Mothers in the woods: Multitrophic interactions and oviposition preference in the bronze bug *Thaumastocoris peregrinus*, a pest of *Eucalyptus*.

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(Caetano Veloso, Manhatã)

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Chapter 1

Introduction and thesis outline

From 1990 to 2015 global tree plantation area has increased from 167.5 million to 277.9 million hectares (Payn et al., 2015). Uruguay has accompanied this trend, experiencing an explosive growth after the passing of the Forestry Law (15.939) in 1988, leading to an increase of 1000 % of the area devoted to commercial tree plantations from this date to 2010 (Morey & Porcile, 2002; Ligrone, 2011). Currently, it reaches a million hectares, 800,000 of which are planted with monospecific stands of *Eucalyptus* (Boscana et al., 2016). *Eucalyptus*-based forestry benefited in the beginning from a relatively benign sanitary situation, given the distance from the area of origin of the genus (Jeffries & Lawton, 1984). However, the expanding area and the elapsed time have promoted the introduction of pests and diseases from the center of origin of *Eucalyptus*, threatening plantation productivity (Wingfield et al., 2008, 2013). Moreover, the introduction of invasive pests has been further enhanced in the last two decades by an increased global trade of seeds, plants and wood packaging material (Humble, 2010; Paine et al., 2011; Wingfield et al., 2013).

Common pest management practices in forestry plantations include tree breeding, silvicultural management and chemical and biological control. Above all these strategies, classical biological control, defined as 'the intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control' (Eilenberg et al., 2001) represents one of the major ways forward for managing the global problem of invasive pests in tree plantations (Garnas et al., 2012), with many successful examples in forestry systems (Hanks et al., 1996; Cordero Rivera et al., 1999; Hodkinson, 1999; Protasov et al., 2007). However, implementing a classical biological control program is not exempt of difficulties, arising from many complex interacting factors (Hokkanen & Sailer, 1985; Hokkanen & Lynch, 2003). In order to establish a successful biological control program, research efforts must be invested in understanding the biology of both the pest and the potential biocontrol agents, as well as in developing effective rearing systems for both (Etzel & Legner, 1999).

In recent years, an increasing number of studies have demonstrated that, because individuals species are embedded in a multitrophc context, behaviors such as oviposition-site selection by insects, are affected by bottom-up factors such as the quantity and quality of the host plant, and top-down factors such as the availability of enemy-free oviposition sites (Turlings et al., 1990; Bernays & Chapman, 1994; Dicke, 2000; Dicke & van Loon, 2000; Dicke et al., 2009). Chemical cues play an important role in these interactions, transferring information within the multitrophic system to organisms of the same or different trophic levels (Turlings et al., 1990, 1995; Dicke, 2000; Arimura et al., 2005; Dicke et al., 2009). For instance, marking and/or oviposition deterring pheromones can regulate egg distribution in herbivorous insects (Ditrick et al., 1983; Hilker & Klein, 1989; Blaakmeer et al., 1994; Hilker & Meiners, 2002), while parasitoid-attractive synomones can be released by the host plant in response to egg deposition (Vinson, 1976; Strand & Vinson, 1982, 1983; Colazza et al., 2004; Hilker & Fatouros, 2015). Despite the fact that such interactions among trophic levels may be of great importance in long-term systems such as plantation forestry, multitrophic studies in forestry systems are scarce. The characterization of the cues involved, and the understanding of their roles in regulating multitrophic interactions, may increase the probability of success of a biological control program.

The aim of this thesis was to investigate the factors affecting the oviposition behavior of an herbivorous true bug (*Thaumastocoris peregrinus*), within a multitrophic system comprised of its host plant (*Eucalyptus* spp.), a common co-occurring sap-feeder (*Glycaspis brimblecombei*) and a specialist egg parasitoid (*Cleruchoides noackae*). Based on the information obtained, this study also pursued potential improvements in the management of *T. peregrinus* in *Eucalyptus* plantations. To achieve this, I focused on the preference-performance hypothesis, and tested preference-performance correlations assessing the effects of host-plant quality, the presence of conspecifics, or the infestation by a potential competitor. I also investigated host-selection behavior of the parasitoid, testing its responses towards different contact cues. I addressed the following research questions:

(1) Is oviposition behavior of *Thaumastocoris peregrinus* affected by host-plant characteristics?

(2) Is oviposition behavior of *T. peregrinus* affected by conspecifics?

(3) Is oviposition affected by the presence of a co-occurring sap-feeder?

(4) What cues does the egg parasitoid *Cleruchoides noackae* exploit to locate its host?

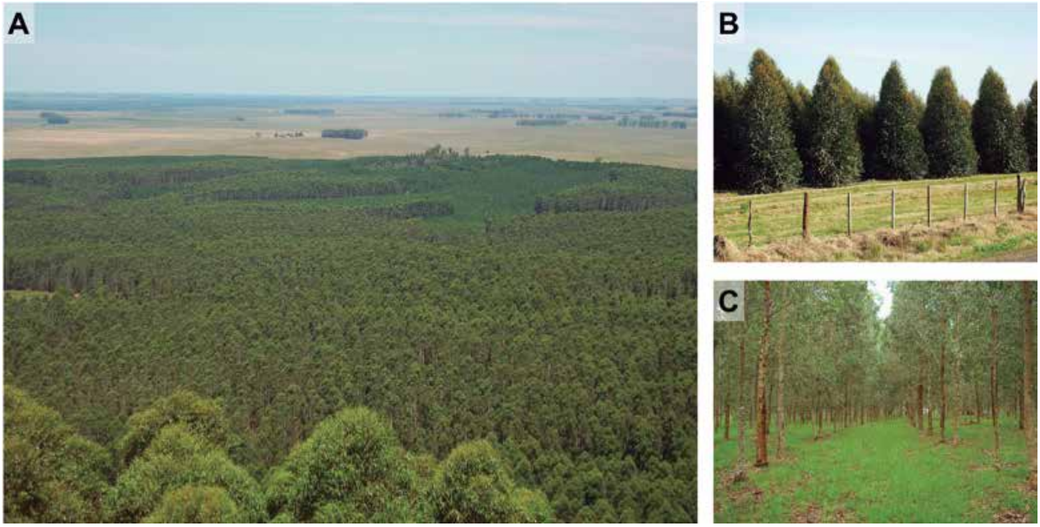

Figure 1.1. *Eucalyptus*: (A) Commercial plantation of *E. grandis*; (B) Clonal plantation of *E. grandis*; (C) *E. tereticornis*.

Study system

The Plant (Figure 1.1)

The genus *Eucalyptus*, contains more than 500 species all endemic to Australia and New Guinea, more than 200 of which occur in Southeast Australia (Brooker & Kleinig, 1990). Eucalypts, also known as gum trees in Australia, are the most important dicotyledon species planted in forestry worldwide, particularly in the southern hemisphere (Paine et al., 2011). Eucalypts used in forestry are not deciduous species, so leaves may remain on a mature tree for two or even three years before falling (Brooker & Kleinig, 1990). Heteroblasty, i.e. the simultaneous presence of different leaf developmental stages, such as juvenile or adult leaves, is a remarkable characteristic of *Eucalyptus* species (Gras et al., 2005). Juvenile leaves usually differ in their morphology, phyllotaxy and wax content relative to adult leaves, providing an extra niche differentiation to herbivores (Brennan et al., 2001; Nahrung & Allen, 2003; Gras et al., 2005; Velikova et al., 2008).

In Uruguay, 68% of the total area occupied by eucalypt plantations is planted as a source of fiber for the growing pulp industry (Boscana et al., 2016). The extant area is planted for timber, energy, or serve as shade and shelter for cattle. In the latter case, red gums such as *Eucalyptus tereticornis* Smith are usually employed (Balmelli & Resquin, 2005). This species is a medium-sized to tall forest tree that occurs originally along the coast and adjacent hills of eastern Australia, from eastern Victoria to northern Queensland and in New Guinea. It is characterized by a slightly marked heteroblasty, juvenile leaves are slightly discolorous bluish green and exhibit opposite phyllotaxy, whereas adult leaves are concolorous green, moderately reticulate, and show alternate phyllotaxy (Brooker & Kleinig, 1990). In Uruguay, hybridization between this species and *E. camaldulensis* Dehnh. is very common. In this thesis, I mostly used *E. tereticornis* as the reference species, including also in the experiments the rose gum (*Eucalyptus grandis* Hill ex Maiden), the Tasmanian blue gum *E. globulus* Labill., and the Dorrigo white gum *Eucalyptus benthamii* Maiden & Cambage, all currently planted in Uruguay in high productivity stands for pulp production.

The incidence of native herbivore entomofauna to eucalypt stands is of no significance in Uruguay and other subtropical and temperate regions in South America (Paine et al., 2011). Thus, most of the insects attacking eucalypts in Uruguay are introduced Australian insects. Eucalypts may counteract herbivore attack by a plethora of constitutive chemical defenses, such as components of the epicuticular leaf waxes or substances that provide leaf toughness. The defensive role of induced defenses, in turn, is a subject of debate (Ohmart & Edwards, 1991; Rapley et al., 2007; Henery et al., 2008; Naidoo et al., 2014).

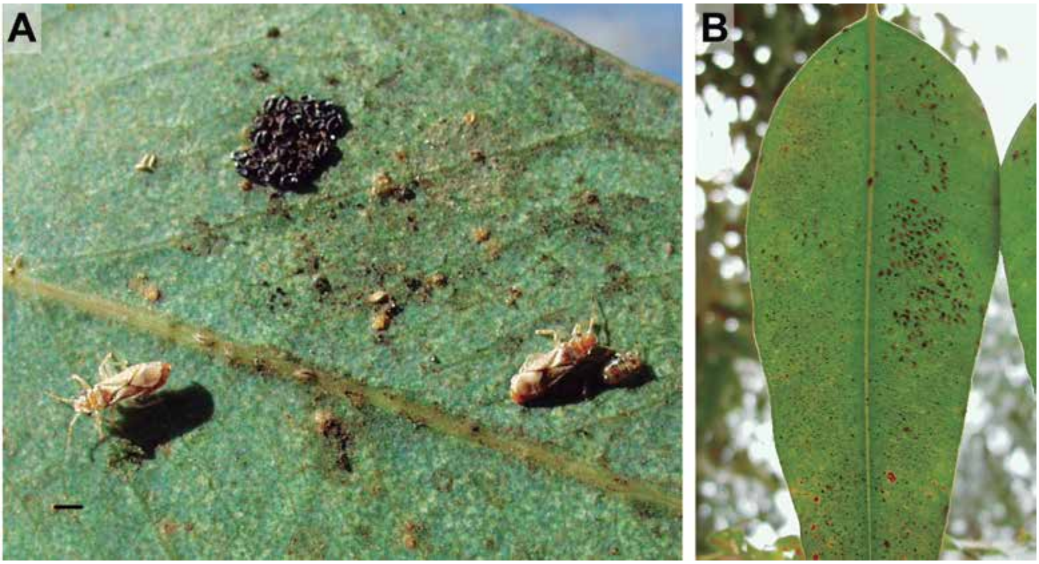

Figure 1.2. The bronze bug, *Thaumastocoris peregrinus*: (A) Eggs and adults (Black bar: 1 mm); (B) Nymphs aggregated on a leaf.

The herbivore (Figure 1.2)

The bronze bug *Thaumastocoris peregrinus* Carpintero and Dellapé (Heteroptera: Thaumastocoridae), originally restricted to Australia, has become one of the most important invasive pests of *Eucalyptus* tree plantations worldwide (Noack & Rose, 2007; Wilcken et al., 2010). In less than 15 years it has reached Africa (Jacob & Neser, 2005), South America (Carpintero & Dellapé, 2006; Martínez & Bianchi, 2010; Wilcken et al., 2010; Ide et al., 2011; Benítez Díaz et al., 2013), North America (Jiménez-Quiroz et al., 2016), New Zealand (Sopow et al., 2012), Europe (Laudonia & Sasso, 2012; Garcia et al., 2013), and the Middle East (Novoselsky & Freidberg, 2016). It is a small flattened oligophagous bug (1–3 mm long) that feeds on *Eucalyptus* and some *Corymbia* species. The family Thaumastocoridae is a small marginal family that remained poorly studied until the rise of the bronze bug. It is characterized by certain peculiarities in its morphology, such as the presence of strong anteriorly produced mandibular plates, grossly asymmetrical male genitalia, and reduced female genitalia characterized by the absence of an ovipositor and spermatheca (Schuh & Slater, 1995).

The feeding behavior of the bronze bug has been determined as cell rupture feeding (*sensu* Backus et al., 2005), implying the use of the stylets to lacerate plant cells in a small area, concomitantly with the secretion of watery saliva into the ruptured cellular layers and then ingest the resulting 'soup' (Santadino et al., 2017). Insect feeding results in white dots on the leaves, which later turn brown and often fall from the tree. The consequent loss of photosynthetic surface area due to bronzing and defoliation leads to several degrees of tree stress and can even kill the tree (Jacob & Neser, 2005).

The adults lack functional metathoracic scent glands but possess a glandular rectal organ that they quickly extrude posteriorly when disturbed (Martins et al., 2012). A male pheromone has been obtained from this organ (Martins et al., 2012) that has been characterized as an aggregation pheromone (González et al., 2012). Although seasonal dynamics of the bronze bug are not yet clearly understood, it displays a multivoltine life cycle in Uruguay, peaking from late summer to mid-autumn.

The potential competitor (Figure 1.3)

The red gum lerp psyllid, *Glycaspis brimblecombei* Moore (Sternorrhyncha: Aphalaridae), is a small (1-3 mm length as adults) winged sap-sucking herbivorous insect. Nymphs secrete a white conical, sweet-tasting structure called lerp, which serves as a protective cover (Firmino-Winckler et al., 2009). Although they can feed on other species such as *Eucalyptus grandis* and *E. globulus*, they prefer adult shoots and leaves of *E. camaldulensis* and *E. tereticornis*, on which they show higher survival rates, shorter nymphal development times, and lay more eggs (Firmino-Winckler et al., 2009). Their preference for adult old leaves is related to a decreased content of leaf epicuticular waxes relative to juvenile leaves (Woodburn & Lewis, 1973; Brennan & Weinbaum, 2001). Psyllids are considered phloem feeders, and some species can induce premature senescence on *Eucalyptus* leaves (Moore, 1964; Steinbauer et al., 2014). Despite causing important damage in other parts of South America (Santana & Burckhardt, 2007), it has remained as a pest of relatively low importance in Uruguay since its detection in 2004 (Bianchi & Sánchez, 2004). In sub-tropical climate conditions, the red gum lerp psyllid exhibits a multivoltine cycle, with higher population densities between August and December (FAO-MGAP, 2006). Reared on *E. tereticornis* at 26 °C, eggs hatch in 8 days, nymphs develop in two weeks, and adult longevity ranges from 8 to 17 days (Firmino-Winckler et al., 2009).

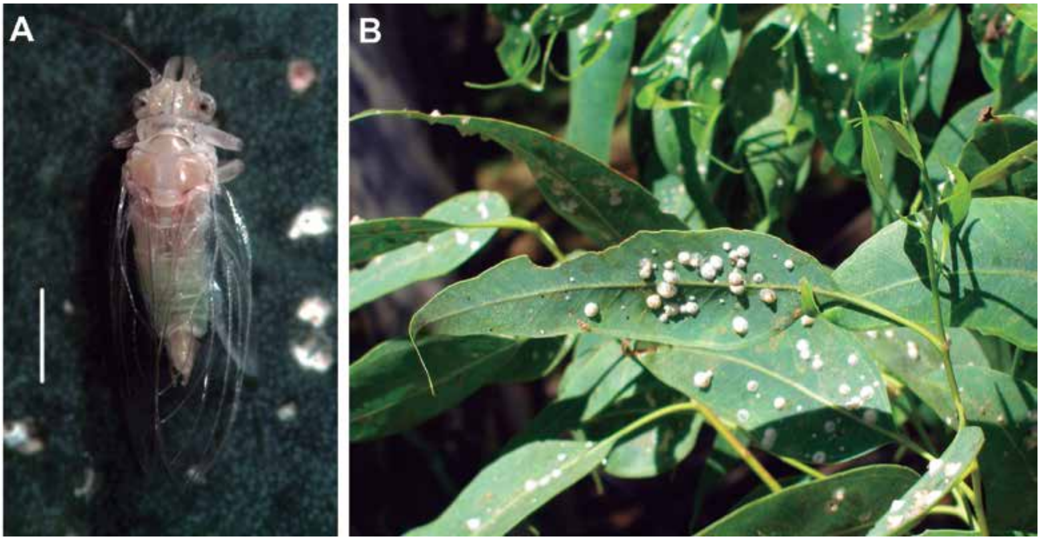

Figure 1.3. The lerp psyllid, *Glycaspis brimblecombei*: (A) Adult (White bar: 1 mm); (B) Lerps on leaves of *Eucalyptus tereticornis*.

Figure 1.4. The egg parasitoid *Cleruchoides noackae*: Female adult (Black bar: 0.1 mm).

The parasitoid (Figure 1.4)

The egg parasitoid *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae) is a tiny wasp (0.5 mm length) that was first recovered from *Thaumastocoris* spp. eggs in New South Wales, Australia (Lin et al., 2007). Under laboratory conditions, adult female lifespan is about 2 days when supplemented with honey, and it parasitizes eggs of up to 3 days old (Mutitu et al., 2013; Souza et al., 2016). Arrhenothokous parthenogenesis has been confirmed for *C. noackae*; unfertilized eggs produce males and fertilized eggs produce females (Mutitu et al., 2013). An analysis of the genetic diversity of *C. noackae* suggests a relatively low dispersal ability (Nadel et al., 2012). The biological and ecological traits of *C. noackae* make it a candidate for biological control of *T. peregrinus*. Therefore, several countries have started biological control programs with this species as one of the few management alternatives for the bronze bug (Jaques, 2010; Nadel & Noack, 2012; Mutitu et al., 2013; Souza et al., 2016).

Outline of the thesis

In Chapter 2 the multitrophic factors affecting oviposition behavior in true bugs are reviewed and discussed. Particular features of this taxon such as feeding habits, aggregation and nymphal movement are considered in comparison to the more studied order Lepidoptera. Also, the current models for host location of egg parasitoids are revisited, taking into account particular features of the Heteroptera.

In Chapter 3, I describe the design of the rearing setup for the bronze bug and I analyze the main biological parameters of this species.

Chapter 4 investigates the contact cues utilized by *T. peregrinus* females for the selection of feeding and ovipositing sites, and the concurrent effect of oviposition preference on the performance of the offspring, within the theoretical framework of the 'mother knows best' paradigm.

In Chapter 5, I address the effect of the previous colonization by and intra-guild potential competitor, *G. brimblecombei*, on the feeding and oviposition preference of *T. peregrinus* females, and the resulting performance of bronze bug neonates.

In Chapter 6, I describe a rearing and release scheme for the main natural enemy of the bronze bug, the parasitoid wasp *C. noackae*, in the context of the biological control of the bronze bug.

Chapter 7 addresses the contact cues involved in oviposition-site selection by *C. noackae* focusing of the response by the wasp to short-range contact cues.

Finally, in Chapter 8 I integrate the findings of this thesis towards a model for oviposition selection of true bugs in general, and I discuss their implications for developing management strategies for the bronze bug in *Eucalyptus* plantations in particular.

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Chapter 2

Behavioral ecology of oviposition-site selection in herbivorous true bugs

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Abstract

Optimal oviposition theory predicts that female herbivores will prefer to oviposit on those plants that maximize offspring performance, also known as the 'mother knows best' paradigm. This is the general pattern within the insect order Lepidoptera with specialist diets and reduced larval mobility. In that context, mother's decisions are crucial to the development of the offspring. In this review we discuss oviposition-site selection behavior by the Heteroptera, focusing on the particular traits of this taxon in comparison to the most studied holometabolous insects. This review takes a multitrophic perspective and focuses on three main factors affecting the behavioral ecology of oviposition-site selection in true bugs: 1) life-history characteristics of the bugs, including host plant specialization and mobility of larvae; 2) plant characteristics, including morphology and induced responses to feeding and oviposition and 3) interactions with natural enemies, including parasitoid foraging behavior and strategies of bugs to interfere with the activity of parasitoids. New insights to the 'mother knows best' paradigm of the behavioral ecology of oviposition-site selection by insects are provided by comparing the oviposition decisions displayed by true bugs with those of the well-studied Lepidoptera. The review ends with the identification of questions to be addressed in future studies on the behavioral ecology of oviposition by insects.

Keywords: Oviposition, Heteroptera, multitrophic interactions, preference-performance

Introduction

A major paradigm in behavioral ecology is the "mother knows best" paradigm of oviposition decisions of herbivorous insects. The decisions made during oviposition-site selection have especially addressed lepidopteran insect herbivores. Insect taxa dominated by specialist diets and reduced larval mobility like the Lepidoptera tend to exhibit a correlation between female oviposition-site selection and offspring performance (Renwick and Chew, 1994; Thompson and Pellmyr, 1991 but see Mayhew, 1997; Ohsaki and Sato, 1994; Pires et al., 2000). In comparison, particular traits of the true bugs may modulate this correlation, requiring an expansion of the general model developed for lepidopterans. Here we will address the behavioral ecology of oviposition-site selection in true bugs in a multitrophic perspective and by comparing with the knowledge on this aspect of lepidopteran herbivores.

True bugs (Hemiptera: Heteroptera) comprise more than 40,000 species, being one of the most successful groups of non-holometabolous insects, in terms of niche colonization (Weirauch & Schuh, 2011). Representatives of this suborder exploit a wide range of ecological niches, from blood-feeding ectoparasites to predatory, omnivorous, and herbivorous species, in both terrestrial and aquatic ecosystems (Schuh & Slater, 1995). More than half of the terrestrial species of true bugs, particularly within the Pentatomorpha and Cimicomorpha, feed on plants (Cobben, 1978). Unlike the situation for other groups of herbivorous insects, only a limited number of species are specialists, the majority of them presenting broad host-plant ranges, or zoophytophagous habits (Cobben, 1979; Eubanks et al., 2003). Several reviews have addressed the evolution and systematics (Cobben, 1968, 1979; Weirauch & Schuh, 2011), biology, and biodiversity of the Heteroptera (Miller, 1971; Kelton, 1982; Schuh & Slater, 1995; Fauvel, 1999; Wheeler, 2001).

Oviposition-site selection plays a crucial role in true bug–plant interactions, because the majority of the herbivorous heteropterans lay their eggs directly on plants. When trying to explain why insect herbivores select some plants over others for oviposition, evolutionary ecologists have turned to the optimal oviposition theory, also known as the preference–performance hypothesis (Jaenike, 1978). According to this hypothesis, natural selection favors the ability of insect females to select those host plants for oviposition that maximize the performance of their offspring. Although several empirical studies have given support to the preference–performance hypothesis, the majority of them addressing Lepidoptera (Thompson & Pellmyr, 1991; Renwick & Chew, 1994), several other studies show a poor correlation between offspring performance and oviposition-site preference (Ohsaki & Sato, 1994; Mayhew, 1997; Pires et al., 2000). Females face a tradeoff between plant nutritional quality for their offspring and several other factors including physiological state, mobility of immature stages, spatiotemporal patterns of host plant availability, foraging decisions, and predation risk (Price et al., 1980; Thompson, 1988; Dicke, 2000; Janz, 2002; Scheirs & De Bruyn, 2002; Moravie et al., 2006; Kaplan & Denno, 2007).

