., 1975). The resulting response peaks in the insect can be matched to compound peaks from the GC-FID to identify responsive compounds (Struble & Arn, 1984). Responses are amplified and sent to a digital signal acquisition controller which connects to a computer to display and record responses in real time. Alternatively, a sample delivery system can be used to manually puff air through a disposable glass pipette containing an odour cartridge. Compounds from the cartridge are puffed into a stream of humidified air, terminating at the insect preparation. Air and solvent puffs can be used as controls to ensure that the compounds inside the extracts, and not the solvents, are eliciting real responses. The Single Sensillum/Cell Recording technique (SSR/SCR) allows responses from individual olfactory neurons to be measured by inserting tungsten microelectrodes into an antennal sensillum and measuring the impulse frequency generated in response to odours (Jones & Oldham, 1999). SSR can be used to identify different classes of olfactory receptor neurons, and to infer odour-specificity based on the response profiles to host and non-host compounds (Wee et al., 2016). In other words, SSR can help to determine whether or not an insect is capable of distinguishing between different compounds that all elicit neurophysiological responses on the antennae, and this information is useful, for example, for understanding whether or not a parasitoid is capable of distinguishing between different hosts based on their volatile profiles. This technique is capable of showing responses to minute quantities of compounds, and it offers a thorough understanding of the ability of an insect to differentiate between different compounds (Wadhams, 1984).

Electrophysiological techniques are useful for revealing the chemical identities of host-associated kairomones and herbivore-induced plant volatiles which are attractive to parasitoids (Gouinguené et al., 2005; Mumm & Dicke, 2010; K. C. Park et al., 2001). These kinds of experiments are commonly used to isolate and identify odorants worthy of further behavioural testing in arena bioassays or olfactometers (Colazza et al., 2014). Electrophysiological techniques are especially useful to employ once kairomonal activity has been demonstrated between a parasitoid and a host

with crude extracts or whole insects. For example, *Anaphes nitens* (Girault), a mymarid parasitoid of the eucalyptus weevil, *Gonipterus spp.* Schoenherr, was found to be attracted to egg capsules and faeces from its host, and to leaves of *Eucalyptus globulus* Labillardière, in small petri arenas (Branco et al., 2021). Subsequent GC-MS/EAD analysis showed 45 compounds elicited an antennal response, and most of these were found to be emitted by the host plant, but were also present in faeces or on the host. GC-EAD is useful for the simultaneous discovery of an intraspecifc cue in a host insect which also elicits a kairomonal response in a parasitoid (Dweck et al., 2010). Kpongbe et al. (2019) were able to isolate the aggregation pheromone of the African legume pest *Clavigralla tomentosicollis* Stål (Hemiptera: Coreidae), and show that this pheromone was also used by the egg parasitoid *Gryon* sp. as a kairomone. Initial behavioural assays showed only male bugs attracted both sexes of parasitoids, so male headspace extracts were analysed through GC-EAD with both stink bugs and egg parasitoids. Isopentyl butanoate elicited responses in bugs and parasitoids, and attraction of both were demonstrated through behavioural assays (Kpongbe et al., 2019). Zhong et al. (2017) reported electrophysiological and behavioural responses of *Trissolcus japonicus* to (*E*)-2-decenal and tridecane in hexane extracts of *Halyomorpha halys*, and these two compounds are known to be common components of stink bug volatile profiles (Borges & Aldrich, 1992; Moraes et al., 2008).

Qualitative differences in either the volatile profiles of hosts, or the ability of different parasitoids to perceive them, can be responsible for important differences in attraction displayed by parasitoids. Ngumbi et al. (2009) compared the relative strength of EAG responses between the specialist parasitoid *Microplitis croceipes* (Cresson) and the generalist *Cotesia marginiventris* (Cresson), in order to see if each responded more strongly to different parts of the headspace blend taken from plants infested with *Heliothis virescens* (F.), a shared host, or *Spodoptera exigua* (Hübner), a host only of the generalist. The generalist parasitoid showed higher responses to green leaf volatiles, while the specialist showed higher responses to some of the HIPV components induced by herbivory of *H. virescens*. These results suggest even minor qualitative differences in HIPVs induced by herbivore damage can still provide important cues for specialist parasitoids to exploit (Turlings & Erb, 2018). Similarly, Li et al. (2020) used electrophysiological techniques to show how the eulophid parasitoid *Chouioia cunea* Yang could detect six compounds common to three lepidopteran hosts. Interestingly, parasitoids had a clear preference for *Hyphantria cunea* Drury in behavioural bioassays, and all three hosts were selected at different rates (Li et al., 2020). I report a similar situation in chapter four where New Zealand stink bugs were found to have similar profiles of olfactory-active volatile compounds, but present in slightly different ratios. In chapter five I show that, despite similar profiles, *T. basalis* and *T. oenone* had significantly different retention times in arenas contaminated by *C. simplex* or *N. viridula*, although footprint profiles in arenas may be qualitatively different to adult solvent rinses.

Electrophysiological techniques are also useful for teasing out the complexities behind the different responses of parasitoids to the same host or host plant. Ortiz-Carreon et al. (2019) identified

three compounds released by maize plants, whose levels are influenced by herbivory from *Spodoptera frugiperda* (Smith): α-pinene, α-longipinene and α-copaene. They demonstrated that the braconid egg-larval parasitoid *Chelonus insularis* Cresson responded to these compounds in GC-EAD experiments, but that the compounds were not attractive to the parasitoid in isolation, only when blended. The blend of α-pinene and α-copaene was even more attractive than extracts made from damaged maize plant leaves. In contrast, Sun et al. (2020) demonstrated how *Campoletis chlorideae* Uchida was only attracted to herbivore-induced volatiles when they were presented singly, but not in blends. Feeding by *Helicoverpa armigera* (Hübner) induced production of *cis*-jasmone and *cis*-3-hexenyl acetate, and when these compounds were applied to tobacco plants in olfactometer tests, parasitoids preferred single compounds at certain doses rather than a blend of the two (Sun et al., 2020). Other compounds associated with the host habitat or surrounding environment may also play an important role in the ability of a parasitoid to locate its host. For example, Fürstenau et al. (2016) used GC-EAD and EAG recordings to show that the parasitoid *Holepyris sylvanidis* Brèthes responded to two compounds associated with its host *Tribolium confusum* (Jacquelin du Val)—(E)-2-nonenal and 1-pentadecene—and that the addition of uninfested habitat substrate increased their attractiveness to parasitoids. These results suggest the chemical ecological factors mediating interactions between hosts, their plants, and parasitoids are complex, and that different plant-herbivore-parasitoid systems exhibit different relationships among the diverse chemical compounds and the insect and plant species within that system (Hilker & McNeil, 2008). Electrophysiological techniques are therefore highly valuable in disentangling this complexity in order to more easily study the attraction between parasitoids and hosts based on kairomonal activity.

1.5 Conclusions

Classical biological control can be an excellent tool for managing pests, and is likely to be an important long-term strategy for controlling emerging pests such as brown marmorated stink bug, spotted lantern fly, and spotted wing drosophila. But care must be taken to characterise the host-specificity of proposed agents as thoroughly as possible. Pre-release risk assessments are critical for providing regulators with important information on the chances of a proposed BCA causing non-target effects. Traditional physiological host range tests are an essential component for any classical biological control programme, as they provide unambiguous evidence of the list of species an agent can attack and develop in. These results demonstrate whether or not non-target species are recognised as hosts by the agent, and whether or not they are suitable for the agent to develop in. However, by design, physiological host range tests are unable to assess the full suite of host location behaviours normally expressed by a parasitoid in the field. Parasitoids rely on olfaction as the primary sensory modality mediating their interactions with potential hosts. Therefore, the application of chemical ecological techniques to characterise odorants and demonstrate how they shape the host range of parasitoids would offer valuable information for pre-release non-target risk assessments. Some of

these techniques are being incorporated into behavioural work more frequently, but few studies have utilised them within the context of biological control and realised their value for non-target risk assessment. There is a clear gap and opportunity for host range studies to make more explicit use of olfactometry, arrestment studies, and electrophysiology when characterising the biosafety of proposed BCAs.

Behavioural bioassays involving olfactometers and arrestment to substrate-borne volatiles are useful for confirming kairomonal activity before electrophysiological investigations, and for confirming the behavioural functions of compounds identified through electrophysiological techniques. The behavioural methods used should be appropriate for the stage of host location being examined, to ensure a good fit between the experimental protocol and expected behaviour. For example, long to medium range orientation to plant or host volatiles is best understood by using moving air olfactometers, whereas arrestment studies are usually more appropriate for examining shorter range cues such as host or egg extracts that may contain contact kairomones. Arrestment studies in particular have great potential to offer high-quality information directly relevant for non-target risk assessments. By comparing the relative searching motivation between multiple parasitoids on a non-target species, or a single parasitoid on multiple non-target species, it is possible to rank non-target hosts based on the likelihood of a parasitoid or parasitoids finding them in the field. It is also possible to rank parasitoids based on how strong their preferences are to their host-species relative to non-target species. When this information is compared to results from physiological host range testing, it provides unique insight into the risks associated with different parasitoids.

The ecological interactions between BCAs and their hosts, non-target species, plants, and the wider environment are complex and can be difficult to untangle. Electrophysiological techniques such as GC-EAD coupled with chemical analytical techniques such as GC-FID and GC-MS offer a way to investigate and make sense of this complexity, by identifying the chemical compounds eliciting behavioural responses in parasitoids. Tritrophic plant-herbivore-parasitoid systems in particular are very difficult to understand without knowledge of the chemical basis of communication between each trophic layer. The extraction, analysis, and neurophysiological assessment of compounds in host or plant extracts offers a reliable and relatively fast method to investigate these links. Identifying semiochemicals in host and plant extracts can help to identify the attractiveness of non-target species or particular habitats for BCAs, and this information is highly relevant and useful for non-target risk assessments. While electrophysiological responses to compounds do not necessarily mean the compounds have behavioural functions, GC-EAD and EAG recordings are an important and time-saving step in helping to narrow down the list of compounds which deserve further attention in behavioural assays. Electrophysiological methods offer a highly accurate and rapid way to assess parasitoids for their olfactory-active compound response profiles. Results from electrophysiological tests can then be used to design additional behavioural tests to elucidate the behavioural function of responsive compounds.

Studies which integrate physiological host range experiments with electrophysiological or behavioural components offer the most promising strategy for making progress in understanding the chemical basis of host-specificity in parasitoids (e.g. Bin et al. 1993; Colazza et al. 2007; Hedstrom et al. 2017; Fors et al. 2018; Salerno et al. 2019). Greater collaboration between ecologists, chemists, and biological control practitioners would likely result in more integrative work which is able to answer a broader range of questions about non-target risks. Future work should prioritise the combination of approaches to understand how chemistry impacts host specificity. In particular, a greater understanding of olfactory response profiles in parasitoids would help to determine how host volatiles, non-host volatiles, or the absence of these classes of compounds shape the neural basis of odour-specificity of parasitoid BCAs. Olfactometer studies are needed to compare the hierarchical attractiveness of hosts, and to investigate how the production of plant volatiles through herbivory or oviposition may affect non-target risks. Finally, arrestment studies should focus on measuring the attraction of parasitoids to different hosts, their body parts, or the products they produce. They should also be used to investigate the relative search motivation of parasitoids for non-target species, and this information could be directly or indirectly translated into relative risk scores used for pre-release studies.

1.6 Research aims and objectives

This PhD thesis presents the results of work related to a Better Border Biosecurity (B3) project called "Improving risk prediction and reducing uncertainty pre-release for classical biocontrol agents". This project is part of B3 Theme A, Risk Assessment (Intentional Introductions), and aims to improve the tools used to predict the impacts of deliberately introduced BCAs in order to reduce uncertainty for regulators. The key end user of this work is the New Zealand Environmental Protection Authority (EPA), the government agency responsible for regulating activities which affect the environment, including the release of new organisms. As the agency responsible for evaluating applications to release BCAs, the EPA is required to weigh the potential risks and benefits of new introductions, and it relies on scientific evidence in order to make its decisions. Typically, this evidence includes an assessment of a proposed biocontrol agent's physiological host range conducted in containment. Physiological host range testing is a necessary and important step in determining a candidate agent's host-specificity, but by design, these kinds of tests are unable to account for the variety of complex natural filters which influence the expression of host range in the field. Chemical ecological factors are extremely important for determining both the capacity and the likelihood of an agent to seek out and attack non-target hosts. Therefore, the application of chemical ecological techniques to evaluating the attractiveness of non-target species to proposed BCAs would help to reduce uncertainty faced by the regulator when having to make a decision based solely on physiological host range data.

This thesis was originally designed to focus on the chemical ecology of *Trissolcus japonicus* in relation to non-target New Zealand stink bugs. *Trissolcus japonicus* is a BCA of *Halyomorpha*

halys (brown marmorated stink bug, BMSB), a serious horticultural pest native to East Asia but invasive in North America and Europe. The parasitoid has been approved for release in New Zealand should the stink bug arrive, and this pre-emptive (or pro-active) approach to approving a biocontrol agent is believed to be the first example of the pre-approval of a biocontrol agent before the target pest has arrived, supported by scientific host range testing in containment. However, I was unable to import *T. japonicus* from my collaborators at the USDA in Newark, Delaware, for about a year since March 2019, after routine SEM imaging of imported parasitoids revealed unusual structures on their antennae. In order to avoid any biosecurity risks, I ceased importing parasitoids while the Ministry for Primary Industries (MPI) investigated. Ultimately, the identity of these structures was unable to be determined, but I discovered similar structures on parasitoids from my own laboratory colonies of *Trissolcus basalis* and *Trissolcus oenone*, particularly on older specimens, which suggests they may be a natural and/or widespread phenomenon in this group of wasps. MPI provided approval to continue imports in late 2019, but at this time the containment facilities at Plant & Food Research in Auckland were undergoing maintenance and could not be used until they were recommissioned at the start of 2020. I arranged to import more parasitoids but my plans were immediately disrupted by the outbreak and global spread of Coronavirus disease caused by the SARS-CoV-2 virus. New Zealand entered a complete 'level 4' lockdown on the 25th of March, with some restrictions easing on 27 April, and a return to 'level 2' restrictions on 13 May. Auckland entered another 'level 3' lockdown on 12 August which lasted until 30 August, until another lockdown between 14 to 17 February. A final lockdown for Auckland happened between 28 February and 7 March 2021. Disruptions to the project caused by the extended inability to import *T. japonicus* and the cycling of Coronavirus lockdowns meant it was necessary to pivot the project to focus on *T. basalis* and *T. oenone*, two closely related parasitoids which are already present in New Zealand. Thesis chapters are prepared as manuscripts to facilitate submission to scientific journals.

Chapter two presents the results from physiological host range testing of *T. japonicus* in relation to the endemic alpine shield bug *Hypsithocus hudsonae*. Previous host range testing in relation to non-target New Zealand stink bugs was carried out by John Charles and his colleagues at Plant & Food Research, Auckland, but they were unable to find *H. hudsonae* in the field, despite several attempts in visits to Central Otago and Southland. I used no-choice oviposition tests to identify whether or not this species was a physiological host for *T. japonicus*, and I found parasitoids attacked and emerged from this species at high rates. I combined my data with data collected by John Charles and his colleagues in order to present an overview of no-choice oviposition results with *T. japonicus* on non-target New Zealand Pentatomidae, and to compare parasitism rates between different species. I discussed my results in relation to the advantages and limitations of physiological host range testing. Despite high rates of acceptance and emergence, I did not believe *T. japonicus* posed a significant risk to *H. hudsonae* primarily due to the difference in climate and habitat structure

between the parasitoid's preferred habitats and those occupied by the shield bug. This work was published in *Austral Entomology* (DOI: 10.1111/aen.12532).

Chapter three presents the results from retrospective physiological host range testing of *T. basalis*, and physiological host range testing of *T. oenone*, in relation to New Zealand stink bug species. Only limited host range studies were conducted with *T. basalis* before it was introduced into New Zealand in 1949, and the results of this work were lost. Subsequent sporadic host range testing conducted in the 1960s by Ron Cumber lacked sufficient replication, was not always quantitative, and did not include all stink bug species in New Zealand. There were no previous host range testing records for the native *T. oenone*, and very little work had been done on this parasitoid previously. I tested both parasitoids in no-choice oviposition tests to provide an overview of their host range and to compare the hosts they were capable of attacking and developing in. I found both species were able to attack and emerge from all stink bug species tested, except *T. oenone* did not develop in or emerge from *Nezara viridula*. I combined my data with both previous physiological host range studies involving *T. japonicus* in order to summarise and compare the physiological host ranges of all three *Trissolcus* species in relation to New Zealand species of stink bugs. This work will be submitted to *Biological Control* following examination of the thesis.

Chapter four presents the results from electrophysiological experiments with *T. japonicus*, *T. basalis*, and *T. oenone*, in relation to volatile organic compounds associated with New Zealand stink bugs. I made solvent extracts with female stink bugs and tested extracts from eight stink bug species with each of the parasitoids in multiple rounds of electrophysiology experiments. I first used GC-EAD to record parasitoid antennal responses to compounds in solvent extracts. Next I used synthetic standards to confirm responses in another round of GC-EAD experiments with each parasitoid. Finally, I puffed synthetic compounds over the antennae of each parasitoid species to confirm the identity of compounds, and to measure and compare responses between each parasitoid. I found all three parasitoids responded to seven compounds associated with stink bugs, and that the response profile for *T. japonicus* was slightly different to those of *T. basalis* and *T. oenone*. I discussed my results in relation to the importance of identifying compounds which may mediate host specificity in parasitoids, and the utility of electrophysiological techniques for characterising these compounds. This chapter will be submitted to *Journal of Pest Science* following examination of the thesis.

Chapter five presents the results from a series of experiments integrating electrophysiology, open-arena bioassay experiments, and competition experiments between *T. basalis* and *T. oenone* to compare their attraction to, and relative performance on, the Australian pest of solanaceous plants *Cuspicona simplex*. I first conducted electrophysiological experiments with *T. basalis* in relation to solvent extracts of *N. viridula* eggs, in order to tentatively identify candidate compounds as the contact kairomone used by the parasitoid during host acceptance. I then examined arrestment responses and measured searching motivation between *T. basalis* and *T. oenone* on both *N. viridula* and *C. simplex* in order to compare these metrics with oviposition results. I discussed these results in

relation to how arrestment experiments showing searching motivation can be a useful complement to oviposition tests, particularly when parasitoids attack and develop in multiple species at similar rates. Finally, I conducted competition tests between the two parasitoids on *C. simplex* eggs to examine the behaviour and outcomes of extrinsic contests on the egg mass, and to examine the outcomes of intrinsic contests in multiparasitised eggs. I found parasitoids showed similar levels of aggression, but that the native parasitoid parasitized more eggs and won almost all of the larval contests in multiparasitised eggs. This work will be submitted to *BioControl* following examination of the thesis.

      Finally, chapter six is a synthesis of the findings of this work and a discussion of results in relation to the aims of the thesis.

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CHAPTER 2: Pre-emptive host-specificity testing of *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) reveals high parasitism levels against the endemic New Zealand alpine shield bug in laboratory no-choice tests

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Abstract

Brown marmorated stink bug (BMSB), *Halyomorpha halys* (Hemiptera: Pentatomidae), is a serious horticultural pest causing considerable damage to local production and international supply chains as it spreads around the world. The samurai wasp, *Trissolcus japonicus* (Hymenoptera: Scelionidae), is well recognised as the most promising classical biological control against BMSB. The wasp has been conditionally approved for release in New Zealand in the event the stink bug establishes here. Previous host range testing showed all available non-target New Zealand pentatomids except a single exotic species were accepted for oviposition, and that the parasitoid was capable of parasitising the eggs of two native pentatomids at proportions similar to BMSB. Only one New Zealand species of pentatomid, the endemic alpine shield bug *Hypsithocus hudsonae*, was not previously tested owing to the difficulty of collecting it from the field. Here I report the results of no-choice oviposition tests between *H. hudsonae* and *T. japonicus*, conducted in containment, to complement previous physiological host range testing of this parasitoid in New Zealand. Parasitoids emerged from 14 out of 15 egg masses, and in total, from 78 out of 83 eggs (94%). The mean sex ratio was 89% female, and no males emerged from six egg masses. *Hypsithocus hudsonae* is confirmed as a physiological host for *T. japonicus*, and this finding is discussed in relation to the strengths and limitations of physiological host range studies.

2.1 Introduction

Classical biological control programmes can be safe and cost-effective ways to manage the impacts of pests below economically acceptable thresholds (Caltagirone, 1981; Cock et al., 2015; Goldson et al., 2020). However, the introduction of a biological control agent (BCA) into a novel environment brings with it the risk of non-target effects (De Clercq et al., 2011; Follett & Duan, 2000; Louda et al., 2003; Lynch & Thomas, 2000). As a result, a variety of international and local regulations now govern the processes used to import and release BCAs into different jurisdictions (Barratt et al., 2017, 2018; G. A. C. Ehlers et al., 2020; R.-U. Ehlers, 2011; IPPC, 2017). Many countries require that candidate BCAs undergo host-specificity testing to ensure agents are fit for purpose and do not pose unnecessary risks to local biota (Heimpel & Cock, 2018; Hunt et al., 2008, 2011; Sheppard & Warner, 2016). Pre-release risk assessment frameworks emphasise the importance of defining a BCA's physiological (= fundamental) host range, defined as the group of species in the introduced range that are accepted as hosts, and are compatible for development of the agent (Babendreier et al., 2005; Barratt, 2011; Bigler et al., 2006; Van Driesche et al., 2004; van Lenteren et al., 2006). No-choice oviposition tests are commonly used for this purpose because they provide unambiguous evidence of both host acceptance and developmental compatibility (Van Driesche et al., 2004). Physiological host range data can be supplemented with other approaches, such as choice tests to rank host preferences in a stepwise fashion (Murray et al., 2010; Withers & Mansfield, 2005), odour specificity tests such as y-tube olfactometer experiments (Avila et al., 2016), and chemical ecological methods such as those utilising electrophysiology or behavioural tests to determine a response to compounds in a host extract (Olsson & Hansson, 2013).

Brown marmorated stink bug (BMSB), *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), is an invasive horticultural pest native to China, Japan, the Korean peninsula, and Taiwan (Lee et al., 2013). It has emerged as an important pest throughout the world, having first been detected in the United States in 1996 (Hoebeke & Carter, 2003). Since this time BMSB has spread to Canada (Fogain & Graff, 2011), at least 25 European countries (EPPO, 2019; Haye, Gariepy, et al., 2015; Wermelinger et al., 2008), Turkey (Güncan & Gümüş, 2019), Kazakhstan (Temreshev et al., 2018), and Chile (Faúndez & Rider, 2017). BMSB is now considered one of the most destructive invasive pests in its invaded ranges (Leskey & Nielsen, 2018), particularly in stone and pome fruit growing regions in the mid-Atlantic states of the USA and northeastern Italy (Bariselli et al., 2016; Leskey et al., 2012). Climate modelling predicts that the eventual global distribution of BMSB could encompass southeastern regions of South America, southern regions of Africa, parts of Central Asia, much of Southeast Asia and

the Pacific, and Australasia (Fraser et al., 2017; Kriticos et al., 2017; Zhu et al., 2012). Brown marmorated stink bug is therefore an important emerging threat to many important horticultural regions worldwide, if left unchecked.

Trissolcus japonicus (Ashmead) (Hymenoptera: Scelionidae) is an oligophagous egg parasitoid of Pentatomidae and Scutellaridae (Talamas et al., 2013; Talamas, Johnson, et al., 2015). It emerges in high numbers from parasitised BMSB egg masses, and is the most dominant natural enemy associated with the stink bug in its native range (Yang et al., 2009; Zhang et al., 2017). *Trissolcus japonicus* is considered to be the most promising BCA against BMSB in invaded ranges, having been the subject of host-range testing in North America since 2007 (Botch & Delfosse, 2018; Hedstrom et al., 2017; Lara et al., 2016, 2019; Rice et al., 2014; Talamas, Herlihy, et al., 2015), and in Europe more recently (Haye et al., 2020). This is because native natural enemies in North America (Abram et al., 2017; Dieckhoff et al., 2017; Ogburn et al., 2016) and Europe (Costi et al., 2019; Haye, Fischer, et al., 2015; Roversi et al., 2017; Stahl, Babendreier, et al., 2019) emerge in very low numbers from fresh BMSB egg masses, and are considered to be ineffective as augmentative agents. The unexpected discovery of adventive populations of *T. japonicus* in North America (Abram et al., 2019; Hedstrom et al., 2017; Jarrett et al., 2019; Milnes et al., 2016; Morrison et al., 2018; Talamas, Herlihy, et al., 2015) and Europe (Sabbatini Peverieri et al., 2018; Stahl, Tortorici, et al., 2019) illustrates its ability to disperse to new regions, and has made understanding its physiological host-range an important priority for countries where it has not yet been released, or self-established.

New Zealand is considered to be suitable for establishment of BMSB (Fraser et al., 2017; Kriticos et al., 2017; Zhu et al., 2012). Incursions of BMSB into New Zealand could significantly damage important primary industries, jeopardise successful Integrated Pest Management (IPM) programmes through the application of additional pesticides, and have severe cultural and commercial impacts on Māori, who have significant economic and cultural interests in both horticultural production and the natural estate (Ballingall & Pambudi, 2017; Burne, 2019; Teulon et al., 2019; Walker et al., 2017). A significant upswing in detections of BMSB at the New Zealand border since 2014 means the probability of stink bug establishment remains moderate to high (Duthie, 2012, 2015; MPI, 2019; Ormsby, 2018; Vandervoet et al., 2019). A pre-emptive (or proactive) biological control programme (sensu Hoddle et al., 2018) has been underway in New Zealand since 2015 with the aim of finding a suitable classical BCA before BMSB gains a foothold in the country. Previous host range testing with *T. japonicus* showed the egg masses of two native pentatomids (*Cermatulus nasalis nasalis* Westwood and *Glaucias amyoti* Dallas) were parasitised at proportions similar to BMSB, two exotic species at

proportions between 70–80% (*Dictyotus caenosus* Westwood and *Monteithiella humeralis* Walker), three species at proportions below 35% (*Cermatulus nasalis hudsoni* Westwood, *Oechalia schellenbergii* (Guérin), and *Cuspicona simplex* Walker), and one species not at all (*Nezara viridula* (L.)) (Charles et al., 2019). The New Zealand Environmental Protection Authority (EPA) has conditionally approved the release of *T. japonicus* in the event of BMSB establishing in the country (EPA, 2018), and is actively monitoring the results of further host-range testing.

One New Zealand pentatomid species has not previously been tested in physiological host range experiments: the alpine shield bug *Hypsithocus hudsonae* Bergroth. This is the only endemic New Zealand pentatomid species, and its unusual biology makes it an important addition to host range testing. It is classified as 'naturally uncommon' with the qualifier 'range restricted', as it is known from only a handful of at-risk alpine habitats in the lower South Island of New Zealand (Stringer et al., 2012). It has limited capacity to disperse owing to reduced hindwings (Larivière, 1995). *Hypsithocus hudsonae* features in important Hemiptera catalogues and checklists (Larivière, 1995; Larivière & Larochelle, 2004, 2014), but has not been the primary subject of any published research to date. Accordingly, little is known about its natural history, including reproduction, development, ecology, or even host plants. Previous host range testing on *T. japonicus* did not include *H. hudsonae* because attempts to collect it were unsuccessful (Charles et al., 2019). Incorporating *H. hudsonae* into host range testing in New Zealand is therefore an important priority. Here I present the results of no-choice oviposition tests between *T. japonicus* and *H. hudsonae*, to establish whether this endemic pentatomid species falls within the physiological host range of the parasitoid.

2.2 Methods

Collecting and rearing pentatomids

Adult specimens of *H. hudsonae* were collected in December 2017, December 2018, and January 2020, from the Otago and Southland regions of the South Island, New Zealand. In 2017 and 2020, adults were collected from Eyre Mountain (-45.297532, 168.591342) from 1350-1400 m above sea level. Adults were found under rocks, and in association with herbaceous plants including *Poa colensoi* Hook.f, *Wahlenbergia albomarginata* Petterson, *Raoulia buchananii* Kirk, *Anisotome flexuosa* Dawson and relatives, *Epilobium* sp., *Kelleria* sp., *Celmisia prorepens* Petrie, *Celmisia brevifolia* Cockayne and *Plantago lanigera* Hook.f, although no host-feeding was directly observed. In 2018, specimens were collected from Old Man Range (-45.342735,

169.225502) at an altitude of 1500 m. Here, I found the bugs near water sources on cushion plants and mosses, and again, did not observe any feeding on host plants. Voucher specimens of *H. hudsonae* in ethanol were deposited into the New Zealand Arthropod Collection, Manaaki Whenua Landcare Research, Auckland, with accession number NZAC03018492.

Laboratory cultures of pentatomids were established in clear plastic containers (~170 mm H × 210 mm L × 135 mm W) with ventilated lids. Containers were provisioned with a small plastic lid containing moist cotton wool, and sheets of folded wax paper as oviposition substrate. Cages were kept at 20°C (16:8 h L:D). Insects were moved to clean cages containing fresh materials every 2–3 days. I provided a variety of food based on availability, but primarily green beans, raw peanuts, *Coprosma* berries, sweetcorn, blueberries, plantain grass seed heads, dried apricots, and foliage from potted *Hebe odora* plants. I observed a late-instar nymph feeding on a freshly moulted conspecific nymph, so *Spodoptera* larvae were trialled as potential prey, but were not fed upon. Egg masses were collected from cages every 1–3 days and used for experiments. All eggs used for host range testing were obtained from field-collected adults, as I failed to rear *H. hudsonae* through multiple generations in the lab.

Parasitoid shipments

Trissolcus japonicus were shipped from Newark, Delaware, by the USDA-ARS Beneficial Insects Introductions Research Unit. Parasitised BMSB egg masses held in 10-dram plastic vials were air-couriered from Newark to the Plant & Food Research invertebrate containment facility at Mt Albert in Auckland, New Zealand. Egg masses were held in a temperature controlled room at 20°C and 16:8 LD photoperiod while parasitoids emerged and mated. Parasitoids were then transferred to their own plastic vials and provided with a small amount of honey on the lid as a carbohydrate source. All remaining BMSB egg mass material was frozen for 48 h at -20°C and then autoclaved to comply with biosecurity protocols.

No-choice oviposition tests

To prepare egg masses for experiments, fresh (< 72-h-old) masses were taken from the colony and mounted onto a piece of double-sided tape attached to a strip of card (appx. 20 mm x 40 mm). Exposed sticky tape was coated with fine sand (White 200 "Scenic Sand", Activa® products) to avoid trapping parasitoids. Each no-choice oviposition replicate consisted of placing a prepared egg mass into a 10-dram plastic vial, and introducing a single naïve female

parasitoid between 3 and 17 days old (owing to the timing of pentatomid egg availability and parasitoid shipments). No-choice tests were carried out at a constant temperature of 20°C and 16:8 LD photoperiod. After 48 h of exposure, the female parasitoid was removed from the vial and discarded.

I observed each exposure for up to 60 minutes to investigate the link between host acceptance and the developmental fate of attacked eggs. I recorded how long it took each female to make contact with the egg mass, the sequence and duration of each oviposition event (defined as having started when the parasitoid raised her wings until after she marked the egg with the tip of her ovipositor), the total time for the female to oviposit into all the eggs in each mass, and whether there were any aborted attempts (defined as having occurred when a female failed to mark an egg after withdrawing her ovipositor). I examined exposed *H. hudsonae* egg masses every day to record the timing of parasitoid emergence. I recorded the number and sex of emerging parasitoids. Eggs from which neither pentatomid nor parasitoid developed were classified as undeveloped.
Owing to the priority of maximising the number of replicates for host range testing, I used all the egg masses produced after the December 2018 trip in no-choice oviposition tests. Egg masses produced in December 2017 during the attempt to rear the species were used as pseudo-control masses to record pentatomid emergence in the absence of parasitism.