The aim of this review is to address oviposition-site selection by true bugs and discuss this within the context of recent findings in herbivorous insect–plant interactions and in comparison with the more intensely studied Lepidoptera. Apart from specified cases, this review addresses herbivorous true bugs in terrestrial habitats, because that is the most common ecological context in which studies on insect herbivory have been conducted. In the next paragraph, we discuss life-history traits that are characteristic of this taxon and likely to affect optimal oviposition behavior. We focus particularly on the food habits of the group, aggregative behavior, and juvenile mobility. Subsequently, we present the current knowledge on the molecular mechanisms of induced responses of plants to feeding and oviposition by true bugs. We conclude that co-occurrence of feeding damage and oviposition in true bugs affects plant responses in a different way than has been observed in other taxa. Next, we address the role of plant chemicals and structures on the oviposition-site selection. The subsequent section deals with the interaction of ovipositing true bugs and their natural enemies. Particularly, we discuss how the foraging behavior of egg parasitoids of true bugs has affected the evolution of strategies by true bugs to avoid them. Finally, we discuss the main research questions to be addressed to include heteropterans in a comprehensive model of the behavioral ecology of herbivorous insects.

Life-history traits affecting oviposition strategies in true bugs

Host-plant range

Diet breadth can play an important role in oviposition-site selection by herbivorous insects (Jaenike, 1990; Thompson & Pellmyr, 1991; Mayhew, 1997). In general, the relationship between preference and performance tends to be weaker in polyphagous insects when compared to oligophagous or monophagous species (Gripenberg et al., 2010). True bugs contain a large number of generalists and omnivorous species. For instance, herbivorous mirids can feed on more than 25 families of plants, while other species in this family alternate between a phytophagous and a carnivorous diet (Wheeler, 2001). Moreover, the pentatomid bug *Nezara viridula* (L.), a worldwide pest of crops, is able to feed on more than 250 plant species from 30 families (Todd, 1989). Jaenike (1990) pointed out that an insect with a wide host range is under less pressure than a specialist to find host plants, and can prioritize factors other than nutritional quality in selecting oviposition sites, such as the absence of natural enemies or competitors. Some studies on Heteroptera support this idea. For instance, the hawthorn lacebug, *Corythucha cydoniae* (Fitch), a tingid that feeds exclusively on plants in the Rosaceae family preferred to oviposit on less pubescent plants where offspring survival was higher (Schultz & Coffelt, 1987), whereas the polyphagous mirid bug *Lygus hesperus* Knight preferred pubescent phenotypes of cotton for oviposition although pubescence significantly reduced nymphal growth (Benedict et al., 1983). When comparing both results, a factor other than nutritional quality seems to affect mother’s decision in the latter example. Yet, studies on other polyphagous heteropterans showed a strong correlation between preference and performance. For example, the plant bug *Lygocoris pabulinus* (L.) can feed on many plant species from different families but showed an innate preference for potato plants as oviposition site in laboratory and semifield conditions, which in turn was correlated with a better development and realized fecundity of the offspring (Groot et al., 2003). Thus, when considering the total life cycle, *L. pabulinus* turned out to be a specialist in term of oviposition. Moreover, a strong preference-performance correlation has been observed in some predatory heteropterans, including *Orius insidiosus* (Say), whose early stages depend for food on the plant on which they hatched. Female *O. insidiosus* preferred thinner and less pubescent leaves as oviposition sites, which allow the offspring to feed on them and increase survival (Lundgren et al., 2008). To sum up, the pattern seen in true bugs seems to be similar to that observed in other orders: generalists tend to exhibit a weaker correlation between mother's choice and offspring performance than specialists. The case for the zoophytophagous species deserves further research. In the studied species, early stages depend on plant resources to develop, and thus strongly depend on their mother's choice.

Optimal foraging vs optimal oviposition

The mother's oviposition preference–offspring performance correlation can be affected when the selection pressure on the female is in conflict with the requirements of its offspring. For instance, optimal foraging for food, *i.e.* maximizing adult performance, can affect plant selection to a larger extent than optimal oviposition (Scheirs & De Bruyn, 2002). In the majority of the Lepidoptera, mother and offspring feed on different substrates, leading to a conflict between the search for food and for oviposition sites. Thus, optimal oviposition sites can be neglected by females when these sites are far from a nectar source (Thompson & Pellmyr, 1991). In insects whose adult stages feed on the same plants as juveniles, however, optimal foraging may be difficult to separate from optimal oviposition, because oviposition usually takes place at, or in the vicinity of feeding sites. This overlap between feeding sites and oviposition sites has been observed in species from other insect orders, such as in the chrysomelid beetle *Cassida rubiginosa* Müller (Fujiyama et al., 2008). A similar overlap would be expected for true bugs, provided that all life stages feed on the same host plants and usually co-occur in the field. That could be the case for the bronze bug *Thaumastocoris peregrinus* Carpintero and Dellapé, which usually oviposits at the feeding sites (Martínez & Bianchi, 2010; Wilcken et al., 2010) but other cases provide evidence for a tradeoff between oviposition and feeding requirements of the mother. For instance, Ventura and Panizzi (2003) found that although the majority of the females of the alydid *Neomegalotomus parvus* (Westwood) oviposit on dry pods, some of them preferred green pods. Females that oviposited on green pods may have prioritized feeding over oviposition as these activities co-occur (Ventura & Panizzi, 2000). Females of the harlequin bug, *Murgantia histrionica* Hahn, switch between host plants more frequently than do the males, a behavior that has been associated with a strategy called "putting the eggs in many baskets" (English-Loeb & Collier, 1987). By allocating the eggs to different plants, the harlequin bug spreads the risk of losing offspring because of a bad host plant quality (English-Loeb & Collier, 1987). Finally, the stink bug *N. viridula* leaves the feeding sites after mating and oviposits on undamaged plants (Kiritani et al., 1965). We will discuss the evolutionary significance of this behavior in the context of the avoidance of predation and parasitism, later on in this review. Considered as a whole, these examples suggest that although there is not likely to be a conflict between oviposition and foraging, given that heteropteran females usually feed on the same resource as their offspring, in some taxa oviposition triggers a new set of behaviors that may affect the female's foraging strategy.

Modes of Oviposition

Heteropterans exhibit a large diversity of egg morphologies and modes of oviposition (see reviews by Cobben, 1968; Hinton, 1981; Javahery, 1994; Lundgren, 2011; Southwood, 1956). Herbivorous species lay eggs either on the plant surface (termed epiphytic oviposition), as is the case for the Pentatomorpha, or insert them into the plant tissues (endophytic oviposition), a strategy observed in the majority of the Cimicomorpha (Tallamy & Schaefer, 1997). Epiphytic eggs have a thicker chorion and are generally laid in batches (Goula, 2008). From the perspective of oviposition-site selection, it is important to note that laying eggs in batches may have advantages in terms of protection of the eggs against desiccation, or to reduce the searching time of the ovipositing female (Hinton, 1981; Courtney, 1984; Clark & Faeth, 1998). On the other hand, egg batches are more apparent to natural enemies, so physiological and behavioral adaptations have evolved to protect them (Tallamy & Schaefer, 1997; Janz, 2002). Endophytic oviposition results in a lower risk of desiccation and a better protection from natural enemies (Goula, 2008). From the perspective of oviposition-site selection, however, inserting an egg in the plant requires plant tissues to be hard enough to resist oviposition as well as to support the eggs (Wheeler, 2001). These requirements may reduce the potential oviposition sites for a true bug that displays endophytic oviposition.

Aggregation in Early Stages and Oviposition-Site Selection

Many true bug species show aggregative behavior in the early developmental stages (Schuh & Slater, 1995). In the majority of the species studied, the process of aggregation is mediated by pheromones (Aldrich, 1988). Wertheim et al. (2005) evaluated the benefits and costs related to pheromone-mediated aggregation in nonsocial arthropods. They concluded that aggregation may include benefits like the improvement of local abiotic conditions, a more efficient use of the host plant or a more effective defense against natural enemies than isolated individuals (Wertheim et al., 2005). On the other hand, competition for food and mates is likely to be more intense in aggregated patches and aggregation also increases the risk of pathogen infection and the apparency to natural enemies. Several studies on aggregation by both true bugs (e.g. Bongers, 1968; Bongers and Eggermann, 1971; Lockwood and Story, 1986; Tullberg et al., 2000) and lepidopterans (e.g. Allen, 2010; Clark and Faeth, 1998; Denno and Benrey, 1997; Fordyce, 2003; Stamp, 1980) have presented support for these conclusions. Larval aggregation has evolved independently in several taxa within the Lepidoptera, leading to a numerical dominance of these species in the communities (Fitzgerald, 1993). Lepidopteran species that exhibit larval aggregation are batch layers (Stamp, 1980; Courtney, 1984). These egg clusters are generally the product of a single female. Indeed, butterflies and moths usually reject to oviposit on plants already occupied by conspecifics, which is mediated by both visual and chemical cues from eggs and larvae (Schoonhoven, 1990; Renwick & Chew, 1994; Blaakmeer et al., 1994). On the other hand, nymphal aggregation in true bugs can be observed in species that display different oviposition behaviors. Stink bugs usually lay egg clutches and follow a pattern similar to that described for Lepidoptera. Thus, nymphs from a single egg batch usually feed in aggregations during the first instar (Kiritani et al., 1965; Todd, 1989; Zahn et al., 2008) and female pentatomids generally avoid colonized plants when selecting oviposition sites (Todd, 1989; Zahn et al., 2008). Other pentatomorphan taxa like the Alydidae and Coreidae can lay single eggs. In some species within these families, aggregation may be achieved by early nymphal aggregation after hatching (Leal et al., 1995; Ventura & Panizzi, 2003; Nakajima et al., 2010; Ventura et al., 2011). Some mirid females, however, seem to be attracted toward plants damaged by conspecifics. For example, the mirid bugs *L. pabulinus* and *L. hesperus* prefer to oviposit on plants damaged by conspecifics (Groot et al., 2003; Blackmer et al., 2004). Thus, nymphal aggregation can be observed in plants carrying eggs from different mirid females. Additionally, other true bug females cannot only lay eggs on the same plant, but also place them adjacent to the eggs of their conspecifics, leading to the formation of communal egg batches (Figure 2.1). For instance, *T. peregrinus* egg batches contain hundreds of eggs laid by several females (Martínez & Bianchi, 2010; Wilcken et al., 2010). In some cases, this aggregated oviposition can be the consequence of a restricted availability of suitable substrates. This is the case for the open sea bug *Halobates sobrinus* White that depends on floating debris or a dead bird to oviposit, leading to the formation of big batches up to 70,000 eggs produced by more than 800 females (Cheng & Pitman, 2002). Although the latter is an extreme example, it illustrates the versatility of the oviposition behavior within the Heteroptera. To sum up, feeding aggregation by early stages in Heteroptera does not necessarily rely on egg clustering as occurs within the Lepidoptera but on several oviposition strategies that may also include sharing a common host plant as oviposition site, or even the formation of communal egg batches.

Figure 2.1. Communal oviposition of *Thaumastocoris peregrinus* on damaged leaves of *Eucalyptus tereticornis.*

Immature Mobility

The mobility of the juvenile stages is another factor that may affect oviposition decisions. Nymphs of true bugs can be highly mobile, even in the early stages. For instance, in Uruguay, first instar nymphs of *T. peregrinus* have been found in yellow sticky traps located as far as 15 m away from the vegetation (G. Martínez, personal observation). Nymphs of the mirid *Lygus rugulipennis* L. can change their spatial distribution pattern in response to the quality of the host plants (Hannunen & Ekbom, 2001, 2002). This ability of true bug nymphs to move away from the plant on which they hatched and search for other resources affects an inherent assumption of the preference–performance hypothesis, that is, a strong dependence of the offspring on the food source provided by the mother when selecting an oviposition site. This has been recorded for most of the Lepidoptera studied, in which larvae have a reduced mobility and are not able to select their food. Thus, the quality of the food source chosen by the mother when selecting an oviposition site can have a major impact on growth and development of the offspring in these species (Thompson & Pellmyr, 1991). Some recent studies, however, show that even in this context, early larvae may also play a role in host plant selection, especially when they are mobile (Soler et al., 2012). Thus, neonates may have a more active role in selecting the source of food in the Heteroptera, provided the relatively higher mobility of the nymphs, together with the ability to relocate to other host plants. In fact, females from several species within the Pentatomorpha oviposit on nonhost plants together with host-plants. This behavior has been studied for the pentatomid *Palomena angulosa* Motschulsky (Wada & Hori, 1997), the coreid *Acanthocoris sordidus* Thunberg (Nakajima & Fujisaki, 2010), and the alydid *Riptortus pedestris* (F.) (=*Riptortus clavatus*) (Leal et al., 1995; Nakajima et al., 2010). In these three species, oviposition on nonhost plants is related to a complex of traits including a nonfeeding first instar nymph, an increased locomotion ability of the second instar nymph, and the use of conspecific cues to locate a suitable source of feeding. Nakajima and Fujisaki (2010) suggested that oviposition on nonhost plants by *A. sordidus* implies a tradeoff between lower egg mortality and higher nymphal mortality. Indeed, ovipositing on nonhost plants reduced egg disappearance due to ground-living predators, and also reduced the attack by the egg parasitoid *Ooencyrtus nezarae* Ishii. On the other hand, second instar nymphs of *A. sordidus* hatching on nonhost plants risked failure of reaching suitable plants. The nature of the cues exploited by *A. sordidus* nymphs to locate a host plant was not determined in this study, although an attraction toward plants with adults was recorded. On the other hand, *R. pedestris* is attracted to a male pheromone that is only released when food is available (Leal et al., 1995). In conclusion, the high mobility of the juveniles in the Heteroptera forms part of a suite of traits that allow the females to (partly) transfer the selection of suitable food to their offspring.

Plant traits affecting oviposition behavior by true bugs

Mechanisms of plant defense

Induced plant responses to insect damage have been widely studied in the last decades (for reviews see Dicke and Baldwin, 2010; Dicke and Hilker, 2003; Haukioja, 1991; Heil, 2008; Hilker and Meiners, 2010; Karban and Baldwin, 1997; Walling, 2000). Overall, chewing insects have been shown to induce the jasmonic acid (JA)-signaling pathway, while phloem feeders mainly induce the salicylic acid (SA) pathway (Walling, 2009; Pieterse et al., 2009). These signaling pathways often crosstalk, allowing plants to adjust the defense responses depending on the feeding-guild of the attacker (Pieterse & van Loon, 1999; Engelberth et al., 2001; Kessler & Baldwin, 2002; Pieterse & Dicke, 2007; Koornneef & Pieterse, 2008). More recently, it has been shown that plants can also react to the presence of insect eggs (Hilker & Meiners, 2002a; b; Schröder et al., 2005; Hilker & Meiners, 2006; Fatouros, Dicke, et al., 2008; Hilker & Meiners, 2010). Oviposition may induce histomorphological changes in the plant surface that hamper egg attachment to the plant (Doss et al., 2000; Little et al., 2007), as well as the synthesis of toxic compounds that negatively affect egg development (Seino et al., 1996; Yamasaki et al., 2003). Furthermore, plants carrying eggs can emit volatiles that attract natural enemies of the eggs (Meiners & Hilker, 2000; Wegener et al., 2001; Hilker et al., 2002; Colazza, Fucarino, et al., 2004; Colazza, McElfresh, et al., 2004; Fatouros, Dicke, et al., 2008). Hilker and Meiners (2006, 2010) pointed out that induced responses to oviposition by herbivorous Lepidoptera and Hymenoptera allow the plant to anticipate to future feeding damage. In true bugs, however, oviposition can hardly be dissociated from feeding damage due not only to the fact that adult females usually feed on the oviposition site, but also due to a tendency of the females to aggregate (Fujisaki, 1980; Hannunen & Ekbom, 2001; Ventura & Panizzi, 2003; Wilcken et al., 2010).

Feeding-induced plant defenses to lacerate-and-flush true bugs

Caterpillars and aphids have been extensively used as models of species that induce JA- and SA-mediated plant responses (Karban & Baldwin, 1997; Walling, 2009). This means that caterpillars and aphids modify plant characteristics through different signal-transduction pathways with different consequences for the plant's phenotype (Kessler & Baldwin, 2002; Dicke et al., 2009). Conversely, true bugs have been less studied and, if they are mentioned, they are either included with other sucking feeders or suggested as JA-inducers. All true bugs are sucking insects with haustellate mouthparts, but most of them are cell-content feeders that employ the lacerate-and-flush feeding strategy (Miles, 1972; Cobben, 1978). They produce damage to the plant tissues by injecting digestive enzymes directly into the plant cells (Backus, 1988). Some studies suggest that plants respond to feeding by true bugs in a similar way as to leaf chewers. For example, the harlequin bug *M. histrionica* induces an increased emission of volatiles in *Brassica oleracea* L. (Conti et al., 2008), the green stink bug *N. viridula* in *Zea mays* L. (Williams et al., 2005), *Vicia faba* L. and *Phaseolus vulgaris* L. (Colazza, McElfresh, et al., 2004), the Western tarnished plant bug *L. hesperus* in *Z. mays* (Rodriguez-Saona et al., 2002) and the mirid bug *Tupiocoris notatus* (Distant) (=*Dicyphus minimus*) in *Nicotiana attenuata*

Torr ex S. Watson (Kessler & Baldwin, 2001). The emission of volatile fatty acid derivatives was induced in *Z. mays*, *N. attenuata* and *B. oleracea* by *L. hesperus*, *T. notatus*, and *M. histrionica*, respectively. These compounds are derived from the octadecanoic pathway and are, thus, connected to JA responses (Feussner & Wasternack, 2002). The emission of terpenes was also increased as a consequence of feeding in the majority of the systems studied (but see Conti et al., 2008). The change in the volatile emission by the plant following feeding damage by true bugs seems to be a combination of a response to mechanical damage made by the mouthparts and a specific elicitor contained in the saliva, only reported for caterpillars (Turlings et al., 1993; Mattiacci et al., 1995). The role of heteropteran salivary compounds in eliciting plant responses has been already confirmed in the bitrophic systems *Gossypium hirsutum* L.-*L. hesperus*, *Z . mays*-*L. hesperus*, and *Z. mays*-*N. viridula* (Rodriguez-Saona et al., 2002; Williams et al., 2005). On the other hand, some studies suggest that the SA pathway could also be induced by true bugs. For instance, the emission of methyl salicylate (the volatile methyl ester of SA (MeSA)) has been observed as a consequence of feeding by *L. rugulipennis* on *V. faba*, leading to the conclusion that an SA-mediated response is induced as a consequence of feeding by this mirid bug (Frati et al., 2009). A combined effect of feeding and oviposition cannot be discarded in this study, as the highest concentration of MeSA was found in plants containing both feeding damage and *L. rugulipennis* females. Heidel and Baldwin (2004) found that although feeding by *T. notatus* on *N. attenuata* plants induced the emission of volatile blends that resemble those triggered by caterpillars, it downregulated the expression of proteinase inhibitor-related genes, and followed a general pattern closer to an SA- rather than a JA response. In sum, heteropterans induce plant responses and although the evidence collected to date is not conclusive about the underlying signaling, the data suggest that the response is partially similar to that to leaf chewers and to sap sucking insects.

Oviposition-induced plant responses

Several studies have assessed induced responses of plants to oviposition by true bugs. First, egg deposition has been found to affect primary metabolism. For instance, oviposition by *M. histrionica* reduced the photosynthetic rate and stomatal conductance in *B. oleracea* after 24 h (Velikova et al., 2010). Second, oviposition by Heteroptera also modifies the plant volatile spectrum. For instance, broad bean (*V. faba*) plants damaged by feeding and oviposition by female *L. rugulipennis* released larger amounts of terpenoids and MeSA than healthy plants or plants only exposed to males, i.e. with feeding damage but without eggs (Frati et al., 2009). Plant responses to oviposition alone can be assessed in Pentatomidae by removing the mouthparts of mated females. This technique has been used with *N. viridula* and *M. histrionica* (Bin et al., 1993; Colazza, Fucarino, et al., 2004; Colazza, McElfresh, et al., 2004; Conti et al., 2008). For both species, the data show that oviposition alone modifies the volatile blend emitted, but to a lesser extent than the feeding does. Finally, it has been suggested in other taxa that the response to oviposition observed in plants depends on elicitors present in the egg (Little et al., 2007; Bruessow et al., 2010), in oviduct secretions (Meiners & Hilker, 2000; Wegener et al., 2001; Hilker et al., 2002, 2005), or in substances transferred by the males during mating (Fatouros, Broekgaarden, et al., 2008). Similarly, an insect-related compound is expected to be involved in the induced plant response to oviposition by true bugs, especially in species with epiphytic oviposition that do not cause any injury with the ovipositor. These elicitors remain to be characterized.

Interaction between feeding and oviposition affecting induced plant responses

When feeding and oviposition by true bugs are compared to the much better studied lepidopteran model, the most striking difference that emerges concerns the interaction between oviposition and feeding-induced plant responses. In the Lepidoptera, where oviposition and feeding occur sequentially, the mother might manipulate plant defenses, by inducing antagonistic effects in the egg-carrying plant, that benefit the performance of their offspring. For instance, oviposition by *Pieris brassicae* upregulated genes involved in SA biosynthesis in *Arabidopsis thaliana* L. (Little et al., 2007). Consequently, SA levels increased gradually in leaf discs of *A. thaliana* carrying egg batches of *P. brassicae*, reaching more than 10-fold higher levels than in control leaves after four days. Furthermore, the induction of JA-regulated genes was inhibited in leaf discs treated with egg extracts of *P. brassicae* (Bruessow et al., 2010). Heteropteran females, however, usually feed on the plant before ovipositing (Todd, 1989; Wheeler, 2001; Groot et al., 2003). This simultaneous occurrence of oviposition and feeding may select for synergistic responses in the plant. In fact, a combination of feeding and oviposition may not only enhance volatile emission but also result in additional changes in the emission profile, different from those observed in plants with either feeding damage or eggs. For instance, the emission of the sesquiterpene (E)-ß-caryophyllene was enhanced by a combination of oviposition and feeding by *N. viridula* and *M. histrionica* on *P. vulgaris* and *B. oleracea*, respectively, while plants only carrying eggs did not differ in the level of emission of this compound compared to healthy plants in *P. vulgaris*, or even no emission could be recorded in *B. oleracea* (Colazza, Fucarino, et al., 2004; Colazza, McElfresh, et al., 2004; Conti et al., 2008; Velikova et al., 2010). In the latter example, the production of green leaf volatiles and glucosinolates by the plant in the scenario of combined feeding and oviposition was also detected (Conti et al., 2008). Furthermore, in the majority of the studied species within the Pentatomorpha, feeding starts after the second instar, so there is a time interval between oviposition and feeding by hatching nymphs that might reach up to 72 h (e.g. Zahn et al., 2008). On the other hand, oviposition by cimicomorphan bugs is usually endophytic. As this mode of oviposition inflicts mechanical damage to the plant tissue, it is more likely to trigger a JA-mediated response. For example, a JA-mediated response has been observed in tomato plants following oviposition by the omnivorous bug *Orius laevigatus* (Fieber) (Puysseleyr et al., 2010). In any case, induced plant responses to oviposition by true bugs cannot be dissociated from feeding-induced responses. In order to better understand the interactions between oviposition-induced and feeding-induced responses in plants by true bugs, it is essential to enhance the knowledge of the molecular mechanisms of the defenses induced by lacerate-and-flush feeding bugs in their host plants.