2.3 Data analysis

I combined my dataset with data from Charles et al. (2019) to compare no-choice egg parasitism by *T. japonicus* against *H. hudsonae*, and the eight other New Zealand pentatomid taxa tested by those authors. I calculated 'percentage egg parasitism' as the proportion of eggs from which a parasitoid emerged, excluding empty eggs within each mass. This differs from the way parasitoid development was reported by Charles et al. (2019), as those authors excluded egg masses which were not accepted by a female (i.e. they excluded egg masses from which no parasitoids emerged). Similarly, I report mean pentatomid emergence and mean proportion of undeveloped eggs across all egg masses within each treatment, not just from masses which were parasitised to some degree. The number of eggs in each mass reported by Charles et al. (2019) already exclude empty eggs, whereas none of my *H. hudsonae* egg masses contained any empty eggs. I calculated 'percentage mass acceptance' as the proportion of egg masses from which at least one wasp successfully emerged. I tested whether there were differences in percentage egg parasitism between the different pentatomid species with a binomial generalised linear mixed

effect model (GLMM). I included the source of the data as a random effect with two levels (data collected by us and data collected by Charles et al. 2019). I calculated *post hoc* pairwise comparisons to identify which combinations of pentatomid species were significantly different in their probability of parasitoid emergence, and corrected for multiple comparisons using the false discovery rate correction (Table 1). I calculated estimated marginal means and confidence intervals for each pentatomid species, and back-transformed these onto the original scale to examine the probability of emergence. All analyses were performed in R (R Core Team, 2020).

Table 1: Post-hoc pairwise comparisons between pentatomid species for probability of *Trissolcus japonicus* parasitoid emergence.

contrast	estimate	SE	z.ratio	*p*-value
Cermatulus nasalis hudsoni – *C. nasalis nasalis*	-2.52	0.18	-14.23	<0.01
C. nasalis hudsoni – Cuspicona simplex	-0.53	0.18	-2.94	<0.01
C. nasalis hudsoni – Dictyotus caenosus	-1.55	0.20	-7.88	<0.01
C. nasalis hudsoni – *Glaucias amyoti*	-3.80	0.20	-18.86	<0.01
C. nasalis hudsoni – *Hypsithocus hudsonae*	-4.12	0.49	-8.43	<0.01
C. nasalis hudsoni – *Monteithiella humeralis*	-2.11	0.22	-9.66	<0.01
C. nasalis hudsoni – Nezara viridula	29.46	110009.52	0.00	1.00
C. nasalis hudsoni – Oechalia schellenbergii	0.23	0.18	1.23	0.29
C. nasalis nasalis – C. simplex	1.98	0.10	19.23	<0.01
C. nasalis nasalis – D. caenosus	0.97	0.13	7.59	<0.01

C. nasalis nasalis – G. amyoti	-1.28	0.14	-9.43	<0.01
C. nasalis nasalis – H. hudsonae	-1.61	0.47	-3.45	<0.01
C. nasalis nasalis – M. humeralis	0.40	0.16	2.52	0.02
C. nasalis nasalis – N. viridula	31.97	110009.52	0.00	1.00
C. nasalis nasalis – O. schellenbergii	2.74	0.11	24.88	<0.01
C. simplex – D. caenosus	-1.01	0.13	-7.60	<0.01
C. simplex – G. amyoti	-3.27	0.14	-23.11	<0.01
C. simplex – H. hudsonae	-3.59	0.47	-7.68	<0.01
C. simplex – M. humeralis	-1.58	0.16	-9.58	<0.01
C. simplex – N. viridula	29.99	110009.52	0.00	1.00
C. simplex – O. schellenbergii	0.76	0.12	6.51	<0.01
D. caenosus – G. amyoti	-2.25	0.16	-14.06	<0.01
D. caenosus – H. hudsonae	-2.58	0.47	-5.44	<0.01
D. caenosus – M. humeralis	-0.57	0.18	-3.12	<0.01
D. caenosus – N. viridula	31.00	110009.52	0.00	1.00
D. caenosus – O. schellenbergii	1.77	0.14	12.76	<0.01

G. amyoti – H. hudsonae	-0.32	0.48	-0.68	0.64
G. amyoti – M. humeralis	1.69	0.19	9.02	<0.01
G. amyoti – N. viridula	33.26	110009.52	0.00	1.00
G. amyoti – O. schellenbergii	4.03	0.15	27.46	<0.01
H. hudsonae – M. humeralis	2.01	0.48	4.16	<0.01
H. hudsonae – N. viridula	33.58	110009.52	0.00	1.00
H. hudsonae – O. schellenbergii	4.35	0.47	9.27	<0.01
M. humeralis – N. viridula	31.57	110009.52	0.00	1.00
M. humeralis – O. schellenbergii	2.34	0.17	13.80	<0.01
N. viridula – O. schellenbergii	-29.23	110009.52	0.00	1.00

2.4 Results

Collecting and rearing pentatomids

During the first field trip to collect *H. hudsonae* in December 2017, I collected 25 adults from Eyre Mountain and attempted to rear them through a complete generation. Adults laid a total of ten egg masses between 29 December 2017 and 20 January 2018. I observed nymph emergence from all egg masses. From a total of 94 eggs, 41 nymphs emerged (58.5%) but only one developed through to adult successfully, with the majority developing to second or third instar. Total nymph emergence was 58.5% across masses (mean 64.5%, range 20–100%). Undeveloped eggs did not change in appearance from when they were laid and were assumed to have been infertile. Owing to the difficulty of collecting and rearing this species, I used data from these unexposed egg masses as a negative pseudo-control treatment.

In December 2018, I collected 11 adults from Old Man Range. These insects produced 15 egg masses in the lab between 24 December 2018 and 4 January 2019. Masses contained 2 to 11 eggs (mean = 5.5). These egg masses were exposed to *T. japonicus* in the no-choice oviposition experiment reported here. Eggs from which neither pentatomid nor parasitoid emerged were assumed to have been infertile or unviable. During this trip I observed a significant number of *H. hudsonae* adults feeding on bird droppings, probably from *Haematopus unicolor* Forster (variable oystercatcher) observed in the area. I collected some bird droppings, and once back at the lab in Auckland I observed stink bugs would readily feed on them. However, I only tested this once with pentatomids from this trip.

In January 2020 I returned to Eyre Mountain and collected 30 adults and 24 nymphs. These specimens ultimately produced only two egg masses, and I was unable to use them in experiments owing to the closure of containment facilities for maintenance at this time.

Table 2: Mean number of *Trissolcus japonicus* eggs, proportion of pentatomid emergence, parasitoid sex ratio, and proportion of undeveloped eggs for each treatment within no-choice oviposition tests against New Zealand pentatomid species. Control treatments refer to pentatomid egg masses allowed to develop and hatch in the absence of parasitism, whereas No-Choice treatments refer to pentatomid egg masses exposed to *T. japonicus* in no-choice oviposition tests.

Species	Treatment	Egg masses	Mean number of eggs	Mean pentatomid emergence	Mean female wasp sex ratio	Mean proportion undeveloped
Cermatulus nasalis hudsoni	Control	3	21.3	33.3%	-	66.7%
C. nasalis hudsoni	No-Choice	11	21.2	46.4%	87.5%	35.8%
C. nasalis nasalis	Control	47	29.0	83.8%	-	16.2%
C. nasalis nasalis	No-Choice	42	28.2	10.5%	84.3%	10.7%
Cuspicona simplex	Control	72	9.0	74.7%	-	25.3%
C. simplex	No-Choice	71	11.1	41.9%	75.6%	29.6%
Dictyotus caenosus	Control	7	8.7	56.8%	-	43.2%
D. caenosus	No-Choice	26	13.2	15.9%	82.1%	32.2%
Glaucias amyoti	Control	50	13.7	96.5%	-	3.5%
G. amyoti	No-Choice	70	13.7	4.6%	87.3%	4.7%
Hypsithocus hudsonae	Control*	10	9.4	64.5%	-	35.5%
H. hudsonae	No-Choice	15	5.5	6.7%	89.0%	2.8%
Monteithiella humeralis	Control	30	11.4	90.9%	-	9.1%
M. humeralis	No-Choice	19	11.4	19.7%	89.0%	14.2%

Nezara viridula	Control	35	53.6	43.5%	-	56.5%
N. viridula	No-Choice	34	59.8	49.0%	0.0%	51.0%
Oechalia schellenbergii	Control	36	28.1	93.9%	-	6.1%
O. schellenbergii	No-Choice	36	26.4	77.0%	91.9%	6.6%

*Due to logistical constraints, control replicates for the *H. hudsonae* treatment, in which nymph emergence was recorded in the absence of parasitism, were conducted after a previous collection event in a different year. Data relating to pentatomid species other than *H. hudsonae* comes from: Charles JG, Avila GA, Hoelmer KA, et al. (2019) Experimental assessment of the biosafety of *Trissolcus japonicus* in New Zealand, prior to the anticipated arrival of the invasive pest *Halyomorpha halys*. *BioControl* 64(4): 367–379. DOI: 10/ggrtkp. Data taken from Charles et al. is analysed and presented differently to how it is presented in their original article

Acceptance and Parasitism

Trissolcus japonicus accepted 93% of *H. hudsonae* egg masses, and parasitized a mean of 91% of eggs within masses. When combined with similar data from Charles et al. (2019), percentage parasitism varied significantly between pentatomid species (df = 8, F = 1287.7, *p* < 0.001). *Hypsithocus hudsonae* placed a close second out of nine New Zealand pentatomid taxa with respect to the highest measures of parasitism for both of these parameters (Figure 1). The probability of parasitoid emergence was significantly different among all combinations of pentatomid species except *C. nasalis hudsoni* and *O. schellenbergii*, and *H. hudsonae* and *G. amyoti* (Table 1). None of the comparisons between *N. viridula* was significant owing to the absence of parasitism observed for this species. Plotting estimated marginal means showed *T. japonicus* had the highest probability of emerging from *H. hudsonae* and *G. amyoti*, while *C. nasalis hudsoni* and *O. schellenbergii* represented the lowest probability of emergence (Figure 2).

Parasitoids made contact with egg masses after a mean of 185 ± 303 seconds (range: 2–1221 seconds), and each oviposition event took a mean of 197 ± 37 seconds (range 154–321 seconds). To complete oviposition for each mass, parasitoids took a mean of 374 seconds ± 182 seconds for each egg present. Within the single egg mass from which no parasitoids emerged, all three eggs gave rise to pentatomid nymphs. Two eggs within attacked masses remained undeveloped. The mean sex ratio of emerging parasitoids was 89% female, which was similarly high for the other pentatomid species tested (Table 2).
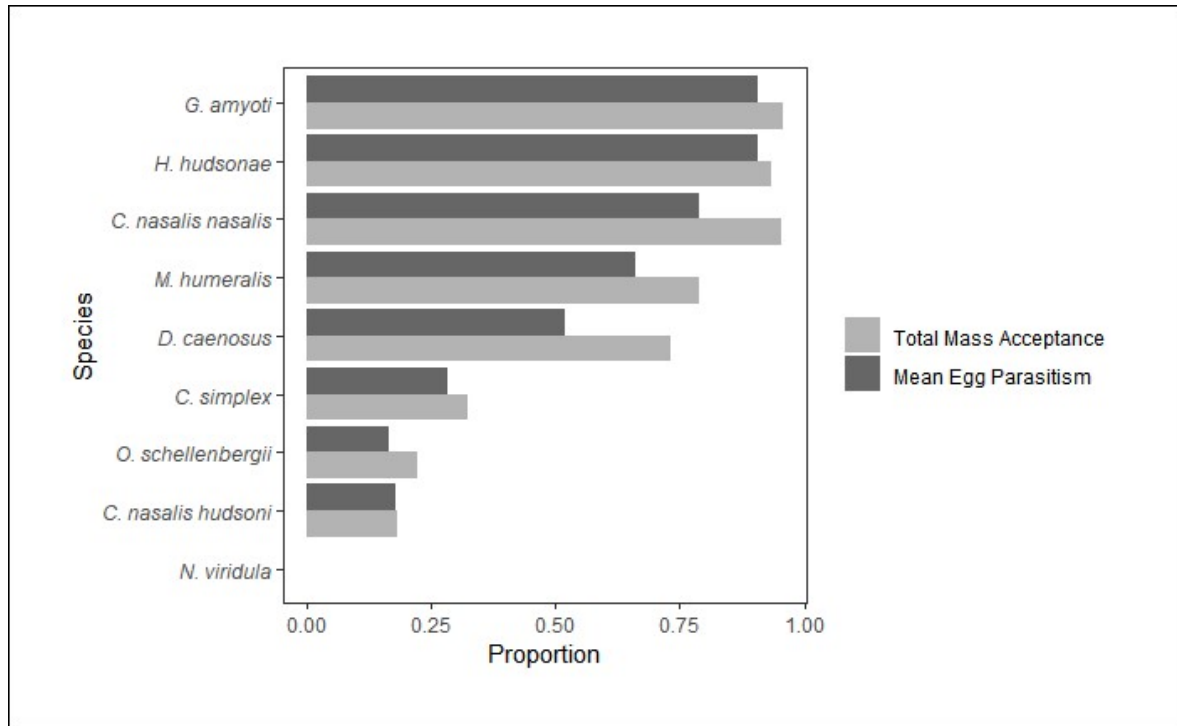
Figure 1: Total mass acceptance and mean egg parasitism for each pentatomid species exposed to *Trissolcus japonicus* in no-choice oviposition experiments.

Following exposures, I observed a female ovipositing into at least one egg from all but one egg mass, and this was the only mass from which no parasitoids emerged. For attacked masses, I directly observed all except two eggs to be attacked by a parasitoid (each from a different mass) within the initial 60-minute observation period. One of these gave rise to a parasitoid, while the other was ultimately classified as undeveloped. I observed a single incidence of superparasitism where a female went through a successful pattern of oviposition twice on the same egg, and a parasitoid ultimately emerged from this egg. I observed three occasions where the female aborted her oviposition attempt but went on to complete oviposition successfully (spread between two eggs), and parasitoids ultimately emerged from both these eggs.
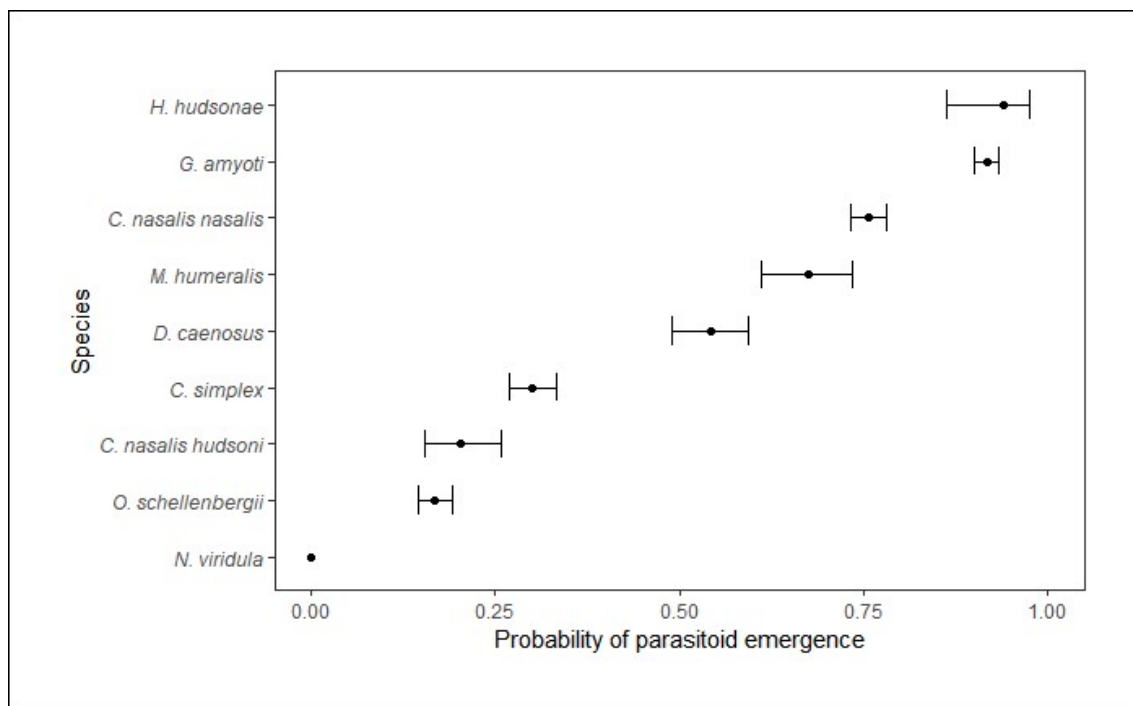
Figure 2: Probability of parasitoid emergence (with 95% confidence intervals) for each pentatomid species exposed to *Trissolcus japonicus* in no-choice oviposition experiments

2.5 Discussion

Host suitability

To determine whether the endemic New Zealand pentatomid *H. hudsonae* was a physiological host for *T. japonicus*, I exposed 15 egg masses to the parasitoid in small-arena no-choice oviposition experiments. I found that female *T. japonicus* readily attacked *H. hudsonae* eggs. Parasitoids accepted over 90% of masses offered to them, and mean percentage parasitism was very high, at 91%. These results unambiguously classify *H. hudsonae* as a physiological host of *T. japonicus*, and show it is one of the most highly parasitized New Zealand pentatomid species in no-choice oviposition tests. Within attacked masses, only two eggs did not develop into parasitoids. Although I exposed parasitoids to egg masses for 48 hours, I can confidently compare my results to those obtained during 24-hour exposures because I directly observed almost all oviposition events occurring within the initial observation period for each egg mass. I collected data on the timing of events in the acceptance and oviposition process, and offspring sex ratios, to complement emergence data from my experiments. Behavioural observations help to clarify links between host acceptance (the extent to which the parasitoid oviposits into host eggs) and host suitability (the

proportion of host eggs which support successful development of the parasitoid) (Barton Browne & Withers, 2002).

Overall, the sex ratio was strongly female-biased, and in six out of 15 egg masses, no males were produced at all. This is an unusual result, as it runs counter to the common finding that scelionid parasitoids produce at least one male offspring in each host egg mass, owing to a 'males first' strategy (Austin et al., 2005; Cumber, 1964; Eberhard, 1975; Johnson, 1984, 1987). *Trissolcus* parasitoids encounter relatively stable numbers of aggregated hosts, which females are usually able to monopolise, while emerging offspring typically copulate with siblings prior to dispersing (Johnson, 1987). Accordingly, local mate competition predicts a flexible strategy where the sex ratio should tend toward females as the number of hosts increases (Hamilton, 1967; Strand, 1988; Waage, 1982). This prediction has been experimentally confirmed in *T. basalis* by Colazza & Wajnberg (1998), who showed that females altered the sex ratio of their brood by changing the sequence in which they allocate sex to their offspring, as the size of the host patch increases. The proportion of female offspring increased up to egg masses containing 32 eggs, after which it decreased slightly for larger egg masses. Females were found to start their sequences with males, then lay a certain number of female eggs, before switching back to progressively allocating more male offspring near the end of the sequence, potentially because of sperm depletion (Colazza & Wajnberg, 1998).

Previous testing

Charles et al. (2019) reported the acceptance and development of *T. japonicus* on other New Zealand pentatomid species under similar laboratory conditions. They reported high egg mass acceptance for *Glaucias amyoti* Dallas, *Cermatulus nasalis nasalis* Westwood, and the target host *H. halys*. Interestingly, they reported much lower percentage parasitism for the target host than those reported here for *H. hudsonae*, which suggests *H. hudsonae* eggs may provide a more favourable environment for development. Taken together, my results, and those reported by Charles et al. (2019), provide a comprehensive assessment of the physiological host range of *T. japonicus* in relation to New Zealand pentatomid species.

Laboratory host range testing conducted with *T. japonicus* overseas shows wide discrepancies in the ability of the parasitoid to attack and develop in non-target pentatomid species. In China, where *T. japonicus* and *H. halys* are native, the parasitoid attacked egg masses from seven out of eight non-target pentatomid species (Zhang et al., 2017). Mean

percentage egg parasitism was greater than 70% for all species tested, suggesting high host suitability among Chinese Pentatomidae, as might be expected. These results are similar to those collected in Europe, where all 11 non-target pentatomid species were attacked in no-choice tests conducted by Haye et al. (2020), and all but three pentatomids were attacked in tests conducted by Sabbatini-Peverieri et al. (2021). Mean percentage egg parasitism was also relatively high at 83.2%, although no parasitoids emerged from the eggs of *N. viridula*. Host range testing in the Western United States shows *T. japonicus* will attack the eggs of around two thirds of the non-target North American pentatomid species presented to it. However, it achieves a relatively low mean percentage egg parasitism between 25 and 35% in these species (Hedstrom et al., 2017; Lara et al., 2019). In the Eastern United States, *T. japonicus* attacked only 3.3–10% of the egg masses produced by three non-target species (Botch & Delfosse, 2018). Taken together, these results suggest that Chinese, European, and New Zealand pentatomid species are more attractive and more suitable for the development of *T. japonicus* than those from North America. This makes sense as the North American fauna would probably be more distantly related to faunas from the other three regions, although exploring this idea further is difficult because of a lack of phylogenetic analyses of sufficient resolution within the Pentatomidae (McPherson, 2018).

One explanation for the high proportion of successful parasitoid development and high sex ratios may be the lack of an immune defence in this pentatomid species. Even closely related host species can vary widely in the efficacy of their immune response to shared egg parasitoids (Reed et al., 2007). For example, the eggs of two species of Californian eucalyptus longhorned borers, *Phoracantha semipunctata* F. and *P. recurva* Newman (Coleoptera: Cerambycidae), both exhibit a wound-healing response to oviposition by the egg parasitoid *Avetianella longoi* Siscaro (Hymenoptera: Encyrtidae) (Reed et al., 2007). But only *P. recurva* eggs initiate a cellular encapsulation response to destroy parasitoid eggs and larvae. It is possible *H. hudsonae* eggs lack the cellular processes necessary to encapsulate parasitoid eggs, or that the parasitoid is able to effectively suppress this kind of immune response (Abdel-latief & Hilker, 2008).

The lack of male emergence in my study cannot be explained by the low numbers of eggs in the masses I exposed to parasitoids, as males are often laid early in the sequence (Colazza & Wajnberg, 1998). *Trissolcus* species are known to produce more females up until the age of around 5 days (Powell & Shepard, 1982), but I always observed female-biased sex ratios regardless of the mother's age. I also cannot explain this result as an outcome of the way parasitoids were confined together prior to experiments (Strand, 1988). Local mate

competition, influenced by the effect of host quality, may have played a role in the low numbers of male *T. japonicus* emerging from *H. hudsonae* (Charnov et al., 1981; Werren & Simbolotti, 1989). Overall, high proportions of emergence coupled with strongly female-biased sex ratios indicate *H. hudsonae* is at least a highly suitable physiological host for *T. japonicus*.

Implications for non-target effects

Based on no-choice oviposition test results, the risk of non-target effects may be greater in Europe and New Zealand than in North America if the relative performance of arthropod BCAs on target and non-target species in laboratory tests predicts the risk of non-target attack in the field, as it does for weed BCAs (Paynter et al., 2015). CLIMEX modelling predicts the potential distribution of *T. japonicus* in New Zealand would extend to most of the North Island, most of the South Island excluding the west coast, and could be much greater than the potential distribution of *H. halys* (Avila & Charles, 2018). Overlap with the endemic *H. hudsonae* would occur only in areas predicted to be climatically suitable for *T. japonicus*. BMSB is often associated with human environments during its initial dispersal phase, before expanding to wetland, forest, and agricultural habitats (Leskey & Nielsen, 2018; Wallner et al., 2014). Therefore it seems unlikely that parasitoids would venture far from high densities of BMSB associated with agroecosystems and their surrounding vegetation (Lee et al., 2013; Leskey et al., 2012), or other non-target pentatomids in their food web (Todd et al., 2020). *Trissolcus japonicus* favours arboreal habitats (Herlihy et al., 2016), so it seems unlikely to expand into high-altitude environments dominated by low herbage and cushion plants. Overall, the high percentage parasitism I observed against *H. hudsonae* does not imply there is a high risk of attack in the field, and when climate modelling, parasitoid habitat preferences, and BMSB dispersal behaviour are taken into account, I believe the risk of non-target effects on *H. hudsonae* by *T. japonicus* is low. *Trissolcus japonicus* shares a similar physiological host range with *T. basalis* and *T. oenone*, overlapping with all known hosts of each, except for *N. viridula*, which *T. oenone* has also never been recorded parasitising (Cumber, 1964). Additional no-choice experiments are currently underway to update the physiological host range of *T. basalis* and *T. oenone* with quantitative data to explore this further.

    Trissolcus japonicus accepted *H. hudsonae* eggs at very high proportions, unambiguously demonstrating this pentatomid species falls within the parasitoid's

physiological host range. A major strength of no-choice oviposition tests is that a negative result offers unambiguous evidence for the test species being outside the host range of the proposed agent, while a positive result offers clear evidence that the agent is able to attack and develop on the test species (Babendreier et al., 2005; Murray et al., 2010; van Lenteren et al., 2006). However, care needs to be taken in how positive results are interpreted. Confirming a non-target species as a physiological host of a proposed biological control agent does not necessarily mean this species will be attacked in the field. No-choice tests are designed to be simple confinement experiments to maximise the chance of attack, to minimise the risk of a potential physiological host going unnoticed. As a result, they remove a variety of filters which are present in the natural environment and which exert considerable influence on the process parasitoids use to find their hosts (Babendreier et al., 2005; Van Driesche et al., 2004; van Lenteren et al., 2006).

Semiochemicals associated with plants and potential hosts are important cues which parasitoids use to make decisions about which hosts to search for, and ultimately, which hosts to parasitise (Colazza & Wajnberg, 2013; Godfray, 1994; Hilker & McNeil, 2008; Meiners & Peri, 2013). Scelionid egg parasitoids are known to use kairomones associated with adult hosts, and the adhesive material surrounding eggs, as important host location and acceptance cues (Bin et al., 1993; Conti et al., 2003; Strand & Vinson, 1982; Tognon et al., 2018). Like other *Trissolcus* species, *T. japonicus* is able to detect kairomones from adult pentatomids on both natural and artificial substrates, and displays different arrestment responses for different pentatomid species (Boyle et al., 2020; Colazza et al., 1999; Conti et al., 2004). Studies on BCAs and their potential for non-target effects are increasingly incorporating chemical ecology methods. Studies assessing odour-specificity in moving-air experiments (Avila et al., 2016; Park et al., 2019), arrestment responses to contact or volatile chemical cues (Colazza et al., 2009; Tognon et al., 2017, 2018), and electrophysiology help us to understand which specific compounds are capable of eliciting behavioural responses (Zhong et al., 2017). Ultimately, a better understanding of the chemical ecology of candidate BCAs in relation to non-target species will provide important information for pre-release risk assessments, and could become an important complement to traditional oviposition tests.

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CHAPTER 3: Retrospective host specificity testing shows *Trissolcus basalis* (Wollaston) and the native *Trissolcus oenone* (Dodd) (Hymenoptera: Scelionidae) have overlapping physiological host ranges in New Zealand

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Abstract

Trissolcus japonicus Ashmead (Hymenoptera: Scelionidae) was recently approved for release in New Zealand against brown marmorated stink bug *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), subject to the pestiferous stink bug establishing here. However the existing host-parasitoid complex between New Zealand pentatomids, the introduced biological control agent *Trissolcus basalis* Wollaston, and the native pentatomid parasitoid *Trissolcus oenone* Dodd, is poorly understood. Host range testing was never undertaken before *T. basalis* was released in New Zealand in 1949 against green vegetable bug (*Nezara viridula* [L.]), and subsequent work in the 1960s was only of a qualitative nature. I conducted no-choice oviposition tests between the two resident *Trissolcus* species and all available New Zealand pentatomid species to characterise the physiological (=fundamental) host ranges of these parasitoids. I present the results of the first retrospective host specificity study on *T. basalis* in New Zealand. My results show *T. basalis* attacks and develops in all nine pentatomid taxa I exposed it to (including the endemic alpine species *Hypsithocus hudsonae* Bergroth), while *T. oenone* attacks and develops in seven out of eight pentatomid species I tested it against (and its capacity to attack *H. hudsonae* remains unknown). Parasitism efficiencies for all treatments exceeded 60%, while development times were similar for both parasitoids regardless of host. I discuss the importance of physiological host range testing for understanding potential non-target effects, and I examine my results in the context of potential competition between introduced parasitoids for non-target species.

3.1 Introduction

Classical biological control has proven to be an effective and economical way to manage the impacts of pests (Caltagirone, 1981; Cock et al., 2016; Stiling & Cornelissen, 2005; Van Driesche et al., 2018; Walker et al., 2017). However, introducing a biological control agent into a new environment may lead to unintended consequences for non-target species (Barratt, 2011; Boettner et al., 2000; Follett & Duan, 2000; Louda et al., 2003; Lynch & Thomas, 2000). A growing awareness of non-target effects has encouraged the development and adoption of regulations governing the import and release of natural enemies (Barratt et al., 2017; G. A. C. Ehlers et al., 2020; R.-U. Ehlers, 2011; IPPC, 2017). At the same time, biological control researchers have been instrumental in pushing for broader acceptance of standardised methods to assess the host specificity of agents before they are released (Barratt et al., 2000; Bigler et al., 2006; van Lenteren et al., 2003, 2006a). The combination of these approaches has led to a gradual shift away from the use of generalist biological control agents over the last few decades, towards those with genus- or species-level host specificity (Van Driesche & Hoddle, 2016).

Laboratory testing forms an essential component of risk assessments for candidate biological control agents (Babendreier et al., 2005; Barratt et al., 2010; van Lenteren et al., 2006b). No-choice tests are commonly recommended as a first step to define the list of species which support the successful development of the agent through its entire life cycle [i.e. it's physiological (=fundamental) host range] (Van Driesche et al., 2004; Van Driesche & Murray, 2004). These tests aim to maximise the probability of attack by confining the agent with a test species for an extended period of time in order to confirm whether or not it is a physiological host (Murray et al., 2010; Withers & Mansfield, 2005). Consequently, no-choice tests provide clear evidence of whether or not a candidate biological control agent can attack and develop on a potential host species, and are therefore routinely employed for pre-release risk assessments (Bigler et al., 2006; Van Driesche et al., 2004; van Lenteren et al., 2006b). However, many historical releases of biological control agents were conducted in the absence of such testing (Van Driesche & Hoddle, 2016). Carrying out retrospective host range studies following release can provide important information about the specificity of agents, fill lingering knowledge gaps about risks to non-target species, and may help to predict non-target risks posed by closely related agents currently being considered for release

(Avila et al., 2016; Cameron et al., 2013; Haye et al., 2005; Hinz et al., 2014; Louda et al., 2003).

Many species of herbivorous stink bugs (Hemiptera: Pentatomidae) are considered to be important crop pests around the world due to the damage they cause by feeding on plant tissues and vectoring plant pathogens (McPherson, 2018). Accordingly, some stink bug pests have only become invasive recently, while others have been the targets of biological control programmes for some time (Conti et al., 2021). Brown marmorated stink bug *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) has recently emerged as one of the most important crop pests in many parts of the world (Leskey & Nielsen, 2018), sparking a biological control response centred on research into the host specificity of its most promising natural enemy, *Trissolcus japonicus* (Hymenoptera: Scelionidae) (Buffington et al., 2018; Rice et al., 2014). Scelionid egg parasitoids are commonly employed against invasive stink bugs as biological control agents due to their parasitism efficiencies and acceptable host-specificities (Austin et al., 2005). *Nezara viridula* (L.) is perhaps the most well-known pentatomid pest, having spread through most of the warmer parts of the world on the back of expanded crop production (J. W. Todd, 1989). *Trissolcus basalis* (Wollaston) is closely associated with *N. viridula* in the Americas, Europe, the Middle East, South Asia, and has been deliberately introduced against the stink bug in Hawaii, Australia, New Zealand, and other Pacific Islands (Jones, 1988). *Trissolcus basalis* was reported to have been broadly successful at suppressing local *N. viridula* populations, but this view has since given way to the consensus that past introductions of *T. basalis* were only one component among many factors contributing to declines in the stink bug in these areas (Abram et al., 2020).