Plant cues exploited in oviposition-site selection by true bugs

Plant volatiles

Plant odors can be used by female true bugs as cues to locate oviposition sites. For instance, a series of studies have found sex-related differences in the exploitation of plant cues by Miridae, suggesting a role of plant odors in oviposition-site selection in this family. Females of *Lygus lineolaris* and *L. pabulinus* showed stronger responses to plant volatiles than males in electroantennographic (EAG) studies (Chinta et al., 1994; Groot et al., 1999). Females of *L. hesperus* were significantly more attracted to plant volatiles, especially terpenoids, than males in Y-tube olfactometer tests although female antennae showed a lower EAG response to alfalfa volatiles than antennae of conspecific males (Williams et al., 2010). Plant odors can trigger different responses in insects, depending on their host-plant range. In fact, plant secondary compounds that usually act as repellents for generalist species can serve as attractants for specialists (Meijden, 1996; Schoonhoven et al., 2005). Thus, specialist species can exploit characteristic secondary metabolites of their host plants as cues or "token stimuli" to locate oviposition sites (Dicke, 2000). Although the majority of true bugs are polyphagous (Backus, 1988), some species exhibit narrower host plant ranges. Additionally, plants undergoing feeding or oviposition stress become more apparent in a landscape of undamaged plants, due to the enhanced emission of volatile compounds (Dicke, 2000). Insect herbivores can, in turn, exploit this information when selecting the sites for feeding or oviposition (Halitschke et al., 2008). Plant volatiles induced by feeding and oviposition usually act as oviposition repellents in other insect orders (e.g. Craig et al., 1988; Renwick & Chew, 1994; Hilker & Meiners, 2011), but many true bugs are attracted to these cues (Groot et al., 2003; Blackmer et al., 2004). However, pentatomorphan bugs tend to avoid colonized plants when selecting oviposition sites.

Plant anatomy

Plant surfaces can also affect oviposition-site selection by true bugs. For instance, the omnivorous true bug *O. insidiosus* Say (Anthocoridae) preferred to oviposit on leaf petioles with thinner epidermis in an *in vitro* biassay and the neonate nymphs feed on the plant on which they hatched (Lundgren et al., 2008). In this example, oviposition-site selection shows a direct correlation with the fitness of the offspring. Similarly, particular physical characteristics of the plant can provide protection against natural enemies (Udayagiri & Welter, 2000). Yet, some heteropterans exhibit a preference for certain oviposition surfaces that cannot be fully explained by the resultant offspring performance. For example, the bronze bug *T. peregrinus* prefers to lay eggs on roughened surfaces, even on paper (Noack & Rose, 2007), a behavior that has been also observed in other members of this family (Hill, 1988). In addition, *L. lineolaris* prefers to oviposit on moist tissue paper over green beans, a preference that has been used for rearing purposes (Snodgrass & McWilliams, 1992) as well as the use of cotton balls instead of green leaves by some species of Pentatomidae (Silva & Panizzi, 2007). The question remains to what extent the biomechanics of the oviposition may affect the selection of particular surface patterns as oviposition sites by true bugs.

Behavioral decisions related to egg parasitization

Foraging patterns of egg parasitoids of true bugs

Eggs of true bugs are subjected to intense predation and parasitism in natural and anthropogenic ecosystems (Schuh & Slater, 1995; Schaefer & Panizzi, 2000; Wheeler, 2001). For instance, egg predation and egg parasitization represent a major contribution to the natural control of Pentatomidae in crops (Tillman, 2011). Natural enemies of insect herbivores develop in a multitrophic environment and, consequently, they have evolved the ability to exploit information from the first and the second trophic levels to find their potential prey or hosts. In the last decades, much attention has been paid to the use of particular chemical cues, also called infochemicals (*sensu* Dicke & Sabelis, 1988) from plants and herbivores by carnivorous insects (reviewed by Vet & Dicke, 1992; Fatouros, Dicke, et al., 2008; Colazza et al., 2009; Dicke & Baldwin, 2010). Vet & Dicke (1992) stated that the use of infochemicals in making foraging decisions by predators and parasitoids of herbivores implies a reliability-detectability dilemma: infochemicals originating from the host plant are easy to detect but do not represent the presence of the host, while those generated by the hosts are highly reliable indicators of prey but are, in general, hard to detect because they are emitted in much lower quantities. Three possible solutions to this dilemma are proposed: (1) to exploit information from other stages of the host/prey as infochemical detour; (2) to rely on infochemicals that result from the interaction of the herbivore with the host plant (herbivore-induced plant volatiles), or (3) to link highly detectable plant cues to more reliable host cues through associative learning (Vet & Dicke, 1992). The majority of the studies on natural enemies of Heteroptera have focused on egg parasitoids, particularly those species that can be used in biological control programs. Consequently, information is available on the foraging strategies of many egg parasitoids of true bugs (Figure 2.2). Egg parasitoids face some particular challenges concerning the nature of their hosts. Unlike other stages, eggs are inconspicuous, release few chemical cues, and tend to have short duration in the environment (Fatouros, Dicke, et al., 2008). These characteristics affect the foraging pattern of egg parasitoids to a strong dependence on the infochemical detour, particularly the use of adult cues (Fatouros, Dicke, et al., 2008). General patterns regarding the use of infochemicals by egg parasitoids, including those that parasitize eggs of true bugs, have been reviewed by Fatouros, Dicke, et al. (2008) and Colazza et al. (2009). Hence, we summarize in the following paragraphs some particular features of the searching patterns developed by egg parasitoids of true bugs, and we discuss how this may have affected oviposition decisions of female heteropterans.

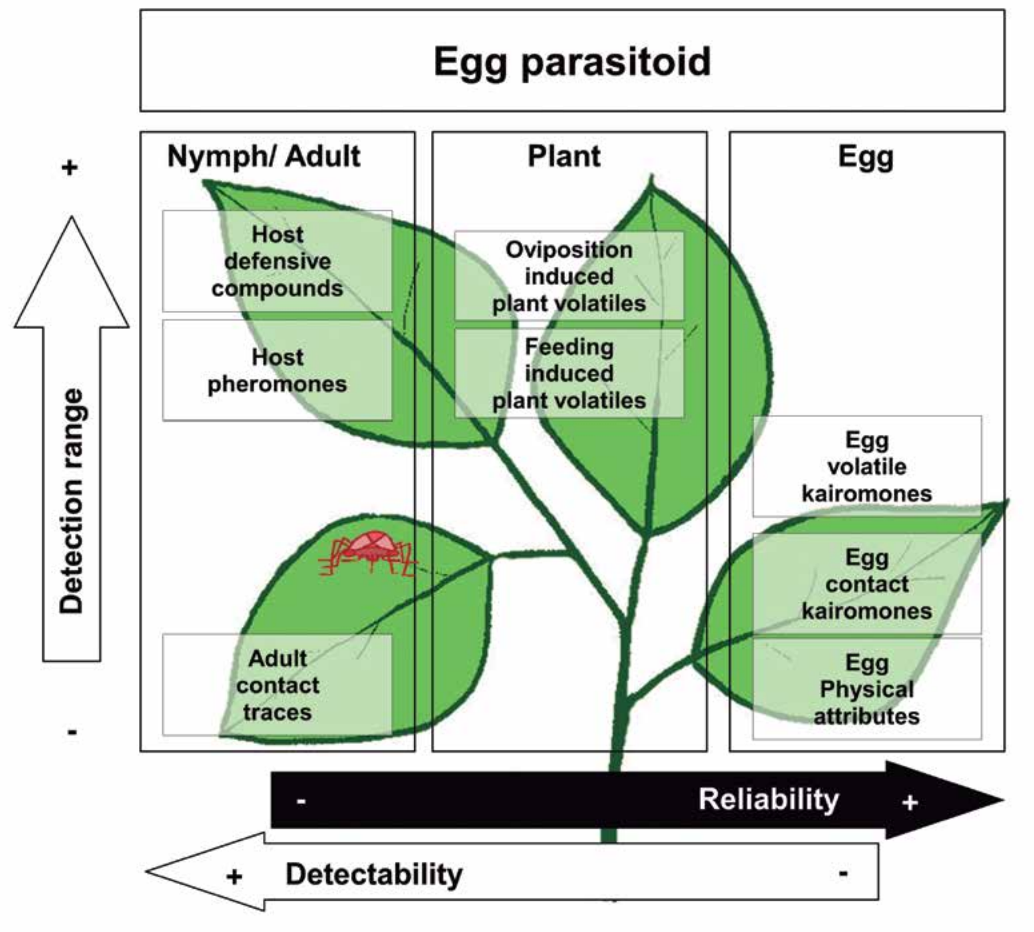
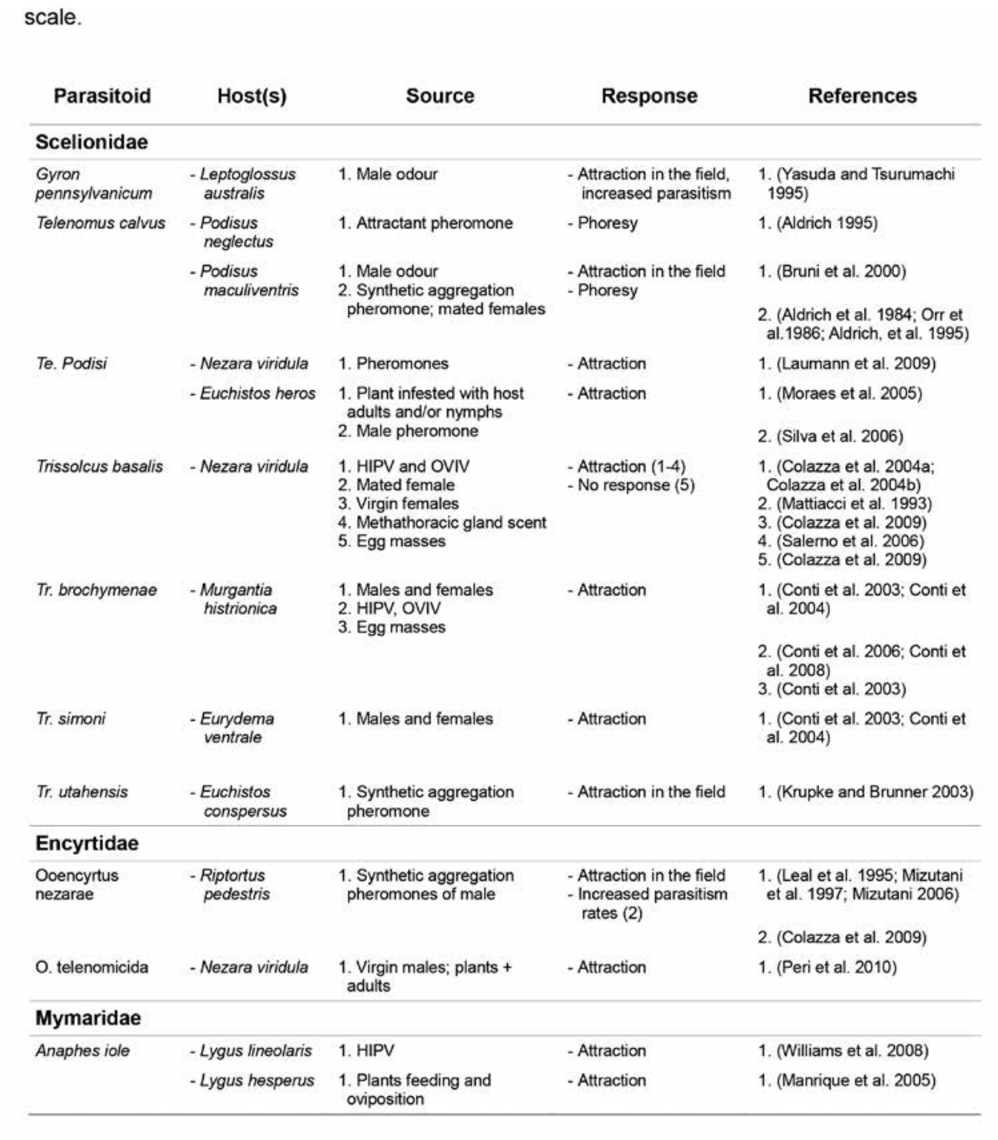


Figure 2.2. Schematic representation of the chemical cues exploited by egg parasitoids of true bugs, ordered according to their reliability-detectability (horizontal axis) and their detection range (vertical axis).

Information exploited by egg parasitoids of true bugs

Egg parasitoids respond to several chemical cues that can be detected at a certain distance from the host plant (volatiles) or after landing on the host plant (non-volatiles). In this context, we will refer to these cues as long-range and short-range cues, respectively. Several studies have described cues exploited by egg parasitoids of true bugs at long range (Table 2.1) and short range (Table 2.2). Infochemicals related to heteropteran eggs present some particular characteristics regarding their reliability and detectability, when compared with other insect orders. First, information from host plants can be reliable to locate eggs of monophagous herbivore species, such as the majority of the Lepidoptera, but it may not be useful to locate eggs of polyphagous species, which is most common within the Heteroptera.

Table 2.1. Cues exploited by egg parasitoids of true bugs to locate their hosts at a long-range scale.

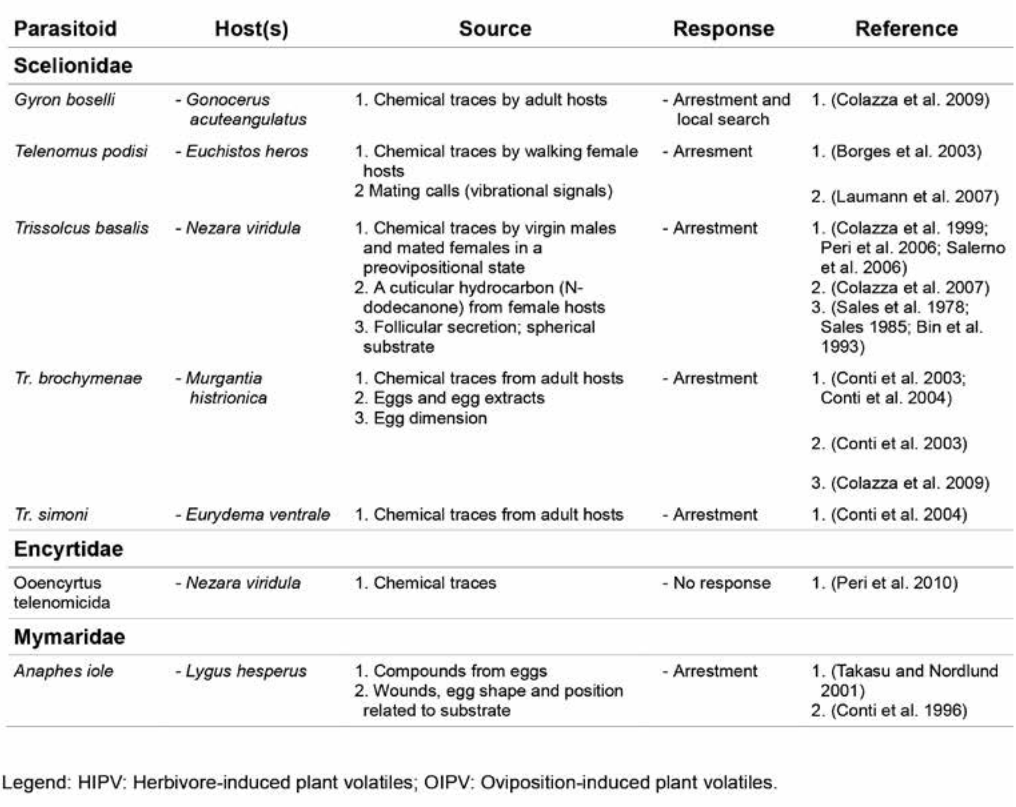


scale.

Parasitoid	Host(s)	Source	Response	References
Scelionidae				
Gyron pennsylvanicum	- *Leptoglossus australis*	1. Male odour	- Attraction in the field, increased parasitism	1. (Yasuda and Tsurumachi 1995)
Telenomus calvus	- *Podisus neglectus*	1. Attractant pheromone	- Phoresy	1. (Aldrich 1995)
	- *Podisus maculiventris*	1. Male odour 2. Synthetic aggregation pheromone; mated females	- Attraction in the field - Phoresy	1. (Bruni et al. 2000) 2. (Aldrich et al. 1984; Orr et al.1986; Aldrich, et al. 1995)
Te. Podisi	- *Nezara viridula*	1. Pheromones	- Attraction	1. (Laumann et al. 2009)
	- *Euchistos heros*	1. Plant infested with host adults and/or nymphs 2. Male pheromone	- Attraction	1. (Moraes et al. 2005) 2. (Silva et al. 2006)
Trissolcus basalis	- *Nezara viridula*	1. HIPV and OVIV 2. Mated female 3. Virgin females 4. Methathoracic gland scent 5. Egg masses	- Attraction (1-4) - No response (5)	1. (Colazza et al. 2004a; Colazza et al. 2004b) 2. (Mattiacci et al. 1993) 3. (Colazza et al. 2009) 4. (Salerno et al. 2006) 5. (Colazza et al. 2009)
Tr. brochymenae	- *Murgantia histrionica*	1. Males and females 2. HIPV, OVIV 3. Egg masses	- Attraction	1. (Conti et al. 2003; Conti et al. 2004) 2. (Conti et al. 2006; Conti et al. 2008) 3. (Conti et al. 2003)
Tr. simoni	- *Eurydema ventrale*	1. Males and females	- Attraction	1. (Conti et al. 2003; Conti et al. 2004)
Tr. utahensis	- *Euchistos conspersus*	1. Synthetic aggregation pheromone	- Attraction in the field	1. (Krupke and Brunner 2003)
Encyrtidae				
Ooencyrtus nezarae	- *Riptortus pedestris*	1. Synthetic aggregation pheromones of male	- Attraction in the field - Increased parasitism rates (2)	1. (Leal et al. 1995; Mizutani et al. 1997; Mizutani 2006) 2. (Colazza et al. 2009)
O. telenomicida	- *Nezara viridula*	1. Virgin males; plants + adults	- Attraction	1. (Peri et al. 2010)
Mymaridae				
Anaphes iole	- *Lygus lineolaris*	1. HIPV	- Attraction	1. (Williams et al. 2008)
	- *Lygus hesperus*	1. Plants feeding and oviposition	- Attraction	1. (Manrique et al. 2005)

To our knowledge, no study on egg parasitoids of true bugs has yet recorded attraction toward volatiles from healthy plants. A better strategy for egg parasitoids of true bugs is to rely instead on feeding-induced or oviposition-induced plant volatiles, *i.e.* solution 2 of the reliability-detectability dilemma. Indeed, after Colazza et al. (2004a) first reported the attraction of the egg parasitoid *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae) to plant volatiles induced by oviposition by *N. viridula* on *V. faba* plants, similar findings have been reported in other egg parasitoids of herbivorous true bugs (Conti et al., 1996; Manrique et al., 2005; Conti et al., 2006, 2010). One of the most remarkable features of the true bugs is the chemical apparency of their mobile stages. In fact, true bugs are characterized by the presence of abdominal glands in the juveniles and metathoracic glands in the adults; both gland

Table 2.2. Cues exploited by egg parasitoids of true bugs to locate their hosts at a short-range scale.



Parasitoid	Host(s)	Source	Response	Reference
Scelionidae				
Gyron boselli	- *Gonocerus acuteangulatus*	1. Chemical traces by adult hosts	- Arrestment and local search	1. (Colazza et al. 2009)
Telenomus podisi	- *Euchistos heros*	1. Chemical traces by walking female hosts 2 Mating calls (vibrational signals)	- Arresment	1. (Borges et al. 2003) 2. (Laumann et al. 2007)
Trissolcus basalis	- *Nezara viridula*	1. Chemical traces by virgin males and mated females in a preovipositional state 2. A cuticular hydrocarbon (N-dodecanone) from female hosts 3. Follicular secretion; spherical substrate	- Arrestment	1. (Colazza et al. 1999; Peri et al. 2006; Salerno et al. 2006) 2. (Colazza et al. 2007) 3. (Sales et al. 1978; Sales 1985; Bin et al. 1993)
Tr. brochymenae	- *Murgantia histrionica*	1. Chemical traces from adult hosts 2. Eggs and egg extracts 3. Egg dimension	- Arrestment	1. (Conti et al. 2003; Conti et al. 2004) 2. (Conti et al. 2003) 3. (Colazza et al. 2009)
Tr. simoni	- *Eurydema ventrale*	1. Chemical traces from adult hosts	- Arrestment	1. (Conti et al. 2004)
Encyrtidae				
Ooencyrtus telenomicida	- *Nezara viridula*	1. Chemical traces	- No response	1. (Peri et al. 2010)
Mymaridae				
Anaphes iole	- *Lygus hesperus*	1. Compounds from eggs 2. Wounds, egg shape and position related to substrate	- Arrestment	1. (Takasu and Nordlund 2001) 2. (Conti et al. 1996)

Legend: HIPV: Herbivore-induced plant volatiles; OIPV: Oviposition-induced plant volatiles.

types are specialized in the secretion of volatile compounds that are repellent to predators (Aldrich, 1988). These defensive compounds constitute easily detectable, as well as highly reliable odor sources that are commonly exploited by egg parasitoids (Aldrich, 1988). In addition, heteropterans are known to emit a diversity of infochemicals involved in intraspecific communication: sex, aggregation, and alarm pheromones. Eavesdropping on the chemical communication system of their hosts is a common infochemical detour employed by egg parasitoids of stink bugs (Leal et al., 1995; Mizutani et al., 1997; Mizutani, 2006), but an empirical study failed to find a significant attraction of the egg parasitoid *Anaphes iole* Girault to pheromones of *L. hesperus* in a Y-tube olfactometer, when compared to clean air controls. A possible explanation for this can be found by comparing this system with the Pentatomidae. Stink bugs usually lay egg in batches. Furthermore, many parasitoids of the Pentatomidae exhibit phoresy, i.e. they can be transported to the oviposition sites by mounting the adult stink bugs (Aldrich, 1988). On the other hand, *L. hesperus* lays solitary eggs inserted into the plant and *A. iole* does not display phoretic behavior. Infochemicals from adult and nymphal stages of the hosts are also exploited by the egg parasitoids of true bugs at a short range to locate the eggs once they have landed on the plant surface. Although no studies have reported attraction of egg parasitoids to the long-range cues emitted by eggs of true bugs, *Trissolcus* spp. and *A. iole* have been shown to respond to contact cues from eggs of their hosts (Conti et al., 1996, 1997; Takasu & Nordlund, 2001; Conti et al., 2010).

Strategies developed by true bugs to avoid egg parasitization

The subtle mechanisms of host location displayed by egg parasitoids impose a great pressure on heteropterans. Particularly, true bugs face a tradeoff between the use of chemical communication and defense, and the risk of parasitism. Adaptive responses against egg parasitization in the Heteroptera can be grouped into three different strategies aimed at (1) avoiding long-range chemical cues; (2) preventing egg parasitoids to access the eggs, and (3) protecting the offspring by guarding the eggs.

Avoid Infochemical-Dense Patches.

One way to protect the eggs from being found by parasitoids is to select oviposition sites that are free from long-range chemical cues. This is commonly the case for pentatomorphans that lay epiphytic clusters of eggs. For instance, *N. viridula* females usually disperse after mating and oviposit on healthy plants (Kiritani et al., 1965). Similarly, those species that oviposit on nonhost plants may benefit by protecting their eggs from being found by natural enemies. This has been reported by *A. sordidus* (Nakajima & Fujisaki, 2010). Another way to avoid long-range cues is by reducing or avoiding the emission of chemical cues that can be eavesdropped by egg parasitoids. Aldrich (1988) suggested that the emission of long-range attractant pheromones by true-bug males rather than females, which is a common pattern in the Pentatomorpha, may have evolved as an adaptation to prevent parasitoids from following mated females to locate their hosts. This strategy can be adaptive if the females disperse after mating, as is the case for *N. viridula*. Consequently, the main egg parasitoid of *N. viridula*, *T. basalis*, has evolved to discriminate the sex of the chemical residues of tarsi left by their host and is attracted only by residues of females (Colazza et al., 2007). In contrast, *Trissolcus brochymenae* (Ashmead) cannot discriminate the sex of adult stages of its host *M. histrionica* (Conti et al., 2003). Unlike *N. viridula*, female adults of *M. histrionica* usually stay in the vicinity of males (English-Loeb & Collier, 1987).

Avoid parasitoid accessibility to eggs

Many true bugs select oviposition sites that are inaccessible to egg parasitoids. For instance, the water strider *Aquarius paludum insularis* Motschulsky usually lays eggs in the water, on aquatic plants, near the water surface. When reared in the presence of a high density of its egg parasitoid *Tiphodytes gerriphagus* Marchal, *A. paludum insularis* preferred deeper oviposition sites in a water tank (Hirayama & Kasuya, 2009). Furthermore, females exposed to a high density of parasitoids maintained the preference for deeper oviposition sites for more than 20 days after the parasitoids had been removed, even when deep oviposition increased the nymphal mortality due to oxygen deprivation and posed mortality risks to the ovipositing females (Hirayama & Kasuya, 2010). This example illustrates the importance of parasitism pressure on oviposition-site selection by true bugs. Host accessibility is also a critical factor for parasitoids of endophytic eggs, so insect females can decide to insert their eggs in plant structures that prevent the parasitoid from reaching them. Evidence of the use of plant structures as protection against natural enemies has been found in the plant bug *L. hesperus*.

This bug prefers to lay eggs in strawberry fruits (*Fragaria* spp.) rather than in other plant parts. This preference is related to a lower parasitization by *A. iole* on strawberry fruits, because the parasitoid wasp cannot penetrate the fruit receptacle (Udayagiri & Welter, 2000). Finally, egg aggregation can also hamper the accessibility of the parasitoid to the eggs. For instance, parasitism of eggs of *Apiomerus crassipes* (F) is limited to the batch edges (Swadener & Yonke, 1973). In the majority of the cases however, egg batches are exposed to attack by parasitoids, and this may explain why parental care is observed in many species of herbivorous true bugs that lay egg batches.

Parental care

Several taxa within the Heteroptera exhibit parental care (reviewed by Tallamy & Wood, 1986; Tallamy & Schaefer, 1997; Goula, 2008) . Tallamy & Schaefer (1997) propose that egg guarding is an ancestral trait in the Heteroptera that tends to be maladaptive to the females. Overall, egg guarding by females constraints the oviposition period and usually the guarding mothers live shorter due to a higher predation rate (Tallamy & Schaefer, 1997). An alternative decision for females of species that exhibit maternal care is egg dumping, defined as placing the eggs under the care of conspecifics (Tallamy, 2005). Egg dumping may reduce predation risk in females and thus increases fecundity as these females live longer. A semi-field experiment showed that eggs of *Gargaphia solani* Heidemann dumped on conspecific batches suffered greater mortality, but their mothers avoided predation more successfully than the guarding females and showed higher total fecundity than the guarding mothers (Tallamy & Horton, 1990).

In summary, top-down factors seem to be an important force that shapes oviposition-site selection by true bugs. Foraging behavior of egg parasitoids of true bugs has evolved to exploit the chemical apparency of the true bugs as an infochemical detour strategy; so many true bug species have developed strategies in order either to avoid ovipositing in volatile-dense environments or to decrease the level of emission, notably those species that display epiphytic oviposition. Endophytic eggs seem to be more protected but a general strategy in true bugs exhibiting this behavior is to rely on plant anatomy features in order to prevent the parasitoids from accessing the eggs. Egg guarding has shown to be a costly strategy but females exhibiting this behavior can still have an alternative solution in dumping their eggs on other egg batches and thus maximizing their realized fecundity.

Concluding remarks and future perspectives

In this review, we have summarized the current knowledge on oviposition-site selection by true bugs focusing on plant-herbivore, herbivore–herbivore, and plant–herbivore–parasitoid interactions. Despite the remarkable importance of this taxon in terms of economical damage and biological control, many questions remain to date. First, although research on preference–performance correlations in true bugs is still scarce, it reveals some differences with the more intensively studied Lepidoptera with monophagous habits and restricted larval mobility and dispersal. Studies to date on true bugs indicate that the relevance of plant quality in oviposition-site selection is lower in the Heteroptera. An interesting system to study is constituted by those species that select nonhost plants for oviposition. To what extent is this behavior an adaptive trait? What are the consequences for the fitness of the offspring? How have those traits associated with this behavior, i.e. a highly mobile second instar nymph, a nonfeeding first instar nymph, and a resource-related emission of an attractive pheromone by adults, evolved?

The theoretical framework developed so far to explain the processes underlying the interaction between feeding- and oviposition-induced responses in plants only takes into account insects that do not feed at the oviposition site. The fact that, unlike the situation for butterflies and moths, feeding damage and oviposition usually co-occur in true bugs poses new questions toward the understanding of the molecular processes underlying the interactions between plants and ovipositing insect herbivores. More research is needed to unravel the plant responses to feeding damage by lacerate-and-flush insects such as heteropterans, as a first step toward elucidating plant–true-bug interactions. Oviposition-induced responses present new challenges because of the coexistence of at least two different oviposition modes in true bugs but a model of plant responses to epiphytic and endophytic oviposition is still in its infancy.

Another aspect that requires further investigation is the role of chemical cues from plants and con-specific true bugs in oviposition-site selection by true bugs. In lepidopteran insects, avoiding the plants already colonized by other insects seems to be common. Information available to date suggests a greater diversity of behaviors in herbivorous Heteroptera, notably between the Cimicomorpha and the Pentatomorpha. Herbivory has evolved from zoophytophagous habits in the former whereas it seems to be a basal trait of the latter. How are those different patterns associated with the evolutionary history of these two taxa?

Foraging strategies of many egg parasitoids of true bugs from a tritrophic perspective have been extensively reported, showing a common pattern of exploitation of host-related pheromones and defensive allomones. The high degree of specificity, reported in some cases, opens an interesting field of research toward the use of these infochemicals for the pest management. Additionally, egg parasitoids of true bugs do not seem to exploit cues from healthy host plants to locate their host, unlike the egg parasitoids of more specialized insects, like some butterflies. On the other hand, research on predators of true bugs is still scarce.

In the last two decades, the understanding of the oviposition decisions made by insects has been enriched with new perspectives, particularly in a multitrophic context. From the multitrophic perspective, the “mother knows best” paradigm now implies not only a role for the plant’s nutritional quality, but also several bottom-up and top-down factors discussed in the present review. True bugs are the great study subjects to understand the implication of the preference–performance hypothesis because of their behavioral diversity. Furthermore, the existence of phylogenetically related predators and prey co-occurring in the same habitat, as is the case for some mirid bugs, provides a unique opportunity to better integrate the factors contributing to oviposition-site selection, and toward a comprehensive understanding of the behavioral ecology of oviposition in herbivorous insects.

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Chapter 3

Life-history analysis of the bronze bug *Thaumastocoris peregrinus* in a newly designed mass rearing strategy

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Abstract

The bronze bug, *Thaumastocoris peregrinus* Carpintero et Dellape (Heteroptera: Thaumastocoridae), is one of the most important emerging pests of *Eucalyptus* LHeritier plantations worldwide. In the development of strategies to control this pest, establishing effective rearing protocols is fundamental to future research programs. We assessed life-history parameters of the bronze bug in a newly designed mass rearing strategy. Separated units were set up to contain different developmental stages. Egg production by females reared on commonly found *Eucalyptus grandis* Hill ex Maiden and *E. tereticornis* Smith was evaluated in order to determine which plant species to use in rearing. Females laid more eggs on *E. tereticornis* than on *E. grandis*, so the former species was chosen for the rearing. A cohort of 207 eggs was followed in Petri dishes until the last individual died or reached the adult stage. We followed egg production by 15 adult couples from the original cohort. Preparation of 150-200 dishes with hatching eggs per week allows for an average production of 7,500 eggs per week. Under our rearing conditions, eggs started hatching on day six, and the first adults were obtained 23 days after oviposition. Almost half of the eggs did not hatch, and the highest nymphal mortality was recorded in the second instar, while the lowest mortality occurred in the last instar. We discuss the relevance of this mass rearing strategy, both within the context of basic behavioral studies of *T. peregrinus*, and as a tool for the mass rearing of the biological control agent, *Cleruchoides noackae* Lin et Huber.

Keywords: rearing protocols, Eucalyptus, forestry, *Cleruchoides noackae,* biological control.

Introduction

The bronze bug *Thaumastocoris peregrinus* Carpintero et Dellape is a major pest of *Eucalyptus* LHeritier plantations worldwide. This small true bug started to disperse from its Australian origin in the beginning of the 2000s invading South Africa (Jacob & Neser, 2005), Argentina (Noack & Coviella, 2006), Brazil (Wilcken et al., 2010), Uruguay (Martínez & Bianchi, 2010), Chile (Ide et al., 2011), New Zealand (Sopow et al., 2012), Italy (Laudonia & Sasso, 2012), and Portugal (Garcia et al., 2013). Management techniques proposed to control this insect include chemical control (Noack et al., 2009), the use of entomopathogenic fungi (Mascarin et al., 2012), and parasitoid wasps (Nadel & Noack, 2012) for biological control. The egg parasitoid *Cleruchoides noackae* Lin et Huber (Hymenoptera: Mymaridae) was identified in Australia from specimens collected in a rearing colony of *T. peregrinus* (Lin et al., 2007) and has been proposed as a candidate for a biological control program (Nadel et al., 2012; Nadel & Noack, 2012). This parasitoid has been released in Chile (SAG, 2010), Brazil (IPEF, 2012), Uruguay (INIA, 2013), and South Africa (Anonymous, 2013). In order to evaluate the parasitoid's biological characteristics and to support the large-scale implementation of biological control of *T. peregrinus* by *C. noackae,* a continuous supply of fresh eggs of the parasitoid's host is essential. However, developing a successful mass rearing methodology for *T. peregrinus* has proven to be difficult (Nadel & Noack, 2012).

The first rearing method described for this insect involved the use of leaf sections attached to entomological pins inside inverted polypropylene vials with caps half-filled with water, generating a 'fluid barrier' (Noack & Rose, 2007). Similarly, Soliman et al. (2012) used Petri dishes with leaf sections floating on water, a setup that allowed them to estimate the duration of different stages and the fecundity of females when reared on different *Eucalyptus* genotypes and hybrids. The water barrier was necessary to maintain the insects on the leaf section, because the juveniles are very mobile. However, the use of Petri dishes for mass production of eggs is costly in terms of labor, time and resources. Here, we describe a mixed method for mass rearing of *T. peregrinus* by using Petri dishes and mesh screen cages. In addition, we provide quantitative information on several life history parameters, obtained during the development of this rearing method.

Materials and Methods

Origin of the colony

Adults and 4th-5th instar nymphs of *T. peregrinus* were collected from natural populations in different plantations in Uruguay in the summer of 2010: a commercial plantation of *E. grandis* Hill ex Maiden (31°53'56.40"S; 55°48'29.80"W), a seed orchard of *E. globulus* Labillardiere (34°39'49.62"S; 56°20'23.23"W), and a shadow and shelter plantation of *E. tereticornis* (31°44'19.24"S; 55°58'43.75"W). The specimens collected were placed in a mesh screen cage (founder cage) (0.5 x 0.5 x 1.0 m) containing a seedling of *Eucalyptus* spp and were left to oviposit. The cages were located in a climate-controlled room at 20 ± 5 °C, 55 ± 10 % RH (mean ± SEM throughout) and provided with natural daylight.

Egg production on different Eucalyptus species

The most abundant eucalypt species in the region are rose gum (*E. grandis*) and red gum (*E. tereticornis; E. camaldulensis* Dehnhardt and hybrids). In order to decide which species to use in the rearing experiments, 60 males and 75 females of *T. peregrinus* were left to oviposit in cages containing either *E. grandis* or *E. tereticornis* branches, with six replications of each treatment. The total number of eggs from each cage was counted after three days and egg production rates were compared by a Wilcoxon rank sum test by using R statistical software (R Development Core Team, 2009).

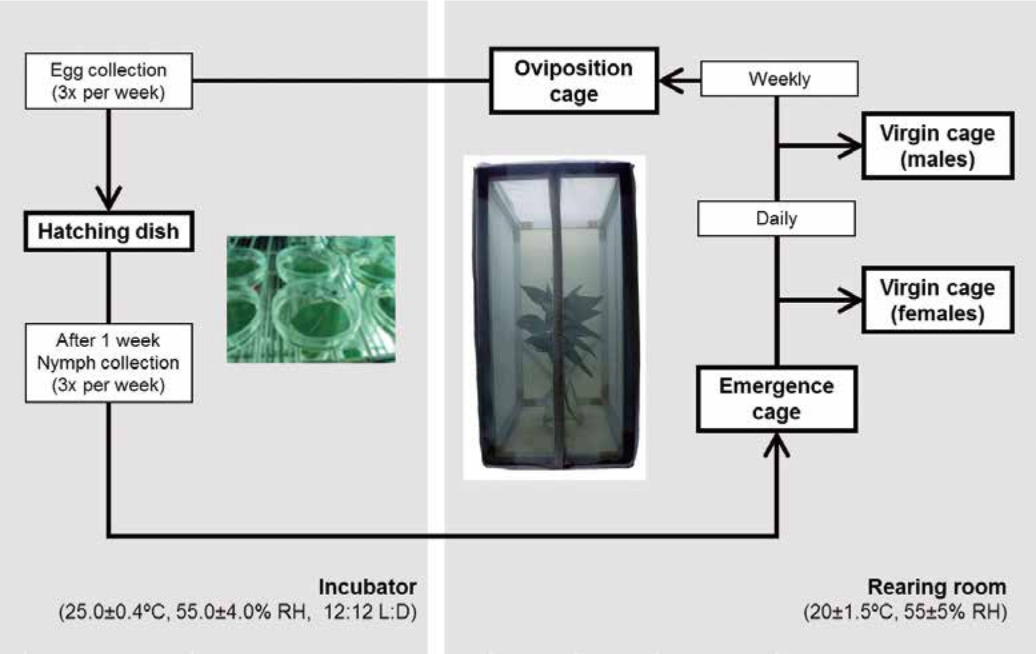


Figure 3.1. Rearing scheme developed for *Thaumastocoris peregrinus*.

After two generations in the founder cage, adult *T. peregrinus* were transferred to smaller mesh-screen cages (0.35 x 0.50 x 0.70 m), hereafter called oviposition cages (Figure 3.2a). Each oviposition cage contained approximately 80 females and 60 males, in accordance with the sex ratio observed in field collections during summer, which was around 0.75 m/f (Gonzalo Martínez, unpublished). Fresh *E. tereticornis* branches were provided for food and oviposition. Branches were previously cleaned with 5% sodium hypochlorite solution and rinsed with distilled water to decrease the pathogen load, then placed in an Erlenmeyer flask with distilled water (Figure 3.2b). Branches were replaced every two days and the insects were counted, sexed and relocated to the new branches with the help of a paintbrush. Simultaneously, eggs laid in old leaves were collected and counted. Egg-carrying leaves were detached from the branch, and leaf sections containing eggs were cut out carefully. These leaf sections were placed in Petri dishes ('hatching dishes') (5.5 cm diameter) and incubated in a rearing chamber set at 25.0 ± 0.5 °C, 55.0 ± 1.5 % RH, and 12:12 L:D. Each hatching dish contained up to 10 eggs placed onto a larger leaf disc, cut out from a mature *Eucalyptus* spp leaf, which was floating on water and covered most of the dish base (Figure 3.2c). Eggs were checked each day for hatching. After molting, second instar nymphs were transferred with a paintbrush to a mesh screen cage (maturation cage) similar to that used for adults, and provided with fresh *E. tereticornis* branches. The maturation cages were checked daily for adult emergence. Recently molted adults, recognized by their whitish integument, were sexed and transferred to separate cages for virgin males and virgin females ('virgin cages'), with characteristics similar to the 'maturation' and 'oviposition cages'. If newly emerged adults had their cuticle already sclerotized, they were not considered virgins and they were transferred to oviposition cages with mixed sexes.

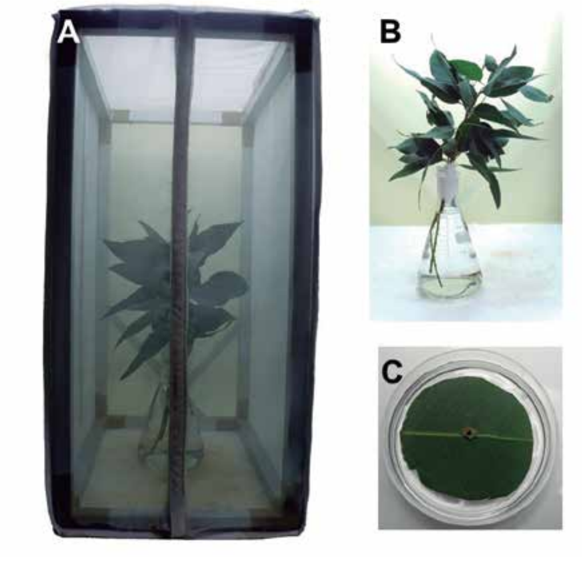


Figure 3.2. Different rearing units used for the rearing of *Thaumastocoris peregrinus*: (A) Mesh screen cage; (B) Branches used within the cages; (C) Hatching dish with a patch of eggs of *T. peregrinus* in the center.

Life cycle at rearing conditions

A life table was constructed after following a cohort of 207 eggs from the hatching dishes in the rearing conditions described above. A life table represents quantitatively the main characteristics of the age-specific reproduction and mortality (Southwood, 1978). After hatching, each nymph was transferred individually to a Petri dish and observed each day at the same hour. All Petri dishes were incubated at 25.0 ± 0.5 ºC, 55.0 ± 1.5 % RH and 12:12 L: D. Survival and molting events were recorded until the last individual had died or reached the adult stage. This methodology was chosen to compare the results with other studies (Soliman et al., 2012). A horizontal life table (i.e.: grouping individuals into the same instar) was built by calculating the following variables:

a_o: Total number of living individuals at the beginning of the experiment

a_x: Total number of living individuals observed at the beginning of each instar

$l_x=(a_x/a_o)$: Proportion of the individuals of the original cohort that remained alive at the beginning of each instar

$d_x=(l_x-l_{x+1})$: Proportion of the original cohort that died during each instar

$q_x=d_x/l_x$: Stage-specific mortality rate

$K_x=\log_{10}(a_x) - \log_{10}(a_{x+1})$: Killing force

Once the adult stage had been reached, individuals from the original cohort were placed as male-female couples into new Petri dishes, and egg production was recorded daily until the last female had died.

Simultaneously, the productivity of the rearing strategy was measured by computing the total number of eggs produced per week and per cage. The number of eggs laid by individual females was estimated by dividing the total number of eggs by the number of females alive during that week.

Results

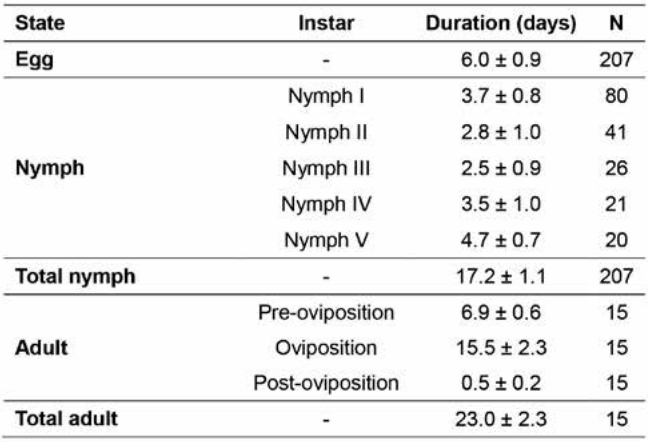
A total of 516 adults and 407 nymphs were collected in the field and used for rearing the first generations in three founder cages. The rearing system consisted of six oviposition cages, three maturation cages, two virgin cages (one for each sex), and an average number of 250 egg-hatching dishes in two incubator chambers. Three technicians spent an average of six hours per day performing rearing tasks; including counting and sexing individuals, mounting hatching dishes, preparing and changing branches, and processing data.

Egg production on different Eucalyptus species

Females laid significantly more eggs on *E. tereticornis* than on *E. grandis* (Wilcoxon rank sum test; W = 0, p = 0.002, n = 6, Figure 3.3), so we decided to use this species in all rearing activities.

Life cycle at rearing conditions

Table 3.1. Duration of the stages of *Thaumastocoris peregrinus* in the rearing conditions. N represents number of individuals.



State	Instar	Duration (days)	N
Egg	-	6.0 ± 0.9	207
Nymph	Nymph I	3.7 ± 0.8	80
	Nymph II	2.8 ± 1.0	41
	Nymph III	2.5 ± 0.9	26
	Nymph IV	3.5 ± 1.0	21
	Nymph V	4.7 ± 0.7	20
Total nymph	-	17.2 ± 1.1	207
Adult	Pre-oviposition	6.9 ± 0.6	15
	Oviposition	15.5 ± 2.3	15
	Post-oviposition	0.5 ± 0.2	15
Total adult	-	23.0 ± 2.3	15

A total of 113 nymphs hatched from the 207 eggs, most of them during the sixth day after egg laying. The total duration of the nymphal stages of *T. peregrinus* was 17.2 ± 1.1 days (Table 3.1), which were divided in five instars as recorded in previous studies (Noack & Rose, 2007; Soliman et al., 2012) (Figure 3.4). Higher nymph mortality rates were recorded in the early instars, with the second instar showing the highest stage-specific mortality rate (q_x = 0.488) (Table 3.2). The last nymphal instar lasted longest (4.7 ± 0. 7 days) (Table 3.1) and exhibited the lowest mortality rate (q_x = 0.05) (Table 3.2).

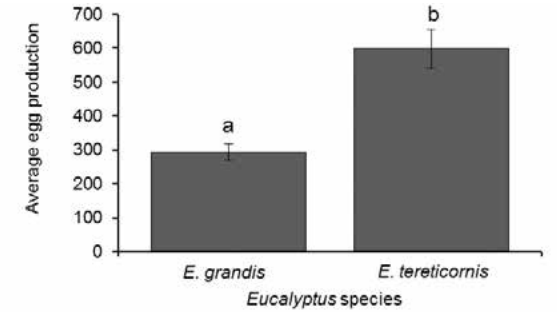


Figure 3.3. Egg production of *Thaumastocoris peregrinus* after three days in rearing cages containing 75 females and 50 males. Branches of two *Eucalyptus* species were used as feeding and oviposition substrates. Different letters indicate significant differences (Wilcoxon rank-sum test; p= 0.002; n= 6).