Green vegetable bug was first reported as a pest in New Zealand in 1944 (Cumber, 1949). The Department of Scientific and Industrial Research imported around 300 *T. basalis* parasitoids from Australia into New Zealand to serve as rearing stock in 1948. Around 48,000 adult *T. basalis* were eventually released in the North Island of New Zealand (Cumber, 1950). Post-release surveys over the next 3 years showed the parasitoid had successfully established, and was already having a significant impact on pest populations around release sites (Cumber, 1953). While the parasitoid appears to have been successful in suppressing *N. viridula*, its physiological host range was never established before it was released, and the parasitoid has received little research attention in New Zealand. *Trissolcus basalis* is known to attack non-target New Zealand pentatomids (Clarke, 1990; Cumber, 1964; Loch & Walter,

1999), but levels of host acceptance and parasitism efficiency have never been quantitatively assessed. In addition to *T. basalis*, two other *Trissolcus* species are known from New Zealand: the endemic *Trissolcus maori* Johnson, a parasitoid of species in the Acanthosomatidae, and the native pentatomid parasitoid *Trissolcus oenone* (Dodd), which is also known to occur in Australia (Cumber, 1964; N. F. Johnson, 1991). Cumber (1964) observed *T. oenone* attacking *C. nasalis*, *O. schellenbergi*, *M. humeralis*, *C. simplex*, *D. caenosus*, and *G. amyoti*, but did not observe any attacks on *N. viridula*. He did not report the proportion of masses or eggs from which parasitoids successfully emerged. A single Australian study investigated the influence of temperature on the development and survivorship of *T. oenone* reared on *Biprorulus bibax* Breddin in the laboratory (James & Warren, 1991), but New Zealand *Trissolcus* species have not been the primary subject of any further research to date.

The New Zealand pentatomid fauna is relatively depauperate compared to other regions of the world, and consists of only six phytophagous species and two predatory species (Table 1) (Larivière, 1995; Larivière & Larochelle, 2014). The predatory species *Cermatulus nasalis* Westwood (Hemiptera: Pentatomidae) is divided into three subspecies: the endemic *C. nasalis turbotti* restricted to the Three Kings Islands, the endemic and predominantly southern *C. nasalis hudsoni*, and the native and predominantly northern *C. nasalis nasalis*. The other predatory species, *Oechalia schellenbergii* Guérin, is native and widespread. Both predatory species are generalists, but are considered to be beneficial as they are often observed feeding on horticultural pests. The phytophagous species *Hypsithocus hudsonae* Bergroth, the alpine shield bug, is endemic to New Zealand, known from only a handful of mountain sites in the lower South Island, and is classified as 'naturally uncommon' and 'range restricted' (Stringer et al., 2012). Very little is known about its basic biology or ecology as it has never been the primary subject of any research, likely due to the difficulties in finding, collecting, and rearing it. One phytophagous species is native, *Glaucias amyoti* Dallas, while the remaining four are introduced: *Monteithiella humeralis* Walker, which is observed feeding primarily on *Pittosporum* and *Coprosma* fruits; *Dictyotus caenosus* Westwood, which favours the seeds of grasses such as *Plantago*; the Southern green stink bug, *N. viridula*; and *Cuspicona simplex* Walker, a pest on cultivated nightshades.

The host ranges of New Zealand pentatomid parasitoids are currently of interest due to the recent conditional approval of *Trissolcus japonicus* Ashmead (Scelionidae:

Hymenoptera) to be released in New Zealand as a classical biological control agent against the brown marmorated stink bug, should the stink bug establish here (EPA, 2018). Pre-release host specificity testing showed all pentatomids in New Zealand except *N. viridula* are physiological hosts of *T. japonicus* (Charles et al., 2019), including the endemic alpine shield bug, *Hypsithocus hudsonae* Bergroth (Saunders et al. 2021). It is therefore important to understand the physiological host ranges of each resident *Trissolcus* species before a third is introduced. This will help to forecast potential intraguild competition amongst biological control agents (Stahl et al., 2020), and help to predict how the addition of a new parasitoid may influence non-target effects on the local pentatomid fauna (Konopka et al., 2019; J. H. Todd et al., 2020). Here I present the results of retrospective no-choice oviposition tests for the two pentatomid parasitoids, *T. basalis* and *T. oenone*, against eight out of ten New Zealand pentatomid taxa.

3.2 Methods
Rearing of pentatomids

Pentatomid colonies were established from field-collected specimens, mostly from the Auckland region. Pentatomid laboratory cultures were established in clear plastic containers (~170 mm H × 210 mm L × 135 mm W) with ventilated lids. Containers were provisioned with moist cotton wool to regulate humidity, and sheets of folded wax paper as oviposition substrate. Containers were kept in a temperature controlled room at 20-25°C (16:8 h L:D cycle) depending on the size and condition of the colony. Insects were moved to clean containers containing fresh materials every 2-4 days. Egg masses were collected from cages once every 1-3 days and placed inside a ventilated petri dish containing filter paper and moist cotton wool. Food was provided once nymphs moulted to second instar. I provided a variety of food based on published literature and availability, but primarily: *Pittosporum* and *Coprosma* fruits for *M. humeralis* and *G. amyoti*, *Solanum* fruits and tomatoes for *C. simplex*, green beans and raw peanuts for *N. viridula*, *Plantago* seed heads for *D. caenosus*, and *Spodoptera* larvae from an existing laboratory culture for *C. nasalis nasalis*, *C. nasalis hudsoni*, and *O. schellenbergii*. *Hypsithocus hudsonae* were observed feeding on green beans, raw peanuts, *Coprosma* berries, sweetcorn, blueberries, plantain grass seed heads, dried apricots, and foliage from potted *Hebe odora* plants (Saunders et al. 2021). *Cermatulus*

nasalis turbotti was excluded from the study owing to its restricted range on the Three Kings Islands and the difficulty of collecting specimens, while *Hypsithocus hudsonae* was excluded due to the difficulty in rearing this species in captivity.

At the end of the laying season in April, *G. amyoti*, *M. humeralis*, and *C. nasalis hudsoni* were moved to a 14-16°C dark incubator and provided with less food to encourage diapause. After 8 weeks they were moved back to the regular feeding and temperature regimen to encourage the resumption of egg laying. *C. nasalis nasalis* and *C. simplex* didn't require exposure to lower temperatures as they kept laying sporadically through the winter. To feed the two phytophagous species during their artificial winter and from June-November, *Coprosma* and *Pittosporum* fruits were collected in early April and stored at 4°C in kiwifruit

Table 1: Biostatus, distribution, and hosts of New Zealand Pentatomidae.

Species	Subfamily	Biostatus	Distribution (including commonness)	Hosts
Cermatulus nasalis hudsoni	Asopinae	Endemic	New Zealand. Found throughout the South Island in montane to subalpine areas.	Alpine insects and their larvae on low vegetation and shrubs.
Cermatulus nasalis nasalis	Asopinae	Native	Australia, East Timor, and New Zealand. Common throughout the North and South Islands in lowland to subalpine areas.	Primarily Lepidoptera and Coleoptera larvae on a wide range of introduced and native plants.
Cermatulus nasalis turbotti	Asopinae	Endemic	New Zealand. Known only from coastal lowland areas on the Three Kings Islands.	Insects and their larvae on mānuka.
Cuspicona simplex	Pentatominae	Exotic	Australia and New Zealand. Common throughout the North Island and the top half of the South Island.	Exclusively Solanaceae. Pest on cultivated nightshades.
Dictyotus caenosus	Pentatominae	Exotic	Australia, New Caledonia, and New Zealand. Common throughout the North and South Islands in lowland to subalpine regions.	Closely associated with introduced low herbs and grasses, particularly *Plantago*. Occasional pest on blackberries and lucerne.
Glaucias amyoti	Pentatominae	Native	Australia, East & West Timor, Palau, Papua New Guinea, and New Zealand. Common throughout the North Island and top of the South Island in lowland to montane regions. Found on the Kermadec Islands.	Closely associated with *Coprosma*, and to a lesser extent, *Pittosporum* and other native and introduced trees.
Hypsithocus hudsonae	Pentatominae	Endemic	New Zealand. Known from only a few alpine sites in Central Otago and the Southern Lakes Districts.	Unknown but speculated to include alpine shrubs and tussocks. Observed feeding on droppings from *Haematopus unicolor* Forster (variable oystercatcher) at Old Man Range, Southland.
Monteithiella humeralis	Pentatominae	Exotic	Australia and New Zealand. Common throughout the North Island and top of the South Island in lowland to montane regions.	Closely associated with *Pittosporum*, and to a lesser extent, *Coprosma*.
Nezara viridula	Pentatominae	Exotic	Almost worldwide. In New Zealand, common throughout lowland to montane regions of the North Island and top half of the South Island. Found on the Kermadec Islands.	Polyphagous pest on a wide range of vegetable crops.
Oechalia schellenbergii	Asopinae	Native	South Pacific, Australia, Philippines, and New Zealand. Common throughout lowland to montane regions in the North Island and top of the South Island.	Primarily the larvae of Lepidoptera and Coleoptera.

trays lined with plastic sheets. Most fruit stored this way remained viable until December, when new fruits were available. New colonies of *D. caenosus* had to be established each season as they did not resume laying eggs after entering an initial diapause.

Rearing and identification of Trissolcus *parasitoids*

A colony of *T. basalis* was established from parasitised *N. viridula* egg masses collected on *Cleome* from Kelmarna Gardens, Auckland, in February 2019. This colony was reared through approximately 15 generations in the lab before being used in experiments. A colony of *T. oenone* was established from parasitised *C. simplex* egg masses collected on *Coprosma* (near *Solanum*) from the suburb of Mt Albert, Auckland, in November 2019. This colony was reared through approximately 3 generations in the lab before being used for experiments. Parasitoid colonies were maintained in a temperature controlled room at 25°C (16:8 h L:D cycle) on the eggs of the species they were originally collected in. Fresh (<24h old) or stored host eggs (i.e., at 10°C for up to 2 weeks) were used to maintain the colonies. Following exposure, the female parasitoid was removed after 1-3 days and the resulting progeny usually started to emerge after 11-13 days, with males emerging first. I separated the two *Trissolcus* species based on the following characters: *T. basalis* femur and scape orange, and mesopleural foveae (row of foveae at posterior margin of mesopleuron) the same size throughout; *T. oenone* femur and scape darkened, and mesopleural foveae increasing in size from top to bottom.

No-choice oviposition tests

I performed no-choice oviposition tests with New Zealand pentatomid species in order to determine the physiological host range of each parasitoid species. Fresh (<24h old) egg masses were mounted onto a piece of double-sided tape attached to a strip of card (20mm x 40mm). Exposed sticky tape was coated with fine sand (White 200 "Scenic Sand", Activa® products) to avoid trapping parasitoids. Each no-choice oviposition replicate consisted of a prepared egg mass which was placed into a plastic screw-top vial (length 60mm, diameter 28mm), before introducing a single naïve female parasitoid which was between one and five days old. No-choice tests were carried out at 20°C and 16:8h L:D cycle in order to be comparable with previous host range testing experiments conducted with *T. japonicus*

(Charles et al. 2019; Saunders et al. 2021). After 24h the female parasitoid was removed from the vial. I recorded the date of emergence, number and sex of parasitoids, and waited two weeks from the initial emergence date to dissect any unhatched eggs to determine their fate. I classified eggs as being empty, containing an unemerged pentatomid nymph, containing an unemerged parasitoid, as undeveloped (undifferentiated jelly-like material), or as containing an emerged pentatomid or parasitoid. I conducted experiments with a total of 35 parasitoid-pentatomid treatments, and aimed for a minimum of 20 replicates for each treatment. However, I achieved fewer replicates for six treatments due to the low availability of egg masses for these species.

3.3 Data analysis

I pooled my dataset with no-choice results reported by Charles et al (2019) and Saunders et al. (2021) to compare no-choice oviposition tests between *T. basalis*, *T. oenone*, and *T. japonicus*, with New Zealand Pentatomidae. Following Bin & Vinson (1991), I report 'discovery efficiency' (defined as the proportion of egg masses which were discovered, or parasitized to some degree, by the parasitoid), and 'parasitism efficiency' (defined as the mean proportion of parasitized eggs within discovered masses). I also define 'undeveloped' eggs as those containing neither developing pentatomid or parasitoid, and typically these eggs were filled with a yellow or brown jelly-like substance. While I classified egg masses as parasitized through the presence of emerged or unemerged parasitoids in my dataset, egg masses were only counted as parasitized through the presence of emerged parasitoids in the Charles et al. dataset (as they did not record the contents of unhatched eggs). However, there were very few egg masses which contained unemerged parasitoids without also including emerged parasitoids, so this change didn't meaningfully affect my results. It should be noted that Charles et al. (2019) group unemerged parasitoids, unemerged pentatomids, and undeveloped eggs together as 'unhatched eggs', while I recorded each of these separately. The number of eggs in each mass reported by Charles et al. already excludes empty eggs, whereas I recorded this separately and exclude them for calculations used to compare the two datasets.

I tested for differences in parasitism efficiency between the different parasitoid and pentatomid species with a binomial GLMM. I calculated post-hoc pairwise comparisons to identify which combinations of parasitoid and pentatomid treatments were significantly

different in their probability of parasitoid emergence, and corrected these for false discovery rate for multiple comparisons. I calculated estimated marginal means and confidence intervals for each combination, and back-transformed these onto the original scale to plot the probability of emergence. I also tested whether the time for parasitoid development differed significantly based on pentatomid host or parasitoid species with a Poisson GLMM. I compared results from *T. basalis* and *T. oenone*, but was only able to include *T. japonicus* development time in *H. hudsonae* as Charles et al. (2019) didn't record development time. To examine the duration of development for each parasitoid species within each pentatomid species, I calculated estimated marginal means and confidence intervals, and back-transformed these onto the original scale. I removed treatments with fewer than eight replicates to aid in readability, as these treatments produced very large confidence intervals (*N. viridula* × *T. japonicus*, *N. viridula* × *T. oenone*, *C. nasalis hudsoni* × *T. oenone*, *H. hudsonae* × *T. basalis*, *C. nasalis nasalis* × *T. oenone*). All analyses were performed in R 4.0.2 (R Core Team, 2020).

3.4 Results

No-choice oviposition tests

In total, *Trissolcus basalis* discovered 91% of the egg masses it was exposed to, and achieved a mean parasitism efficiency of 87%. *Trissolcus oenone* discovered a total of 65% of the egg masses it was exposed to, and achieved a mean parasitism efficiency of 93%. Finally, *T. japonicus* discovered a total of 57% of the egg masses it was exposed to, and achieved a mean parasitism efficiency of 86% (Figure 1). Mean female sex ratios were highest in *T. oenone* (81%), followed by *T. japonicus* (74%), and *T. basalis* (70%).

Mean parasitism efficiency differed in the combined dataset based on a significant interaction between species of pentatomid and species of parasitoid (F = 1741.7, df = 15, p < 0.001). The highest probability of emergence was *T. oenone* from the eggs of *M. humeralis*, while the lowest probability to emerge was *T. basalis* from the eggs of *N. viridula* (Figure 2; Table 2). *Trissolcus oenone* and *T. japonicus* failed to develop or emerge from the eggs of *N. viridula* at all. *Trissolcus basalis* and *T. oenone* were equally likely to emerge from the three exotic species *M. humeralis*, *D. caenosus*, and *C. simplex*, and from the native *G. amyoti*, while *T. japonicus* generally had a lower probability of emerging from non-target species

than either of the two resident parasitoids (Figure 1). All combinations of parasitoids and pentatomids had a mean parasitism efficiency over 75% except for *T. japonicus* on

Table 2: Total discovery efficiency, mean pentatomid nymph emergence, and mean parasitism efficiency for pentatomid species exposed to three egg parasitoids.

Pentatomid species	Treatment	n	Pentatomid nymphs	Discovery efficiency	Parasitism efficiency	Parasitoid female sex ratio	Unhatched
Cermatulus nasalis hudsoni	Control	43	29.93%	NA	NA	NA	70.07%
Cermatulus nasalis hudsoni	*Trissolcus basalis*	19	0.00%	89.47%	87.17%	80.53%	12.83%
Cermatulus nasalis hudsoni	*Trissolcus japonicus*	11	46.40%	18.18%	17.79%	87.45%	35.81%
Cermatulus nasalis hudsoni	*Trissolcus oenone*	1	0.00%	100.00%	88.00%	90.91%	12.00%
Cermatulus nasalis nasalis	Control	78	83.31%	NA	NA	NA	16.69%
Cermatulus nasalis nasalis	*Trissolcus basalis*	35	0.00%	100.00%	90.37%	82.76%	9.63%
Cermatulus nasalis nasalis	*Trissolcus japonicus*	42	10.52%	95.24%	78.76%	84.34%	10.72%
Cermatulus nasalis nasalis	*Trissolcus oenone*	4	57.18%	25.00%	25.00%	93.75%	17.82%
Cuspicona simplex	Control	149	81.72%	NA	NA	NA	18.55%
Cuspicona simplex	*Trissolcus basalis*	54	2.38%	96.30%	91.19%	77.22%	6.43%
Cuspicona simplex	*Trissolcus japonicus*	71	41.87%	32.39%	28.52%	75.57%	29.61%
Cuspicona simplex	*Trissolcus oenone*	41	2.20%	97.56%	94.88%	83.47%	2.93%
Dictyotus caenosus	Control	39	54.26%	NA	NA	NA	47.03%
Dictyotus caenosus	*Trissolcus basalis*	32	4.05%	93.75%	90.53%	84.15%	5.42%
Dictyotus caenosus	*Trissolcus japonicus*	26	15.90%	73.08%	51.92%	82.13%	32.17%
Dictyotus caenosus	*Trissolcus oenone*	8	0.00%	100.00%	92.91%	84.51%	7.09%
Glaucias amyoti	Control	104	95.17%	NA	NA	NA	6.49%
Glaucias amyoti	*Trissolcus basalis*	60	29.69%	68.33%	61.24%	90.88%	9.07%
Glaucias amyoti	*Trissolcus japonicus*	70	4.56%	95.71%	90.74%	87.26%	4.70%
Glaucias amyoti	*Trissolcus oenone*	33	63.10%	30.30%	29.10%	90.57%	7.80%
Hypsithocus hudsonae	Control	10	64.46%	NA	NA	NA	35.54%
Hypsithocus hudsonae	*Trissolcus basalis*	2	0.00%	100.00%	58.33%	50.00%	41.67%
Hypsithocus hudsonae	*Trissolcus japonicus*	15	6.67%	93.33%	90.56%	89.03%	2.78%
Monteithiella humeralis	Control	100	91.73%	NA	NA	NA	8.87%

Monteithiella humeralis	*Trissolcus basalis*	52	3.69%	96.15%	92.38%	78.44%	3.93%
Monteithiella humeralis	*Trissolcus japonicus*	19	19.74%	78.95%	66.09%	89.03%	14.18%
Monteithiella humeralis	*Trissolcus oenone*	29	3.45%	96.55%	94.51%	78.70%	2.04%
Nezara viridula	Control	70	47.38%	NA	NA	NA	50.97%
Nezara viridula	*Trissolcus basalis*	30	10.50%	76.67%	49.36%	84.01%	40.14%
Nezara viridula	*Trissolcus japonicus*	34	49.04%	0.00%	0.00%	NA	50.96%
Nezara viridula	*Trissolcus oenone*	31	47.13%	0.00%	0.00%	NA	52.87%
Oechalia schellenbergii	Control	89	85.77%	NA	NA	NA	18.12%
Oechalia schellenbergii	*Trissolcus basalis*	58	4.99%	96.55%	91.28%	86.11%	3.73%
Oechalia schellenbergii	*Trissolcus japonicus*	36	76.99%	22.22%	16.46%	91.89%	6.55%
Oechalia schellenbergii	*Trissolcus oenone*	23	21.86%	69.57%	56.59%	79.55%	21.55%

O. schellenbergii (74%) and *D. caenosus* (71%), and *T. basalis* on *N. viridula* (64%) and *H. hudsonae* (58%). On average, 30% of eggs in the control treatment remained unhatched. The mean proportion of unhatched eggs across each of the parasitoid treatments was lower, with *T. japonicus* having the highest at 21%, followed by *T. oenone* (16%) and *T. basalis* (15%). Eleven out of 35 treatments in the combined dataset resulted in proportions of unhatched eggs exceeding 25% (Table 2). Four of these were control treatments for *C. nasalis hudsoni*, *D. caenosus*, *H. hudsonae*, and *N. viridula*, while two treatments were composed of *T. oenone* and *T. japonicus* on *N. viridula*, an incompatible host. A finer comparison showed that unhatched eggs in control masses tended to be evenly split between unemerged pentatomids and undeveloped eggs, whereas unhatched eggs in masses exposed to wasps tended to have much lower proportions of unemerged pentatomids, and instead were mostly split between unemerged parasitoids and undeveloped eggs (Figure 3).
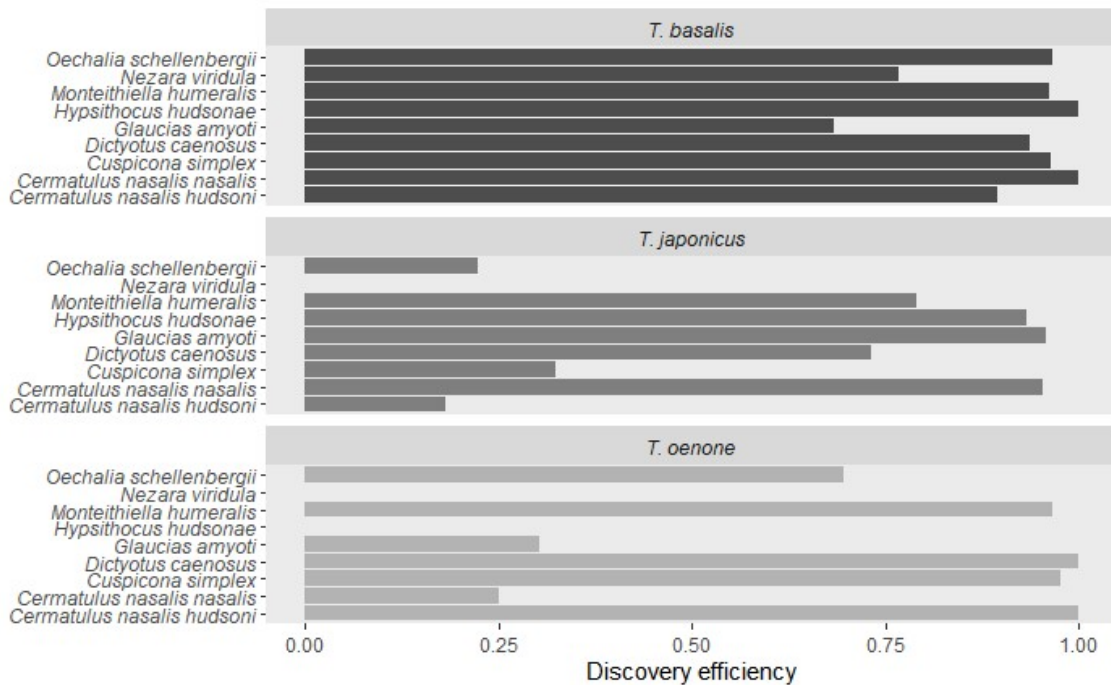
Figure 3: Total discovery efficiency of three parasitoids in relation to egg masses from New Zealand pentatomid species.
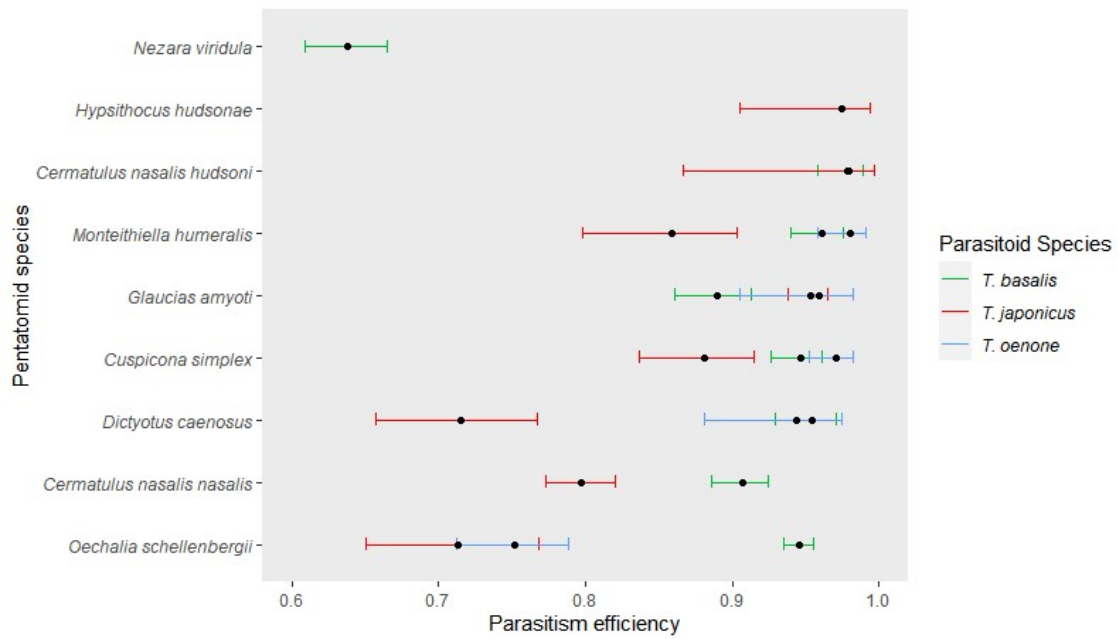
Figure 4: Expected mean parasitism efficiencies (with confidence intervals) for three egg parasitoids exposed to New Zealand stink bug species.
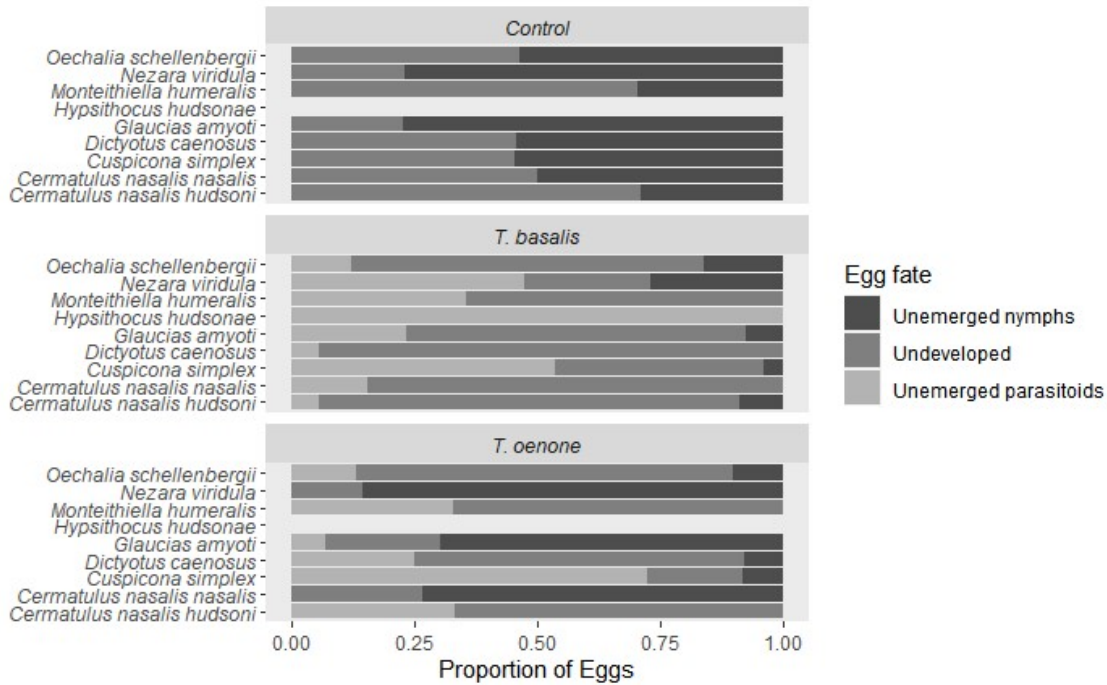
Figure 5: Mean proportions of unemerged nymphs, undeveloped eggs, and unemerged parasitoids in pentatomid eggs in control treatments versus those exposed to two resident egg parasitoids.

I found a marginal trend suggesting a difference in parasitoid development time between *T. basalis* and *T. oenone* based on pentatomid species (df = 8, F = 14.56, p = 0.068). Each parasitoid took a mean of between 16 and 22 days to develop inside the eggs of each pentatomid species tested (Figure 4). Overall, the fastest development time was demonstrated by *T. japonicus* in *H. hudsonae* eggs (16.8 days). Of the two resident parasitoids, *T. basalis* had the fastest mean development time in the exotic *D. caenosus* eggs (18.5 days), while both resident parasitoids took the longest to develop in native *G. amyoti* eggs (20.8 days for *T. oenone* and 21.7 days for *T. basalis*).
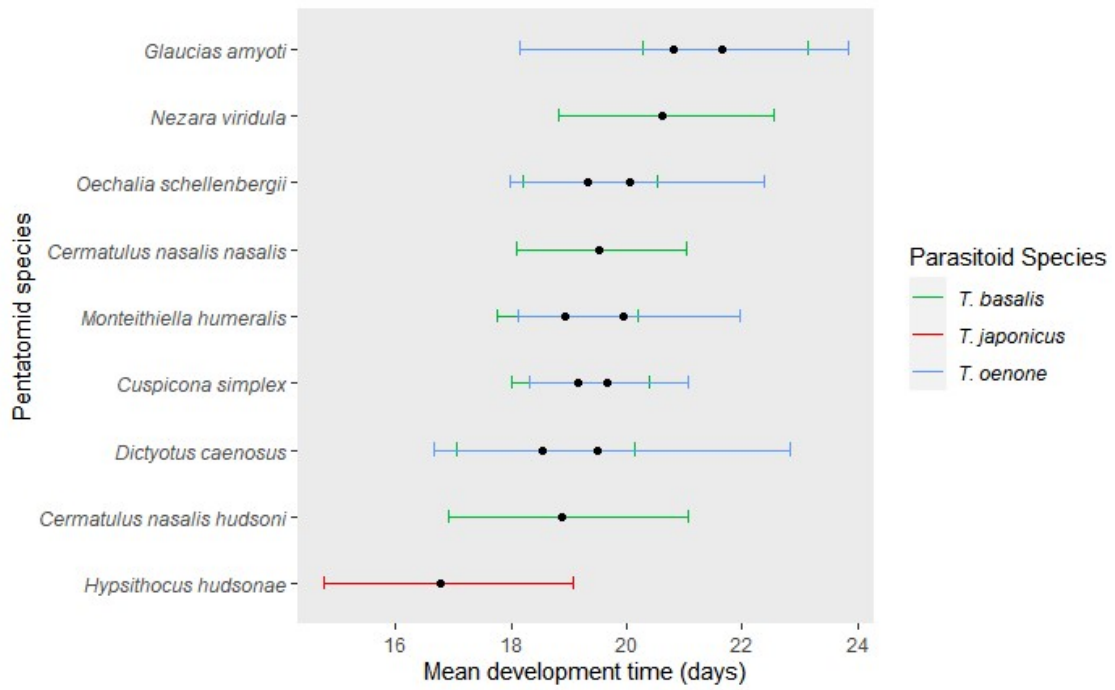
Figure 4: Mean development time for three egg parasitoids inside different pentatomid hosts.