Egg production

For the reproductive period we followed egg production of 15 adult couples (Table 3.1). As we obtained less than 30 individuals from the original cohort, couples were completed by using individuals from the general rearing that had emerged on the same day. The conditions during this part of the study were not the same as in the general rearing, because the couples were placed in Petri dishes instead of mesh screen cages, in order to follow individual couples. Nonetheless, the results provide a rough estimate of life span and egg production rates that can be expected in the rearing conditions. The average total duration of the adult stage was 23.0 ± 2.3 days, with a pre-oviposition period of 6.9 ± 0.6 days. The females laid eggs for 15.5 ± 2.3 days, and died soon after egg laying ended (post-oviposition period: 0.5 ± 0.2 days). Daily egg production during the reproductive period was 2.5 ± 0.1. After day 29 only one couple was alive, and hence data on its egg production were not included (Figure 3.5).

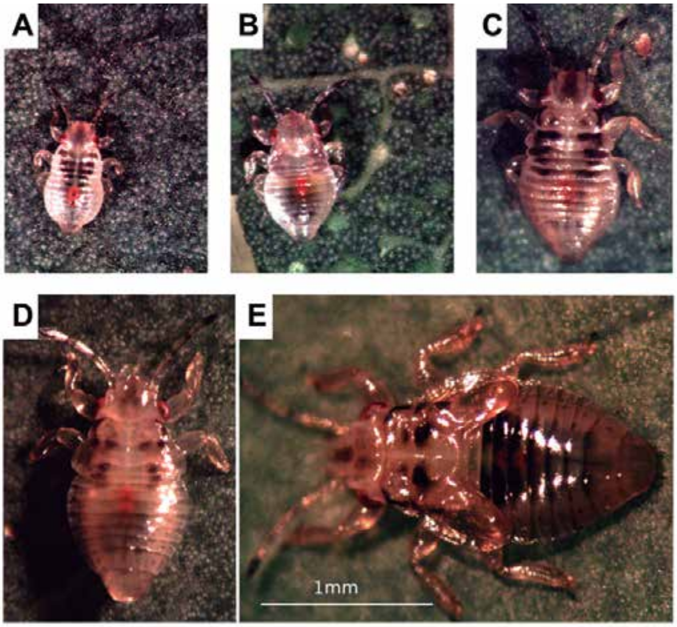
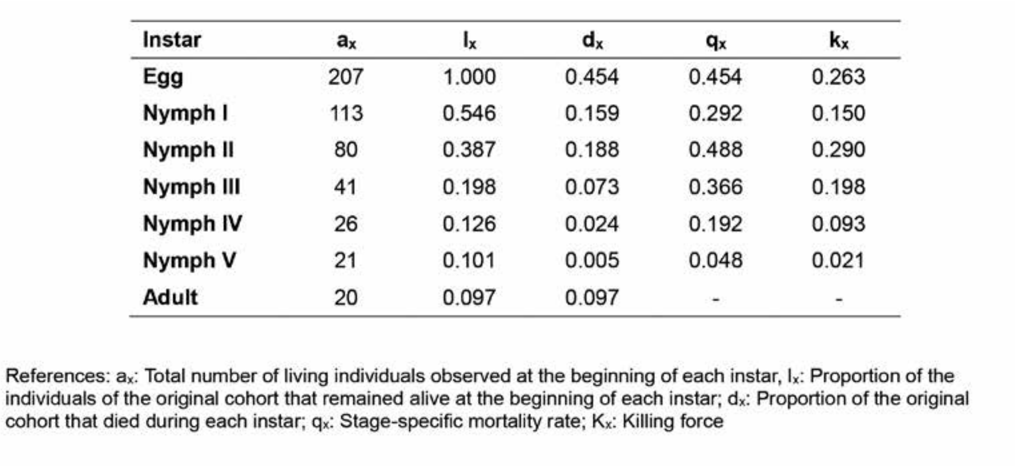


Figure 3.4. Nymphal stages of *Thaumastocoris peregrinus*. (A) Nymph I; (B) Nymph II; (C) Nymph III; (D) Nymph IV; (E) Nymph V.

Figure 3.6 shows the weekly egg production in the rearing colony of *T. peregrinus* during 2012, when the rearing scheme presented here was fully implemented. The first weeks showed the lowest production, which coincided with moving to a new building with better thermal insulation. After week 10, egg production stabilized between 1000 and 1500 eggs per cage per week, showing sporadic peaks such as those observed during weeks 10, 17 and 46. Not only a higher total production of eggs but also a higher number of eggs laid per female was recorded during these peaks, which in turn coincided with longer periods without manipulation because of holidays, suggesting that stress due to manipulation (changing of branches, counting of individuals) may have an effect on egg productivity.

Table 3.2. Life table for *Thaumastocoris peregrinus* in the rearing conditions (n= 207).



Instar	a_x	l_x	d_x	q_x	k_x
Egg	207	1.000	0.454	0.454	0.263
Nymph I	113	0.546	0.159	0.292	0.150
Nymph II	80	0.387	0.188	0.488	0.290
Nymph III	41	0.198	0.073	0.366	0.198
Nymph IV	26	0.126	0.024	0.192	0.093
Nymph V	21	0.101	0.005	0.048	0.021
Adult	20	0.097	0.097	-	-

References: a_x: Total number of living individuals observed at the beginning of each instar, l_x: Proportion of the individuals of the original cohort that remained alive at the beginning of each instar; d_x: Proportion of the original cohort that died during each instar; q_x: Stage-specific mortality rate; K_x: Killing force

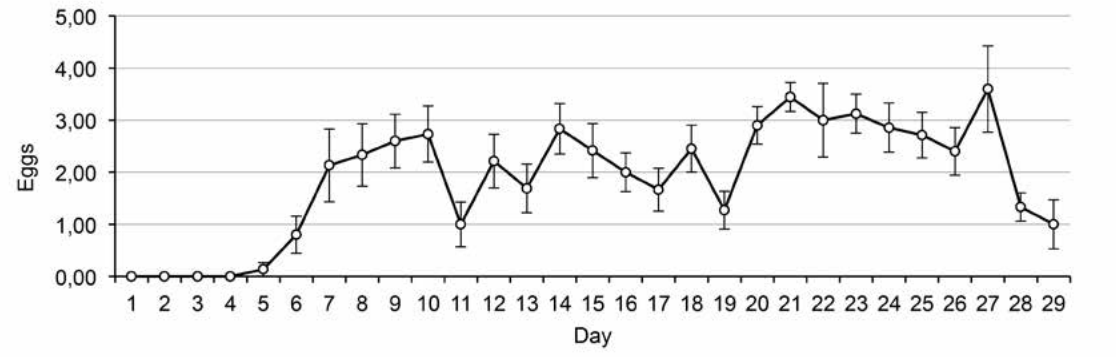


Figure 3.5. Average daily egg production by individual *Thaumastocoris peregrinus* couples in Petri dishes (initial n= 15).

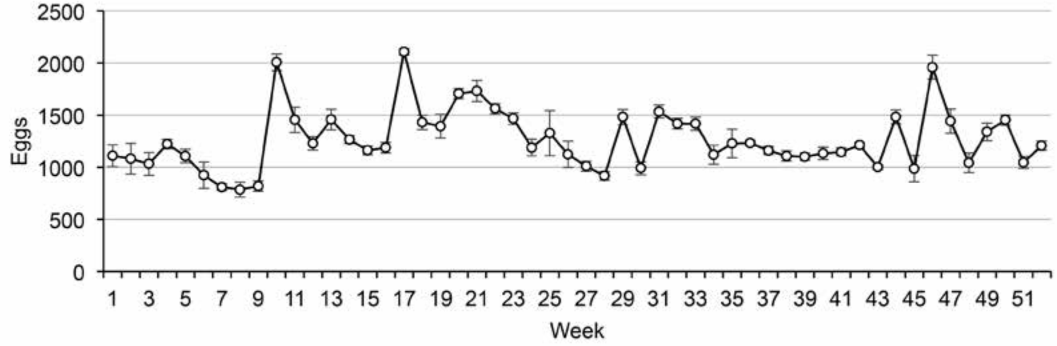


Figure 3.6. Average weekly egg production by *Thaumastocoris peregrinus* per cage, once the rearing scheme had been fully implemented (n=6).

Discussion

The rearing method presented here allowed for a successful production of *T. peregrinus* eggs at a large scale; which can be both used as a source for new colonies (if needed for additional studies) or as hosts for biological control agents. By preparing 150-200 hatching dishes per week, which can be accomplished by two people, part-time, an average production of 7,500 eggs can be obtained. The eggs were collected within 48 h after oviposition, which coincides with the time window for parasitization by the egg parasitoid *Cleruchoides noackae* (Mutitu et al., 2013).

An efficient rearing method requires plant material that not only optimizes egg production, but is also easy to obtain and manipulate. In our study, a larger number of eggs were produced when *T. peregrinus* females were offered *E. tereticornis* instead of *E. grandis*. Although to our knowledge no reports are available on the fecundity of the bugs when feeding on *E. tereticornis,* a previous study showed higher oviposition rates on *E. grandis* than on *E. camaldulensis* (Soliman et al., 2012), a species closely related to *E. tereticornis*, with which hybrids are usually formed in Uruguay and Argentina. Hence, the eucalypt species has an impact on *T. peregrinus* oviposition rates, and therefore is an important factor for rearing optimization.

Under the rearing conditions used in this study, it took 23 days for eggs to develop into adults, including an average of 6 days for egg hatching. Since first-instar nymphs are highly susceptible to fungal attack, which in turn may be favored by water condensation inside the Petri dish, the frequency of checking hatching dishes for newly emerged nymphs was important for improving survival. The duration of the immature stages in our study was somewhat shorter than that previously reported for *T. peregrinus* in the field (Jacob & Neser, 2005; Bouvet & Vaccaro, 2007). The relative duration of the immature instars was similar to that reported by Soliman et al. (2012) for a laboratory study: the first, fourth and fifth instars lasting longer than the second and third. While mortality varied among stages and instars, highest mortality occurred during the egg stage, since almost half of the eggs did not hatch. This can be due to several causes such as desiccation of the leaf on which they had been laid, drowning in condensation water, or the fact that *T. peregrinus* can lay unfertilized eggs just as recorded for other heteropterans (Schuh & Slater, 1995). Previous studies have reported variable hatching rates for eggs obtained *in vitro*, from 22 % (Noack & Rose, 2007) to 80 % (Soliman et al., 2012) and even 95 % (Mutitu et al., 2013). It is hence evident that factors affecting egg viability should be studied and eventually improved, for example by dissecting unhatched eggs and preventing desiccation or excessive water. Early nymphs showed greater susceptibility to manipulation, which led us to wait for at least one molt in the dish before touching the individuals. Later nymphal instars showed significantly less mortality, and were hence easily manipulated. In our study in Petri dishes, pre-oviposition time lasted about a week, which is shorter than the ten days reported on *E. camaldulensis* (Soliman et al., 2012).

The total duration of the adult stage was 23.0 ± 2.3 days, slightly more than half of what has been previously reported (Bouvet & Vaccaro, 2007; Martínez & Bianchi, 2010; Wilcken et al., 2010; Soliman et al., 2012). Such reduction in adult lifespan could be a consequence of the experimental setup, including the choice of the *Eucalyptus* species, which could have reduced the post-ovipositional period in our experiment. The total egg production per female in individual Petri dishes was 39.5 ± 6.1, which falls between the productivity reported for females feeding on *E. camaldulensis* and *E. grandis* (23 and 75 eggs/female, respectively) (Soliman et al., 2012).

Egg production has been maintained within the range of 1000 to 1500 per cage per week, or ranging from 11 to 24 per female per week. These values correspond to a daily oviposition rate between 1.6 and 3.4 eggs per female, which matches the daily egg production we observed for individual *T. peregrinus* in Petri dishes (2.5 ± 0.1), as well as previous reports (Noack & Rose, 2007; Soliman et al., 2012). The egg production in the rearing increased during several periods when the cages remained uninspected, suggesting that manipulation of the individuals due to manual transfer from old branches to new ones negatively affected reproductive parameters. An alternative way of transferring the insects to new feeding leaves is currently under investigation.

The importance of developing a reliable rearing scheme for the bronze bug is evident given the rapid spread of this pest in the world, and the need for a continuous egg supply to develop a successful biological control program with the egg parasitoid *C. noackae*. Furthermore, a constant supply of individuals is particularly required in order to continue basic biological studies of both the pest and the parasitoid. Finally, the weekly counting of the products obtained from each rearing unit (whether eggs, juveniles or adults) paves the way for assessing the health of the colony, and for identifying the sources of eventual rearing problems.

Acknowledgements

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Chapter 4

Oviposition preference but not adult feeding preference matches with offspring performance in the bronze bug *Thaumastocoris peregrinus*

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Abstract

Optimal foraging and optimal oviposition are two major forces leading to plant selection by insect females, but the contribution of these forces to the host-selection process has been little studied for sucking herbivores. We studied feeding and oviposition behavior of a global pest, the bronze bug, *Thaumastocoris peregrinus* Carpintero & Dellapé (Heteroptera: Thaumastocoridae), by using dual-choice bioassays to evaluate the preference of females between host species, developmental leaf stage, or prior plant exposure to conspecifics. We assessed the link between these preferences and the performance of the offspring, by comparing survival and developmental time of nymphs reared on the various treatments. Finally, we compared the composition of the leaf wax of healthy and damaged leaves, and tested the effects of leaf wax on female preference behavior. Using healthy adult leaves of *Eucalyptus tereticornis* Sm. (Myrtaceae) as a reference, we found that females prefer to feed on *Eucalyptus grandis* W. Hill ex Maiden and *E. tereticornis* adult leaves that had been previously damaged by female conspecifics, whereas they reject juvenile leaves of *E. tereticornis* as food. Females also prefer to oviposit on leaves previously damaged by conspecifics but they rejected *E. grandis* as oviposition substrate. Nymphal performance varied among leaf treatments, suggesting a correlation with oviposition preference (but not feeding preference). Epicuticular wax extracts from damaged leaves contained higher concentrations of long-chain, saturated linear alkanes, aldehydes, and alcohols than extracts from undamaged leaves. However, a choice assay failed to demonstrate an oviposition preference based on leaf surface wax chemistry. We discuss these findings in the context of the preference-performance relationship.

Key words: reference-performance linkage, true bugs, host selection, mother-knows-best hypothesis, leaf epicuticular wax, forestry pest, Heteroptera, Thaumastocoridae, dual-choice assay

Introduction

Gravid female insects face two major challenges: finding food and adequate substrates for their eggs. Optimal foraging theory predicts that adult insects prefer to feed on those host plants that provide the best adult performance (Stephens & Krebs, 1986). On the other hand, optimal oviposition theory predicts that insect females should choose to oviposit on those host plants that maximize the fitness of their offspring, resulting in a positive correlation between oviposition-site preference and offspring performance (Jaenike, 1978). The latter has been the leading thought for explaining host-plant selection by phytophagous insects, even when empirical data on various species do not show a correlation between female oviposition choice and offspring performance (Thompson, 1988; Thompson & Pellmyr, 1991; Mayhew, 1997). In some cases, this lack of correlation can be explained by not only considering the intrinsic properties of plants, but also the wider effects on offspring performance such as the likelihood of mortality due to natural enemies (Ohsaki & Sato, 1994; Dicke, 2000).

There are several scenarios, however, in which the choice of a host plant may be better explained by optimal feeding opportunities (Scheirs & De Bruyn, 2002). If females feed as adults, their fecundity may not be as strongly dependent on larval resources as in species that do not feed in the adult stage, leading to a weaker link between preference and performance (Gripenberg et al., 2010). For instance, adults that exploit a different feeding resource than their offspring may choose to oviposit on suboptimal offspring hosts if these are closer to a mother's food source (Murphy, 1983; Renwick & Chew, 1994). However, if insects feed on the same resource during all stages, it becomes difficult to discriminate feeding preferences from oviposition preferences. In this case, adult performance on the host may be the main factor leading to host selection if oviposition sites are found in the close vicinity of adult feeding sites (Jaenike, 1986; Fujiyama et al., 2008).

In plants like *Eucalyptus* spp. (Myrtaceae), which exhibit heteroblastic development (Potts & Wiltshire, 1997), leaf chemistry or toughness, particularly differences between leaf developmental stages (juvenile vs. adult leaves), may be relevant factors mediating host-plant selection, possibly affecting feeding and oviposition choices. In line with this, several insect species that feed on *Eucalyptus* foliage show uneven distributions of immature and adult stages with respect to leaf developmental stage (Brennan et al., 2001; Brennan & Weinbaum, 2001a,b; Lawrence et al., 2003).

Herbivorous true bugs usually exploit the same resources throughout their lifetime, and exhibit broad host-plant ranges. Sometimes oviposition can be linked to female foraging activities (Ventura & Panizzi, 2003), but in several other cases oviposition-site selection can be attributed to other factors such as plant structure (Benedict et al., 1983; Ventura & Panizzi, 2003), inaccessibility to natural enemies (Udayagiri & Welter, 2000; Hirayama & Kasuya, 2009), or competitor-free space (Kiritani et al., 1965; English-Loeb & Collier, 1987). Understanding the behavioral patterns involved in host-plant selection is important to gain ecological knowledge of herbivorous insects. Such understanding is relevant in species of economic importance, and may be used in the development of sustainable management strategies (Cook et al., 2007). However, it has been poorly investigated in herbivorous sucking feeders and particularly in true bugs.

Here, we studied feeding and oviposition preferences, and their link to nymphal performance, for an important pest of *Eucalyptus* spp. grown in plantations worldwide, the bronze bug, *Thaumastocoris peregrinus* Carpintero & Dellapé (Heteroptera: Thaumastocoridae) (Nadel & Noack, 2012). We used dual-choice assays to evaluate the preference of females for leaves of three *Eucalyptus* species, two developmental leaf stages, and the effect of prior exposure of plants to bronze bug conspecifics. In addition, we determined the performance of nymphs on the different substrates offered to the ovipositing females. Finally, as female preference may be determined by the chemistry of leaf epicuticular lipids, we compared wax composition and tested the preference of females for wax extracts of leaves with previous damage by the bronze bug.

Materials and methods

Insects and plants

The bronze bug *T. peregrinus* feeds on the leaves of plants in the genus *Eucalyptus*, including the species most extensively used for commercial forestry: *Eucalyptus grandis* W. Hill ex Maiden, *Eucalyptus globulus* Labill., *Eucalyptus camaldulensis* Dehnh., *Eucalyptus urophylla* S.T. Blake, and hybrids (Soliman et al., 2012). Adult females live ca. 45 days and usually lay eggs in communal patches (Martínez et al., 2014). Eggs hatch after 6 days at 20-25 ºC, and nymphs go through five instars before reaching the adult stage. Male and female adults of *T. peregrinus* were obtained from a stable indoor mass rearing maintained on *E. tereticornis* (INIA-Tacuarembó; Martínez et al., 2014), within 2 days of their last molt. They were allowed to mate in a cage consisting of an aluminum framework (35 × 50 × 70 cm) covered with mesh screen, and fed with fresh shoots of *E. globulus* in Erlenmeyer flasks, with new shoots provided every other day. We provided *E. globulus* prior to the preference bioassays in order to feed the insects with a food source that would not be included in the experiments. The insects were left in this cage for 1 week prior to the experiments, and the females were considered to have mated during this period (Noack & Rose, 2007; Soliman et al., 2012; Martínez et al., 2014).

Leaves of different *Eucalyptus* species were obtained from trees with adult foliage (fully extended leaves, between 2 and 4 years old), planted at INIA Experimental Station (31°44'23.84"S, 55°58'44.74"W). Juvenile leaves of *E. tereticornis* were detached from saplings produced from seeds of the same trees that we used as sources of adult leaves.

Preference

We assessed feeding and oviposition preference of mated *T. peregrinus* females in an experimental arena consisting of a Petri dish (5.5 cm diameter) containing two freshly-cut leaf squares (2 × 2 cm). One of the squares corresponded to the treatment being assessed, the other was in all cases cut from a healthy adult leaf of *E. tereticornis*, which we defined as our control given the observed preference for this plant species, both under rearing conditions and in the field (Martínez et al., 2014). The abaxial side of the leaf squares was used as contact surface for the insects, and the two squares were joined with adhesive tape affixed to the adaxial side. A white paper strip (2 × 0.5 cm) was attached to the center of the arena as a neutral release area. The experimental arena was delimited by distilled water both to prevent the bugs from escaping and as moisture medium for the leaf squares (Soliman et al., 2012; Martínez et al., 2014). To do this, the arena was slightly elevated from the bottom of the Petri dish with two pieces of cotton swabs, and water was added up to the height of the leaf squares.

We assessed the feeding and oviposition preference of *T. peregrinus* females between paired options consisting of *E. tereticornis* adult leaves vs. *E. grandis* or *E. benthamii* adult leaves or *E. tereticornis* juvenile leaves. Using the same experimental design, we also evaluated the effect of previous presence of conspecifics of the bronze bug on the preference of the females. In this case, we tested a healthy (= undamaged) adult leaf of *E. tereticornis* against leaves that had been exposed to different densities of insects for 1 week. In order to obtain such leaves, saplings of the same origin and age were either exposed to five mated females (low density), 70 or more females (high density), 70 or more males, or no insects as the control. The level of high density was decided based on densities observed in field surveys (Martínez & Gómez, 2014).

A slightly modified arena was used to test the role of leaf epicuticular waxes on the preference of females. In this case, we used a rectangular (2 × 4 cm) leaf piece cut out of a healthy *E. tereticornis* adult leaf, and two squares of filter paper (1 × 1 cm) that were affixed onto the leaf piece and topically treated with leaf epicuticular wax extracts (see section: extraction and analysis of epicuticular lipids below). The preference of females was evaluated for wax extracts from undamaged and damaged *E. tereticornis* adult leaves. Damaged leaves were obtained by exposing saplings to 25 couples of *T. peregrinus* for a week. The extracts were applied in dichloromethane (10 µl) at a dose of 50 µg cm^{-2}, which is the calculated surface concentration of wax on *E. tereticornis* leaves (calculated after Diao et al., 2010).

All preference experiments were conducted with individual, 1-week-old mated females, initially gently placed in the center of the arena with a paint brush. Each female was used once and then discarded. The Petri dishes were kept at 25 ± 5 °C, 55 ± 10% r.h. (mean ± SEM throughout), and L12:D12 for 48 h, after which oviposition and feeding were assessed by counting eggs and fecal drops, respectively.

Nymphal performance

Recently laid eggs (ca. 10) were placed on a leaf square (3 × 3 cm) floating on distilled water in a Petri dish (5.5 cm diameter). Immediately after hatching, neonates were transferred individually to separate Petri dishes containing leaf squares (3 × 3 cm) belonging to one of the following treatments: undamaged adult leaves of *E. tereticornis*, *E. grandis*, or *E. benthamii*, or adult leaves of *E. tereticornis* previously exposed for 2 days to 100-130 adult *T. peregrinus*. Leaf squares were renewed every other day, and nymphs were observed daily at the same hour, until the last individual reached the adult stage or died. In total 400 eggs were followed (100 per treatment). Causes of death were grouped into one of the following categories: disease, drowning, and dead during molting.

Extraction and analysis of epicuticular lipids

Epicuticular wax extracts were obtained from undamaged and damaged *E. tereticornis* adult leaves (leaf fresh weight: 0.61 ± 0.04 vs. 0.52 ± 0.04 g, respectively; n= 10 per treatment; t= 1.591, d.f.=18, P=0.129). We used damaged leaves exposed to 25 couples of *T. peregrinus*, as described in section 'Preference'. The leaves were individually dipped in dichloromethane for 30 ss with vortex agitation. The solvent was left to evaporate at ambient temperature, and the remaining material was weighed and re-dissolved in dichloromethane to obtain a concentration of 2 mg ml^{-1}. A portion (1 ml) of the extract was used for chemical analysis after adding a known amount of n-tridecane as internal standard, and the remaining extract was used for oviposition preference bioassays.

Wax extracts were analyzed by gas chromatography and mass spectrometry (GC-MS) using a Shimadzu QP-2010 machine equipped with a non-polar column (RTx-5ms, 30 m, 0.25 µm, 0.25 mm i.d.; Restek, Bellefonte, PA, USA) and a constant carrier flow of 1 ml helium per min. The temperature of the GC oven was set from an initial 40 ºC for 5 min, then heated at 8 ºC per min up to 300 ºC, and held for 15 min. The injector temperature was set to 250 ºC and the interphase temperature was 310 ºC. Injection (1 µl) was done in splitless mode, and mass spectral data were collected in the scan mode at 70 eV (mass range 30-550, ion source 200 ºC). Linear retention indices (RI) were calculated from the relative retention times of the separated extract components with those of a blend of n-alkanes that was injected immediately after the samples. The main compounds in the extracts were therefore identified on the basis of their retention indices and mass spectra, using the mass spectral database NIST 08. Quantification was done by comparison of the TIC (Total Ion Chromatogram) peak areas relative to that of the internal standard (n-tridecane).

Data analysis

In the dual-choice preference assays, female preference was analyzed by calculating the proportion of fecal drops and eggs in the tested treatment, relative to the total number of fecal drops and eggs (treatment + control), following Grostal & Dicke (1999). Hence, feeding and oviposition preference indices were established for each replicate, so that zero represents a complete preference for the control (*E. tereticornis* undamaged adult leaves), 0.5 represents no preference, and 1 means a complete preference for the treatment under evaluation. The calculated indices were transformed by the arcsine-square root transformation, and the resulting data were analyzed by a Student's t-test, under the null hypothesis of no preference (H_0: $\mu = \text{arcsine} \sqrt{0.5}$). Mortality was extremely low during the preference bioassays, with only one female found dead in 240 replicates (n= 80 per treatment). Some females did not feed or oviposit during the experiment, and they were hence excluded from the analysis.

Nymphal performance was analyzed by comparing survival and duration of the nymphal stage. The survival curve for the control treatment (unexposed leaf of *E. tereticornis*) was compared to the other treatments by the log-rank test (Peto & Peto, 1972). The developmental time for the first instar and the total nymphal stage was compared among treatments by means of a Kruskal-Wallis H test, followed by pairwise comparisons by the Wilcoxon rank-sum test. Differences in the cause of nymphal death were assessed by X^2 tests. Quantitative differences in leaf weight, wax extract yields, and epicuticular wax components in damaged and healthy leaves were evaluated by a Student´s t-test for independent samples. All statistical analyses were run in R v.3.2.0. (R Development Core Team, 2011).

Results

Preference

When females were offered a choice between healthy adult leaves of *E. tereticornis* vs. *E. grandis*, they preferred to feed on the latter (t = -2.557, d.f. = 78, P = 0.01), whereas no feeding preference was detected between *E. tereticornis* vs. *E. benthamii* (t = -1.027, d.f. = 78, P>0.05). Females of *E. tereticornis* preferred adult leaves over juvenile leaves (t = 7.235, d.f. = 73, P<0.001; Figure 4.1). Oviposition preference was not in line with feeding preference. In the assays in which the treatment sample was taken from a plant species other than *E. tereticornis*, more than half of the females did not lay eggs. Females displayed an oviposition preference for *E. tereticornis* adult leaves over *E. grandis* (t = 2.685, d.f. = 24, P = 0.013; Figure 4.1). No oviposition preferences were found between *E. tereticornis* adult leaves (control) vs. leaves of *E. benthamii* or juvenile *E. tereticornis*.

Mortality of females was higher in the arena when damaged samples were compared, reaching 22.5% (n = 65 per treatment). When the females were offered a choice between *E. tereticornis* adult leaves with vs. without previous damage by conspecifics, they preferred to feed on the former if the conspecifics were female. This preference was apparent both when the leaves were taken from plants that had been exposed to a relatively low (t = -2.847, d.f. = 49, P = 0.006) or high (t = -2.439, d.f. = 51, P = 0.01) density of females. Interestingly, no preference was found when the leaves had been exposed to males (t = -1.070, d.f. = 43, P = 0.29; Figure 4.2). Yet, females laid more eggs on leaves previously damaged by the higher density of females (t = -4.767, d.f. = 42, P<0.001) and males (t = -4.369, d.f. = 31, P<0.001). No oviposition preference was found in the experiment with leaves damaged by a low density of conspecific females (t = 0.0104, d.f. = 35, P = 0.99; Figure 4.2).

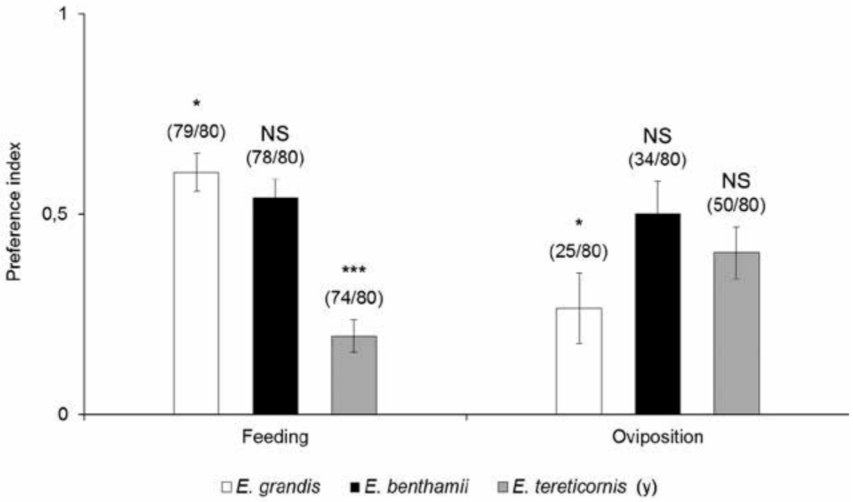


Figure 4.1. Mean (± SEM) feeding and oviposition preference index of *Thaumastocoris peregrinus* females in dual choice assays with three *Eucalyptus* food plant species and undamaged adult *Eucalyptus tereticornis* leaves as control. The '(y)' behind *E. tereticornis* denotes juvenile leaves. Numbers above bars (A/B) indicate the number of valid replicates (A) and the total number of replicates performed (B). An index below 0.5 indicates preference for feeding or ovipositing on the control plant. Asterisk indicate significant deviations from 0.5 (t-test: *0.01<P<0.05; ***P<0.001; ns, not significant).

Nymphal performance

The survival of nymphs was affected by the identity of the food plant. Survival curves were different for individuals reared on *E. tereticornis* compared to *E. grandis* (X^2 = 12.6; 1 d.f; P <0.001) and *E. benthamii* (X^2 = 20.4, both d.f. = 1, P<0.001) and the difference was already apparent after the first instar (Figure 4.3). Nymphs performed similarly on damaged and healthy *E. tereticornis* leaves during the first three instars, but lower survival rates were observed on damaged leaves in the fourth and fifth instars, which were reflected in different survival curves (X^2 = 4.2, d.f. = 1, P = 0.041; Figure 4.3). Causes of death were different among treatments (X^2 = 14.9, d.f. = 6, P = 0.021; Table 4.1). The main causes of death were drowning in the surrounding water and disease by various pathogens, mostly entomopathogenic fungi. Fewer individuals died during molting, especially on *E. benthamii*. Drowning was particularly important for individuals reared on exposed *E. tereticornis*.

The duration of the first instar was different among treatments. Nymphs reached the second instar earlier when reared on *E. tereticornis*, irrespective of whether the leaves had been damaged by conspecifics or not (H = 43.069, d.f. = 3, P<0.001; Figure 4.4). Considering the whole nymphal stage, nymphs reared on healthy *E. tereticornis* and *E. benthamii* developed faster, whereas nymphs on damaged leaves of *E. tereticornis* developed slower (H = 45.076, d.f. = 3, P<0.001; Figure 4.4). The time needed for nymphs to reach the second instar and complete their development was longer on *E. grandis*.

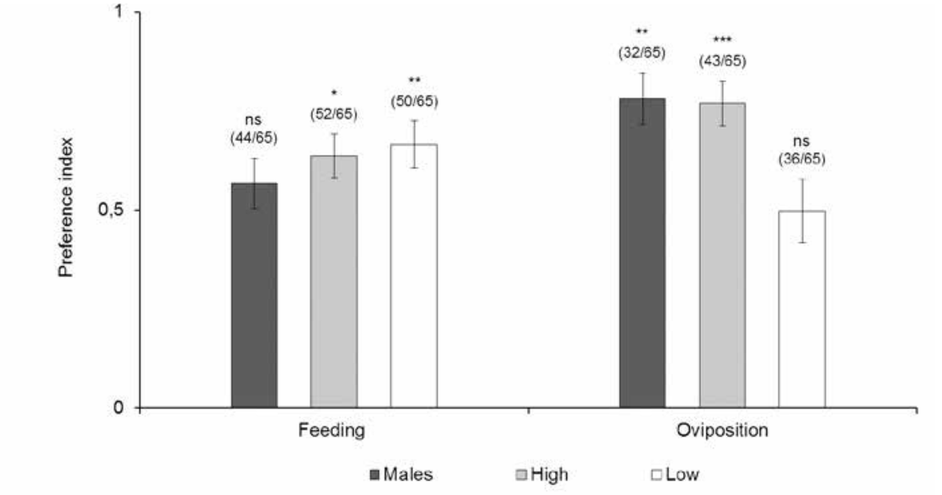


Figure 4.2. Mean (± SEM) feeding and oviposition preference index of *Thaumastocoris peregrinus* females in dual choice assays with *Eucalyptus tereticornis* adult leaves previously damaged by conspecifics with undamaged adult *E. tereticornis* leaves as control. The leaves were taken from plants that had been exposed to two density levels of *T. peregrinus* females (high = 70 per plant; low = five per plant) or to 70 males per plant. Numbers above bars (A/B) indicate the number of valid replicates (A) and the total number of replicates performed (B). An index below 0.5 indicates preference for feeding or ovipositing on the control plant. Asterisks indicate significant deviations from 0.5 (t-test: *0.01<P<0.05, **0.001<P<0.01, ***P<0.001; ns, not significant).

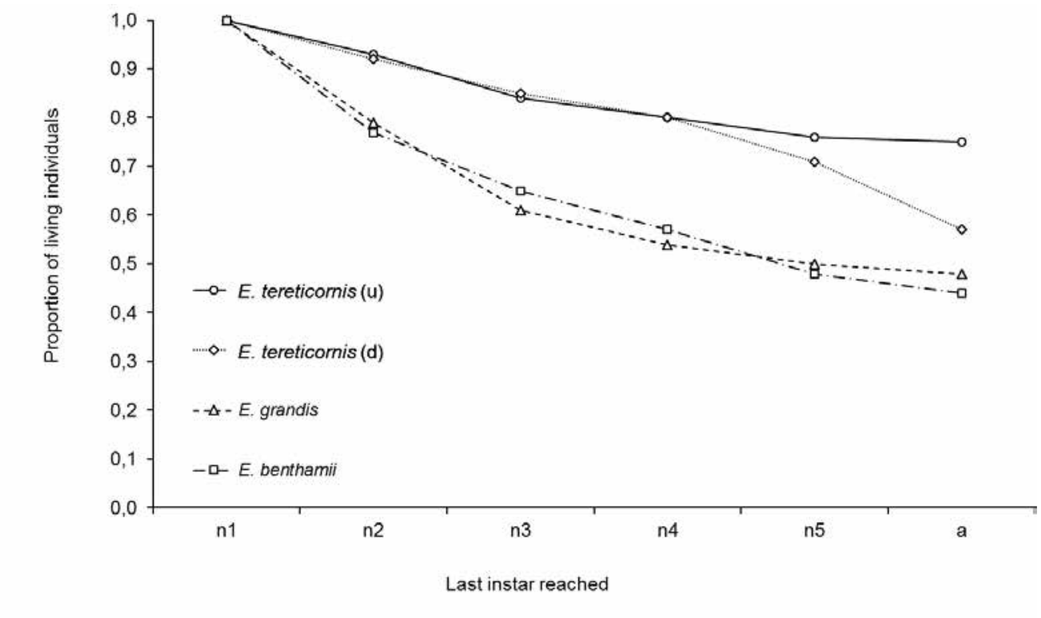
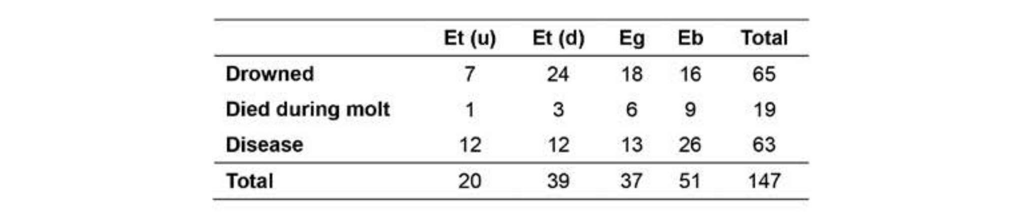


Figure 4.3. Survival of *Thaumastocoris peregrinus* nymphs reared on three *Eucalyptus* food plant species. Dots indicate the proportion at the beginning of each instar (initial n = 100). (u), Undamaged leaves; (d), leaves previously damaged by conspecifics; n1-n5, nymphal stages 1-5; a, adult.

Table 4.1. Number of *Thaumastocoris peregrinus* nymphs that died during development on leaf discs of three *Eucalyptus* food plant species, and their cause of death. A total of 100 nymphs initiated development in each treatment. Et, *Eucalyptus tereticornis*; Eg, *E. grandis*; Eb, *E. benthamii*; 'u', undamaged leaves; 'd', damaged leaves.



	Et (u)	Et (d)	Eg	Eb	Total
Drowned	7	24	18	16	65
Died during molt	1	3	6	9	19
Disease	12	12	13	26	63
Total	20	39	37	51	147

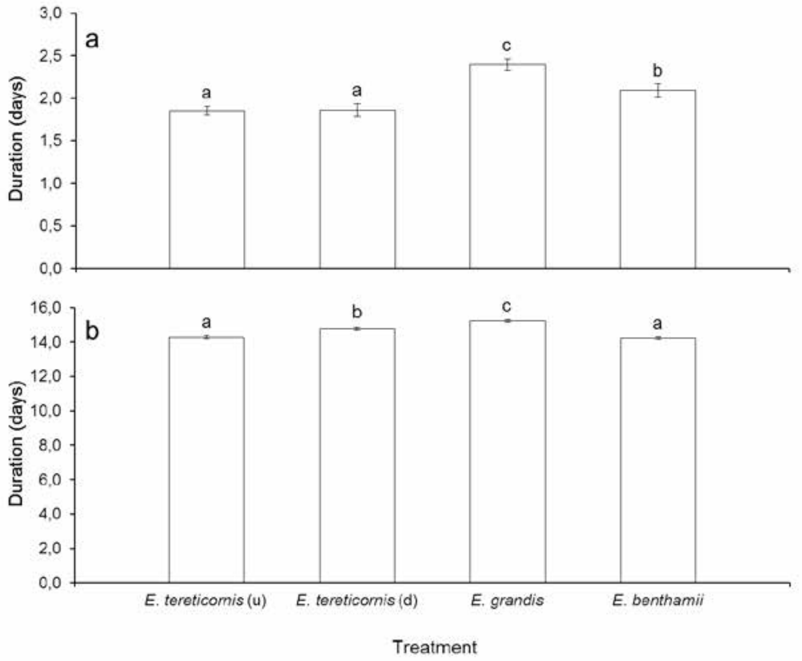


Figure 4.4. Duration (days) of (A) first nymphal instar and (B) total nymphal stage of *Thaumastocoris peregrinus* on different treatments (initial n = 100). Bars within a panel capped with different letters are significantly different (Wilcoxon test: P<0.01). (u), Undamaged leaves; (d), leaves previously damaged by conspecifics.

Epicuticular wax extracts

Undamaged and damaged *E. tereticornis* leaves yielded similar amounts of epicuticular wax extracts (11.2 ± 1.3 and 10.2 ± 1.5 mg g^{-1} leaf fresh weight, respectively; n = 10 per treatment; t= 0.504, d.f.=18, P=0.62). GC-MS analysis of the extracts indicated similar chromatographic profiles, but significant differences in the amounts of their main components. These were typical wax constituents, including saturated linear hydrocarbons, primary alcohols, and aldehydes, which eluted from the column between 33 and 43 min (Figure 4.5). The identification of the compounds was based both on fragmentation patterns (Table S1) and linear retention indices (Table 4.2). Saturated linear hydrocarbons (1-3, 5 in Figure 4.5 and Table 4.2) showed the typical fragmentation patterns of n-alkanes, with the expected molecular ions for n-pentacosane (1, m/z 352) and n-heptacosane (3, m/z 380), and retention indices showing a series of n-alkanes

(Table 4.2). Compounds 2 and 5 showed identical fragmentation patterns but no molecular ions, given the small amounts in the samples, and were identified by their retention indices as n-hexacosane and n-octacosane, respectively.

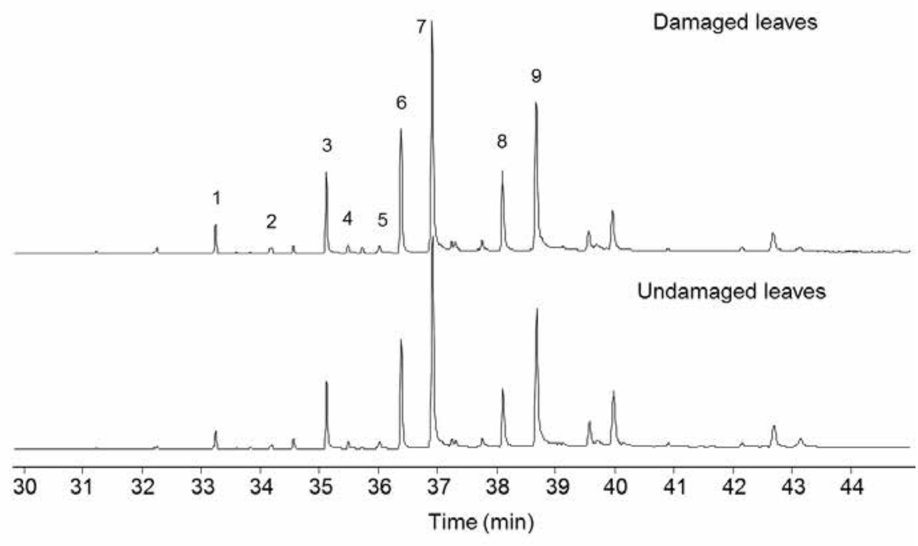


Figure 4.5. Partial chromatographic profiles (TIC) of epicuticular wax extracts of leaves of *Eucalyptus tereticornis* after herbivory by *Thaumastocoris peregrinus* (damaged) or undamaged. See Table 4.2 for identification of the numbered compounds, non-labelled peaks did not differ between treatments and were not identified.

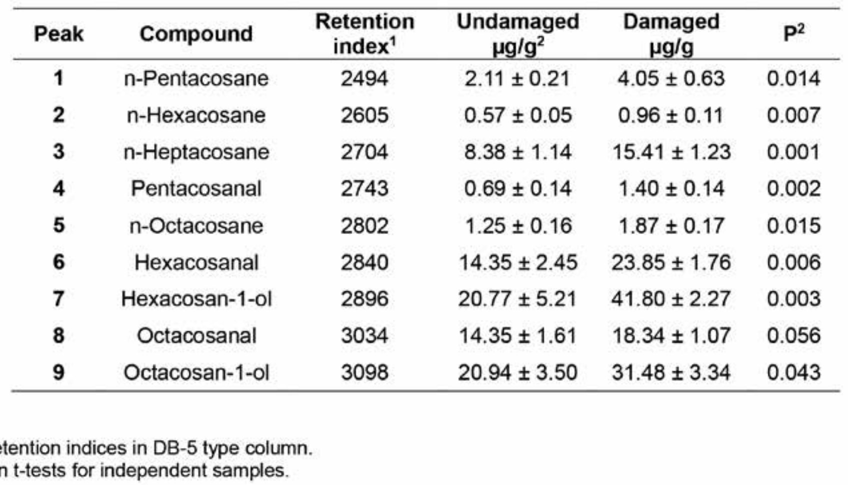
The wax extracts also contained long-chain oxygenated components, including three linear saturated aldehydes (4, 6, 8 in Figure 4.5) and primary alcohols (7, 9). The former were identified as pentacosanal, hexacosanal, and octacosanal, respectively. These compounds showed the typical fragmentation pattern characterized by the $(M\text{-}H_2O)^{\bullet+}$ ion, and the C_nH_{2n+2} and C_nH_{2n-2} ion series. Hexacosanal (6) and octacosanal (8), which were present in larger amounts, also showed the expected molecular ions as well as the typical losses of 28 $[(M\text{-}CO)^{\bullet+}]$ and 46 $[(M\text{-}C_2H_4)^{\bullet+}]$ mass units. The retention indices of the three aldehydes also supported the identification (Table 4.2). Alcohols 7 and 9 were identified as hexacosan-1-ol and octacosan-1-ol, both of which showed mass spectra characterized by the $(M\text{-}H_2O)^{\bullet+}$ and m/z 31 $(CH_2OH)^+$, both typical of primary alcohols. Both alcohols also indicated the loss of 28 mass units corresponding to the loss of H_2O and C_2H_4, resulting in monounsaturated, unbranched long-chain hydrocarbons that characterize the remaining ions in the mass spectra. The presence of C_{26} and C_{28} primary alcohol/aldehyde pairs was used to corroborate the expected difference in retention indices of 60 units (Table 4.2), as reported for other similar pairs (Adams, 2007).

The quantitative analysis based on TIC peak areas showed that most of the components in the volatile fraction of the leaf wax (1-7, 9) were significantly more abundant in leaves with previous damage by *T. peregrinus*. Concentration doubled for some compounds, reaching up to 40 μg g^{-1} of leaf fresh weight for the major one, hexacosan-1-ol (Table 4.2). The aldehyde hexacosanal

(8) showed a non-significant increase, but was nonetheless identified and quantified due to its relevant amount in the extracts. Minor components that showed no quantitative differences between herbivory treatments were not included in the analysis (unlabeled peaks in Figure 4.5).

The choice assays for oviposition preference with the wax extracts showed no significant differences between treatments. Females of *T. peregrinus* laid on average 2.6 ± 0.3 eggs on the filter paper treated with wax from damaged leaves, and 2.3 ± 0.3 on the control filter papers treated with wax from healthy leaves. The calculated oviposition preference index was 0.53 ± 0.04, close to the expected index of 0.5 for 'no preference' (t = 0.668, d.f. = 79, P = 0.51).