3.5 Discussion

Parasitism and development

No-choice oviposition tests provide unambiguous evidence of a biological control agent's physiological host range due to their simple design, and for this reason, are recommended as a first step in assessing host specificity (Babendreier et al., 2005; Van Driesche et al., 2004; van Lenteren et al., 2006a). Conducting retrospective no-choice tests is especially valuable in cases where host specificity testing was never completed prior to release of the agent (Avila et al., 2016; Cumber, 1953). This study integrates the results from several studies to quantitatively understand the physiological host ranges of two resident scelionid parasitoids and one approved biological control agent in relation to the New Zealand pentatomid fauna (Charles et al., 2019; Saunders et al., 2021).

My results show that both resident *Trissolcus* species share a very similar physiological host range with each other, and with the conditionally approved biological control agent *T. japonicus*. *Trissolcus basalis* successfully parasitized and emerged from all pentatomid species it was exposed to, while *T. oenone* failed to emerge from the introduced pest, *N. viridula.* Across all treatments, *T. basalis* and *T. oenone* achieved a higher mean discovery and parasitism efficiency than *T. japonicus*. My finding that the two resident parasitoids parasitized a higher proportion of egg masses, and a higher proportion of eggs within masses, may be explained by the fact that *T. japonicus* has not had an opportunity to adapt to the chemical ecological cues or immunological defences associated with these novel hosts (Abdel-latief & Hilker, 2008; Vinson & Iwantsch, 1980).

Within *Nezara viridula* was the only species of pentatomid not parasitized by *T. japonicus* in my study or previous studies (Haye et al., 2020; Lara et al., 2019; Sabbatini-Peverieri et al., 2021). It was also not accepted by *T. oenone* in my study, so I can therefore rule out *N. viridula* as a physiological host of *T. oenone*. However, I was unable to expose *T. oenone* to *H. hudsonae*, so the suitability of this endemic pentatomid species as a host for *T. oenone* remains unclear. The mean parasitism efficiency of *T. basalis* on *N. viridula* was only 64%, which was surprising, given *N. viridula* is considered to be the primary host of *T. basalis* (Austin et al., 2005; N. F. Johnson, 1987). This result may be explained by my observation that *T. basalis* parasitoids often struggle to chew through the eggs of *N. viridula* in the laboratory when they are not provided with a piece of moist cotton inside the rearing vials,

particularly at the study temperature of 20°C. An alternative explanation may be that the eggs produced by the *N. viridula* colony are harder than wild produced eggs as a result of the age of the colony. In either case, to keep testing protocols comparable between the three datasets, I decided not to add moist cotton to any vials when parasitoids were emerging, as I did to *T. basalis* emerging in colony vials. I also observed a very low proportion of pentatomid emergence from *C. nasalis hudsoni* in the control treatment, and most unhatched eggs were classified as undeveloped. This is most likely due to unfavourable rearing conditions as this species is predominantly an alpine species, so a constant temperature of 20°C may have been too high for optimal development and reproduction.

Competition and non-target effects

Considering the high degree of overlap in physiological host ranges between the three species of *Trissolcus* parasitoids examined here, there is the potential for interspecific competition on non-target species, and to a lesser extent, target species. Most available pentatomid egg collection records were observed between December and April (Larivière, 1995), which indicates that competition between each resident *Trissolcus* species could occur during this period. However, no information is available on the seasonal abundance of *T. basalis* and *T. oenone* in New Zealand, and therefore, the potential magnitude of competition between the two parasitoid species remains unknown. *Trissolcus basalis* was recently recorded emerging from *H. halys* eggs in the United States and Europe (Balusu et al., 2019; Zapponi et al., 2021). This latest finding adds another species to the list of North American egg parasitoids already known to attack *H. halys*, which now numbers 19 species from four genera (Abram et al., 2014; Rice et al., 2014). Some of the implications of this competition have already been investigated, including the finding that the native North American egg parasitoid *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) accepts the eggs of *H. halys* for oviposition, but fails to develop in or emerge from them (Abram et al., 2014). Similarly, *T. basalis* was attracted to adult *H. halys* volatiles and readily oviposited into the stink bug eggs, even though around 95% of parasitoids failed to develop (Peri et al., 2021). In these scenarios, *H. halys* acts as an 'evolutionary trap' for the native parasitoid (Abram et al., 2014). Interestingly, failed parasitism did account for around a 25% reduction in stink bug development compared to controls in the North American study, an example of parasitoid-

induced host egg abortion occurring in a non-host species (Abram et al., 2016). The possibility of *H. halys* eggs acting as an evolutionary trap for the native *T. oenone* in New Zealand deserves further research attention.

When female egg parasitoids encounter a host patch at the same time, they may compete for control of the resource through aggressive physical contests (Cusumano et al., 2016). Intraspecific competition has been well studied in some scelionid parasitoids, such as *T. basalis* (Field, 1998; Field & Calbert, 1998), but there are fewer studies on the mechanisms shaping the outcomes of contests between different species of parasitoids. Recent work suggests that a variety of factors may influence the result of interspecific contests, including relative female body size, egg load, ownership status of the egg mass, and innate aggression levels (Mohamad et al., 2011). If interspecific competition results in multiparasitism, then parasitoid larvae compete for egg resources. Important factors mediating larval competition include the order and timing of oviposition, and the development time of each parasitoid (Cusumano et al., 2016). Based on my results it is unclear if either of the two resident *Trissolcus* species would be expected to consistently win the larval contest in multiparasitised eggs, as both have very similar development times. However, these interactions are worth exploring further, because competitive dynamics between introduced biological control agents and native parasitoids may influence the efficacy of biological control, for example if neither parasitoid developed in multiparasitised eggs.

One important factor mediating competition between egg parasitoids is the ability to detect, recognise, and exploit semiochemicals associated with hosts or the plants they feed on (Conti & Colazza, 2012). For example, *T. basalis* was better at locating host patches than its competitor *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae), when both were released into a field containing naturally laid *N. viridula* sentinel eggs (Peri et al., 2014). This is likely due to the fact that *T. basalis* is capable of detecting and exploiting a higher number of volatile kairomones associated with *N. viridula* (Colazza et al., 1999), including more reliable cues such as synomones released by plants in response to stink bug oviposition (Colazza et al., 2004). In contrast, *O. telenomicida* relies primarily on cues associated with virgin adult male stink bugs, which represent a far less reliable cue to the presence of suitable host patches (Peri et al., 2011).

The link between parasitism levels and population impact remains one of the most important knowledge gaps for many case studies of classical biological control (Van Driesche & Hoddle, 2016). The degree to which egg parasitism levels detected in no-choice laboratory trials on target or non-target pentatomid species translates into population level impacts is just one such knowledge gap, and it requires careful study. *Trissolcus basalis* was initially implicated in the decline of the native Koa bug in Hawaii by Howarth (1991) based on circumstantial evidence in his influential paper on non-target effects. But a subsequent study that took a more holistic view of mortality factors associated with the native insect found that parasitism by *T. basalis* was relatively low in the majority of study sites, and that predation by introduced ants had a much higher impact (M. T. Johnson et al., 2005). Recent matrix modelling of stink bug life cycles suggests that percent egg parasitism does not directly translate to an equal reduction in population growth rates, and consequently, even high parasitism rates should not be conflated with rates of suppression (Abram et al., 2020). Despite enduring attack by a suite of natural enemies in its native range, including high parasitism levels by *T. japonicus* (Zhang et al., 2017), *H. halys* remains a common pest requiring ongoing management in some areas and on some crops (Lee et al., 2013). Similarly, despite high parasitism levels against *N. viridula*, *T. basalis* has had mixed success in suppressing its primary host in different regions of the world (Jones, 1988). For example, it has generally provided good control in New Zealand (Cumber, 1964), but despite sometimes achieving very high parasitism levels against *N. viridula*, the stink bug remains a common pest in Australia and Central Italy (Clarke, 1990; Colazza & Bin, 1995). If some oligophagous egg parasitoids are able to achieve very high parasitism levels in some regions of the world without substantially reducing the populations of their target species, then it may also be true that oligophagous egg parasitoids could reach high parasitism levels on non-target species without substantially affecting their populations (Abram et al., 2020). The ability for non-target species to withstand attacks by biological control agents is partly governed by where the agent sits on the specificity spectrum: a specialist agent which attacks only one species would be expected to have a much higher impact on their target (and cause no non-target effects in the process), while the less host-specific a biological control agent is, the more it is able to spread its attacks amongst a wider pool of hosts, of which the target pest may be only one (Follett & Duan, 2000; Van Driesche & Hoddle, 2016). However, just because a biological control agent may be able to attack a relatively wide pool of hosts, it

does not necessarily follow that it will choose to locate such hosts (or even be capable of detecting them) (Park et al., 2018). Bridging the gap between host use and host impact, particularly among pentatomid egg parasitoids, remains an important avenue for future research.

My study shows that *T. basalis*, *T. oenone*, and *T. japonicus* overlap in their physiological host ranges, except for *N. viridula* which is not a host of *T. oenone* or *T. japonicus*. While the two resident parasitoids were able to exploit New Zealand pentatomid hosts more efficiently than *T. japonicus*, all three parasitoids achieved parasitism efficiencies over 50% in laboratory no-choice tests. I found moderate evidence to suggest a slight discrepancy in development time for *T. basalis* and *T. oenone* based on the pentatomid host, but at 20°C, the difference between shortest and longest development time was only a few days. Although physiological host range tests are an essential component of pre-release risk assessments for classical biological control agents, the results they provide should not be extrapolated to how parasitoids will behave under field conditions. To better understand ecological host range, and in order to improve pre-release risk assessments for classical biological control agents, it will be necessary to incorporate a wider variety of experiments, for example those used to assess chemical ecological interactions between biological control agents and potential non-target species. Electrophysiology and behavioural responses to host-associated kairomones are emerging as useful complementary methods to traditional oviposition tests when forecasting risks to non-target species, and I am currently investigating these kinds of tests in relation to the *Trissolcus*-Pentatomidae complex in New Zealand. Establishing baseline parasitism levels for New Zealand pentatomid species will also be an important research priority in order to understand how a hypothetical future release of *T. japonicus* may affect competition and non-target dynamics between parasitoids.

3.6 References

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CHAPTER 4: Integrating chemical ecology and behavioural bioassays to understand host preferences in egg parasitoids

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4.1 Abstract

Current approaches to assessing the non-target risks associated with biological control agents are conservative, and they often rely on physiological host range experiments conducted in quarantine laboratories. By their nature, tests such as no-choice oviposition experiments offer robust evidence of a parasitoid's ability to attack and develop in a host. But they also exclude many important chemical cues in the natural environment, which play a key role in the ability of parasitoid to locate its host. These cues act as a filter through which a list of potential physiological hosts is reduced to a list of those hosts which are actually attacked in the field (ecological host range). Host-associated semiochemicals trigger and extend the duration of host-searching behaviour in parasitoids, and are therefore important cues mediating host location, and ultimately, the expression of ecological host ranges. I conducted a series of experiments with *Trissolcus basalis* Wollaston, a biological control agent introduced into New Zealand in 1949, and *Trissolcus oenone* (Dodd), a native parasitoid of pentatomids, in order to better understand the chemical basis mediating differences in host-specificity between these parasitoids. First, I recorded the antennal responses of *Trissolcus basalis* to egg extracts of *N. viridula* made with hexane or acetone to tentatively identify potential contact kairomones used by this parasitoid to recognise and accept hosts. I then compared the searching motivation of *T. basalis* and *T. oenone* in open arena arrestment bioassays contaminated with footprint compounds from *Nezara viridula* (L.) and *Cuspicona simplex* (Walker). *Trissolcus basalis* spent four times longer searching for *N. viridula* than *C. simplex*, while *T. oenone* spent four times longer searching for *C. simplex* than *N. viridula*. Finally, I conducted competition experiments to assess factors important to determining the outcomes of extrinsic and intrinsic contests between these parasitoids when they are simultaneously exposed to *C. simplex* egg masses. The native parasitoid, *T. oenone*, was the superior competitor in extrinsic contests, parasitizing a higher proportion of eggs than *T. basalis*. *Trissolcus oenone* also dominated intrinsic contests by emerging from over 90% of multiparasitised eggs. Integrating these techniques with no-choice oviposition tests provides highly relevant and complementary information for assessing the non-target risks of biological control agents, and I discuss my

results in the context of combining behavioural and chemical ecological techniques for pre-release risk assessments of classical biological control agents.

4.2 Introduction

Hymenopteran parasitoids are widely used as classical biological control agents because they are often highly effective at finding and killing their hosts (Godfray, 1994; Jervis, 2005; Vinson, 1984). Before a parasitoid can be released, authorities in the receiving jurisdiction usually require an assessment of the risks posed to non-target species (Follett & Duan, 2000; Gerard & Barratt, 2020; Heimpel & Cock, 2018; Hoddle, 2016). The scientific basis for host range testing emphasises a risk-based approach which begins with assessing physiological host range through the use of no-choice tests, before proceeding to choice tests and field cage tests (Bigler et al., 2006; Van Driesche et al., 2004; van Lenteren et al., 2006). However, regulators may only have access to the results of laboratory-based oviposition tests when deciding whether or not to approve the release of an agent, especially during pre-release studies which are necessarily more constrained in their testing regimes (Van Driesche & Murray, 2004). In these cases, decision makers rely on the classification of the parasitoid's physiological (=fundamental) host range under laboratory conditions being an accurate proxy for the parasitoid's ecological (=realised) host range eventually expressed in the field (Withers et al., 2021). While there are cases where physiological host range in the lab has predicted ecological host range in the field (Paynter et al., 2015), this is not usually the case for biocontrol agents of arthropods (Haye et al., 2005). While no-choice oviposition tests provide strong and unambiguous evidence showing which non-target species are physiological hosts (Murray et al., 2010), they are not designed to evaluate the earlier steps in the host location process, generally involving long and short range chemo-orientation (Colazza et al., 2014; Van Driesche & Murray, 2004).

There is increasing interest in how the chemical basis of attraction of biological control agents to their hosts may affect both their ability to suppress target pest populations, and their ability to detect and attack non-target species (Avila et al., 2016; Benelli et al., 2013; Jordon-Thaden & Louda, 2003; I. Park et al., 2018). The earlier stages in the host location process are characterised by a reliance on chemical cues associated with hosts or their habitat (Vinson, 1976, 1984; Wajnberg & Colazza, 2013). Semiochemicals are a major class of naturally derived chemical cues which stimulate parasitoids to search for another cue, or a stronger or more attractive one (Bell & Cardé, 1984; Vinson, 1977; Wajnberg & Colazza, 2013). Semiochemicals are classified into those eliciting intraspecific (pheromones) or interspecific (allelochemicals) responses (Brown et al., 1970; Nordlund & Lewis, 1976).

When hosts build shelter, release waste, communicate with each other, or forage, they are likely to release products containing kairomones, a class of allelochemical which elicit a behaviourally or physiologically adaptive response in the receiver (Dicke & Sabelis, 1988). Herbivory and oviposition can also stimulate plants to produce herbivore induced plant volatiles, which can be attractive to parasitoids (Turlings & Erb, 2018). Whereas long range volatile compounds associated with plants act as attractants to draw parasitoids into an appropriate habitat, host-associated kairomones act more as stimulants to prolong and intensify searching behaviour once they have found a suitable habitat (Colazza et al., 2014; Vinson, 1998). The ability of a parasitoid to detect and respond to such cues varies, based on the genetic make-up of the individual parasitoid, the kind of experience it has previously accrued, and by the complex processes of short-term conditioning or long-term learning, whereby parasitoids are able to respond to novel cues after associating them with innate cues (Lewis et al., 1990; Vet et al., 2003; Vet & Groenewold, 1990).

Stink bugs (Hemiptera: Pentatomidae) are a diverse and widespread group, and some species are important pests in horticultural regions around the world (Conti et al., 2021; McPherson, 2018). Brown marmorated stink bug (*Halyomorpha halys* Stål), a serious horticultural pest native to East Asia, has recently spread through North America and Europe, causing millions of dollars of damage in lost crop yields (Leskey & Nielsen, 2018; McPherson, 2018). Natural enemy surveys in East Asia showed the egg parasitoid *Trissolcus japonicus* Ashmead (Hymenoptera: Scelionidae) is the most dominant natural enemy against BMSB, and it has therefore been the subject of host range testing in the United States since 2007 (Buffington et al., 2018; Rice et al., 2014), in New Zealand since 2015 (Charles et al., 2019; Saunders et al., 2021), and more recently in Europe (Haye et al., 2020). Egg parasitoids in the family Scelionidae are commonly employed as biological control agents against pentatomid pests (Austin et al., 2005; Orr, 1988), and research into their efficacy has a long history. For example, green vegetable bug, *Nezara viridula* (L.), is one of the most well-known and one of the most widespread crop pests in the world (J. W. Todd, 1989). *Trissolcus basalis* (Wollaston) is the most dominant natural enemy of *N. viridula*, being closely associated with it in Europe, the Americas, the Middle East, South Asia, and deliberately introduced for the purpose in Hawaii, Australia, New Zealand, and other parts of the Pacific (Clarke, 1990; Colazza & Bin, 1995; Jones, 1988). Research on the chemical ecology of the interaction between *N. viridula* and *T. basalis* has revealed important insights into the host

selection and host location processes used by egg parasitoids (Bin et al., 1993; Colazza et al., 1999).

A long-stated goal of chemical ecology has been the application of its techniques and results to enhance the suppression of pests (Lewis et al., 1990). A better understanding of how natural enemies detect and respond to semiochemicals, and in particular, how certain compounds influence host preferences, could help to improve the way classical biological control agents are screened for non-target risks before release (I. Park et al., 2018; Salerno et al., 2006). Electroantennogram studies measure the DC potential of an insect's olfactory receptor neurons in relation to a blend of compounds in a host extract, or to single compounds puffed over the antennae (Schneider, 1957). This technique has long been used to study intraspecific responses to pheromones (Olsson & Hansson, 2013), but more recently, interest has grown in the use of electrophysiological methods to identify kairomones which may play a role in the host-specificity of biocontrol agents (Iacovone et al., 2016; Li et al., 2017; K. C. Park et al., 2001). On the other hand, behavioural tests in open arenas have been used to confirm parasitoid attraction to host volatiles (Colazza et al., 1999), and to demonstrate how parasitoids discriminate between different host sexes (Colazza et al., 2007) and between different physiological stages of host development (Peri et al., 2006; Salerno et al., 2019). Similarly, arrestment studies can also be used to compare the strength of attraction between different hosts (Conti et al., 2004), and this information could be used to estimate the relative risk posed by a biological control agent in finding and attacking non-target species.

Along with research on host-location and preferences, research on potential competition among the natural enemies of hosts can inform and direct the most fruitful combinations of parasitoids for release in biological control programs. Interspecific competition between biological control agents and/or native parasitoids can occur both extrinsically (between females exploiting a host resource) and intrinsically (between larvae competing for the same host resources) (Cusumano et al., 2016; Harvey et al., 2013). Female *Trissolcus* egg parasitoids locate pentatomid egg masses by exploiting semiochemicals associated with host plants, adult stink bugs, and the surface of the eggs themselves, before going through a sequence of steps leading ultimately to oviposition or rejection of the egg (Colazza et al., 1999; Field & Keller, 1999; Iacovone et al., 2016). During this process, female egg parasitoids must choose between further exploiting the host patch, or defending it from intruders, who are capable of exploiting it even after the resident female has laid eggs

and left the patch (Field & Calbert, 1998; Vinson, 1998). The ability of a biocontrol agent to respond to extrinsic and intrinsic competition has important implications for the efficacy of the agent, the fitness of native parasitoids, and for populations of non-target species which may be attacked by both parasitoids (Cusumano et al., 2012; Konopka et al., 2017; Peri et al., 2014).

The chemical ecology of stink bug parasitoid host ranges, and potential competition arising between introduced and native parasitoids, are currently of interest in New Zealand where *Trissolcus japonicus* Ashmead has been the subject of host range testing as part of a pre-emptive classical biocontrol programme against the brown marmorated stink bug, should the bug establish in New Zealand (EPA, 2018). The Environmental Protection Agency made this decision on the basis of physiological host range testing against non-target Pentatomidae (Charles et al., 2019), the results of which were subsequently updated to include the endemic alpine shield bug, *Hypsithocus hudsonae* Bergroth (Saunders et al., 2021). Complementary work has since provided a holistic overview of the physiological host ranges of the two resident pentatomid egg parasitoids: *Trissolcus basalis* (introduced against *N. viridula* in 1949); and *Trissolcus oenone* (Dodd) (native to New Zealand and Australia) (Chapter three). In short, all three parasitoids attack and develop in all Pentatomidae at parasitism efficiencies exceeding 60%, with the exception of the non-host association between *T. japonicus* and *T. oenone* with *N. viridula*, and an untested association between *T. oenone* and *H. hudsonae*. The potential future introduction of *T. japonicus* into New Zealand, and the relatively broad, overlapping host ranges of these three parasitoids, have stimulated interest in characterising potential ecological interactions between them (J. H. Todd et al., 2020), and investigating the chemical ecology linking them to their hosts.

In this study, I conducted three different kinds of experiments in order to examine the chemical ecology of host preferences in *T. oenone* and *T. basalis* in relation to *N. viridula* and *Cuspicona simplex* Walker (Hemiptera: Pentatomidae), a known host of both parasitoid species. First, I conducted electrophysiological experiments with *T. basalis* and solvent volatile extracts of *N. viridula* eggs in order to compare the magnitude of antennal responses between extracts made with hexane and acetone, and to identify candidate chemical compounds acting as contact kairomones facilitating host acceptance in the parasitoid. Second, I conducted arrestment bioassays with both parasitoids in open arenas contaminated with footprint volatiles from each pentatomid species, and compared parasitoid retention time

against control arenas uncontaminated by stink bugs. The purpose of these bioassays was to determine the strength of attraction between each parasitoid and pentatomid, and to test whether this would be lowest for *T. oenone* and *N. viridula*, a non-host association. Finally, I conducted competition experiments by releasing individual females from each parasitoid species onto egg masses of *C. simplex* simultaneously, and recording behavioural interactions relevant to extrinsic competition. I also collected data on the outcome of larval contests in multiparasitised eggs to examine intrinsic competition between an introduced biological control agent and a native parasitoid. The broader goal of this work was to generate multiple lines of evidence to assess the chemical ecological basis of host preferences in these parasitoids, and to test the integration of a wider variety of methods to assess host specificity, with a view towards applying these methods more generally to classical biological control agents in pre-release risk assessments. Ultimately, a better understanding of how and why parasitoids prefer to attack some hosts and not others can be used to design pre-release host range testing regimes which provide more certainty to decision makers by considering aspects of the parasitoid's ecological host range.

4.3 Methods

Insect rearing and identification

Stink bug colonies were started with wild-collected specimens from Auckland, and were housed in clear plastic containers measuring 170 mm × 210 mm × 135 mm with ventilated lids at 20-25°C (16:8 h L:D). I provisioned rearing containers with moist cotton and fanned wax paper for oviposition substrate. *Solanum* fruits and tomatoes were provided as food for *C. simplex*, and green beans and raw peanuts for *N. viridula*.

Trissolcus basalis was reared from parasitised *N. viridula* egg masses naturally laid on *Cleome* plants in Auckland, in February 2019. *Trissolcus oenone* was reared from parasitised *C. simplex* egg masses naturally laid on *Coprosma* (near *Solanum*) plants in Auckland, in November 2019. Parasitoids were reared at 20-25°C (16:8 h L:D) on the eggs of the species they were originally collected in. Fresh eggs (<24h old), or eggs stored at 10°C for no longer than 2 weeks, were used to maintain the colonies.

Extract preparation and chemical analysis

I prepared stink bug egg solvent extracts to examine *T. basalis* for antennal responses to compounds associated with eggs from its primary host, *N. viridula*. I used acetone as the solvent because egg extracts prepared with acetone are known to retain bioactive compounds responsible for eliciting oviposition behaviour in *T. basalis* (Bin et al., 1993). I also made a hexane extract to compare the chemical composition and strength of antennal responses with the acetone extract. For the acetone extract, 0.8g of <48h old egg masses were immersed in 2ml of solvent (0.2g of eggs per 1ml of solvent) in a glass vial for 96 hours, before transferring the extract to a clean vial and freezing at -20°C until required for analyses. For the hexane extract, 0.25g of <48h old egg masses were immersed in 1ml of solvent in a glass vial for 24 hours, before transferring the extract to a clean vial and freezing at -20°C until required for analyses. Each of the extracts were made with different immersion times because the original intention was to make three different immersion replicates for each solvent (24h, 48h, 96h). Unfortunately I ran out of time and usable stink bug eggs, but had already made the two extracts used here. Each extract was analysed on a gas-chromatograph (GC, Agilent 7890B) coupled to a mass-spectrometer (MS, Agilent 5977A). A 1µl sample was injected into the GC in splitless mode and carried with helium gas at a flow rate of 1.6 mL/min. The GC column was non-polar (Agilent DB-5 ms) and measured 30m × 0.25mm ID × 0.25µm film thickness. The temperature program started at 40°C and was held for 2 minutes, then was increased to 250°C at a rate of 4°C/min, followed by a 10 degree per min ramp to 280 degrees, then held for 10 minutes. The transfer line was kept at 250°C.

Electrophysiological recordings

I recorded electroantennograms to measure *T. basalis* antennal responses to *N. viridula* egg extracts made with acetone and hexane. I anaesthetised each female wasp with carbon dioxide gas before removing its head and the distal tip of one of its antennae with a fine scalpel under a stereomicroscope (x20 mag). I positioned each specimen between two silver wire electrodes sheathed by glass capillaries pulled to fine points. Glass capillaries were trimmed with a ceramic cutter and filled with Ringer's solution. The severed head was positioned into contact with the reference electrode and the severed antenna was positioned into contact with the recording electrode using a Sutter Instruments micromanipulator. I concentrated each extract by 10× under a gentle stream of argon gas before applying a 10-µL aliquot to a 5 × 25mm strip of filter paper (Whatman No. 1; Whatman, U.K.) and allowed the

solvent to evaporate for 10s before placing the paper inside a glass Pasteur pipette (146 mm; Fisher Scientific Co., Pittsburgh, Pennsylvania) to form an odour cartridge. I inserted the tip of each odour cartridge pipette into a 2mm diameter hole in a glass airflow tube containing a charcoal-filtered and humidified air stream with a flow rate of 400ml/min. The antennal preparation was positioned in front of the air stream. The recording electrode was connected to an electroantennogram detector (EAD, IDAC 4, Syntech Research and Equipment, Hilversum, Netherlands) and I used Autospike software (v3.9, Syntech Research and Equipment, Hilversum, Netherlands) to record antennal responses. I wrapped the wide end of odour cartridge pipettes in aluminium foil when not in use to prevent evaporation of test compound, and I used each cartridge less than 10 times. For the control air cartridge, I kept the filter paper blank, and for the solvent control cartridges, I applied a 10-μL aliquot of neat hexane or acetone. I used a different parasitoid for each recording, and captured six recordings showing consistent responses for each extract with *T. basalis*.
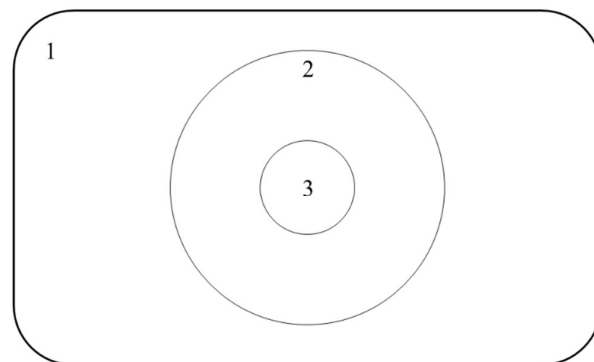
Figure 6: Schematic diagram of open arenas used for arrestment experiments. 1. Upturned tray. 2. Filter paper (21mm diameter). 3. Inner zone of paper where stink bugs were confined under a small petri dish lid (60mm diameter, marked in pencil).

Open arena arrestment bioassays

I conducted arrestment bioassays with *T. basalis* and *T. oenone* to measure how long each parasitoid remained arrested by the odours of adult *N. viridula* and *C. simplex*. Arrestment experiments were conducted in a 25 °C room lit with fluorescent lights, between the hours of 8am and 3pm. I used an open arena design inside a mesh cage (59cm L × 59cm W × 59cm D)

consisting of a plastic tray (46cm L × 34cm W) turned upside down with a piece of filter paper (21mm diameter) on top (Figure 1). Each piece of filter paper had a 60mm diameter circle in the centre of the paper. Five female stink bugs were taken from rearing cages and confined under a small petri dish inside the 60mm circle for three hours prior to experiments. Single parasitoids were held in glass vials in the room for at least 30 minutes prior to experiments in order to acclimate. Stink bugs were removed from the paper and placed back into the colony. A single parasitoid was then released into the centre of the filter paper by gently tapping the bottom of the vial while holding it upside down approximately 30mm from the surface of the paper. Parasitoids which immediately flew away without walking on the paper at all were recaptured and discarded, including for control replicates, while parasitoids which remained on the paper for at least enough time to walk had their retention time recorded. I recorded the time parasitoids were retained in contact with the inner circle of the paper, which I defined as having ended once they moved approximately 5mm past the line, as parasitoids were typically 'pulled' back into the circle during their searching as they zig-zagged around the circle. I recorded the total time parasitoids remained in contact with the filter paper, which I defined as having ended once the parasitoid reached the outer margin of the paper or flew away. Finally, I recorded whether or not the parasitoid displayed arrestment behaviour characteristic of scelionid egg parasitoids (described in Colazza et al., 1999). I used at least 30 replicates of each parasitoid exposed to each stink bug and control treatment. The control treatment consisted of arenas as described above but pieces of filter paper were not contaminated by stink bugs.
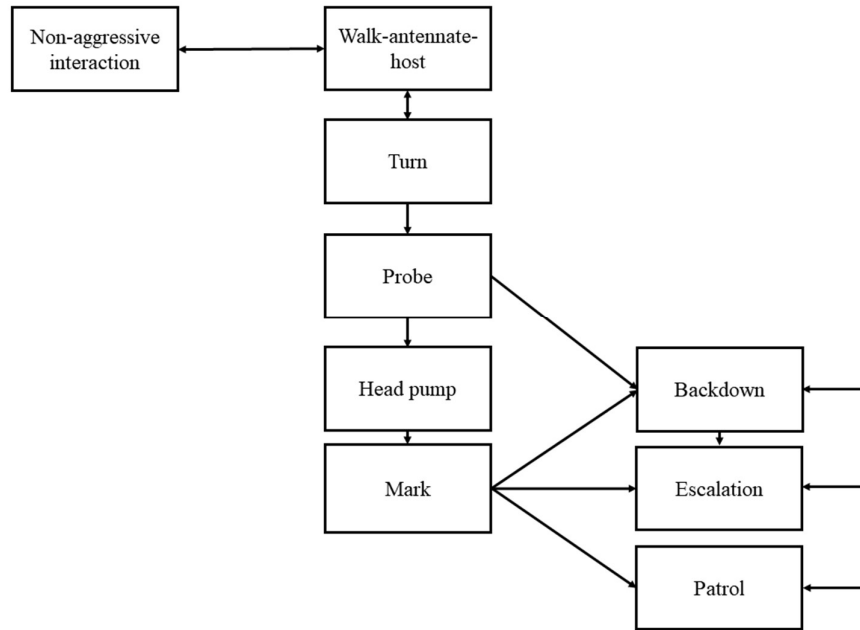
Figure 7: Ethogram showing generalised model of behaviours exhibited by parasitoids in competition experiments.