Table 4.2. Epicuticular wax components in *Eucalyptus tereticornis* leaves and their mean (± SEM, n= 10) concentration [µg equivalents of tridecane (internal standard) / g leaf fresh weight] in undamaged leaves and leaves infested with *Thaumastocoris peregrinus* (damaged).



Peak	Compound	Retention index[1]	Undamaged µg/g[2]	Damaged µg/g	P[2]
1	n-Pentacosane	2494	2.11 ± 0.21	4.05 ± 0.63	0.014
2	n-Hexacosane	2605	0.57 ± 0.05	0.96 ± 0.11	0.007
3	n-Heptacosane	2704	8.38 ± 1.14	15.41 ± 1.23	0.001
4	Pentacosanal	2743	0.69 ± 0.14	1.40 ± 0.14	0.002
5	n-Octacosane	2802	1.25 ± 0.16	1.87 ± 0.17	0.015
6	Hexacosanal	2840	14.35 ± 2.45	23.85 ± 1.76	0.006
7	Hexacosan-1-ol	2896	20.77 ± 5.21	41.80 ± 2.27	0.003
8	Octacosanal	3034	14.35 ± 1.61	18.34 ± 1.07	0.056
9	Octacosan-1-ol	3098	20.94 ± 3.50	31.48 ± 3.34	0.043

[1] Linear retention indices in DB-5 type column.
[2] Based on t-tests for independent samples.

Discussion

The preference bioassays demonstrate that *T. peregrinus* females can discriminate among *Eucalyptus* species, and that they prefer to feed on adult leaves over juvenile ones. However, they appear to utilize different criteria for selecting a host plant for oviposition. Whereas *E. grandis* was preferred as food source when tested against *E. tereticornis*, the opposite was true when oviposition preference was assessed. Similarly, leaf developmental stage clearly determined food selection, but oviposition was not different between juvenile and adult *E. tereticornis* leaves. Similar results have been found in other insects that oviposit on their food plants, for example, the eucalypt-specialist leaf beetle *Chrysophtharta agricola* (Chapuis) (Chrysomelidae) (Lawrence et al., 2003; Nahrung & Allen, 2003), the herbivorous coccinellid beetle *Henosepilachna niponica* (Lewis) (Fujiyama et al., 2008), and the mirid bug *Lygocoris pabulinus* (L.) (Groot et al., 2003). In these examples, the dissimilar preference for oviposition and feeding substrates has been attributed to different nutritional requirements of immature stages and adults, a scenario that cannot be discarded for *T. peregrinus*. Alternatively, leaf chemistry or toughness, as well as the difference in these attributes between adult and juvenile foliage in *Eucalypus*, may affect feeding and oviposition choices and, consequently, the preference of adults and nymphs of *T. peregrinus* with respect to leaf developmental stage, as has been observed in other insects feeding on *Eucalyptus* (Brennan et al., 2001; Brennan & Weinbaum, 2001a,b; Lawrence et al., 2003).

Host-plant selection by *T. peregrinus* females was positively affected by the previous presence of conspecifics. It is well known that some insects use chemical cues released by conspecifics, or by the plant itself, to select a host plant that has already been colonized (Schoonhoven et al., 2005). The present study demonstrates that *T. peregrinus* females prefer to feed on leaves previously colonized by other females. Oviposition preference, in turn, linked with previous damage by both males and females, suggesting again different criteria for the selection of feeding and oviposition substrates by the females. Rather than the nutritional value, the aggregation of the neonates may be the factor accounting for the female selecting previously colonized leaves for oviposition. Aggregation at early stages can be beneficial for the offspring's use of food resources and defense against natural enemies (Tullberg et al., 2000; Wertheim et al., 2005). Indeed, the neonates are highly mobile and can easily relocate within the branch (Wilcken et al., 2010), and they tend to aggregate under field and laboratory conditions (H Groba, G Martínez & A González, unpubl.).

Previous colonization by conspecifics may result in the production of volatile or non-volatile chemical cues by the plant. Recently, Martins & Zarbin (2013) showed damage by *T. peregrinus* results in the release of volatiles by leaves of *E. benthamii*, which deter females in olfactometer tests. Although this result seems in contradiction with our findings in preference bioassays, our experimental design aimed at testing compounds at the surface of the leaves, as it consisted of a closed system with the insect walking on the leaf squares. Non-polar compounds from epicuticular waxes have been shown to mediate oviposition-site selection in other insects, such as *Ostrinia nubilalis* (Hübner) on maize plants (Udayagiri & Mason, 1997) and psyllids on eucalypt hosts (Brennan et al., 2001; Brennan & Weinbaum, 2001a,b). Therefore, we chose to analyze the composition of the epicuticular wax of damaged and undamaged leaves as potential chemical cues. Our chemical analysis indicated that the amount of wax produced by the leaves did not increase after herbivory by *T. peregrinus*, as wax extraction yields were not different between damaged and undamaged leaves. Yet, most of the components of the volatile fraction (i.e., components suitable for GC analysis) of the epicuticular wax extracts were more concentrated in damaged leaves, suggesting a defensive metabolic response by the plant that increased the production of long-chain hydrocarbons, alcohols, and aldehydes. However, female oviposition preference did not correlate with these chemical changes in the epicuticular wax of damaged leaves, as oviposition rates did not differ. This result suggests that other chemical or physical cues may be responsible for female preference for damaged leaves, or it may stem from an unsuitable experimental design based on an artificial oviposition substrate (filter paper) for testing the wax extracts. New experimental designs or different wax extract concentrations should be considered, because the identification of chemical cues exploited by an economically important insect to select its host plant can help develop sustainable management programs by using semiochemicals (Nadel et al., 2012).

As an essential component of the fitness implications of oviposition-site selection, we evaluated the performance of nymphs in the various host-plant substrates. We focused on nymphal development and survival, and found a weak link with feeding and oviposition preferences. Instar-specific mortality of *T. peregrinus* bugs on *E. tereticornis* displayed a similar pattern to that observed in previous studies, with higher mortality rates during the first and fourth instars (Soliman et al., 2012; Martínez et al., 2014). We did not conduct a performance experiment with juvenile *E. tereticornis* leaves, because *T. peregrinus* nymphs do not develop on juvenile *Eucalyptus* leaves (data not shown). In general, *T. peregrinus* performance was better on *E. tereticornis* than on the other two *Eucalyptus* species. *Eucalyptus grandis* proved to be suboptimal food for *T. peregrinus* nymphs, with higher mortality rates, particularly in the early instars, and longer development time. These results are clearly in line with oviposition preferences, and therefore the rejection of *E. grandis* as a substrate for oviposition can be considered a 'mother knows best' decision, in which the nutritional value of the host plant may play a role. In the case of *E. benthamii* the results were not as clear; whereas the bugs experienced higher mortality during the first few instars and slower development during the first instar, the total development time was as short as on *E. tereticornis*. Consistent with this, female *T. peregrinus* did not exhibit differences in feeding and oviposition preferences for *E. benthamii* when compared to *E. tereticornis*. It is noteworthy that, according to a national monitoring network in Uruguay, *E. benthamii* is the preferred host among timber and pulp production stands, in which *E. tereticornis* is underrepresented (Martínez & Scoz, 2010; Martínez & Gómez, 2012, 2014). Finally, nymphal performance on damaged *E. tereticornis* was similar to that on healthy leaves during early development, but mortality and development time increased in the late instars, indicating again a loose correlation with feeding and oviposition preferences.

Oviposition-site selection is a hierarchical process that operates at several spatial scales, each of which provides different cues that the females may respond to (Vinson, 1976; Visser, 1988; Martínez et al., 2013; Dickins et al., 2013). In our study, host-plant preferences for oviposition and feeding by *T. peregrinus* females were not tightly correlated, suggesting that different criteria mediate each of these selection steps. Only in some situations, oviposition preferences could be ascribed to the preference-performance hypothesis, but in others this link was not clear or even contradictory. It remains to be studied whether feeding preferences correlate with increased female survival, which in turn should affect their overall fitness. In addition, we assessed only bottom-up effects related to the quality of the host plant, whereas insects are exposed to a broader context that includes multitrophic effects (Dicke, 2000; Schoonhoven et al., 2005). Future studies should consider the host-plant selection by female *T. peregrinus* in a tritrophic context, particularly including parasitoids such as *Cleruchoides noackae* Lin & Huber (Hymenoptera: Mymaridae), which plays an important role in the ecology of *T. peregrinus*.

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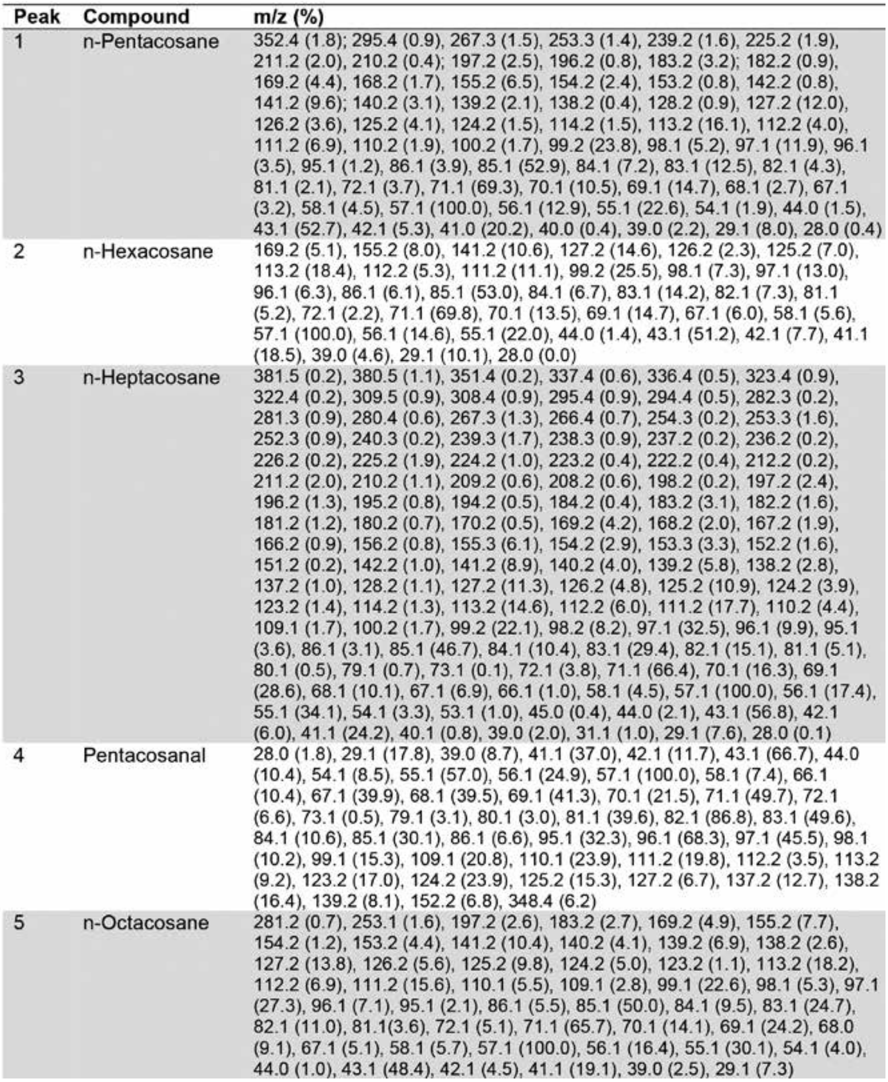
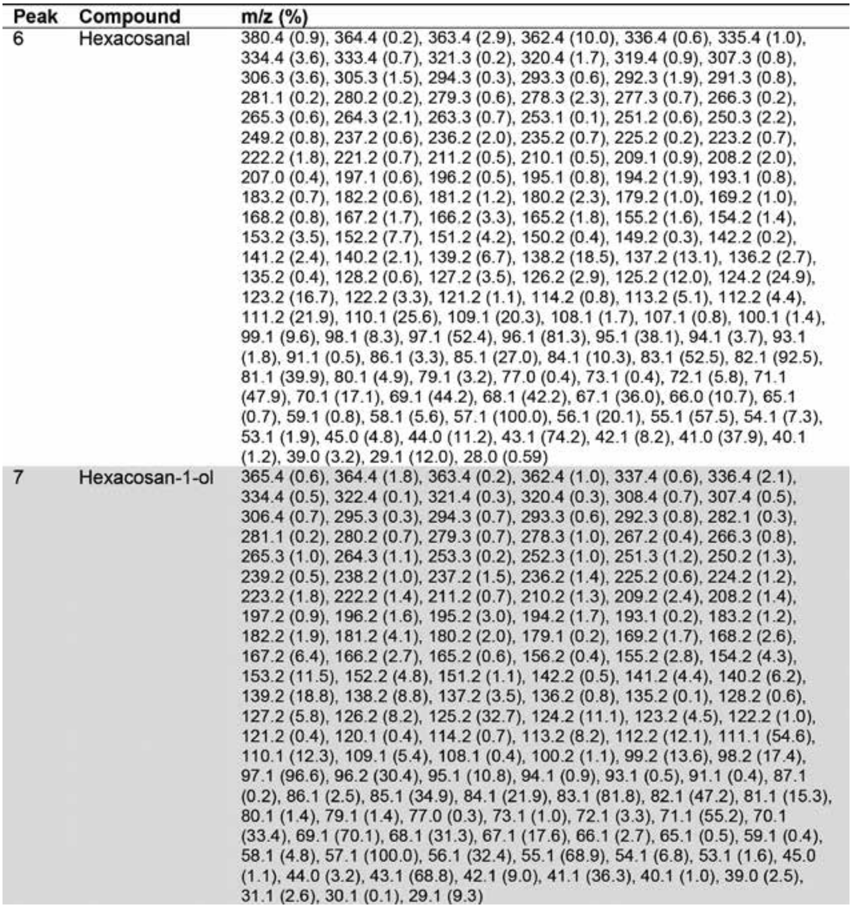
Supporting information

Table S1. Epicuticular wax components in *Eucalyptus tereticornis* leaves and their mean (± SEM, n= 10) concentration [µg equivalents of tridecane (internal standard) / g leaf fresh weight] in undamaged leaves and leaves infested with *Thaumastocoris peregrinus* (damaged).



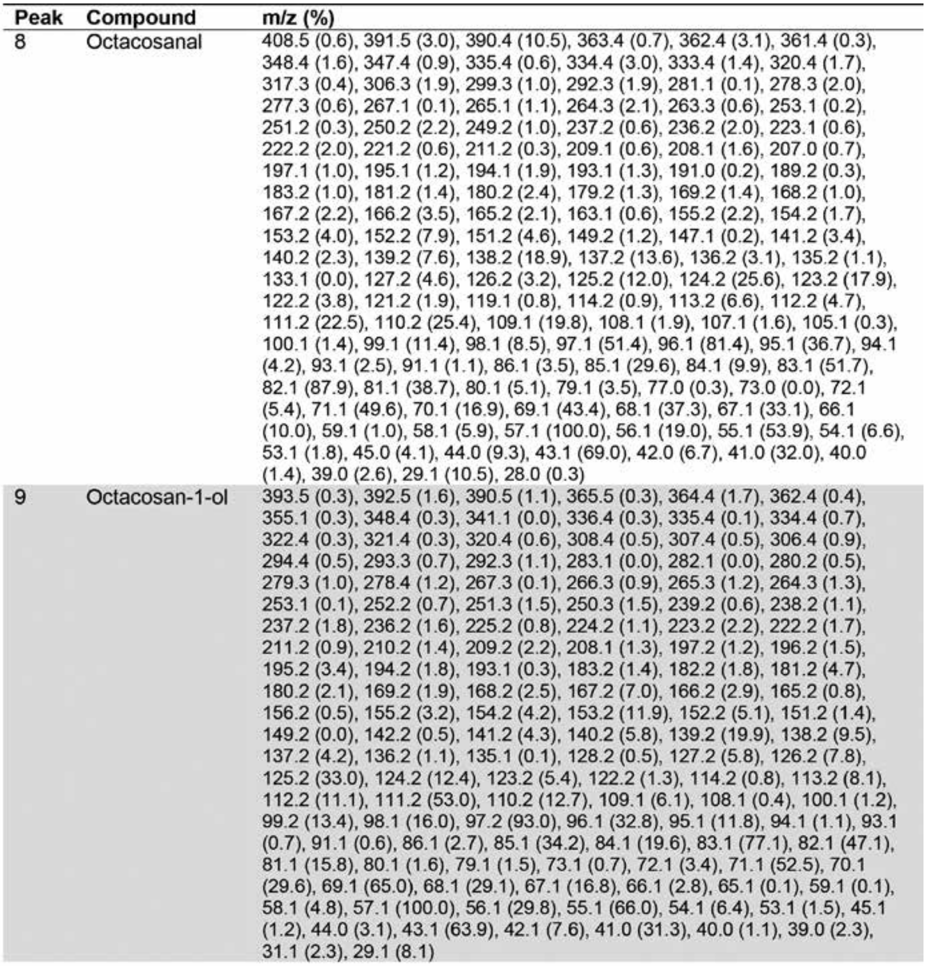
Peak	Compound	m/z (%)
1	n-Pentacosane	352.4 (1.8); 295.4 (0.9), 267.3 (1.5), 253.3 (1.4), 239.2 (1.6), 225.2 (1.9), 211.2 (2.0), 210.2 (0.4); 197.2 (2.5), 196.2 (0.8), 183.2 (3.2); 182.2 (0.9), 169.2 (4.4), 168.2 (1.7), 155.2 (6.5), 154.2 (2.4), 153.2 (0.8), 142.2 (0.8), 141.2 (9.6); 140.2 (3.1), 139.2 (2.1), 138.2 (0.4), 128.2 (0.9), 127.2 (12.0), 126.2 (3.6), 125.2 (4.1), 124.2 (1.5), 114.2 (1.5), 113.2 (16.1), 112.2 (4.0), 111.2 (6.9), 110.2 (1.9), 100.2 (1.7), 99.2 (23.8), 98.1 (5.2), 97.1 (11.9), 96.1 (3.5), 95.1 (1.2), 86.1 (3.9), 85.1 (52.9), 84.1 (7.2), 83.1 (12.5), 82.1 (4.3), 81.1 (2.1), 72.1 (3.7), 71.1 (69.3), 70.1 (10.5), 69.1 (14.7), 68.1 (2.7), 67.1 (3.2), 58.1 (4.5), 57.1 (100.0), 56.1 (12.9), 55.1 (22.6), 54.1 (1.9), 44.0 (1.5), 43.1 (52.7), 42.1 (5.3), 41.0 (20.2), 40.0 (0.4), 39.0 (2.2), 29.1 (8.0), 28.0 (0.4)
2	n-Hexacosane	169.2 (5.1), 155.2 (8.0), 141.2 (10.6), 127.2 (14.6), 126.2 (2.3), 125.2 (7.0), 113.2 (18.4), 112.2 (5.3), 111.2 (11.1), 99.2 (25.5), 98.1 (7.3), 97.1 (13.0), 96.1 (6.3), 86.1 (6.1), 85.1 (53.0), 84.1 (6.7), 83.1 (14.2), 82.1 (7.3), 81.1 (5.2), 72.1 (2.2), 71.1 (69.8), 70.1 (13.5), 69.1 (14.7), 67.1 (6.0), 58.1 (5.6), 57.1 (100.0), 56.1 (14.6), 55.1 (22.0), 44.0 (1.4), 43.1 (51.2), 42.1 (7.7), 41.1 (18.5), 39.0 (4.6), 29.1 (10.1), 28.0 (0.0)
3	n-Heptacosane	381.5 (0.2), 380.5 (1.1), 351.4 (0.2), 337.4 (0.6), 336.4 (0.5), 323.4 (0.9), 322.4 (0.2), 309.5 (0.9), 308.4 (0.9), 295.4 (0.9), 294.4 (0.5), 282.3 (0.2), 281.3 (0.9), 280.4 (0.6), 267.3 (1.3), 266.4 (0.7), 254.3 (0.2), 253.3 (1.6), 252.3 (0.9), 240.3 (0.2), 239.3 (1.7), 238.3 (0.9), 237.2 (0.2), 236.2 (0.2), 226.2 (0.2), 225.2 (1.9), 224.2 (1.0), 223.2 (0.4), 222.2 (0.4), 212.2 (0.2), 211.2 (2.0), 210.2 (1.1), 209.2 (0.6), 208.2 (0.6), 198.2 (0.2), 197.2 (2.4), 196.2 (1.3), 195.2 (0.8), 194.2 (0.5), 184.2 (0.4), 183.2 (3.1), 182.2 (1.6), 181.2 (1.2), 180.2 (0.7), 170.2 (0.5), 169.2 (4.2), 168.2 (2.0), 167.2 (1.9), 166.2 (0.9), 156.2 (0.8), 155.3 (6.1), 154.2 (2.9), 153.3 (3.3), 152.2 (1.6), 151.2 (0.2), 142.2 (1.0), 141.2 (8.9), 140.2 (4.0), 139.2 (5.8), 138.2 (2.8), 137.2 (1.0), 128.2 (1.1), 127.2 (11.3), 126.2 (4.8), 125.2 (10.9), 124.2 (3.9), 123.2 (1.4), 114.2 (1.3), 113.2 (14.6), 112.2 (6.0), 111.2 (17.7), 110.2 (4.4), 109.1 (1.7), 100.2 (1.7), 99.2 (22.1), 98.2 (8.2), 97.1 (32.5), 96.1 (9.9), 95.1 (3.6), 86.1 (3.1), 85.1 (46.7), 84.1 (10.4), 83.1 (29.4), 82.1 (15.1), 81.1 (5.1), 80.1 (0.5), 79.1 (0.7), 73.1 (0.1), 72.1 (3.8), 71.1 (66.4), 70.1 (16.3), 69.1 (28.6), 68.1 (10.1), 67.1 (6.9), 66.1 (1.0), 58.1 (4.5), 57.1 (100.0), 56.1 (17.4), 55.1 (34.1), 54.1 (3.3), 53.1 (1.0), 45.0 (0.4), 44.0 (2.1), 43.1 (56.8), 42.1 (6.0), 41.1 (24.2), 40.1 (0.8), 39.0 (2.0), 31.1 (1.0), 29.1 (7.6), 28.0 (0.1)
4	Pentacosanal	28.0 (1.8), 29.1 (17.8), 39.0 (8.7), 41.1 (37.0), 42.1 (11.7), 43.1 (66.7), 44.0 (10.4), 54.1 (8.5), 55.1 (57.0), 56.1 (24.9), 57.1 (100.0), 58.1 (7.4), 66.1 (10.4), 67.1 (39.9), 68.1 (39.5), 69.1 (41.3), 70.1 (21.5), 71.1 (49.7), 72.1 (6.6), 73.1 (0.5), 79.1 (3.1), 80.1 (3.0), 81.1 (39.6), 82.1 (86.8), 83.1 (49.6), 84.1 (10.6), 85.1 (30.1), 86.1 (6.6), 95.1 (32.3), 96.1 (68.3), 97.1 (45.5), 98.1 (10.2), 99.1 (15.3), 109.1 (20.8), 110.1 (23.9), 111.2 (19.8), 112.2 (3.5), 113.2 (9.2), 123.2 (17.0), 124.2 (23.9), 125.2 (15.3), 127.2 (6.7), 137.2 (12.7), 138.2 (16.4), 139.2 (8.1), 152.2 (6.8), 348.4 (6.2)
5	n-Octacosane	281.2 (0.7), 253.1 (1.6), 197.2 (2.6), 183.2 (2.7), 169.2 (4.9), 155.2 (7.7), 154.2 (1.2), 153.2 (4.4), 141.2 (10.4), 140.2 (4.1), 139.2 (6.9), 138.2 (2.6), 127.2 (13.8), 126.2 (5.6), 125.2 (9.8), 124.2 (5.0), 123.2 (1.1), 113.2 (18.2), 112.2 (6.9), 111.2 (15.6), 110.1 (5.5), 109.1 (2.8), 99.1 (22.6), 98.1 (5.3), 97.1 (27.3), 96.1 (7.1), 95.1 (2.1), 86.1 (5.5), 85.1 (50.0), 84.1 (9.5), 83.1 (24.7), 82.1 (11.0), 81.1(3.6), 72.1 (5.1), 71.1 (65.7), 70.1 (14.1), 69.1 (24.2), 68.0 (9.1), 67.1 (5.1), 58.1 (5.7), 57.1 (100.0), 56.1 (16.4), 55.1 (30.1), 54.1 (4.0), 44.0 (1.0), 43.1 (48.4), 42.1 (4.5), 41.1 (19.1), 39.0 (2.5), 29.1 (7.3)

Table S1. (Cont.).