Competition experiments

I exposed *T. basalis* and *T. oenone* to *C. simplex* egg masses to observe the outcomes of aggressive encounters between single females from each species (extrinsic competition) and to record which species emerged from multiparasitised eggs (intrinsic competition). I mounted a fresh (<24h) *C. simplex* egg mass onto a 40mm x 20mm card with double sided

Table 1: Behaviours observed during competition experiments with *Trissolcus basalis* and *Trissolcus oenone*. Modified from Field (1998).

Behaviour	Description
Walk-antennate-host	Walking over the egg mass while drumming antennae over the surface of the eggs.
Turn	Turns in a circle while standing on the top of a single egg, drumming the sides with antennae.
Probe	Extending wings, everting ovipositor, and then inserting ovipositor into an egg.
Pump	Pumping the head up and down during oviposition.
Mark	Sweeping the everted ovipositor in a figure of eight pattern over the surface of an egg straight after withdrawing ovipositor.
Non-aggressive interaction	An encounter between the parasitoids which did not result in agonistic behaviour, for example, brushing past each other, or inspecting one another at close range.
Patrol	Rapid movement over the surface of the egg mass and around the outside of the mass, often in response to perceiving a competitor in the vicinity.
Backdown	An aggressive encounter in which the aggressor makes physical contact with the receiver, at which point one of the parties immediately moves away from the other. Examples of contact include charging into the opponent, biting the wings, legs, antennae, or head of the opponent, or attempting to pull the opponent away from the egg it is currently ovipositing into.
Escalation	An extended aggressive encounter in which neither party backs down until after an intense physical contest, usually involving between five and twenty seconds of fighting.

sticky tape, and used white Scenic Sand (Activa Products) to cover any exposed tape. I placed the card into a plastic screw top vial (length 60mm, diameter 28mm), introduced a female parasitoid (the focal species), and then screwed the lid back on. As soon as the focal species began oviposition, I immediately removed the lid and introduced a female parasitoid from the other species (the intruder), before replacing the lid. I placed the vial under a microscope fitted with an LED ring light to observe interactions between the two females. I recorded the time it took for the focal individual to make contact with the egg mass, how long it took for the focal individual to oviposit successfully in five eggs (indicated by marking the egg with her ovipositor), the sequence of oviposition for both focal and intruder parasitoids, and the total time taken for all eggs to have been oviposited in by both females, or until the

parasitoids reached a 'stalemate' in their competition where one parasitoid guarded the egg mass and did not let the other approach. I also recorded the frequency and sequence of a variety of reproductive and agonistic behaviours exhibited by the focal species (Table 1; Figure 2; behaviours modified from Field, 1998). At the end of behavioural assays, the two parasitoids were removed and the pentatomid egg mass was kept for 10-11 days at 25°C before freezing the egg mass just prior to parasitoid emergence. I did this in order to confirm the outcome of intrinsic contests by dissecting the eggs and identifying developing parasitoids.

4.4 Data analysis

I tentatively identified compounds in *N. viridula* egg extracts by analysing GC-MS extracts with MassHunter WorkStation 2015 software and the mass spectral library NIST Mass Spectral Search Program version 2.4, 2020. I also compared the Kovats retention index (KI) (Kováts & Weisz, 1965) of each compound with a hydrocarbon series (C8 to C28) using the same temperature program and column type as the extracts.

I normalised responses in electroantennogram recordings in relation to a set of standard compound ((*E*)-2-decenal) responses made throughout each recording, and present averaged responses to the two extracts from multiple recordings.

I tested if there were differences in retention times between the two parasitoids when exposed to volatiles of each pentatomid with a Poisson GLM, and I included the dates of experiments as a random effect. I calculated estimated marginal means and confidence intervals for each combination of arrestment treatment and back-transformed these onto the original scale to examine differences in retention time.

For competition assays, I tested whether *T. basalis* and *T. oenone* differed in the number of oviposition attempts or time it took to successfully parasitise five eggs with two sample t-tests. I tested whether parasitoids differed in the number of times they initiated a backdown encounter based on the number of eggs in the mass with a Poisson GLM, with date as a random effect. I also tested whether there was a difference between the number of eggs each parasitoid species attacked before aggression was initiated, based on the number of eggs in each host patch, the number of offspring each parasitoid invested into the mass, or the time it took the focal species to successfully mark its first five eggs.

All analyses were performed in R 4.0.2 (R Core Team, 2020).

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Table 2: Compounds tentatively identified in acetone and hexane extracts with *N. viridula* eggs based on NIST library matches.

Extract	Compound	Peak area
Acetone	Oleic Acid	245185017
Acetone	*2-Pentanone, 4-hydroxy-4-methyl-	72620191
Acetone	n-Hexadecanoic acid	51886811
Acetone	Octadecanoic acid	25244143
Acetone	trans-9-Octadecenoic acid, pentyl ester	9439859
Acetone	*Docosane, 5-butyl-	6422359
Acetone	Catechol	5446892
Hexane	Eicosane, 1-iodo-	5249133
Acetone	Pentacosane	4163841
Acetone	Hexadecenoic acid, Z-11-	3743110
Acetone	Hexacosane	3268936
Acetone	Tetracosane	2810966
Acetone	Decanedioic acid, bis(2-ethylhexyl) ester	2350087
Hexane	*2-methyloctacosane	2266206
Acetone	3-Methylheptacosane	2075731
Hexane	Nonadecane	1982216
Hexane	*Tricosane	1872925
Acetone	9-Octadecene, 1-methoxy-, (E)-	1798525
Acetone	*(1R, 2S)-2-(Hydroxymethyl)spiro[cyclopropane-1,3'-indol]-2'(1'H)-one	1714452
Acetone	*E-6-Octadecen-1-ol acetate	1640829
Acetone	(Z)-9-octadecen-4-olide	1579544
Acetone	9-Octadecenoic acid (Z)-, methyl ester	1557001
Acetone	Tricosane	1498941
Acetone	*Oxime-, methoxy-phenyl-_	1232961
Acetone	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	1211412
Acetone	Phthalic acid, di(2-propylpentyl) ester	1203384
Acetone	Propanal, 3-(methylthio)-	1197558
Acetone	Benzeneacetaldehyde	1175101
Hexane	Tricosane	1000357
Acetone	Cyclotrisiloxane, hexamethyl-	873536
Acetone	1,3-Dimethylpentalongin	849015
Acetone	trans-Z-.alpha.-Bisabolene epoxide	733901
Hexane	*Tetracosane	665121
Acetone	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)-	546030
Acetone	*Decanedioic acid, didecyl ester	524641
Acetone	Ethanone, 1-(2-methyl-1-cyclopenten-1-yl)-	494374
Acetone	Cyclotetrasiloxane, octamethyl-	416287
Acetone	Hexadecanoic acid, methyl ester	406012

Acetone	9-Octadecenoic acid (Z)-	404394
Hexane	Pentatriacontane	348253
Acetone	*3-Hexen-2-one, 5-methyl-	255139
Acetone	2-Propenoic acid, dodecyl ester	176608
Acetone	4-nitrophthalamide	168762
Acetone	2-Hydroxy-2-cyclopenten-1-one	136481
Acetone	1-[2-[[(1,1-Dimethylethyl)imino]methyl]-3-furanyl-1-propanone	121217
Hexane	9-(Methylthio)-8H-acenaphtho[1,2-c]pyrrole-7-carboxylic Acid	115033
Acetone	Ethyl (2'-nitro-3'-oxo-6',7'-dihydro-3H,5H-benzo[ij]quinolizin-1'-yl)-cyanoacetate	101725

4.5 Results

Electroantennography

I detected seven compounds in the hexane extract, 39 in the acetone extract, and of these only two (tetracosane and tricosane) were common to both (Table 2). Based on peak area, the most dominant compounds in the acetone extract (within the alkane series) were oleic acid, n-hexadecanoic acid, and octadecanoic acid.

   T. basalis showed higher responses to the acetone extract ($F = 202.42$, $df = 4$, $p < 0.001$) (Figure 4). Mean normalised responses of *T. basalis* to *N. viridula* extract were 519 units (SD = 207 units) for the 96 hour acetone extract and 287 units (SD = 145 units) for the 24 hour hexane extract. Air (mean = 100 units), hexane solvent (mean = 73 units, SD = 39 units), and acetone solvent (mean = 107 units, SD = 40 units) control treatments elicited very low responses (Figure 4).

Table 3: Proportion of parasitoids arrested, mean retention times in open arenas contaminated by pentatomid hosts or uncontaminated controls, and proportion of parasitoids which exit arenas by walking (as opposed to flying).

Parasitoid	Pentatomid	n	Arrested	Mean retention time (s)	SD retention time (s)	Exited by walking
T. oenone	*C. simplex*	36	89%	80	61	70%
T. oenone	*N. viridula*	42	71%	21	11	52%
T. basalis	*N. viridula*	72	54%	149	73	53%

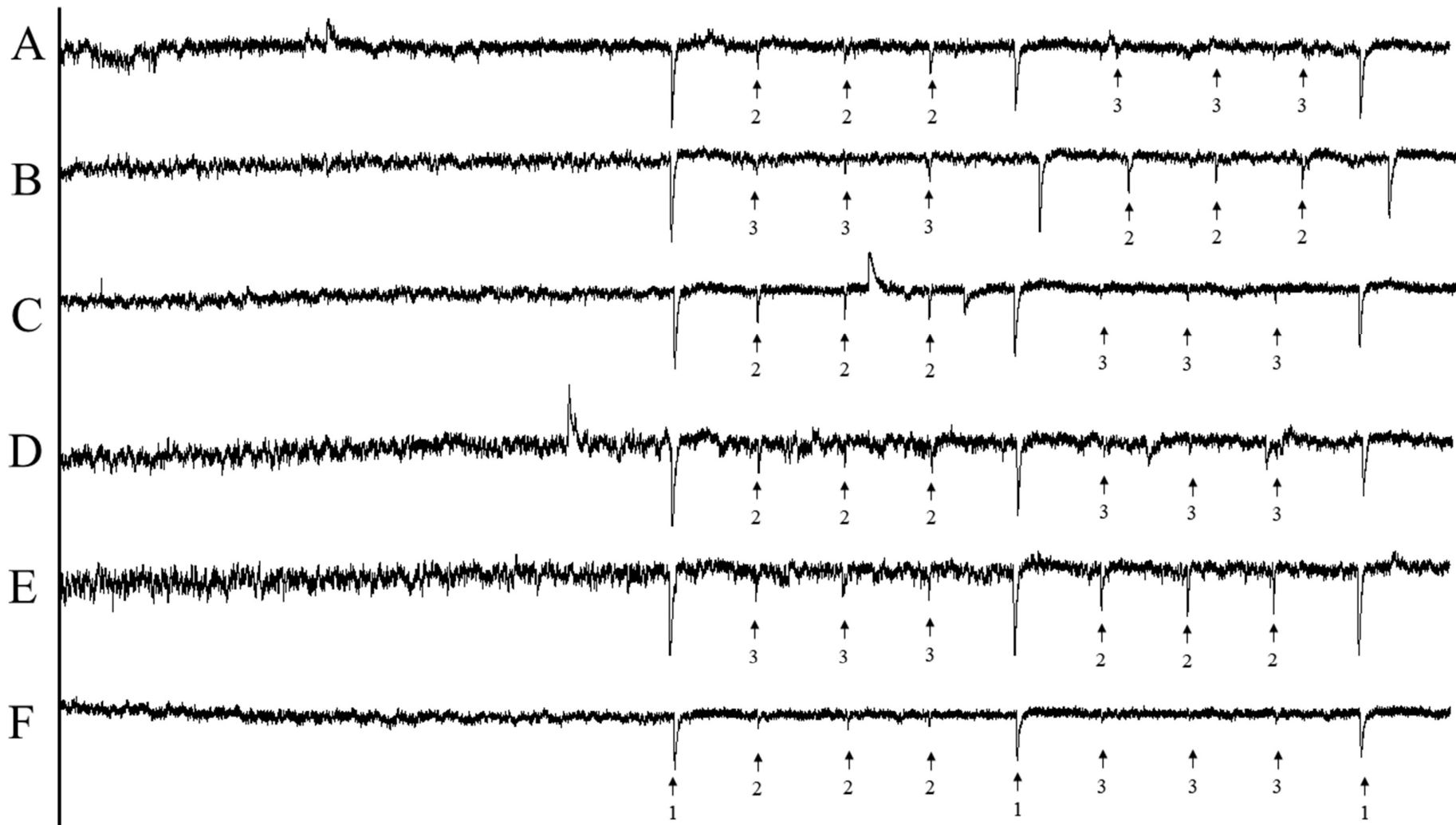| *T. basalis*	*C. simplex*	67	33%	36	33	25%
T. basalis	Control	30	0%	3	5	3%
T. oenone	Control	41	0%	5	10	37%

Figure 3: Electroantennograms showing responses of *Trissolcus basalis* to sets of three puffs of *Nezara viridula* egg extracts made with acetone (2) and hexane (3), with standard responses to (*E*)-2-decenal (1).
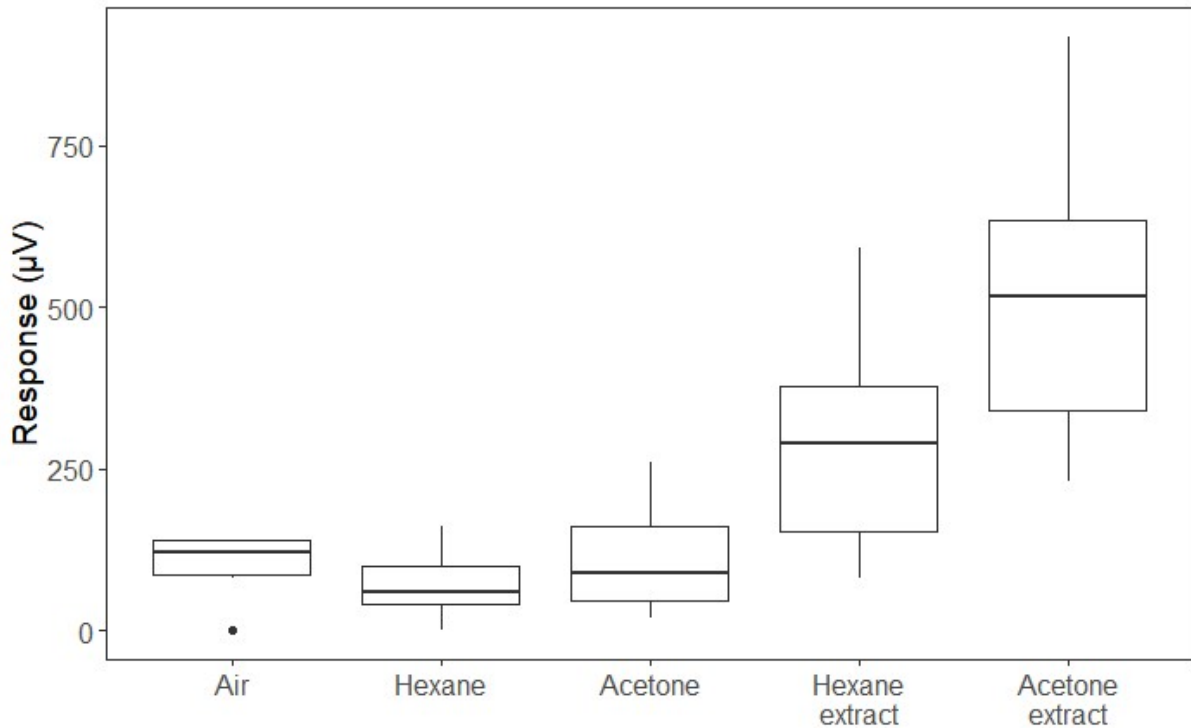
Figure 4: Boxplots showing mean electroantennogram responses of *Trissolcus basalis* to air, hexane, acetone, and *Nezara viridula* egg extracts made with hexane or acetone.

Arrestment bioassays

For open arena arrestment experiments, successfully 'arrested' wasps followed a typical sequence, depicted as an ethogram in Figure 2, with behaviours defined in Table 1. *T. oenone* was arrested in a greater proportion of replicates for both pentatomid treatments than *T. basalis* (Table 3). Filter paper contaminated by *C. simplex* arrested *T. oenone* in 89% of replicates and *T. basalis* in 54% of replicates, whereas paper contaminated by *N. viridula* arrested *T. oenone* in 71% of replicates and *T. basalis* in only 33% of replicates. Neither parasitoid showed any arrestment in control arenas. Parasitoids were significantly more likely to leave control arenas by flying, whereas they were more likely to leave contaminated arenas by walking to the edge of the paper (F = 66.286, df = 1, p < 0.001).

Mean total retention time (i.e. the sum of inner and outer zones) differed based on a significant interaction between species of pentatomid and species of parasitoid (F = 63.76, df = 1, p < 0.001). *Trissolcus basalis* spent four times longer in arenas contaminated by *N. viridula* (mean = 149s, SD = 73s) than *C. simplex* (mean = 36s, SD = 33s), whereas *T. oenone* spent four times longer in arenas contaminated by *C. simplex* (mean = 80s, SD = 61s)

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than *N. viridula* (mean = 21s, SD = 11s) (Figure 5; Figure 6). Mean retention times in the inner zone of the filter paper also differed based on a significant interaction between species of pentatomid and species of parasitoid (F = 63.5156, df = 1, p < 0.001). On average, both parasitoids spent the majority (75-82%) of their time in the inner zones of the filter paper contaminated by pentatomids. Both parasitoids spent less than 6 seconds on average in control arenas.
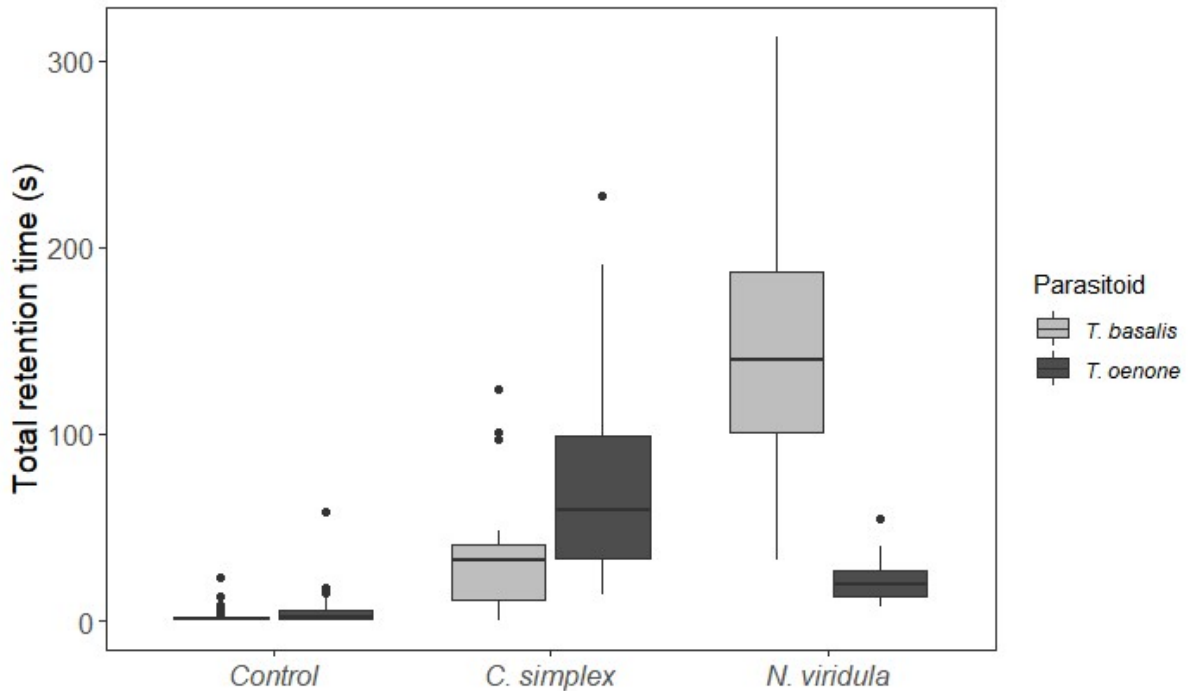
Figure 5: Boxplots showing mean retention times for *Trissolcus basalis* and *Trissolcus oenone* in open arenas contaminated with footprint compounds from *Cuspicona simplex* or *Nezara viridula*, compared to uncontaminated control arenas.

Competition experiments

In control experiments, *T. basalis* developed in or emerged from a mean of 97% (SD = 6%) of *C. simplex* eggs and 60% (SD = 34%) of *N. viridula* eggs, and this difference was significant (F = 7.4915, df = 1, p < 0.01). *Trissolcus oenone* developed in or emerged from a mean of 90% (SD = 26%) of *C. simplex* eggs and 0% of *N. viridula* eggs. There was no significant difference in the percent emergence of the two parasitoids from *C. simplex* eggs (F = 0.8031, df = 1, p > 0.1).

T. basalis successfully oviposited in 67% of the *C. simplex* eggs available in competition assays, while *T. oenone* successfully oviposited in a total of 85% of eggs. Just over half (51%) the eggs in masses used in competition assays were successfully parasitised by both parasitoids. *T. basalis* only developed in 25% of the eggs it attacked, while *T. oenone*
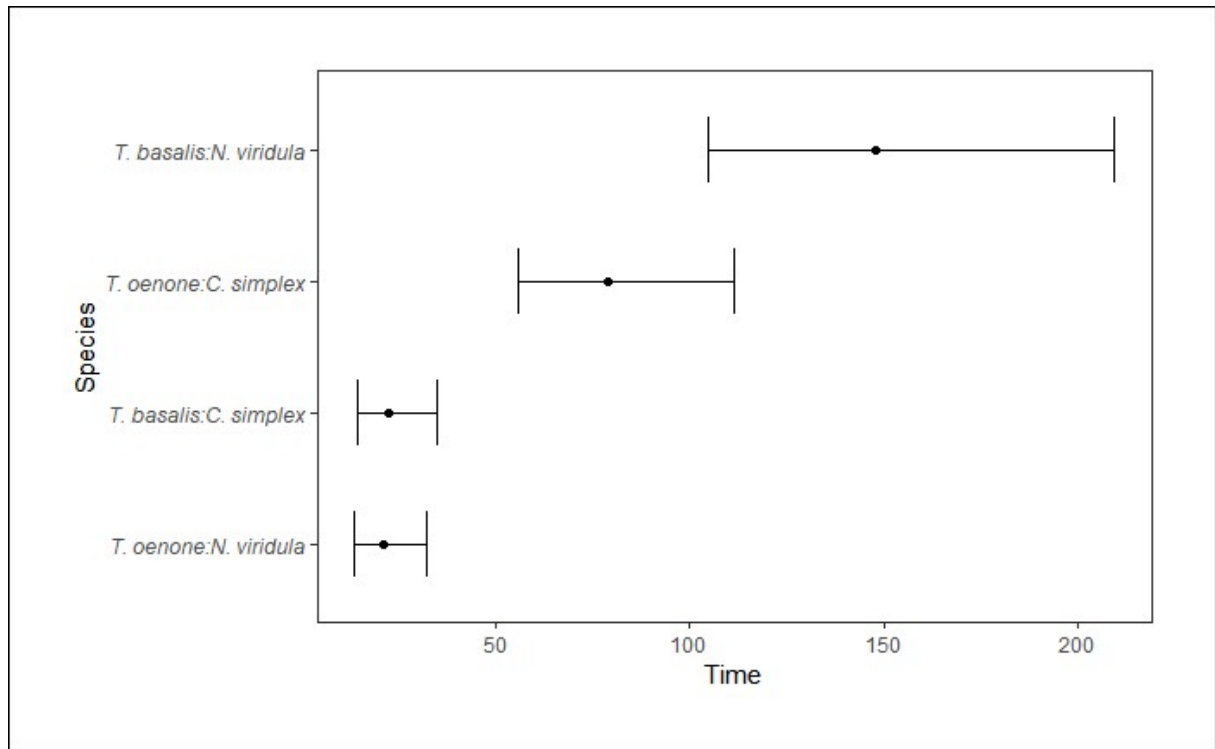
Figure 6: Expected mean retention times (seconds) for combinations of parasitoids and pentatomids in arrestment experiments.

developed in 94% of the eggs it attacked. Of the eggs attacked by both parasitoids, *T. oenone* won the intrinsic contest and developed in almost all (91%) of these.

I observed parasitoids move through a standard sequence of behaviours. Frequencies of behavioural variables recorded during competition assays were similar for both parasitoid species. There was no difference between the two parasitoids in either the number of oviposition attempts (t = 1.6842, df = 18, p = 0.1094) or time it took to mark five eggs (t = 0.082254, df = 18, p = 0.9354), although *T. basalis* marked a higher proportion of eggs for each oviposition attempt (Table 4). *T. basalis* escalated fights more often than *T. oenone* (F =

51.632, df = 1, p < 0.001), and *T. oenone* received more backdown agonistic encounters than *T. basalis* (F = 16.511, df = 1, p < 0.001). The number of eggs attacked by parasitoids before the first aggressive encounter did not significantly differ based on the parasitoid species, the number of oviposition attempts required for the parasitoid to mark five eggs, or the number of eggs in the mass.

4.6 Discussion

The combination of electrophysiology, arrestment, and competition experiments provides more context for helping to understand the host-specificity of these parasitoids than any one method alone. I found that *T. basalis* antennal responses were stronger to acetone extracts than those made with hexane. I also showed that parasitoids exhibited very different levels of motivation when searching for the two pentatomid hosts tested, and that when both parasitoids attack *C. simplex*, the native parasitoid *T. oenone* is expected to dominate both extrinsic and intrinsic contests.

Electroantennography

Trissolcus basalis antennal responses were 55% higher to acetone extracts than hexane extracts. While I am unable to determine if the stronger responses observed here were due to the solvent or immersion time, previous work has shown that acetone extracts of *N. viridula* eggs elicit probing behaviour in *T. basalis* when applied to glass beads, whereas hexane extracts do not (Bin et al., 1993). This suggests acetone is able to effectively remove a contact kairomone present in the adhesive used by female stink bugs to glue eggs to each other and onto a substrate. Chemical analysis showed the acetone extract used in my study contained far more compounds than the hexane extract, and there was little overlap in compounds between the two extracts.

Michereff et al. (2016) identified a similar blend of compounds in acetone extracts made with *Euschistus heros* (F.), and showed that *Telenomus podisi* Ashmead were attracted to eggs and egg extracts in Y-tube olfactometers. The major compounds identified in *E. heros* extracts were hexadecanoic acids, linoleic acids, octadecenoic acids, octadecanoic acid, and ethyl stearate. This suggests linoleic acids, hexadecanoic acids, and octadecanoic acids make good candidates for contact kairomones as they were common to my extracts and those found to be

attractive to *Te. podisi* (Michereff et al. 2016). However, Tognon et al. (2020) showed *Te. podisi* was attracted to a blend of compounds identified from *E. heros* egg extracts made

Parasitoid	Marks per probe	SD	Walk-Antennate	SD	Probe	SD	Pump	SD	Mark	SD	Patrol	SD	Non-Agonistic	SD	Backdown (Aggressor)	SD	Backdown (Receiver)	SD	Escalation (Aggressor)	SD	Escalate (Reciever)	SD
T. basalis	0.74	0.10	28.50	11.59	20.60	6.70	16.40	4.79	15.10	4.91	11.70	3.74	6.80	8.75	2.20	2.20	6.80	5.71	15.60	17.55	3.40	2.88
T. oenone	0.66	0.18	31.20	17.76	25.90	15.27	19.00	10.77	15.10	3.67	12.20	1.69	3.60	4.45	1.20	2.04	12.50	15.69	4.80	9.22	2.00	2.58

Table 4: Mean frequencies of different behaviours observed during competition experiments between *Trissolcus basalis* and *Trissolcus oenone* on *Cuspicona simplex* eggs.

with a five minute hexane immersion. Hexane extracts contained none of the compounds I or Michereff et al. (2016) identified as dominant compounds in acetone extracts, but instead provoked behavioural responses with a synthetic blend of camphene, β-pinene, limonene and benzaldehyde. It is possible that pentatomid eggs contain several kairomonal compounds with differing polarities, so that both hexane and acetone each remove some and not others. The extraction and identification of kairomonal compounds is an important step in understanding how chemical ecology influences the host-preferences and host ranges of classical biological control agents (see chapter three), but to date, has not typically been included in pre-release risk assessments.

Arrestment bioassays

T. basalis spent much longer searching for its primary host *N. viridula*, than for *C. simplex*, while the reverse was true for *T. oenone*. On average, the native parasitoid spent even less time searching for the non-host *N. viridula* than *T. basalis* spent searching for the less preferred host *C. simplex*. As far as I am aware, these are the first arrestment results reported for an egg parasitoid with a non-host pentatomid species. My results suggest that not only are these parasitoids capable of distinguishing between different species of pentatomids, but they may also be able to distinguish between physiological hosts and non-hosts based solely on the blend of footprint compounds left behind by adult stink bugs.

I reported the proportion of replicates in which arrestment occurred, as I felt this information was important for assessing my method. While the native parasitoid was arrested in over 70% of replicates for each pentatomid, *T. basalis* was arrested in half of the *C. simplex* replicates, and in just a third of *N. viridula* replicates. This could be explained by the different number of generations each parasitoid had been reared through in the lab, as the *T. oenone* colony had only been reared through approximately a quarter of the number of generations the *T. basalis* colony had been reared through. Alternatively, this result could be explained by the method I used to contaminate the filter paper with stink bug volatiles. I decided to leave groups of stink bugs in contact with filter paper for three hours before removing them. It's possible that leaving them on the paper for this long resulted in a volatile profile that was less attractive to parasitoids. Most arrestment studies expose a single stink bug to filter paper for an hour or less (Colazza et al., 2014), but in my preliminary tests I had more consistent results using a larger number of insects over a longer exposure time.

I observed both *T. basalis* and *T. oenone* to have very high parasitism efficiencies (>90%) on *C. simplex* eggs in control oviposition tests between the two parasitoids and *C. simplex* conducted for the competition component of the present work. I previously observed very similar results, where both parasitoids achieved over 90% parasitism efficiencies on *C. simplex* eggs, when I conducted oviposition tests reported in chapter two of this thesis. These results also showed both parasitoids are highly motivated to attack *C. simplex*, as both discovered and attacked over 95% of egg masses. Both parasitoids took a mean of 19-20 days to develop in *C. simplex*, and there was no significant difference in their development times. The results from chapter two clearly demonstrate that *C. simplex* is a highly suitable host for the development of both parasitoids. In the context of pre-release risk assessment, and based solely on these kinds of no-choice oviposition results, both of these parasitoids would be considered to pose the same kind of risk to *C. simplex*, although that risk would still largely be unquantified. No-choice oviposition results unambiguously identify a parasitoid's physiological host range, and will always be an essential first step in experimentally characterising the host ranges of proposed classical biological control agents. However, arrestment studies have the potential to make very important contributions to risk assessments by providing another dimension for assessing risk: motivation to search.

My arrestment results showed that *T. basalis* was highly motivated to search for its preferred host, *N. viridula*, but spent only a quarter of that time searching for *C. simplex*. On the other hand, *T. oenone* was highly motivated to search for *C. simplex*, but spent even less time searching for *N. viridula* than *T. basalis* spent searching for *C. simplex*. These results have two important implications. First, they clearly show that both parasitoids are capable of discriminating between adult hosts based solely on footprint compounds, a fact already known for *T. basalis* and other *Trissolcus* species (Colazza et al., 1999; Conti et al., 2004; Salerno et al., 2006) but as yet undocumented in *T. oenone*. Second, they suggest that *T. oenone* may be able to differentiate between physiological hosts and non-hosts based solely on footprint compound profiles. This is the first study to my knowledge to report arrestment results of a parasitoid in open arenas contaminated by a species which is not a physiological host. Peri et al. (2021) showed that naive female *T. basalis* displayed similar arrestment behaviour in arenas contaminated by the suboptimal host *H. halys*, but that parasitoids spent about a third longer in arenas contaminated by their preferred host, *N. viridula*. In isolation, no-choice oviposition results painted a similar picture for both of these parasitoids in relation

to *C. simplex*. But when arrestment results are presented alongside oviposition results, it becomes clear that *T. basalis* is unlikely to be sufficiently motivated to find and attack *C. simplex* to a high degree. Interestingly, recent parasitoid surveys conducted in Auckland between December 2020 and March 2021 yielded only *T. oenone* specimen in sentinel eggs of *C. simplex* (Pers. Comm., Karina Santos, Plant & Food Research), which may reflect the lack of motivation by *T. basalis* to search for this host as reported here. Overall, it is clear that high discovery or parasitism efficiencies (>90%) do not necessarily translate into high motivation to search for the host.

Experiments overseas with scelionid egg parasitoids have revealed similar discrepancies between no-choice oviposition results and motivation to search in arrestment studies, although they have not been contextualised in terms of their value to pre-release risk assessments. *Telenomus podisi* Ashmead and *Trissolcus urichi* (Crawford) expressed similarly high parasitism rates on *Piezodorus guildinii* (Westood) in no-choice oviposition tests (Cingolani et al., 2014). However, arrestment results showed that, on average, *Tr. urichi* spent about 35% longer searching in arenas contaminated by the host than *Te. podisi* (Cingolani et al., 2019). Another example of the utility of arrestment experiments for assessing the relative performance of different parasitoids (or their potential non-target risks) is provided by Peri et al. (2011), who compared the generalist egg parasitoid *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) and *T. basalis*, in relation to *N. viridula*. They found that *O. telenomicida* was unable to detect or exploit footprint kairomones associated with *N. viridula*. A subsequent two year field and semi-field experiment in Western Sicily showed *T. basalis* was the superior parasitoid against *N. viridula*, and this is likely to be because *T. basalis* can rank adult *N. viridula* hierarchically based on their sex and reproductive status (Peri et al., 2006). These kinds of results would be especially valuable in the context of pre-release risk assessments, as they can inform whether or not a candidate agent is likely to detect and search for hosts in the field, and the relative amount of search effort expended by the parasitoid for different non-target species. Regulators often have to make decisions about whether or not to approve the release of a classical agent based solely on laboratory oviposition data, so incorporating more ecological experiments would help to reduce uncertainty around potential non-target effects (Barratt, 2011; Bigler et al., 2006; van Lenteren et al., 2006).

Competition experiments

Trissolcus oenone successfully parasitized 21% more host eggs than *T. basalis* during competition assays. This was despite *T. basalis* escalating fights more often, and *T. oenone* having to back down from agonistic interactions more frequently than *T. basalis*. In addition, out of the eggs parasitized by both species, *T. oenone* developed in over 90%. Female *T. basalis* marked a higher proportion of probed eggs, suggesting they may have been more strategic in their oviposition attempts, or interrupted less frequently by *T. oenone*. Overall, *T. basalis* was more aggressive during extrinsic contests on the egg mass, but *T. oenone* achieved more parasitism and clearly dominated the intrinsic contest between parasitoids developing inside the same host egg. Female *T. basalis* foraging on the same egg mass initiate their first aggressive encounters in response to a trade-off between defending unparasitised hosts, and the value of offspring previously deposited into the patch (Field & Calbert, 1998). Aggression is typically initiated earlier when host patches are smaller, and when encounter rates and the number of offspring invested are higher. I didn't measure encounter rate, and found no relationship between the onset of aggression and any variables I measured.

I expected the introduced biological control agent *T. basalis* to outperform the native parasitoid in both extrinsic and intrinsic contests, but my results show the native parasitoid is the superior competitor. Cumber (1964) conducted a limited series of competition experiments on several species of pentatomid eggs using females between *T. oenone* and *T. basalis*, which he referred to as 'Species N' and *Asolcus basalis*, respectively. He observed a similar pattern in his experiments where usually several eggs were parasitized before the first aggressive encounter between parasitoids. He reported *T. oenone* to be dominant in both extrinsic and intrinsic contests based on behavioural observations and proportions of emerging parasitoids, but his methods were not always defined or quantified in a reproducible way, and lacked sufficient replication. Cumber (1964) observed a relatively common pattern whereby the dominant individual would chase the other away from the egg mass, and then complete oviposition in time to interrupt the renewed attempt made by the submissive individual. I saw this pattern while observing the two parasitoids on the egg masses and speculate it may offer a clue as to how *T. oenone* was able to lay eggs in a higher proportion of the host patch than *T. basalis*. My results largely confirm Cumber's conclusions by using replicated experiments with different parasitoids on the same species of pentatomid eggs, and

by measuring well-defined behavioural variables. But my results differed from Cumber's in that I found *T. basalis* to be more aggressive during extrinsic contests than *T. oenone*, at least when measuring the variables I selected.

Even when a parasitoid survives the intrinsic contest inside a host egg, it may go on to accrue fitness costs that conspecifics developing in their own eggs do not have to bear (Cusumano et al., 2016). For example, when *T. basalis* and *O. telenomicida* multiparasitised *N. viridula* eggs, surviving *T. basalis* offspring were smaller, took longer to develop to maturity, and females produced fewer oocytes (Cusumano et al., 2015). Interestingly, *T. basalis* did not experience the same detrimental outcomes after surviving intraspecific competition, whereas the reverse was true for *O. telenomicida*: its offspring suffered similar fitness costs but only as a result of intraspecific and not interspecific competition. This is likely because *O. telenomicida* injects substances which directly alter the nutritional profile of host eggs, and indirectly mediate interspecific competition with other egg parasitoids that multiparasitise eggs it has previously attacked (Cusumano et al., 2012). It is possible that venoms or accessory gland products are injected into host eggs by *T. oenone*, or both parasitoids, and these products may provide *T. oenone* with a developmental advantage over *T. basalis*. Competition experiments are useful for revealing the sometimes complex interactions between different parasitoids on the same host, and the information they provide could be used to inform pre-release risk assessments for classical biological control agents. If a candidate agent is less dominant on the egg mass, and especially if it consistently loses the intrinsic contest between larvae, then it is unlikely to displace the native parasitoid.

Egg parasitoids face the prospect of having to locate and attack a host life stage which is often cryptic, and whose quality diminishes over a relatively short period of time (Vinson, 1998). As a result, these parasitoids have evolved adaptations to perceive and exploit chemical cues which exist on a reliability-detectability spectrum (Bin et al., 1993; Colazza et al., 1999; Turlings et al., 1990; Vet & Dicke, 1992): on the one hand, plant volatiles are abundant and easily detectable, but unreliable for conveying information about the presence of hosts; and on the other hand, host-derived kairomones offer a far more reliable cue to indicate the presence and even the reproductive status of potential hosts, but they are sparser and more difficult to detect.

I showed that *N. viridula* egg extracts made with acetone and longer immersion times elicited a stronger response from *T. basalis* antennae than hexane extracts with a shorter

immersion time, which suggests one or several of the compounds I tentatively identified may act as a contact kairomone in the adhesive in stink bug eggs. Electrophysiology studies are useful for determining which compounds associated with a host deserve more attention in behavioural experiments, and I think their wider application to pre-release risk assessments would help to improve the way classical biological control agents are screened for their non-target risks.

I showed that *T. basalis* and *T. oenone* displayed different levels of motivation to search for *C. simplex* when exposed to substrate-borne footprint compounds in open arenas. This is despite both parasitoids showing almost identical discovery and parasitism efficiencies to this host in previous oviposition tests. I believe arrestment studies have major contributions to make to the study of non-target risks associated with classical biological control agents, as they provide a relatively simple and inexpensive method to provide a much-needed behavioural context to the results of oviposition tests. They complement and extend traditional physiological host range testing by offering high quality evidence of a parasitoid's motivation to search for a given species (Conti et al., 2004), and this is particularly useful when oviposition results are similar between different parasitoids, or between different non-target hosts for the same parasitoid.

Finally, I showed that a native parasitoid outcompetes an introduced biological control agent in both exploiting a higher number of eggs in extrinsic contests, and by winning over 90% of intrinsic contests in multiparasitised eggs. Competition assays provide useful information for examining the efficacy of a biological control agent on a target pest in the presence of competition, and also for examining whether an introduced agent may displace a native species. Competition experiments could also be used to test whether the combination of multiple parasitoids has a synergistic effect on non-target species leading to higher parasitism and greater risks of non-target effects. A combination of chemical and behavioural ecological approaches to host-specificity testing will help to better characterise non-target risks associated with classical biological control agents, and will help to reduce uncertainties that remain after traditional oviposition tests during pre-release risk assessments.

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CHAPTER 5: Electrophysiological responses of *Trissolcus japonicus*, *Trissolcus basalis*, and *Trissolcus oenone* (Hymenoptera: Scelionidae) to volatile compounds from New Zealand stink bugs (Hemiptera: Pentatomidae)

5.1 Abstract

Parasitoids employed as biological control agents against insect pests rely heavily on their ability to locate hosts through olfaction. Chemical cues associated with hosts and non-hosts are known to play important roles in host specificity. A better understanding of how and why parasitoids attack some hosts and not others—based on volatile organic compounds found as cuticular hydrocarbons or in defensive secretions—would provide important insights during pre-release risk assessments for classical biological control agents. Electrophysiological techniques such as electroantennograms and gas chromatography coupled with electroantennographic detection (GC-EAD) have been used to identify sex pheromones and semiochemicals of interest between biological control agents and their target hosts. However, the application of these techniques to understanding the chemical ecology between biological control agents and non-target species has been slower. I used multiple rounds of GC-EAD and electroantennography to identify olfactory-active compounds associated with adult New Zealand stink bugs on the antennae of three closely related parasitoid species: the premptively approved biological control agent *Trissolcus japonicus* Ashmead, the biological control agent of green vegetable bug *Trissolcus basalis* (Wollaston), and the native Australasian pentatomid parasitoid *Trissolcus oenone* Johnson. I found eight compounds which all three parasitoids responded to, and were able to identify seven of the compounds as follows: (*E*)-2-decenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, and (*E*)-2-hexenal all elicited strong responses in all three parasitoids, while (*E*)-2-decenyl acetate, *n*-dodecane, and *n*-tridecane elicited weaker responses. I only observed responses to *n*-tridecane when puffing pure compound over parasitoid antennae. I confirmed the identity of compounds through comparing mass spectra in the NIST Mass Spectral Database to Kovats retention indices calculated by comparing compounds to a hydrocarbon series (C8 to C28). I discuss my results in the context of the chemical ecology of stink bugs and their egg parasitoids, and I comment on the importance of

accurately identifying kairomones for understanding attraction of biological control agents to non-target species.

5.2 Introduction

Insect antennae are complex sensory organs which have evolved to distinguish useful stimuli with a high degree of sensitivity (Wee et al., 2016). Olfaction is a critical part of the host-location process for parasitic Hymenoptera (Vet and Dicke 1992; Vinson 1998), so a better understanding of how parasitoids perceive host semiochemicals can offer clues to their likely host specificity (Park et al., 2018). Parasitoid antennal sensilla often contain olfactory receptor neurons attuned to a narrow range of volatile organic compounds associated with their hosts, such as cuticular hydrocarbons or compounds in waste products (Blomquist & Ginzel, 2021; Vet & Dicke, 1992). These compounds elicit behavioural responses relevant to host location, such as an increase in the duration or intensity of searching (Vinson, 1998; Wajnberg & Colazza, 2013). The semiochemistry of plant-herbivore interactions has also been shown to influence parasitoid host-searching within a tritrophic system. For example, feeding and oviposition on bean plants by *N. viridula* causes the release of terpenoids which are attractive to *T. basalis* (Colazza et al., 2004). In another study, Conti et al. (2010) showed that a complex of both host and plant volatiles were used by *Trissolcus brochymenae* (Ashmead) to locate *Murgantia histrionica* (Hahn), probably through detection of volatiles adsorbed into the layer of epicuticular wax on the surface of plant leaves (Frati et al. 2013). Gas chromatography coupled with electroantennographic detection (GC-EAD) is a powerful technique which can be used to identify olfactory-active compounds in a mixture, such as a solvent extract or headspace sample taken from hosts or the plants they feed on (Arn et al., 1975; Gouinguené et al., 2005; Zhu & Park, 2005). In the context of biological control programmes, this technique aids in the identification of kairomones associated with hosts or habitats attractive to hosts (Benelli et al., 2013; Kpongbe et al., 2019; H. Yu et al., 2010). A better understanding of host-parasitoid chemical ecology could be used to improve the efficacy of biological control programmes, for example by using chemical lures to attract parasitoids into a certain area (Lewis & Martin, 1990; Peñaflor, 2019), or even through selecting the most appropriate strain of an agent based on its ability to find and attack the pest population (Barratt et al., 2018).

The GC-EAD technique has long been used to understand the chemical nature of attraction between biological control agents and their target hosts (Olsson & Hansson, 2013). However, the method is rarely applied to understand the chemistry mediating interactions between candidate biological control agents and non-target hosts during pre-release host

specificity testing. Identifying compounds associated with target hosts versus non-target hosts could be a useful tool when assessing the suitability of parasitoids for release. The presence of responsive compounds in the chemical profiles of non-target species is a useful starting point to explore similarities and differences in chemical ecology which translate into differences in host ranges or host preferences (Boyle et al., 2020). This kind of information could be a valuable complement to traditional components of risk assessment, such as no-choice oviposition tests, when evaluating parasitoids for release as biological control agents (Conti et al., 2004), and may be especially valuable in cases where it is too difficult or time-consuming to collect or rear large numbers of non-target species for behavioural tests. Even when a non-target species is confirmed as a physiological host in laboratory testing, it is important to understand if the parasitoid will be motivated to search for the host in the field (Avila et al., 2016). Chemical ecology techniques such as GC-EAD may therefore offer valuable insights into ecological host range during the pre-release risk assessment phase of classical biological control programmes.

Trissolcus japonicus Ashmead (Hymenoptera: Scelionidae) is an oligophagous egg parasitoid of pentatomid stink bugs native to East Asia (Talamas et al., 2013; Yang et al., 2009). It is considered to be the most promising biological control agent against the brown marmorated stink bug (BMSB, _Halyomorpha halys_ Stål) (Hemiptera: Pentatomidae), a polyphagous horticultural pest native to the same regions (Lee et al., 2013; Wang & Wang, 1988; G.-Y. Yu & Zhang, 2007), but recently invasive in large areas in the Americas and Europe (Faúndez & Rider, 2017; Haye et al., 2015; Hoebeke & Carter, 2003; Lee et al., 2013; Leskey & Nielsen, 2018; Wermelinger et al., 2008). BMSB is a high priority pest for biosecurity screening in New Zealand because it presents a serious risk to primary industries, which comprise a significant part of the national economy (Ballingall & Pambudi, 2017; Burne, 2019; Duthie, 2015; Fraser et al., 2017). An application to release _T. japonicus_ in New Zealand has been approved by the Environmental Protection Authority (EPA) in the event the stink bug establishes (EPA, 2018). Physiological host range testing in China has shown _T. japonicus_ to be most often associated with BMSB (Zhang et al., 2017), and similar testing in the US and Europe has generally shown low parasitoid emergence rates in non-target stink bugs (Botch & Delfosse, 2018; Haye et al., 2020; Hedstrom et al., 2017; Lara et al., 2019). However, physiological host range testing in New Zealand showed _T. japonicus_ emerges from one endemic pentatomid species and two native species at proportions similarly high to

BMSB, and additionally shows between 70-80% emergence from two exotic species (Charles et al. 2019; Saunders et al. 2021). Uncertainty therefore remains as to the risk of non-target attack posed by the parasitoid in relation to New Zealand stink bugs.

Scelionid egg parasitoids are known to exploit a variety of chemical cues associated with different life stages of their pentatomid hosts and the plants they feed on (Austin et al., 2005; Bin et al., 1993; Conti & Colazza, 2012; Fatouros et al., 2008). Adult stink bugs leave kairomones on surfaces they have walked on (Colazza et al., 1999; Conti et al., 2003), and their activity can induce plants to release synomones which are attractive to parasitoids (Colazza et al., 2009; Frati et al., 2013; Salerno et al., 2019), but few studies have identified the chemicals responsible (Colazza et al., 2007; Salerno et al., 2012; Zhong et al., 2017). The host-parasite complex of pentatomid bugs and their *Trissolcus* parasitoids in New Zealand is depauperate and restricted to eight species of pentatomids and two parasitoids (Cumber, 1964; Larivière, 1995, 2005; Todd et al., 2020). The New Zealand Pentatomidae consists of the introduced predatory bug *Oechalia schellenbergii* (Guérin), and the native *Cermatulus nasalis* Westwood which is split into three subspecies: the endemic subspecies *C. nasalis hudsoni* Westwood and *C. nasalis turbotti* Westwood, and the native subspecies *C. nasalis nasalis* Westwood. Herbivorous species are represented by the endemic *Hypsithocus hudsonae* Bergroth; the native species *Glaucias amyoti* Dallas; and the introduced species *Dictyotus caenosus* Westwood, *Monteithiella humeralis* Walker, *Cuspicona simplex* Walker, and *Nezera viridula*. *Trissolcus basalis* Wollaston was introduced to New Zealand in 1949 as a biological control agent against *N. viridula* (Cumber, 1949, 1950). It is regarded as an effective biological control agent against its target host but is known to attack most non-target pentatomids (Cumber, 1953, 1964). *Trissolcus oenone* Johnson is a native pentatomid parasitoid which has also been recorded attacking most species of New Zealand pentatomids (Cumber, 1964; Johnson, 1991). Other than an Australian captive rearing study (James & Warren, 1991), *T. oenone* has not been the subject of any research to date.

In this study, I used GC-EAD to measure antennal responses to chemical compounds associated with non-target New Zealand stink bugs in three *Trissolcus* species: a proposed classical biological control agent (i.e., *T. japonicus*), a classical biological control agent released 80 years ago (i.e., *T. basalis*), and a native parasitoid (i.e., *T. oenone*). My primary objective was to describe the volatile compound profiles for each New Zealand stink bug species, and to identify which of these compounds are detected by the antennae of the three

parasitoid species tested. I hypothesised that each pentatomid species would have different compounds and different ratios of compounds in its volatile profile, and that each of the three parasitoid species would respond to different compounds based on their physiological host range.

5.3 Methods

Insect colonies

Pentatomid colonies were established from wild specimens and housed in clear plastic containers (~170 mm H × 210 mm L × 135 mm W) with ventilated lids, which were maintained in a temperature controlled room set between 20-25°C (16:8 h L:D), depending on demand for egg masses. Pentatomids were provisioned with moist cotton, wax paper for oviposition, and food after nymphs had moulted to second instar. I provided *Pittosporum* and *Coprosma* fruits for *M. humeralis* and *G. amyoti*, *Solanum* fruits and tomatoes for *C. simplex*, green beans and raw peanuts for *N. viridula*, *Plantago* seed heads for *D. caenosus*, and *Spodoptera litura* (F.) larvae from an existing laboratory culture for *C. nasalis nasalis*, *C. nasalis hudsoni*, and *O. schellenbergii*. I reared *H. hudsonae* on different combinations of food (Saunders et al. 2021) but was ultimately unsuccessful in rearing eggs through to ovipositing adults. *Cermatulus nasalis turbotti* was excluded due to the difficulty of collecting specimens from its range on the Three Kings Islands.

Colonies of *T. basalis* and *T. oenone* were established from wild specimens collected in parasitised egg masses of *N. viridula* and *C. simplex*, respectively, and were reared on their original hosts in the laboratory. Both parasitoid colonies were maintained in a temperature controlled room between 18-25°C (16:8 h L:D) depending on the need to time emergence with EAD recordings. Fresh pentatomid eggs (<24h old), or eggs stored at 10°C for no longer than two weeks, were used to maintain the colonies. *Trissolcus basalis* was originally collected in naturally laid eggs on *Cleome* from Kelmarna Gardens, Auckland, in February 2019. This colony was reared through approximately 15 generations in the laboratory before being used in experiments. *Trissolcus oenone* was originally collected in naturally laid eggs on *Coprosma* (near *Solanum*) from the suburb of Mt Albert, Auckland, in November 2019. This colony was reared through approximately three generations in the lab before being used for experiments.

Shipments of *T. japonicus* were sourced from the USDA ARS Beneficial Insect Research Unit in Newark, Delaware, and imported into Plant & Food Research containment

facilities in Auckland or Lincoln, Canterbury, for use in experiments. Shipments consisted of parasitised BMSB egg masses held in individual 10-dram plastic vials. Egg masses were kept between 18-25°C while parasitoids emerged and mated.

Extract preparation and chemical analysis

Solvent extracts and a solution of synthetic compounds were used to examine each parasitoid for antennal responses to compounds associated with adult stink bugs. For each extract replicate, four female stink bugs were randomly selected from colony containers and immersed in 1 mL of hexane for five minutes inside a glass vial. Each millilitre of hexane contained 10 µg each of n-decane and ethyl tetradecanoate as internal standards. All stink bugs were assumed to have been mated, and were taken from cages where eggs were being laid. The extract was then transferred to a clean glass vial and kept at -20°C until required for analysis. Each extract was analysed on a gas-chromatograph (GC, Agilent 7890B) coupled to a mass-spectrometer (MSD, Agilent 5977A). A 1 µl sample was injected into the GC in splitless mode, with helium used as the carrier gas at a flow rate of 1.6 mL/min. The GC column was non-polar (Agilent DB-5 ms) and measured 30m × 0.25mm ID × 0.25µm film thickness. The temperature program started at 40°C and was held for 2 minutes, then was increased to 250°C at a rate of 4°C/min, followed by a 10 degree per min ramp to 280 degrees, then held for 10 minutes. The transfer line was kept at 250°C.

Electrophysiological recordings

I used GC-EAD to identify olfactory-active volatile compounds from stink bug extracts on all three *Trissolcus* antennae. I anaesthetised each female wasp with carbon dioxide gas before removing its head and the distal tip of one of its antennae with a fine scalpel under a stereomicroscope. For *T. japonicus*, this process occurred inside laboratories that hold a physical containment level two for invertebrates status (PC2). Each specimen was positioned between two silver wire electrodes sheathed by glass capillaries pulled to fine points. Glass capillaries were trimmed with a ceramic cutter and filled with Ringer's solution (Kaissling, 1995). The excised end of the head was positioned into contact with the reference electrode and the excised antenna was positioned into contact with the recording electrode using a Sutter Instrument micromanipulator (MP-225, Sutter Instrument Co., USA). The GC transfer-line was connected to a glass airflow tube containing a charcoal-filtered and humidified air

stream with a flow rate of 400mL/min. The specimen preparation was positioned in front of the air stream. I used an Agilent 7890A GC with a 30 m x 0.32 mm ID HP-5 capillary column with a 0.25 µm film thickness (Agilent Technologies, CA, USA). The GC is equipped with a flame ionisation detector (FID) coupled to an electroantennogram detector (EAD, IDAC 4, Syntech, Germany). I injected 1 µl samples of extract into the GC port set to 250°C in splitless mode. Samples passed through the column at 1 mL/min and were carried by helium. Gas flow was split in a 1:1 ratio, between the parasitoid antenna and the FID, which was set to 300°C. GC oven temperature was programmed to start at 60°C and held for 1 minute, before increasing to 280°C at a rate of 20°C/min, where it was held for 10 minutes. I used Autospike software (v3.9, Syntech, Germany) to record the FID response as compounds left the GC, and simultaneously, to record the insect's antennal response to each compound. I used a different parasitoid for each recording, and aimed to capture at least five clear recordings showing consistent responses for each stink bug extract with each parasitoid species. For each response, I manually measured the amplitude of each depolarisation event.

Once I had identified responsive compounds in the stink bug extracts, I then conducted another round of GC-EAD recordings with synthetic standards to confirm responsive compounds. I injected one microliter samples of a solution containing a mixture of synthetic standards of each compound I successfully identified (each at a concentration of 0.1 mg/mL) for each parasitoid species. Next I presented each individual test compound to each parasitoid using electroantennogram (EAG) recording to identify antennal responses in at least three recordings. I applied a 10-µL aliquot of each compound solution (at a concentration of 100 mg/mL) to a 5 × 25 mm strip of filter paper (Whatman No. 1; Whatman, UK) and allowed the solvent to evaporate for 10 s before placing the paper inside a glass Pasteur pipette (146 mm; Fisher Scientific Co., Pittsburgh, Pennsylvania) to form an odour cartridge. The pipette tip was inserted into a 2-mm diameter hole in the glass airflow tube 10 cm from the outlet. A 0.1-s pulse of charcoal-filtered air (10 mL s^{-1}) was passed through the wide end of the pipette to carry a puff of compound into the air flow tube and over the insect antenna, using an electronic airflow controller (CS-55; Syntech, Germany). I presented each test compound three times in succession with at least 30 s of time interval between successive stimulations. At the start of each EAG recording with *T. basalis* and *T. oenone* I presented a blank air cartridge and a series of solvent puffs. Before and after presenting test compounds, and after every six compound puffs, I presented a single puff of (*E*)-2-decenal to act as a

standard response to allow for the normalisation of responses. I wrapped the wide end of pipettes in aluminium foil when not in use to minimise evaporation of test compound, and I used each cartridge less than 10 times. For the control air cartridge, I kept the filter paper blank, and for the solvent control cartridges, I applied a 10-μL aliquot of neat hexane or acetone.

Synthetic standards were obtained as follows: (*E*)-2-decenal, (*E*)-2-octenal, (*E*)-2-decenyl acetate, n-decane, and (E)-2-hexenal, *n*-tridecane, *n*-nonadecane and ethyl tetradecanoate were purchased from Sigma Aldrich (St. Louis, MO, USA); and (*E*)-4-oxo-2-hexenal was synthesised at The Institute for Plant & Food Research, Palmerston North, by Barry Bunn (Moreira & Millar, 2005).

5.4 Data analysis

To identify the compounds extracted from the stink bug species, I analysed the extracts by GC-MS using MassHunter WorkStation software 2015 and the mass spectral library NIST Mass Spectral Search Program version 2.4, 2020. The NIST library matches were confirmed by calculating the Kovats retention index (KI) (Kováts & Weisz, 1965) of each compound, which was done by running a hydrocarbon series (C8 to C28) using the same temperature program and column type as the extracts. I further confirmed the compounds by analysing a solution of each of the synthetic compounds against the extracts by comparing the retention time and the mass spectral patterns. To compare the volatile profiles of each pentatomid species, I quantified the compounds using the internal standard method and calculated the proportion of each active compound based on peak area in each stink bug extract versus peak area of the internal standards. To compare the similarity of each pentatomid species based on their volatile compound profiles, I performed nMDS with the quantified compound values and plotted the results in an ordination plot. I excluded *O. schellenbergii* because their extracts had few olfactory-active compounds present.

To compare the magnitude of electrophysiological responses between the three parasitoid species, I calculated mean responses recorded in the two rounds of GC-EAD experiments and one round of EAG experiments. In EAG recordings, I normalised responses in relation to five standard responses to (*E*)-2-decenal (a compound known to be responsive) obtained throughout each recording. All analyses were conducted in R 4.0.2 (R Core Team, 2020).

5.5 Results

From the combination of GC-EAD and EAG experiments, I identified a total of seven compounds which elicited antennal responses from parasitoids: (*E*)-2-decenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, (*E*)-2-hexenal, (*E*)-2-decenyl acetate, *n*-tridecane, and *n*-dodecane (Table 1). In GC-EAD experiments with stink bug solvent extracts, seven compounds elicited clear antennal responses from at least one parasitoid species (Figures 1-4). This included responses to an unknown compound in extracts of *H. hudsonae*, *D. caenosus*, and *N. viridula* with a KI of 1204, eluting between *n*-dodecane and (*E*)-2-decenal, but I was unable to

Table 5: Quantified amounts of compound (ng) per 1µl of solvent stink bug extract for nine New Zealand Pentatomidae taxa.

| Species	(E)-2-hexenal	(E)-4-Oxo-2-hexenal	(E)-2-octenal	n-dodecane	(E)-2-decenal	n-tridecane	(E)-2-decenyl acetate
Cermatulus nasalis hudsoni	9.40	93.74	0.00	2.02	8.96	59.01	98.80
Cermatulus nasalis nasalis	13.79	281.84	5.21	1.61	46.05	65.73	224.84
Cuspicona simplex	593.89	294.24	7.75	19.32	354.00	427.06	93.59
Dictyotuse caenosus	93.37	127.18	115.91	7.43	0.00	164.04	0.00
Glaucias amyoti	583.54	332.04	12.65	42.19	409.62	703.97	312.61
Hypsithocus hudsonae	321.22	151.82	106.88	4.01	12.01	124.60	34.87
Monteithiella humeralis	260.34	28.16	9.00	0.00	374.39	492.76	142.59
Nezara viridula	442.28	171.91	108.00	14.83	215.58	301.81	145.72
Oechalia scellenbergii	0.00	0.00	0.00	0.00	0.00	2.11	0.00
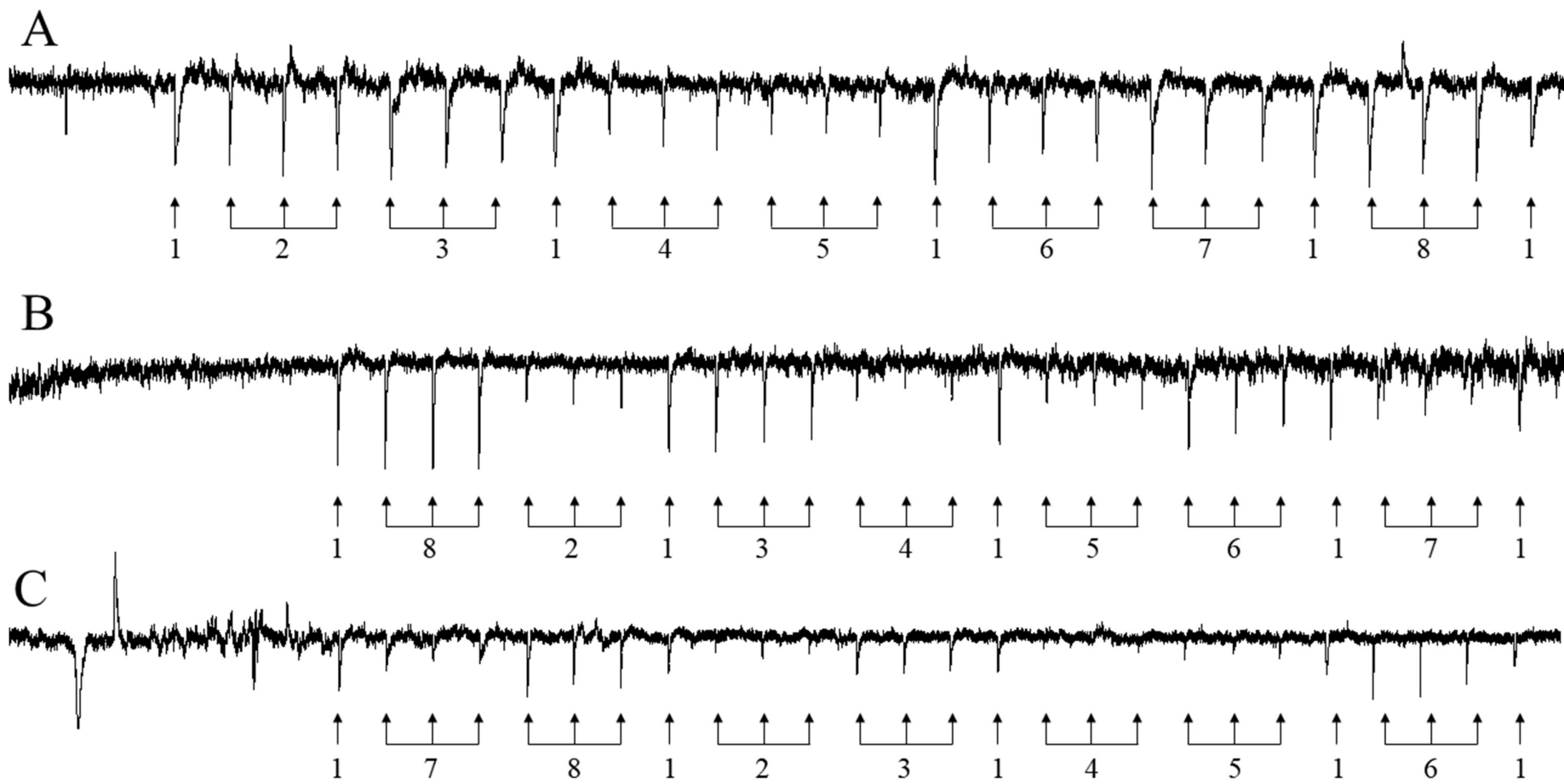
Figure 8: Representative EAG recordings with each parasitoid and each compound suspected to be bioactive from GC-EAD experiments. A. *Trissolcus japonicus*. B. *Trissolcus basalis*. C. *Trissolcus oenone*. 1. (E)-2-decenal (standard). 2. n-dodecane. 3. (E)-2-decenal. 4. n-tridecane. 5. (E)-2-decenyl acetate. 6. (E)-2-hexenal. 7. (E)-4-oxo-2-hexenal. 8. (E)-2-octenal.

identify it based on mass spectra. All three parasitoids responded most strongly to (*E*)-2-decenal and (*E*)-2-octenal, and responses to (*E*)-4-oxo-2-hexenal and (*E*)-2-hexenal were also very strong. I did not observe any responses to n-tridecane in these recordings.
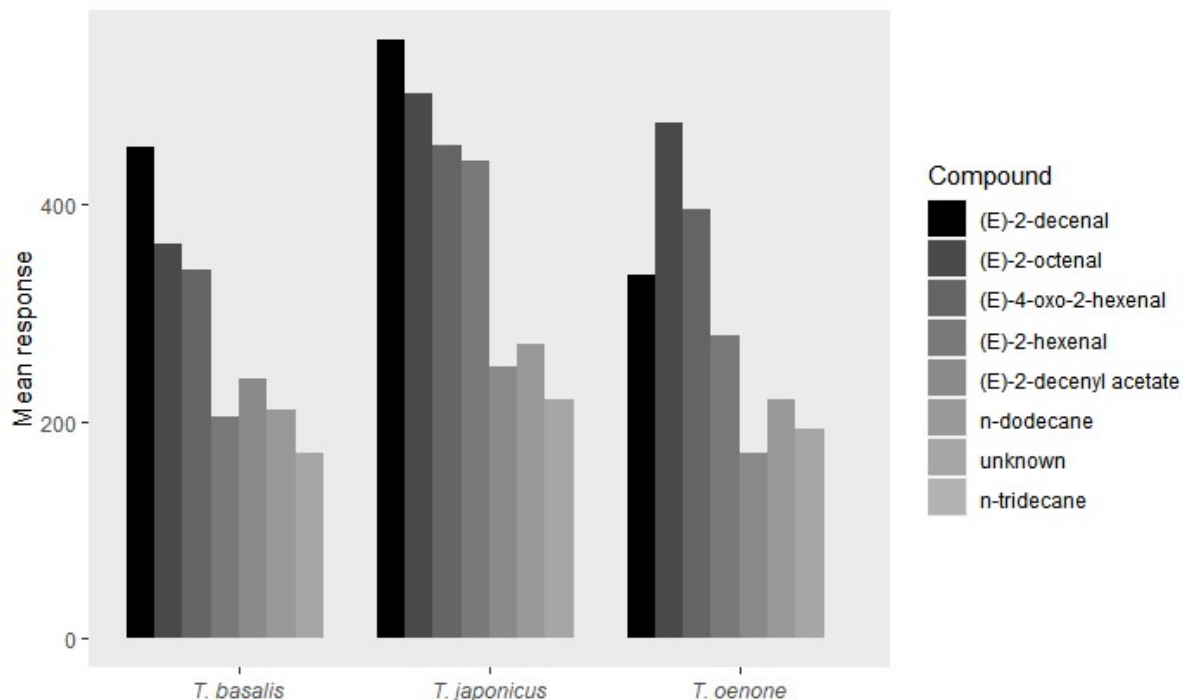

Figure 9: Mean GC-EAD responses to bioactive compounds in stink bug solvent extracts

In the second round of GC-EAD experiments, where each parasitoid was exposed to individual synthetic compounds identified during the previous step, I confirmed responses to all six successfully identified compounds from the first round of EAD (Figure 2). Again, I did not observe any responses to n-tridecane during these experiments. All three parasitoids responded most strongly to (*E*)-2-decenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, while responses to (*E*)-2-decenyl acetate, *n*-tridecane, and n-dodecane were generally weaker. *Trissolcus oenone* failed to respond to (*E*)-2-hexenal in the synthetic blend.
In EAG experiments, parasitoid responses were broadly similar in their relative values as compared with GC-EAD recordings, although responses to (*E*)-4-oxo-2-hexenal for *T. basalis* and *T. oenone* were slightly weaker than expected, based on GC-EAD results. In *T.*

japonicus, there was a less pronounced difference between the group of four compounds which elicited higher responses ((*E*)-2-decenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal,
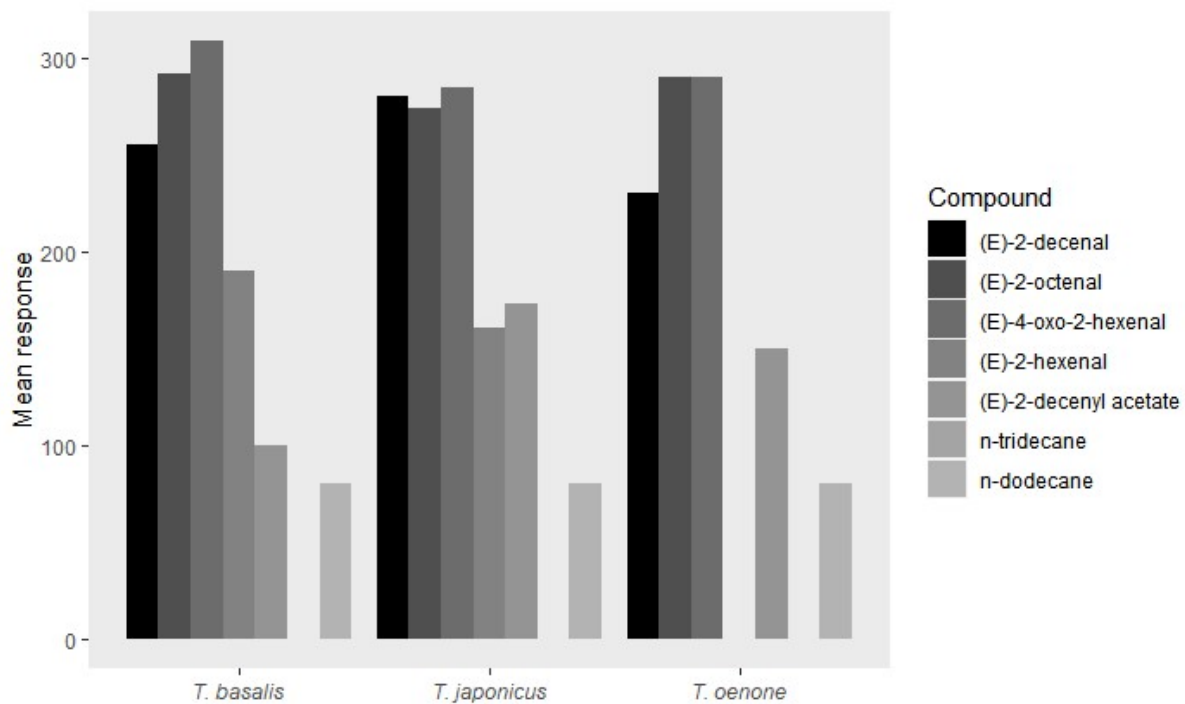
Figure 10: Mean GC-EAD responses to synthetic standards of bioactive compounds tentatively identified from solvent extracts
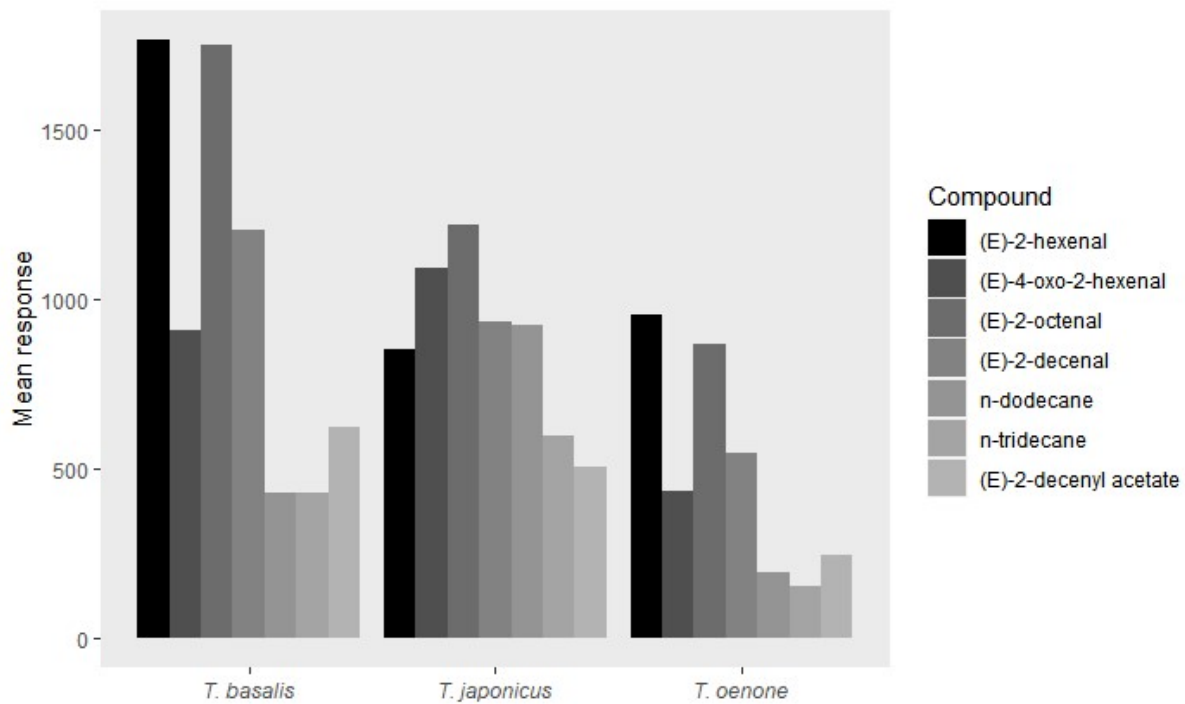
Figure 11: Normalised mean EAG responses to bioactive compounds presented to parasitoids.
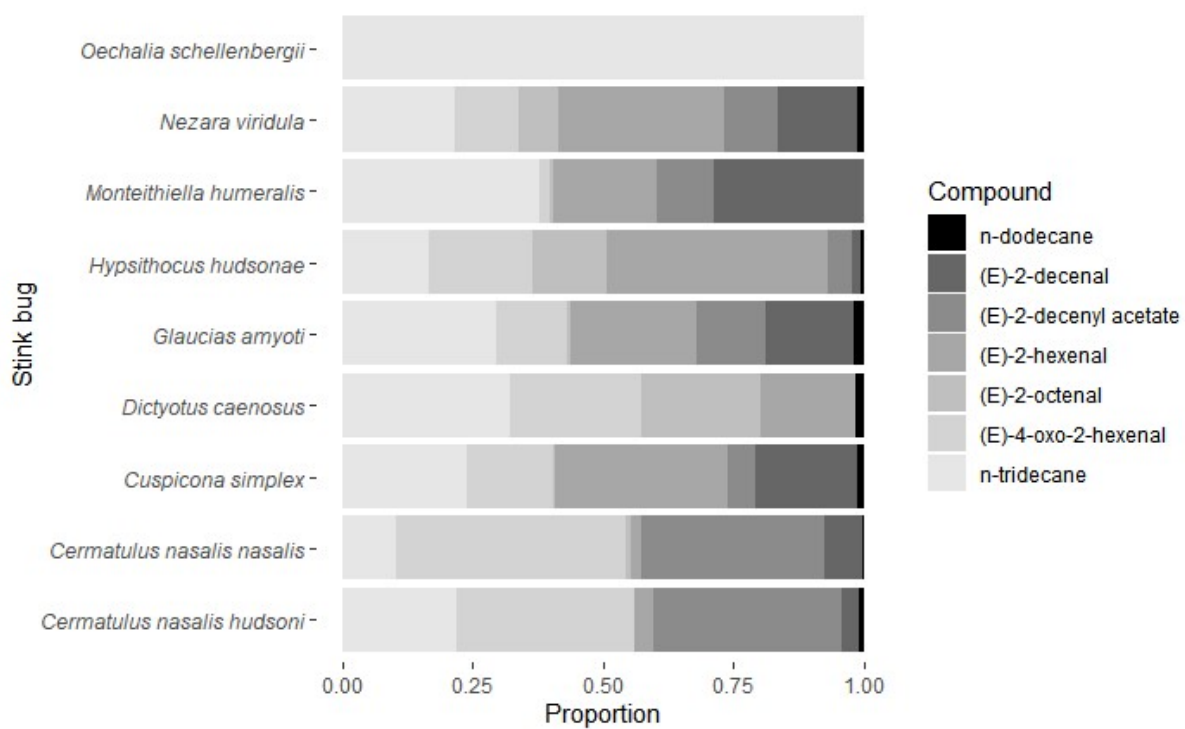

Figure 5: Proportion of bioactive volatile compounds making up stink bug solvent extracts.
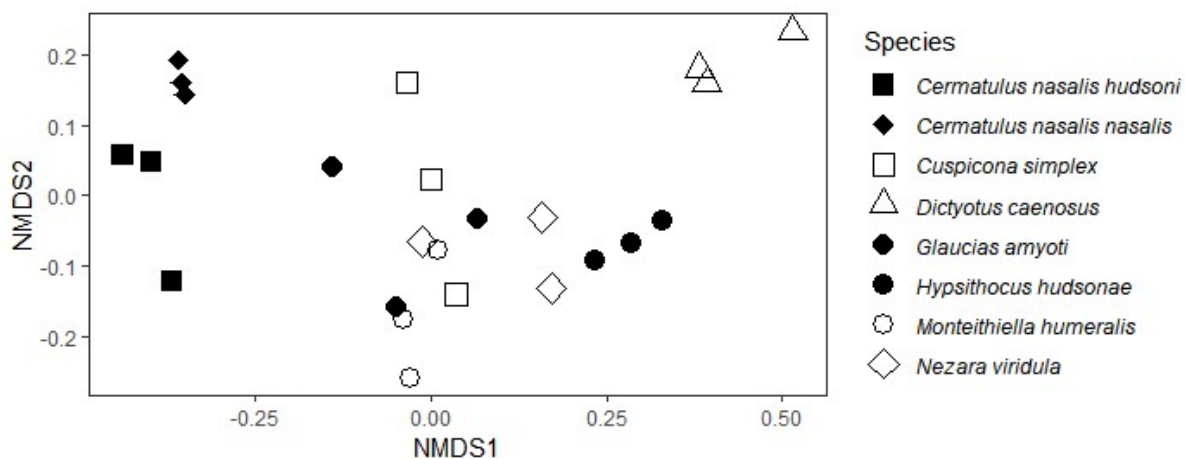
Figure 6: Non-metric Multidimensional Scaling plot showing similarity of pentatomid species based on their volatile profiles, with native or endemic taxa represented by filled shapes.

and (*E*)-2-hexenal) and the group of three compounds which elicited lower responses ((*E*)-2-decenyl acetate, n-tridecane, and n-dodecane).

The comparison of bioactive compounds making up the volatile profile of each pentatomid species showed that most of the seven bioactive compounds were detected within most of the pentatomid extracts, although the relative amounts differed considerably (Figure 5). The only major compound detected in *O. schellenbergii*, which was also found in all pentatomid species, was n-tridecane. Based on the ordination of these results, the two predatory subspecies of *C. nasalis* appeared to form a cluster, while the introduced *D. caenosus* had the least similar profile to other species. The remaining species (*C. simplex*, *M. humeralis*, *N. viridula*, and *G. amyoti*) all overlapped to some degree in the similarity of their volatile profiles, while the extracts of the endemic *H. hudsonae* formed a satellite cluster to this group, appearing to be most similar to *N. viridula* (Figure 6).

5.6 Discussion

I identified seven compounds associated with New Zealand pentatomids which elicited antennal responses in three different species of *Trissolcus* parasitoids: (*E*)-2-decenal, (*E*)-2-

octenal, (E)-4-oxo-2-hexenal, (E)-2-hexenal, (E)-2-decenyl acetate, n-dodecane, and n-tridecane. All three parasitoids responded to all seven compounds in EAG experiments, once these compounds had been identified in two rounds of GC-EAD experiments.

Previous work on the chemical ecology of the Pentatomidae has shown that adult stink bugs produce a range of compounds in their metathoracic glands, and that these compounds can act as defensive allomones and/or pheromones, depending on the receiver (Aldrich, 1988). Common components of defensive secretions include short chain alcohols, aldehydes, and alkanes, and the blend of compounds can change significantly depending on life stage (Borges & Aldrich, 1992; Eliyahu et al., 2012; Tsuyuki et al., 1965). Adults of most stink bug species appear to share much of their defensive chemistry across genera, with the more common compounds including short chain alcohols and their esters (e.g. (E)-2-decenyl acetate), aldehydes (e.g. (E)-2-alkenals and 4-oxo-(E)-2-alkenals), and linear hydrocarbons, of which n-tridecane is reported as one of the most commonly found, and most abundant (Aldrich, 1995; Millar, 2005; Weber, Khrimian, et al., 2017). Brown marmorated stink bug is known to produce all seven of the compounds identified from extracts of New Zealand Pentatomidae, and in fact, these seven compounds constitute the major components in BMSB volatile profiles (Nixon et al., 2018; Zhong et al., 2017).

While I expected more variation in the blends of volatile compounds making up New Zealand stink bug extracts, the variation present appeared to suggest my sampling technique was capable of detecting both qualitative and quantitative differences in volatile blends between species. For example, the two predatory subspecies of *C. nasalis* exhibited very similar chemical profiles for the active compounds I identified. nMDS ordination comparing the similarity of pentatomids based on compounds responsive to parasitoids showed these two native taxa were most similar to each other. Ordination also revealed that the endemic alpine species, *H. hudsonae*, was most similar in its bioactive volatile profile to the cosmopolitan pest *N. viridula*, an unexpected result. It would be worth exploring if the similarity in solvent rinse profile I detected here translates to similar behavioural attraction to adults by *T. basalis*, for example in open arena arrestment bioassays (Conti et al., 2004). It remains unclear why the *O. schellenbergii* extract had far fewer compounds and much lower amounts of compounds than the other pentatomid extracts, as I used the same methodology to extract compounds across all species. Aldrich et al. (1996) were able to identify compounds from male *O. schellenbergii* dorsal abdominal glands, including (E)-2-hexenal, by dissecting

the glands and standing in dichloromethane overnight, but no other investigations into the chemistry of this species appear to have been reported.

My results suggest the ability for *Trissolcus* parasitoids to detect compounds associated with stink bugs is highly conserved, as all three species responded to the same compounds despite widely differing native ranges which contain few (if any) common pentatomid species between them. For example, *Trissolcus basalis* is thought to be native to the Horn of Africa (Jones, 1988), while *T. oenone* is native to Australasia (Johnson, 1991), and *T. japonicus* to Eastern Asia (Talamas et al., 2013). A wide variety of parasitoids are known to exploit the semiochemistry of their hosts in order to locate promising oviposition sites (Godfray, 1994; Vet & Dicke, 1992; Vinson, 1984). When a parasitoid eavesdrops on a semiochemical produced for the benefit of the emitter, it exploits the compound as a kairomone (Nordlund & Lewis, 1976). Natural enemies often have short life spans and short windows during which their hosts are suitable for attack, which means parasitoids need to use reliable cues to locate hosts quickly (Bin et al., 1993). The study of kairomone-mediated host searching in egg parasitoids of pentatomids has started to reveal the identities of attractive compounds associated with stink bugs (Conti & Colazza, 2012; Fatouros et al., 2008; Weber, Khrimian, et al., 2017). Scelionid parasitoids are known to respond to (E)-2-alkenals, and in particular, *T. basalis* is known to be attracted to (E)-2-decenal and (E)-2-hexenal, which are produced by nymphs and adult stink bugs in relatively large quantities (Laumann et al., 2009; Mattiacci et al., 1993). *Telenomus podisi* (F.) is also attracted to (E)-2-hexenal (Vieira et al., 2014), but because this compound is a relatively common plant volatile produced in large amounts by certain crops, it remains unclear whether parasitoids are using the compound as a plant-emitted or host-emitted attractant, or both (Moraes et al., 2008). Both *T. basalis* and *T. podisi* are known to be attracted to (E)-4-oxo-2-hexenal (Laumann et al., 2009), and while this compound is relatively common in the Hemiptera, 4-oxo-(E)-2-alkenals have never been found associated with any other insects (Millar, 2005). *Trissolcus basalis* is known to be attracted to (E)-4-oxo-2-hexenal, as it spent longer in olfactometer arms with this compound present compared to controls (Laumann et al., 2009). However, my understanding of which compounds are attractive or repulsive to scelionid egg parasitoids is still developing, due to a lack of electrophysiological studies to identify responsive compounds, and a paucity of behavioural studies to confirm the kind of behavioural response induced in the parasitoid.

Zhong et al. (2017) recently conducted GC-EAD recordings with female *T. japonicus* in relation to solvent extracts made from *H. halys* females. They showed that female BMSB volatile profiles contain the same seven compounds I found to be bioactive in the three parasitoids I tested, and in BMSB these seven compounds are all major peaks. However, they reported *T. japonicus* antennal responses to just two compounds: n-tridecane and (*E*)-2-decenal. They also reported parasitoid attraction to n-tridecane and parasitoid aversion to (*E*)-2-decenal in Y-tube olfactometer experiments. Malek et al. (2021) investigated *T. japonicus* arrestment responses in open arenas to substrates contaminated by *H. halys* and the suboptimal host *Podisus maculiventris* (Say). While they observed motivated searching behaviour from parasitoids in response to footprint compounds from both species, parasitoids spent longer searching for *H. halys*, and stink bug trails continued to elicit responses in parasitoids 72 hours after they were deposited. GC-MS analyses revealed n-tridecane and (*E*)-2-decenal were deposited by stink bugs, and a 4:1 blend of these compounds prolonged residence times of parasitoids in open arenas while (*E*)-2-decenal alone reduced searching activity. The combination of these results suggests n-tridecane and (*E*)-2-decenal have a kairomonal effect on *T. japonicus*, although there may still be other compounds which influence the host-preferences or host-finding ability of this parasitoid.

I only observed antennal responses to n-tridecane when puffing single compounds over the antennae (likely at higher concentrations than occur naturally), and even then, it was always one of the weakest responses for all three *Trissolcus* species I tested. It is therefore unusual that n-tridecane appears to elicit a kairomonal response in *T. japonicus* on its own. However, it may also enhance the perception of other compounds, and indeed, there is some evidence to suggest that linear hydrocarbons can act as synergists to promote either the evaporation of defensive blends, or the penetration of these blends into the cuticles of other insects (Weber, Morrison, et al., 2017). For example, Eliyahu et al. (2012) found that n-tridecane alone was ineffective at disabling fire ants. However, when combined with other stink bug defensive compounds and applied to crickets, these blends were more effective at deterring ant attacks. In addition, compared to n-tridecane alone, blends of defensive compounds containing n-tridecane caused greater disturbance and agitation when sprayed directly onto ants in biologically meaningful amounts (Eliyahu et al., 2012). Whatever the case, the lack of chemical ecological studies on scelionid egg parasitoids makes it difficult to

draw any firm conclusions at this stage as to the kairomonal function of stink bug defensive compounds in scelionid egg parasitoids.

I originally hypothesised that each pentatomid species would have different compounds and different ratios of compounds in its volatile profile, and that each of the three parasitoid species may respond to different compounds based on their physiological host range. Running GC-EAD experiments with stink bug solvent extracts through was a good first step to identify compounds of interest that could then be used in additional rounds of testing. In order to have greater confidence in my results, I performed another round of GC-EAD with synthetic standards of compounds tentatively identified from solvent extracts. Finally, I presented each test compound to each parasitoid in EAG experiments to confirm that the responses I was seeing were real. It was only during EAG experiments that I observed responses to *n*-tridecane, which underscores the importance of using multiple methods to ensure all possible responses are identified correctly. It is important to follow up chemical identification with thorough behavioural tests before compounds are declared to be kairomones, a step outside the scope of the work presented here. Experiments making use of chemical ecological techniques can complement traditional oviposition tests, and are able to provide high quality information about the ability of parasitoids to detect certain hosts, and their motivation to search for hosts based on these cues (Cingolani et al., 2019; Conti et al., 2004). Ultimately, a better understanding of how semiochemistry mediates host preferences in parasitoids should lead to improved pre-release host range testing procedures for classical biological control agents.

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CHAPTER 6: General discussion and a proposed framework for pre-release non-target risk assessment

6.1 Introduction

Classical biological control programmes will always involve an element of risk because they seek to introduce a new species into a novel environment. Forecasting the risks of biological control agents against non-target species has grown in priority since the 1990s and is now a fundamental component of modern classical biological control research (Barratt et al., 2010). Regulators are tasked with evaluating applications to introduce biological control agents into new environments, and sometimes they have to rely on limited evidence of the potential non-target risks posed by candidate agents to native or economically important taxa (Barratt & Moeed, 2005; Hunt et al., 2011). Results from pre-release host specificity testing are crucial to assess non-target risks posed by biological control agents, and a wider variety of evidence could help to provide much-needed context to traditional approaches (I. Park et al., 2018). Physiological host range testing using no-choice oviposition tests is a necessary but insufficient step for assessing the ecological risks of classical biological control agents (Murray et al., 2010; Withers & Barton Browne, 2004). Oviposition tests are a critical first step for host range testing, as they provide unambiguous evidence to show whether or not a candidate parasitoid recognises non-target species as alternative hosts, and whether or not these species are suitable for parasitoid development (Hoddle, 2016; Van Driesche et al., 2004; van Lenteren, Cock, et al., 2006). However, relying on oviposition tests alone ignores the important chemical ecological cues used by parasitoids to express their ecological host ranges in the field (Conti et al., 2004; Vet & Dicke, 1992; Vinson, 1998). Applying methodologies that specifically measure a parasitoid's ability to sense and act on these chemical cues will provide complementary information and reduce uncertainty for regulators deciding on applications to release biological control agents.

The central aim of this thesis was to test the feasibility and complementarity of several chemical ecological techniques in order to improve the way classical biological control agents are screened for their non-target risks, particularly in the context of pre-emptive (or proactive) biological control programmes. I used the chemical ecology of the interaction between stink bugs (Pentatomidae) and their egg parasitoids in the genus

Trissolcus to explore how useful certain chemical ecological techniques were for assessing host specificity. This was done with a view toward lobbying for these kinds of experiments to be integrated with oviposition tests more frequently during pre-release risk assessment research. I incorporated *Trissolcus japonicus*, a biological control agent pre-emptively (or pro-actively) approved for release in New Zealand against the brown marmorated stink bug, *Halyomorpha halys*, a serious horticultural pest native to East Asia but invasive in North America and Europe (Charles et al., 2019; Leskey & Nielsen, 2018). Logistical constraints meant that I could not include *T. japonicus* in all of my tests so I used the resident biological control agent *Trissolcus basalis* and the native parasitoid of pentatomids *Trissolcus oenone* in my experiments. I was interested in studying the chemical nature of attraction between the different parasitoids and their potential hosts in New Zealand, in order to understand how volatile organic compounds (VOCs) in the cuticular hydrocarbon profiles and defensive secretions of stink bugs mediated attraction and host specificity of their egg parasitoids. In particular, I was interested in assessing:

1. The complete physiological host ranges of *T. japonicus*, *T. basalis*, and *T. oenone*. Physiological host range tests are an important first step in identifying non-target risks associated with classical biological control agents. They are relatively simple and can be completed quickly with laboratory colonies of non-target species, in order to establish which species should be tested further (Bigler et al., 2006). To complement this work, I conducted competition experiments between the two parasitoids already in New Zealand and the nightshade pest *Cuspicona simplex*, as a case study to more thoroughly investigate the interactions between the parasitoids on a single host. Competition between biological control agents and native parasitoids is also an important consideration for non-target risk assessments. Such assessments typically consider the interests of host species which may be directly attacked and parasitized by agents (Barratt, 2011), but there is also the potential for a biological control agent to displace native parasitoids, and this should be investigated if possible. Impacts on native parasitoids may lead to unintended foodweb effects, which can be complex and multi-faceted (Todd et al., 2020), so ideally, non-target risk assessments should include experiments with native parasitoids, where feasible.

2. Arrestment to substrate-borne compounds in open arena bioassays, in order to test whether these experiments could be used to quantify and compare searching motivation between different parasitoids and stink bug hosts. This sort of information would be

incredibly useful to include within non-target risk assessments because it would give a baseline searching motivation for a primary or preferred host, and would then allow comparisons with other non-target species (Conti et al., 2004). If a parasitoid displayed a very high motivation to search for its primary host, and a very low motivation to search for a non-target species, then it would be reasonable to infer the non-target species is unlikely to be pursued in the field (Peri et al., 2014). Because parasitoids rely on chemical cues for virtually all stages of the host location process, the results of arrestment experiments could confidently be translated into risk scores which would aid regulators in their decisions. This kind of information would provide especially useful context when oviposition results are similar between parasitoids on the same host, or between one parasitoid and multiple hosts.

3. Electrophysiological techniques such as gas chromatography coupled with electroantennographic detection (GC-EAD), in tandem with electroantennogram recordings of compound puffs, and the analytical techniques of GC-FID and GC-MS, to identify compounds associated with hosts which are olfactory-active in parasitoids. The ability to identify specific compounds which may have behavioural functions in parasitoids is a rapid and powerful screening technique which allows the practitioner to identify a subset of kairomonal candidate compounds from host extracts for further behavioural testing (K. C. Park et al., 2001; Wee et al., 2016). This saves a lot of time and resources compared to testing every compound in the extract, and it offers far more specific information compared to just testing crude extracts for their behavioural responses. When coupled with physiological host range tests and behavioural assays, electrophysiological techniques greatly aid in the identification of key kairomones mediating parasitism or avoidance of non-hosts (Thanikkul et al., 2017).

This work aimed to contribute to improving the way pre-release host specificity testing of classical biological control agents is conducted in containment facilities, by assessing the usefulness and complementarity of chemical ecological methods, compared to traditional oviposition tests alone, when used in non-target risk assessments. I conducted thorough physiological host range testing between *T. japonicus* and *H. hudsonae* (**Chapter 2**), and between *T. basalis*, *T. oenone*, and almost all New Zealand pentatomid taxa (**Chapter 3**). In **Chapter 4** I integrated electrophysiology of egg extracts, with arrestment studies and competition experiments to compare host specificity between *T. basalis* and *T. oenone*. Finally, I completed multiple rounds of GC-EAD and EAG recordings with all three

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Trissolcus parasitoids against most New Zealand pentatomid taxa in **Chapter 5**. Ultimately, a better understanding of the chemical ecological basis for a classical biological control agent's host specificity will aid decision makers in their evaluation of applications to release new organisms. Additional behavioural and chemical ecological tests provide important information which complements and extends the results of traditional physiological host range testing, and the combination of these approaches will provide regulators with more certainty in their decisions to accept or reject candidate agents for release.

6.2 Physiological host range testing

No-choice oviposition tests are commonly used to assess biological control agents' physiological host ranges before they are released, as they provide unambiguous information about which species are suitable for attack and development (Babendreier et al., 2005; Bigler et al., 2006; van Lenteren, Cock, et al., 2006). Chapter 2 presents the results of no-choice oviposition experiments between *T. japonicus* and the endemic alpine shield bug, *Hypsithocus hudsonae*. This pentatomid species was not included in previous host range testing conducted by Charles et al. (2019), so including it in host range tests was a top priority. As expected, *T. japonicus* accepted (90%) and emerged (>90%) from this species at high rates, showing it is a suitable physiological host for the parasitoid. These results place it a close second to *Glaucias amyoti* in terms of percent parasitism, out of a total of nine pentatomid taxa tested. I exposed egg masses to parasitoids for 48 hours, but observed almost all attacks to occur within an initial one hour period of observation. Results from similar testing overseas suggest pentatomid hosts from Australasia (Charles et al., 2019; Saunders et al., 2021), Europe (Haye et al., 2020), and China (Zhang et al., 2017) are more suitable for development of *T. japonicus* than those from North America (Botch & Delfosse, 2018; Hedstrom et al., 2017). Despite high levels of host acceptance and parasitoid emergence, field attack and impacts on *H. hudsonae* remain unlikely. This is primarily because of climatic and habitat differences between *H. hudsonae*, which occupies high-altitude alpine areas with only low herbage, and *T. japonicus* which prefers arboreal habitats and is associated with habitats attractive to brown marmorated stink bug, such as the edges of cropping systems (Wallner et al., 2014).

In Chapter 3 I presented the results of no-choice oviposition testing between *T. basalis* and *T. oenone* with almost all New Zealand pentatomid taxa. Ron Cumber conducted

a series of host range experiments with these two parasitoids in the 1960s, but his work suffered from low replication, he did not always present quantitative results, and he was unable to include all pentatomid species (Cumber, 1964). *Trissolcus basalis* was introduced in 1949 from specimens mass reared from Australian stock, and the original host range testing was sparse and records were apparently lost (Cumber, 1953). *Trissolcus oenone* was the subject of one study conducted in Australia investigating the effect of temperature on the captive rearing of the parasitoid on *Biprorulus bibax* Breddin (James & Warren, 1991). Applying replicated, quantitative methods to establish the physiological host ranges of these parasitoids was a high priority, due to the pre-approval to release *T. japonicus* in New Zealand. I used similar methods to Charles et al. (2019) and Saunders et al. (2021), so I combined all three datasets to compare the physiological host ranges of these three parasitoids in New Zealand. *Trissolcus basalis* and *T. oenone* accepted and emerged from all species of pentatomid tested, except *N. viridula* was not a physiological host for *T. oenone*, and I was unable to test *H. hudsonae* against *T. oenone*. Parasitoids differed in their discovery and parasitism efficiencies on New Zealand Pentatomidae, but in general, expected mean parasitism efficiencies (percent egg parasitism on discovered masses) were over 60% for all combinations that were sufficiently replicated. *Trissolcus basalis* and *T. oenone* tended to be most similar in expected mean parasitism frequencies, whereas *T. japonicus* tended to have lower expected means. Development times for the two resident parasitoids were very similar, mostly between 19 and 21 days at 20C, and I found no statistically significant difference between them.

In chapter four I investigated extrinsic and intrinsic competition between *T. basalis* and *T. oenone* by exposing a single female from each species to the eggs of *C. simplex* and observing behavioural and developmental outcomes. The native parasitoid *T. oenone* successfully oviposited into 85% of eggs, while the introduced biological control agent parasitized two thirds of the eggs available. Around half of the *C. simplex* eggs were parasitized by both parasitoids, and remarkably, the native parasitoid developed in over 90% of these. From these metrics it is clear that the native parasitoid *T. oenone* can be considered to have won both the extrinsic and intrinsic contest with the introduced biological control agent *T. basalis*. I expected the opposite, as introduced biological control agents are often shown to be superior competitors against native parasitoids when exploiting the same hosts (Murillo et al., 2019; Sithole & Lohr, 2017). It is possible that *C. simplex* is more compatible

and more suitable for the development of *T. oenone,* as both species share a native Australian range, while *T. basalis* was introduced relatively recently in Australia and New Zealand. Even though the native parasitoid appeared to dominate intrinsic contests, it has been shown that the winner of such contests may go on to incur fitness costs as a consequence of surviving competition (Cusumano et al., 2015, 2016). I dissected parasitized eggs to confirm the identities of developing parasitoids, but I did not measure the body size of parasitoids surviving intrinsic contests to compare to control parasitoids. Experiments quantifying the lifetime reproductive capacity of parasitoids surviving competition and those which developed in the absence of competition would help to clarify the longer-term advantages and disadvantages of surviving intrinsic contests in these parasitoids.

No-choice oviposition tests are a critical first step in assessing the host specificity of biological control agents, and some kind of physiological host range testing should always be conducted as part of pre-release risk assessment of biological control agents (Bigler et al., 2006; Van Driesche et al., 2004; van Lenteren, Bale, et al., 2006). They offer very reliable evidence to show which species are recognised as hosts by a parasitoid, and whether or not those species are suitable for parasitoid development (Mansfield & Mills, 2004). If parasitoids emerge at very low rates (<10%), the non-target species can be considered a suboptimal host and it is unlikely that parasitoids will have large impacts on that host in the field, unless oviposition induces considerable non-reproductive mortality or fitness impacts (Abram et al., 2019). Indeed, if parasitoids fail to recognise unfavourable hosts, they themselves could fall into an evolutionary trap and suffer fitness impacts (Abram et al., 2014). However, when parasitism efficiencies are high, it does not necessarily equate to a high non-target risk in the field. The artificial nature of laboratory testing under tightly controlled artificial conditions means that parasitoids are unable to express the full range of host location behaviours they normally rely on (Murray et al., 2010; Withers & Barton Browne, 2004), and they are unable to gather important sensory information from plants, hosts, and the surrounding environment. Olfaction is known to be a critical sensory modality for host location in parasitoids (Vet & Dicke, 1992; Vinson, 1998), whereas important olfactory cues are deliberately excluded from no-choice (and choice) oviposition tests in order to simplify experiments (Van Driesche & Murray, 2004).

Regulators, researchers, and other interested parties should be mindful of the limitations of physiological host range testing (Barratt et al., 2010). Physiological host range

tests are not designed to be robust indicators of non-target risks on their own, but they do offer useful information about which species should be included in further testing, and are complementary to host specificity tests based on the behaviour and chemical ecology of candidate parasitoids.

6.3 Arrestment bioassays

One of the ultimate goals of chemical ecology is the application of its methods to enhance biological control of destructive crop pests (Lewis et al., 1990). The study of how and why natural enemies detect and respond to semiochemicals is directly relevant for understanding the host specificity of biological control agents. My PhD research adds to a growing literature that demonstrates how these chemical ecological approaches can be applied to pre-release risk assessments to help forecast the risks of non-target effects (Avila et al., 2016a; I. Park et al., 2018; Salerno et al., 2006). Host associated kairomones stimulate parasitoids to intensively search the surrounding substrate for more cues as to the presence of potential hosts (Vinson, 1998). Arrestment bioassays measure a parasitoid's motivation to search for the host which has contaminated the arena the parasitoid is released into (Colazza et al., 2014). For the most part, arrestment bioassays are conducted in open arenas so the parasitoid is free to leave once it is no longer interested (Colazza et al., 1999). This means the results can be used to show a hierarchical ranking of searching motivation for each host, which could serve as a proxy for the relative risk of the parasitoid finding these hosts in the field (Conti et al., 2004). If a parasitoid shows little interest in searching for the volatile cues associated with a given host in open arena experiments, it is reasonable to infer the parasitoid will show little interest in searching for that host in the field (Peri et al., 2014). Arrestment bioassays have also been used to demonstrate the preference of parasitoids for hosts based on their sex and physiological state (Colazza et al., 2007; Salerno et al., 2006, 2019). By comparing the results of arrestment bioassays with no-choice oviposition tests and electrophysiology, a more comprehensive picture of the host specificity can be built compared to relying on any one method alone.

In chapter four I conducted open arena arrestment bioassays with *T. basalis* and *T. oenone* to compare their motivation to search for *N. viridula* and *C. simplex*. I confined five female stink bugs in the centre of filter paper arenas for three hours before removing them from the paper and releasing parasitoids. *Trissolcus basalis* spent four times longer on filter

paper arenas contaminated by *N. viridula* than *C. simplex*, while *T. oenone* spent four times longer on arenas contaminated by *C. simplex* than *N. viridula*. Both parasitoid species spent about 80% of their time in the inner zones of the filter paper which were contaminated by stink bugs, and they were far more likely to leave contaminated arenas by walking to the edge, whereas they tended to leave uncontaminated control arenas by flying off the paper after less than 10 seconds. Parasitoids are known to spend longer in the inner zones when they are arrested by host-associated volatiles, and the observation that most parasitoids leave contaminated arenas by walking to the edge is also common (Colazza et al., 1999, 2014; Peri et al., 2006; Salerno et al., 2006). These results clearly show that both parasitoids are capable of distinguishing between the different stink bugs based solely on adult footprint compounds, and that each spend far longer searching for their preferred species. It may also be possible that *T. oenone* is capable of distinguishing between physiological hosts and non-hosts, as it spent even less time searching for *N. viridula* (a non-host) than *T. basalis* spent searching for *C. simplex* (a less-preferred physiological host), but this requires further testing. Nevertheless, to my knowledge, this is the first report of an arrestment study where parasitoids are exposed to a host and non-host.

The real value of arrestment studies for host specificity testing lies in the combination of open arena bioassays with no-choice oviposition tests (Cingolani et al., 2014, 2019). In chapter four I showed both *T. basalis* and *T. oenone* are capable of very high discovery efficiencies and parasitism efficiencies (>90%) on *C. simplex*. On their own, no-choice oviposition tests do not provide fine grain information on non-target risks, except perhaps to indicate incompatibility when parasitism rates are very low (Murray et al., 2010; Withers & Barton Browne, 2004). High parasitism rates indicate the parasitoid is highly capable of recognising the host and developing in the host, but they cannot provide any meaningful information on how likely it is the parasitoid will search for and find the host in the field (Van Driesche et al., 2004; van Lenteren, Cock, et al., 2006). However, when the results of no-choice oviposition tests between *T. oneone* and *T. basalis* on *C. simplex* are paired with arrestment results, it becomes clear each parasitoid shows a very different risk profile for this species. *Trissolcus basalis* is far more motivated to search for *N. viridula*, which is considered to be its primary host, whereas *T. oenone* is far more motivated to search for *C. simplex*. If these observations were collected during a pre-release risk assessment with two candidate biological control agents, they would be highly relevant for the regulator to

consider when making their decision on whether or not to approve one or both of these agents, especially if arrestment studies were conducted with a greater number of non-target species to compare. Recent surveys in Auckland for stink bug egg parasitoids appear to corroborate the searching preferences displayed in chapter four, as only *T. oenone* has been observed parasitising sentinel *C. simplex* eggs (Pers. Comm., Karina Santos, Plant & Food Research). Importantly, my results in chapter three demonstrate how high parasitism rates in physiological host range tests do not necessarily reflect a high motivation to search for the host, but instead, are likely to reflect the close confinement of parasitoids and hosts, which is done deliberately to maximise the chance of classifying a non-target species as a physiological host or not.

6.4 Electrophysiological methods

Olfaction is the most important sensory modality mediating host location in parasitoids, and as such, it plays a key role in the host specificity of classical biological control agents (Avila et al., 2016a; Vet & Dicke, 1992; Vinson, 1998). Identifying which host or plant-associated semiochemicals are attractive to parasitoids is important for understanding how and why parasitoids are attracted to non-target species, and offers clues as to which species may be at greater risk of attack in the field. By using an insect's own neurophysiology, electrophysiological techniques such as GC-EAD and SSR are powerful tools for the identification of olfactory-active compounds (Arn et al., 1975; Kaissling, 1995; Wadhams, 1984). These methods can be used to identify attractive compounds associated with potential hosts (Dweck et al., 2010; Kpongbe et al., 2019), or plant volatiles induced by the feeding or oviposition activity of a herbivore which are attractive to parasitoids (Ortiz-Carreon et al., 2019). These techniques require the extraction and chemical analysis of host semochemicals, and there are a variety of techniques which can be used to obtain samples of analytes from insects or plants (Barbosa-Cornelio et al., 2019; Jones & Oldham, 1999; Reyes-Garcés et al., 2018). Extracts can then be injected into a GC coupled with a parasitoid preparation where the insect is held between microelectrodes to detect depolarisations as a result of exposure to olfactory-active compounds. Another electrophysiological tool, single sensillum recording, can be used to understand the response profile of an insect in order to assess whether or not it is capable of distinguishing between multiple responsive compounds, which is an important element of chemically mediated host specificity (Pickett et al., 2012; Wee et al., 2016).

Electrophysiological tools are a useful part of the process to identify kairomones, especially when paired with physiological host range testing and behavioural bioassays, and the combination of these approaches provides an holistic framework for assessing the chemical basis of host specificity (Benelli et al., 2013; Fors et al., 2018; Milonas et al., 2019; Sabbatini-Peverieri et al., 2021).

In chapter four I exposed *T. basalis* to *N. viridula* egg extracts made with either hexane or acetone, in order to test which solvent produced an extract eliciting stronger antennal responses, and to tentatively identify potential contact kairomones used by *T. basalis* to recognise and accept hosts. Stink bug egg parasitoids are known to exploit kairomones in the adhesive material which glues pentatomid eggs together and to a substrate (Iacovone et al., 2016; Strand & Vinson, 1982), and a previous study showed wasps probe glass beads coated with acetone egg extracts but not when hexane extracts are used (Bin et al., 1993). Building on this work, I used GC-FID and GC-MS to tentatively identify compounds in egg extracts, and electroantennogram recordings to compare the magnitude of responses between extracts made with different solvents. I found many more compounds in the acetone extract and only two compounds were common to both. The most dominant compounds in terms of peak area were oleic acid, n-hexadecanoic acid, and octadecanoic acid. These compounds were dominant components of *Euschistus heros* egg extracts, and Michereff et al. (2016) showed *Te. podisi* were attracted to egg extracts in y-tube olfactometers. However, Tognon et al. (2020) showed that *Te. podisi* was also attracted to extracts made with short immersion hexane extracts, which contained none of the compounds I or Michereff et al. (2016) tentatively identified from acetone extracts. It's possible that both kinds of extracts contain different kairomones attractive to egg parasitoids, but I also found that acetone extracts elicited 50% stronger responses in *T. basalis* than hexane extracts. The combination of these results suggests that acetone is able to extract qualitatively or quantitatively more kairomones than hexane, or kairomones which are more attractive to parasitoids. Identifying compounds acting as contact kairomones is an important goal in host specificity and chemical ecology research into egg parasitoids, but more work is needed to link individual compounds, or blends, with behavioural functions.

In chapter five I tested three scelionid egg parasitoids for olfactory-active compounds associated with nine New Zealand pentatomid taxa, including the endemic alpine shield bug *H. hudsonae*. I prepared hexane body rinse extracts with adult female stink bugs and included

two internal standards in order to quantify the amounts of compounds in samples using GC-FID and GC-MS. I used hexane for adult solvent rinses in chapter five and acetone for egg extracts in chapter four because previous work has shown that *N. viridula* egg extracts made with acetone elicit behavioural responses in *T. basalis* (Bin et al., 1993). Analytes are commonly extracted from adult stink bugs through the use of hexane as the compounds on their bodies are better displaced by a non-polar solvent (Colazza et al., 2007; Fatouros et al., 2008; Weber, Khrimian, et al., 2017). I did two rounds of GC-EAD recordings, first with the extracts, and then with synthetic compunds. I also puffed pure synthetic compounds over the antennae to confirm responses. All three parasitoids responded to a set of seven compounds found among the pentatomid species I tested: (*E*)-2-decenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, and (*E*)-2-hexenal elicited consistently strong responses from parasitoids; while (*E*)-2-decenyl acetate, *n*-tridecane, and *n*-dodecane elicited weaker reponses. All of these compounds are known to be produced by stink bugs as part of their defensive secretions (Borges & Aldrich, 1992; Tsuyuki et al., 1965), and some of these compounds have been shown to elicit behavioural responses in stink bug egg parasitoids (Conti & Colazza, 2012; Fatouros et al., 2008; Weber, Khrimian, et al., 2017). For example, *T. basalis* and *Te. podisi* are known to be attracted to (*E*)-2-hexenal and (*E*)-4-oxo-2-hexenal, while *T. basalis* is also known to be find (*E*)-2-decenal attractive (Laumann et al., 2009; Mattiacci et al., 1993; Vieira et al., 2014). On the other hand, *T. japonicus* has shown an aversion to (*E*)-2-decenal in behavioural tests (Malek et al., 2021; Zhong et al., 2017), suggesting parasitoids use qualitative and quantitative differences in stink bug volatile blends to discriminate between hosts. Linear hydrocarbons may be attractive to parasitoids (Zhong et al., 2017), or they may enhance the dispersal or uptake of other defensive compounds (Eliyahu et al., 2012; Weber, Morrison, et al., 2017). More electrophysiological studies in combination with behavioural tests are needed to draw firmer conclusions about the functions of different compounds associated with stink bugs and other hosts.

6.5 A modified framework for risk assessment

This thesis provides a greater understanding of how and why parasitoids use chemical ecological cues to make decisions about which hosts to attack. More broadly, it demonstrates the value of combining physiological host range experiments with behavioural bioassays and electrophysiological techniques to better characterise the host specificity of parasitoids used

as classical biological control agents, and in order to compare among parasitoids with overlapping niches. The results can be used to inform the selection and interpretation of methods used to assess non-target risks during pre-release host specificity testing. I selected testing procedures based on the needs of pre-emptive biological control programmes. In these types of programmes the target pest has not yet arrived or established. This is the primary reason why I did not include choice tests in the framework, as choice tests are only fully informative when non-target species can be compared with the target pest. The biology of pest and biological control agent also need to be taken into account when designing testing procedures. For example, it is unlikely that a scelionid egg parasitoid would be presented with egg masses from multiple pentatomid species in close proximity on a substrate. However, if the relevant life stages of target and non-target species are likely to be aggregated closely together in space then it may be worthwhile to include choice testing between multiple non-target species when assessing the suitability and safety of candidate agents. The special conditions under which testing must occur in a pre-emptive context also make chemical-ecological approaches more relevant. Without access to target pests, the role of chemical extracts becomes more important. It is far more likely to be acceptable to import extracts made with target hosts, than target hosts themselves if the target pest has not yet established.

There are several valuable opportunities for future work to build on and extend the results presented in this thesis. First, the chemical identification of contact kairomones in the adhesive material of stink bug eggs remains a tantalising research goal. While parasitoids are known to respond to egg extracts in both electrophysiological and behavioural tests, the specific chemical compound(s) responsible for host recognition and acceptance in any pentatomid-parasitoid system are as yet unknown. Understanding whether parasitoids use a species-specific kairomone or if many stink bug species have similar compounds on their egg surfaces would be valuable for non-target risk assessments. The interactions among *T. basalis*, *T. oenone* and *N. viridula* offer a good opportunity to test this idea as *T. oenone* rarely accept *N. viridula* eggs for oviposition, and never develop in this species, suggesting they are responding to the presence of a non-host compound or the absence of a host compound when in contact with these eggs. Another opportunity for future work is characterising the chemical relationship between parasitoids and the compounds left in contaminated arenas by their stink bug hosts. Again, the *T. basalis-T. oenone-N. viridula*

system is ideal for testing this, as parasitoids showed a marked difference in their motivation to search within arenas contaminated by *N. viridula*. Comparing the footprint profile of different stink bugs would help to uncover whether parasitoids are using the presence of different compounds, or slight differences in the ratios or amounts of similar sets of compounds, in order to make foraging decisions. Finally, single sensillum recording is an obvious complement to the other electrophysiological experiments employed in this thesis, and the results would help to shed light on the chemosensory profiles of olfactory receptor neurons in the antennae of scelionid egg parasitoids. Combining the results of SSR with behavioural tests should contribute to a much a better understanding of the chemical basis of host specificity in stink bug egg parasitoids, and could serve as a useful model for future non-target risk assessments in other proposed BCAs.
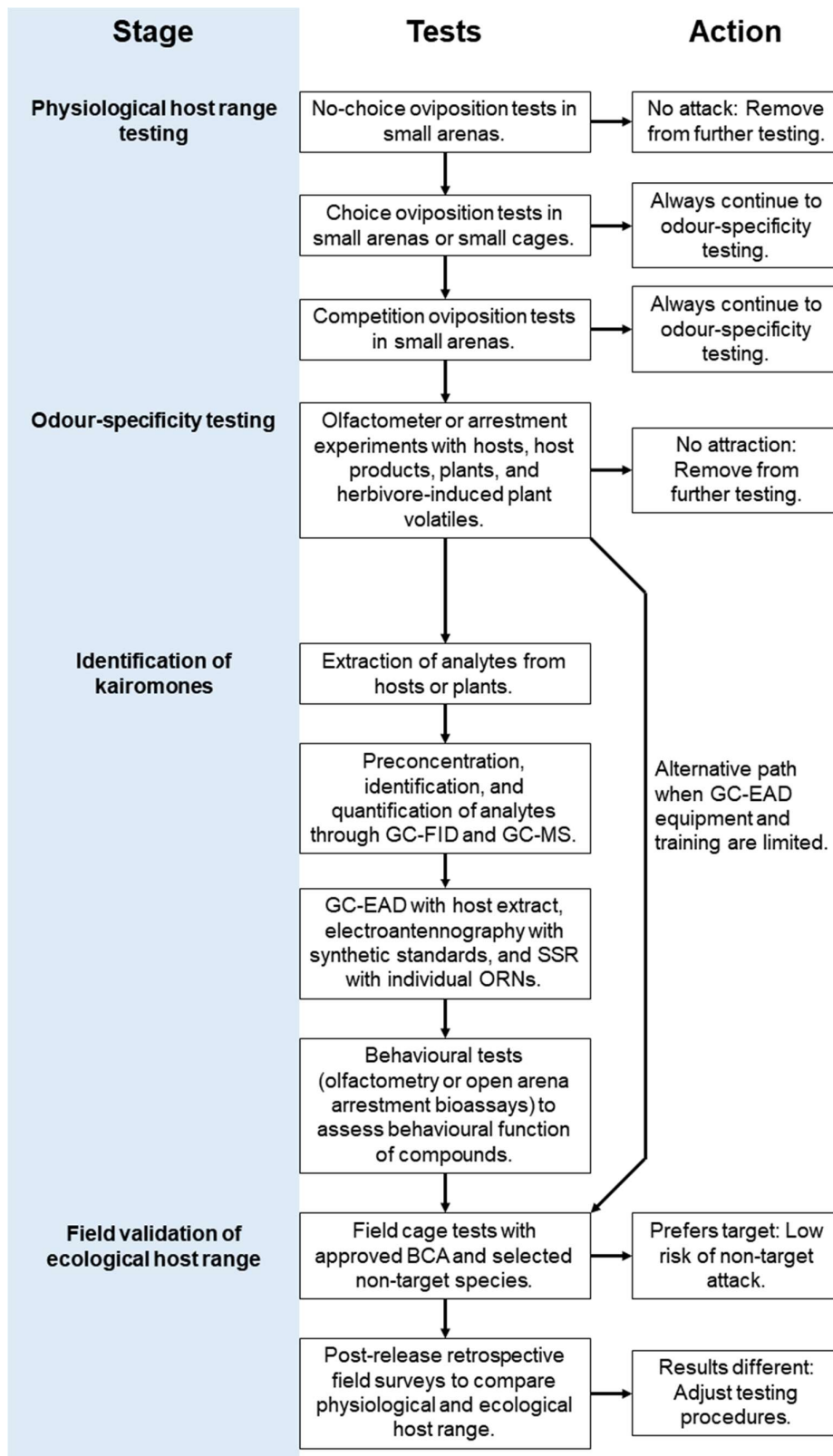
Figure 12: Proposed framework for host specificity testing to assess non-target risks of biological control agents within the context of pre-emptive risk assessments.

Drawing on the lessons and results of this thesis, I propose an expanded framework for integrating chemical-ecological and behavioural methods to evaluate non-target risks posed by classical biological control agents in the context of pre-emptive risk assessments conducted in containment (van Lenteren, Bale, et al., 2006; van Lenteren, Cock, et al., 2006; van Lenteren et al., 2003) (Figure 1). The framework begins with the simplest and easiest tests which relate to the final stage of the host-location process (acceptance and development), and then progresses back through the preceding stages in order to identify the stage at which a non-target species is no longer attractive to a BCA. As testing continues, a wider variety of cues are incorporated into testing in order to move progressively towards increasingly natural test conditions. First, a series of physiological host range tests should be performed in small arenas. No-choice oviposition tests offer unambiguous evidence of the ability for a parasitoid to attack a non-target species, and they show whether or not the host is suitable for development and emergence of parasitoid offspring. Any non-target species which are not attacked, or which are attacked but which do not support the successful development of parasitoids, can be discarded for the purposes of host specificity testing at this stage. However, hosts which are attacked but which do not support successful development or emergence of offspring may still experience a variety of non-reproductive fitness consequences due to parasitoid attack (Abram et al., 2016, 2019), so these non-target effects should be quantified if possible. The next stage involves giving parasitoids a choice between two (or more) non-target species in order to build a hierarchical ranking of host preferences based only on cues offered in small arena laboratory tests. In the absence of further testing, these tests can be used to infer the relative preference of parasitoids attacking and developing in a non-target species compared to the target host. However, the results of choice tests should be interpreted carefully, as they provide little information on the likelihood of a parasitoid finding and exploiting a non-target species in the field. The final stage of physiological host range testing should involve competition tests on selected non-target species between the candidate parasitoid, and either existing BCAs, native parasitoids, or other economically important parasitoids. Competition tests can help to identify non-target effects on competitors sharing the third trophic level with the candidate parasitoid by examining both extrinsic contests (between two parasitoids on the same host patch) and intrinsic contests (between the larvae of two parasitoids in multiparasitised hosts). Existing BCAs and native parasitoids are rarely considered in pre-release non-target risk assessments,

but their exclusion could lead to unintended indirect food-web effects which could be better forecasted during host specificity testing (Todd et al., 2020).

The second stage in the proposed framework is odour-specificity testing. This stage aims to identify whether or not a proposed BCA is capable of recognising odours associated with hosts or their food plants, and whether or not parasitoids are capable of orienting towards these odours as short, medium, and long range cues. The first stage involves measuring attraction to chemicals on the surfaces of hosts themselves, or the products they leave behind on substrates, either through contamination of open arenas, or in olfactometers. Selection of the appropriate method will depend on the biology of the host species and the life stage attacked by the BCA. For example, parasitoids which attack stink bug eggs respond to both solvent extracts of eggs or adults, and whole adults, in both open arena bioassays and olfactometers (Colazza et al., 1999, 2007; Iacovone et al., 2016; Salerno et al., 2006), while a larval parasitoid may respond best to host larvae on plants in olfactometers, or to frass in open arena bioassays (Fors et al., 2018; González et al., 2011; Nurkomar et al., 2017). The next stage involves testing the attraction of parasitoids to host-infested plants to try to determine if BCAs are capable of exploiting plant volatiles induced by the feeding or oviposition activity of hosts (HIPVs and OIPVs). Olfactometers are commonly used to test whether parasitoids are more attracted to plants infested with hosts, compared to hosts in isolation, uninfested plants, or mechanically damaged plants, as control treatments (Thanikkul et al., 2017). Hosts often need to be left to feed and oviposit on plants for several days before indirect plant defences are induced (Turlings & Erb, 2018). Both stages in odour-specificity testing can make use of olfactometers with more than two arms in order to test a wider variety of treatments, and to test a larger number of BCAs at the same time to improve statistical power and reduce the time needed for experiments (Turlings et al., 2004).

The third stage in the proposed framework aims to identify the specific chemical compounds associated with target and/or non-target hosts which elicit kairomonal responses in proposed BCAs. Analytes are first extracted from hosts, their products, or their food plants, through solvent immersion or headspace collection. Internal standards are added to the solvent used to collect the sample. Extracts are then typically preconcentrated through evaporation under an inert gas, before having their constituent compounds quantified and identified through the use of GC-FID and GC-MS techniques. The quantification of compounds makes use of retention indices and the internal standards added earlier, while

identification of compounds makes use of large databases to compare mass spectra of analytes with known compounds. Finally, a number of electrophysiological techniques can be employed in order to understand the response profile of a BCA in relation to olfactory-active compounds in host extracts. GC-EAD depolarisation of olfactory receptor neurons inside the specialised sensilla on the antennae can identify which compounds in the extracts are able to be perceived by the BCA, and based on the magnitudes of responses, which compounds may be important cues mediating host specificity. Electroantennogram recordings can then measure the relative responses of a BCA to synthetic standards of olfactory-active compounds to confirm the identity of responsive compounds. Finally, single sensillum recording can be used to identify how specific, certain types of sensilla are, to certain compounds. Here, the relative importance of compounds can be assessed, based on the specificity and abundance of such sensilla, and the chemosensory profiles associated with hosts versus non-hosts. Together, this information can be used to determine whether there is a set of host-specific volatile cues which are used by the BCA to recognise and select hosts. Electrophysiology techniques can also provide an alternative to odour-specificity testing when hosts are difficult to find or rear in captivity, or when odour-specificity testing requires uneconomical numbers of small-bodied hosts to elicit a response. Once compounds and odour response profiles have been identified through electrophysiological techniques, further rounds of behavioural tests can be used to understand the behavioural function of individual compounds or blends of compounds in host or plant extracts. Alternatively, if access to electrophysiology equipment or training is limited then testing can move from odour-specificity to field cage trials.

The fourth and final stage in the proposed framework involves the field validation of ecological host range. The steps in this stage are intended to be conducted after approval has been given to release an agent, but before releases have gone ahead. In some circumstances these steps could also happen before an agent is released, for example in containment glasshouses. Parasitism tests in field cages are a good way to test how the BCA reacts to non-target species when it is allowed to progress through the steps in the host location progress, but without actually having being released into the environment (Avila et al., 2016b). A greater variety of natural sensory cues are available for BCAs to incorporate into their decisions around which hosts to attack, such as plant cues, climatic variables, and cues derived from the natural expression of host behaviour. Once the BCA has been released, it is

extremely important to conduct retrospective host specificity tests to measure whether field parasitism was predicted accurately by pre-release testing procedures (Haye et al., 2005). The life stages of non-target species attacked by the parasitoid can be collected and reared through to observe incidence of non-target attack, or sentinel non-target hosts can be put out in the field to quantify rates of non-target attack (Barratt, 2004; Pratt et al., 2009). The results of retrospective studies can be used to compare the results from laboratory-based pre-release host specificity testing with ecological host ranges observed in the field, and this information can be used to assess the suitability of laboratory methods in predicting realised host ranges and non-target effects (Barratt et al., 2000, 2010; Paynter et al., 2015, 2020).

Ultimately, the integration of physiological host range testing with behavioural and electrophysiological techniques will accelerate progress towards a better understanding of the host specificity of candidate agents before they are released. Insight into a biological control agent's ecological host range during pre-release studies will provide regulators with more certainty around the scientific basis underpinning their decisions, and help to prevent the approval of potentially dangerous agents, or the unnecessary rejection of reasonably host-specific agents.

6.6 References

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