Peak	Compound	m/z (%)
6	Hexacosanal	380.4 (0.9), 364.4 (0.2), 363.4 (2.9), 362.4 (10.0), 336.4 (0.6), 335.4 (1.0), 334.4 (3.6), 333.4 (0.7), 321.3 (0.2), 320.4 (1.7), 319.4 (0.9), 307.3 (0.8), 306.3 (3.6), 305.3 (1.5), 294.3 (0.3), 293.3 (0.6), 292.3 (1.9), 291.3 (0.8), 281.1 (0.2), 280.2 (0.2), 279.3 (0.6), 278.3 (2.3), 277.3 (0.7), 266.3 (0.2), 265.3 (0.6), 264.3 (2.1), 263.3 (0.7), 253.1 (0.1), 251.2 (0.6), 250.3 (2.2), 249.2 (0.8), 237.2 (0.6), 236.2 (2.0), 235.2 (0.7), 225.2 (0.2), 223.2 (0.7), 222.2 (1.8), 221.2 (0.7), 211.2 (0.5), 210.1 (0.5), 209.1 (0.9), 208.2 (2.0), 207.0 (0.4), 197.1 (0.6), 196.2 (0.5), 195.1 (0.8), 194.2 (1.9), 193.1 (0.8), 183.2 (0.7), 182.2 (0.6), 181.2 (1.2), 180.2 (2.3), 179.2 (1.0), 169.2 (1.0), 168.2 (0.8), 167.2 (1.7), 166.2 (3.3), 165.2 (1.8), 155.2 (1.6), 154.2 (1.4), 153.2 (3.5), 152.2 (7.7), 151.2 (4.2), 150.2 (0.4), 149.2 (0.3), 142.2 (0.2), 141.2 (2.4), 140.2 (2.1), 139.2 (6.7), 138.2 (18.5), 137.2 (13.1), 136.2 (2.7), 135.2 (0.4), 128.2 (0.6), 127.2 (3.5), 126.2 (2.9), 125.2 (12.0), 124.2 (24.9), 123.2 (16.7), 122.2 (3.3), 121.2 (1.1), 114.2 (0.8), 113.2 (5.1), 112.2 (4.4), 111.2 (21.9), 110.1 (25.6), 109.1 (20.3), 108.1 (1.7), 107.1 (0.8), 100.1 (1.4), 99.1 (9.6), 98.1 (8.3), 97.1 (52.4), 96.1 (81.3), 95.1 (38.1), 94.1 (3.7), 93.1 (1.8), 91.1 (0.5), 86.1 (3.3), 85.1 (27.0), 84.1 (10.3), 83.1 (52.5), 82.1 (92.5), 81.1 (39.9), 80.1 (4.9), 79.1 (3.2), 77.0 (0.4), 73.1 (0.4), 72.1 (5.8), 71.1 (47.9), 70.1 (17.1), 69.1 (44.2), 68.1 (42.2), 67.1 (36.0), 66.0 (10.7), 65.1 (0.7), 59.1 (0.8), 58.1 (5.6), 57.1 (100.0), 56.1 (20.1), 55.1 (57.5), 54.1 (7.3), 53.1 (1.9), 45.0 (4.8), 44.0 (11.2), 43.1 (74.2), 42.1 (8.2), 41.0 (37.9), 40.1 (1.2), 39.0 (3.2), 29.1 (12.0), 28.0 (0.59)
7	Hexacosan-1-ol	365.4 (0.6), 364.4 (1.8), 363.4 (0.2), 362.4 (1.0), 337.4 (0.6), 336.4 (2.1), 334.4 (0.5), 322.4 (0.1), 321.4 (0.3), 320.4 (0.3), 308.4 (0.7), 307.4 (0.5), 306.4 (0.7), 295.3 (0.3), 294.3 (0.7), 293.3 (0.6), 292.3 (0.8), 282.1 (0.3), 281.1 (0.2), 280.2 (0.7), 279.3 (0.7), 278.3 (1.0), 267.2 (0.4), 266.3 (0.8), 265.3 (1.0), 264.3 (1.1), 253.3 (0.2), 252.3 (1.0), 251.3 (1.2), 250.2 (1.3), 239.2 (0.5), 238.2 (1.0), 237.2 (1.5), 236.2 (1.4), 225.2 (0.6), 224.2 (1.2), 223.2 (1.8), 222.2 (1.4), 211.2 (0.7), 210.2 (1.3), 209.2 (2.4), 208.2 (1.4), 197.2 (0.9), 196.2 (1.6), 195.2 (3.0), 194.2 (1.7), 193.1 (0.2), 183.2 (1.2), 182.2 (1.9), 181.2 (4.1), 180.2 (2.0), 179.1 (0.2), 169.2 (1.7), 168.2 (2.6), 167.2 (6.4), 166.2 (2.7), 165.2 (0.6), 156.2 (0.4), 155.2 (2.8), 154.2 (4.3), 153.2 (11.5), 152.2 (4.8), 151.2 (1.1), 142.2 (0.5), 141.2 (4.4), 140.2 (6.2), 139.2 (18.8), 138.2 (8.8), 137.2 (3.5), 136.2 (0.8), 135.2 (0.1), 128.2 (0.6), 127.2 (5.8), 126.2 (8.2), 125.2 (32.7), 124.2 (11.1), 123.2 (4.5), 122.2 (1.0), 121.2 (0.4), 120.1 (0.4), 114.2 (0.7), 113.2 (8.2), 112.2 (12.1), 111.1 (54.6), 110.1 (12.3), 109.1 (5.4), 108.1 (0.4), 100.2 (1.1), 99.2 (13.6), 98.2 (17.4), 97.1 (96.6), 96.2 (30.4), 95.1 (10.8), 94.1 (0.9), 93.1 (0.5), 91.1 (0.4), 87.1 (0.2), 86.1 (2.5), 85.1 (34.9), 84.1 (21.9), 83.1 (81.8), 82.1 (47.2), 81.1 (15.3), 80.1 (1.4), 79.1 (1.4), 77.0 (0.3), 73.1 (1.0), 72.1 (3.3), 71.1 (55.2), 70.1 (33.4), 69.1 (70.1), 68.1 (31.3), 67.1 (17.6), 66.1 (2.7), 65.1 (0.5), 59.1 (0.4), 58.1 (4.8), 57.1 (100.0), 56.1 (32.4), 55.1 (68.9), 54.1 (6.8), 53.1 (1.6), 45.0 (1.1), 44.0 (3.2), 43.1 (68.8), 42.1 (9.0), 41.1 (36.3), 40.1 (1.0), 39.0 (2.5), 31.1 (2.6), 30.1 (0.1), 29.1 (9.3)

Table S1. (Cont.).



Peak	Compound	m/z (%)
8	Octacosanal	408.5 (0.6), 391.5 (3.0), 390.4 (10.5), 363.4 (0.7), 362.4 (3.1), 361.4 (0.3), 348.4 (1.6), 347.4 (0.9), 335.4 (0.6), 334.4 (3.0), 333.4 (1.4), 320.4 (1.7), 317.3 (0.4), 306.3 (1.9), 299.3 (1.0), 292.3 (1.9), 281.1 (0.1), 278.3 (2.0), 277.3 (0.6), 267.1 (0.1), 265.1 (1.1), 264.3 (2.1), 263.3 (0.6), 253.1 (0.2), 251.2 (0.3), 250.2 (2.2), 249.2 (1.0), 237.2 (0.6), 236.2 (2.0), 223.1 (0.6), 222.2 (2.0), 221.2 (0.6), 211.2 (0.3), 209.1 (0.6), 208.1 (1.6), 207.0 (0.7), 197.1 (1.0), 195.1 (1.2), 194.1 (1.9), 193.1 (1.3), 191.0 (0.2), 189.2 (0.3), 183.2 (1.0), 181.2 (1.4), 180.2 (2.4), 179.2 (1.3), 169.2 (1.4), 168.2 (1.0), 167.2 (2.2), 166.2 (3.5), 165.2 (2.1), 163.1 (0.6), 155.2 (2.2), 154.2 (1.7), 153.2 (4.0), 152.2 (7.9), 151.2 (4.6), 149.2 (1.2), 147.1 (0.2), 141.2 (3.4), 140.2 (2.3), 139.2 (7.6), 138.2 (18.9), 137.2 (13.6), 136.2 (3.1), 135.2 (1.1), 133.1 (0.0), 127.2 (4.6), 126.2 (3.2), 125.2 (12.0), 124.2 (25.6), 123.2 (17.9), 122.2 (3.8), 121.2 (1.9), 119.1 (0.8), 114.2 (0.9), 113.2 (6.6), 112.2 (4.7), 111.2 (22.5), 110.2 (25.4), 109.1 (19.8), 108.1 (1.9), 107.1 (1.6), 105.1 (0.3), 100.1 (1.4), 99.1 (11.4), 98.1 (8.5), 97.1 (51.4), 96.1 (81.4), 95.1 (36.7), 94.1 (4.2), 93.1 (2.5), 91.1 (1.1), 86.1 (3.5), 85.1 (29.6), 84.1 (9.9), 83.1 (51.7), 82.1 (87.9), 81.1 (38.7), 80.1 (5.1), 79.1 (3.5), 77.0 (0.3), 73.0 (0.0), 72.1 (5.4), 71.1 (49.6), 70.1 (16.9), 69.1 (43.4), 68.1 (37.3), 67.1 (33.1), 66.1 (10.0), 59.1 (1.0), 58.1 (5.9), 57.1 (100.0), 56.1 (19.0), 55.1 (53.9), 54.1 (6.6), 53.1 (1.8), 45.0 (4.1), 44.0 (9.3), 43.1 (69.0), 42.0 (6.7), 41.0 (32.0), 40.0 (1.4), 39.0 (2.6), 29.1 (10.5), 28.0 (0.3)
9	Octacosan-1-ol	393.5 (0.3), 392.5 (1.6), 390.5 (1.1), 365.5 (0.3), 364.4 (1.7), 362.4 (0.4), 355.1 (0.3), 348.4 (0.3), 341.1 (0.0), 336.4 (0.3), 335.4 (0.1), 334.4 (0.7), 322.4 (0.3), 321.4 (0.3), 320.4 (0.6), 308.4 (0.5), 307.4 (0.5), 306.4 (0.9), 294.4 (0.5), 293.3 (0.7), 292.3 (1.1), 283.1 (0.0), 282.1 (0.0), 280.2 (0.5), 279.3 (1.0), 278.4 (1.2), 267.3 (0.1), 266.3 (0.9), 265.3 (1.2), 264.3 (1.3), 253.1 (0.1), 252.2 (0.7), 251.3 (1.5), 250.3 (1.5), 239.2 (0.6), 238.2 (1.1), 237.2 (1.8), 236.2 (1.6), 225.2 (0.8), 224.2 (1.1), 223.2 (2.2), 222.2 (1.7), 211.2 (0.9), 210.2 (1.4), 209.2 (2.2), 208.1 (1.3), 197.2 (1.2), 196.2 (1.5), 195.2 (3.4), 194.2 (1.8), 193.1 (0.3), 183.2 (1.4), 182.2 (1.8), 181.2 (4.7), 180.2 (2.1), 169.2 (1.9), 168.2 (2.5), 167.2 (7.0), 166.2 (2.9), 165.2 (0.8), 156.2 (0.5), 155.2 (3.2), 154.2 (4.2), 153.2 (11.9), 152.2 (5.1), 151.2 (1.4), 149.2 (0.0), 142.2 (0.5), 141.2 (4.3), 140.2 (5.8), 139.2 (19.9), 138.2 (9.5), 137.2 (4.2), 136.2 (1.1), 135.1 (0.1), 128.2 (0.5), 127.2 (5.8), 126.2 (7.8), 125.2 (33.0), 124.2 (12.4), 123.2 (5.4), 122.2 (1.3), 114.2 (0.8), 113.2 (8.1), 112.2 (11.1), 111.2 (53.0), 110.2 (12.7), 109.1 (6.1), 108.1 (0.4), 100.1 (1.2), 99.2 (13.4), 98.1 (16.0), 97.2 (93.0), 96.1 (32.8), 95.1 (11.8), 94.1 (1.1), 93.1 (0.7), 91.1 (0.6), 86.1 (2.7), 85.1 (34.2), 84.1 (19.6), 83.1 (77.1), 82.1 (47.1), 81.1 (15.8), 80.1 (1.6), 79.1 (1.5), 73.1 (0.7), 72.1 (3.4), 71.1 (52.5), 70.1 (29.6), 69.1 (65.0), 68.1 (29.1), 67.1 (16.8), 66.1 (2.8), 65.1 (0.1), 59.1 (0.1), 58.1 (4.8), 57.1 (100.0), 56.1 (29.8), 55.1 (66.0), 54.1 (6.4), 53.1 (1.5), 45.1 (1.2), 44.0 (3.1), 43.1 (63.9), 42.1 (7.6), 41.0 (31.3), 40.0 (1.1), 39.0 (2.3), 31.1 (2.3), 29.1 (8.1)

Chapter 5

Effect of the eucalypt lerp psyllid *Glycaspis brimblecombei* on adult feeding, oviposition-site selection and offspring performance by the bronze bug *Thaumastocoris peregrinus*

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Submitted for publication

Abstract

Oviposition site-selection may be greatly affected by competitive plant-mediated interactions between phytophagous insects but these interactions have been poorly investigated on trees. Here, we evaluated the potential interaction between two invasive pests of *Eucalyptus* trees, the lerp psyllid *Glycaspis brimblecombei* and the bronze bug *Thaumastocoris peregrinus*. We assessed the effect of the co-occurrence of *G. brimblecombei* on the selection of feeding and oviposition sites by *T. peregrinus* females by using dual-choice bioassays. We compared developmental time and survival of the first instar of nymphs reared on healthy *Eucalyptus tereticornis* leaves and on leaves infested with the lerp psyllid, either with or without lerps. Bronze bug females prefer to oviposit on lerp-carrying leaves but we found no difference in feeding preference when compared to healthy leaves. Infestation with the lerp psyllid hampered nymphal performance in terms of developmental time and survival, although the presence of lerps reverted the effect in survival and shortened the duration of the initial instar. These results show an interaction between these two insect species that affect both the oviposition preference of *T. peregrinus* females and the nymphal performance. Although there seems to be a discrepancy between mother preference and offspring performance, feeding on the lerps may prove beneficial to the offspring under long-term conditions and multiple infestation by other pests and diseases.

Keywords: true bugs, preference-performance linkage, Heteroptera, Thaumastocoridae, Aphalaridae, dual-choice assay, intraguild competition, host selection.

Introduction

Interspecific competition is an important factor affecting the fitness of phytophagous insects (Kaplan & Denno, 2007). Competitive interactions are often indirectly mediated by induced changes in plant chemistry, morphology and/or physiology that result from herbivore attack and affect the fitness of a competitor (Karban & Baldwin, 1997; Ohgushi, 2005; Stam et al., 2014). Oviposition-site selection of phytophagous insects is heavily affected by such indirect plant-mediated interactions (Kaplan & Denno, 2007). Plant-mediated interspecific interactions have been reported in insects from the same feeding guild (Denno et al., 2000; Karban et al., 2012) and across feeding guilds (Kessler & Baldwin, 2004; Soler et al., 2012). Furthermore, the outcome of the interaction can change depending on the sequence of arrival of the herbivores onto the plant (Poelman et al., 2008; Soler et al., 2012; Kroes et al., 2016; Huang et al., 2017).

Most of the studies on interspecific competition among phytophagous insects deal with annual plants (Kaplan et al., 2011). Unlike annual plants, trees are exposed to many insect species, occurring either simultaneously or subsequently during the years needed for a rotation cycle in commercial plantation forestry. The study of competitive interactions among phytophagous insects in such long-term systems is particularly interesting, although challenging. Assessing changes in oviposition behavior of an insect due to the co-occurrence of a potential competitor may be a good starting point to determine the effect of interspecific competition among phytophagous insects in these ecosystems.

Among the many insects that attack eucalyptus trees, two that have become important pests worldwide are the red gum lerp psyllid and the bronze bug. The red gum lerp psyllid, *Glycaspis brimblecombei* Moore (Sternorhyncha: Aphalaridae), is a small winged sap-sucking insect that usually lives beneath a white conical sweet-tasting structure called lerp, which is secreted during the nymphal stage (Firmino-Winckler et al., 2009). Although they can feed on other eucalypt species, they are particularly abundant on adult shoots and leaves of red gum trees (*Eucalyptus tereticornis* Smith, *E. camaldulensis* Dehnh.), on which they perform better than on other commercial species like blue the gum (*E. globulus* Labill., *E. maidenii* Muell.) and the rose gum (*E. grandis* Hill ex Maiden) (Hollis, 2004; FAO-MGAP, 2006; Santana & Burckhardt, 2007; Firmino-Winckler et al., 2009). While it is regarded as an important pest in other parts of South America (Santana & Burckhardt, 2007; Benítez Díaz et al., 2013), it has remained as a pest of relatively low importance in Uruguay since its detection in 2004 (Bianchi & Sánchez, 2004). In Uruguay, *G. brimblecombei* is multivoltine, with population peaks between August and December (FAO-MGAP, 2006). The bronze bug, *Thaumastocoris peregrinus* Carpintero et Dellape (Heteroptera: Thaumastocoridae), is an invasive pest of *Eucalyptus* species that has dispersed during the past decade from Australia, its center of origin, to the main eucalypt forestry areas in the world (Nadel & Noack, 2012). In Uruguay, this species shows a preference for red gums as well as blue gums and exhibits a seasonal dynamics with population peaks occurring from March to late April (Martínez & Bianchi, 2010; Martínez & Gómez, 2014; Martínez et al., 2014). Both insect species exhibit epiphytic oviposition and usually lay eggs in batches. Bronze bug females often select oviposition sites elevated from the leaf surface, such as the central nerve of the leaf, deformations on the leaf blade, twigs, or even debris (Wilcken et al., 2010; Nadel & Noack, 2012). Thus, it is common to find bronze bug eggs on eggs of the psyllid, or on the lerps themselves (Ide et al., 2011), which are also frequently fed upon by adults and juveniles (Figure 5.1). To our knowledge, no study has yet investigated the effect of eucalypt infestation with *G. brimblecombei* on preference and performance of *T. peregrinus*.

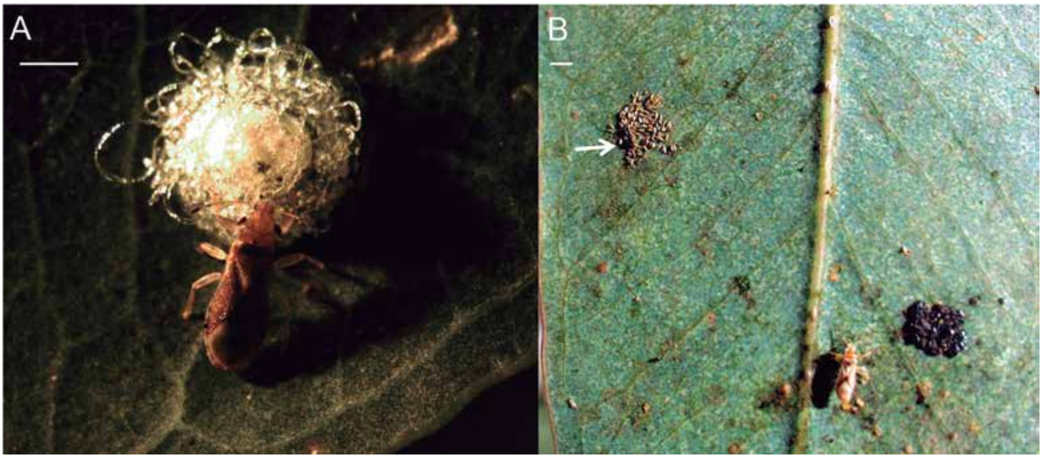


Figure 5.1. (A) Female of *Thaumastocoris peregrinus* feeding on a lerp; (B) Eggs of *T. peregrinus* (arrow) close to *Glycaspis brimblecombei* ovipostures. White bars indicate 1 mm.

The goal of this study was to evaluate the potential intra-guild interaction between these two invasive pests of eucalypt trees, by assessing the effect of the presence of *G. brimblecombei* on the selection of feeding and oviposition sites by bronze bug females, and on the performance of their offspring. We hypothesized, based on field observations, that the co-occurrence of the lerp psyllid provides the bronze bug with an alternative food source that may be beneficial to both the female and her offspring.

Material and methods

Insects

Adults of *T. peregrinus* (1-2 days old) were obtained from a permanent mass rearing at INIA Tacuarembó. They were set to mate in communal mating cages consisting of an aluminum frame (35 x 50 x 70 cm) covered with voile mesh. Each mating cage contained approximately 80 females and 60 males. They were provided with fresh shoots of *E. globulus* in Erlenmeyer flasks. Experimental females were collected from the mating cages after a week, and were assumed to have mated based on previous research on the life cycle of *T. peregrinus* (cf. Martínez et al., 2014, Chapter 3 on this thesis, for details on rearing procedure).

Lerp-carrying leaves were collected in a shelter plantation of *Eucalyptus tereticornis* infested with the lerp psyllid (10-20 lerps per leaf), located at the INIA experimental station (31°44'19.24"S; 55°58'43.75"W).

Preference

Feeding and oviposition preference of mated females was assessed in an experimental arena consisting of a Petri dish (5.5 cm diameter) filled with water and containing two leaf squares (2 cm side) cut out from adult *E. tereticornis* leaves as treatment options. A cotton swab was cut into two halves and attached with adhesive tape to the bottom of the dish to provide a base to the leaf squares and prevent them from floating and touching the walls of the dish (Figure 5.2 A). The leaf squares were then joined by one edge with adhesive tape affixed to the abaxial side, and placed onto the cotton stick at the center of the dish. Finally, an adhesive label (Rotola®, 1.5 x 0.8 cm) was fixed between the leaf squares as a neutral release area (Figure 2B). One bronze bug female was enclosed in each experimental arena and left in a rearing chamber at 25 ± 5 °C, 55 ± 10% relative humidity and L12:D12, for assessing feeding and oviposition preference. Treatments consisted of a leaf square carrying one lerp of *G. brimblecombei* and a square cut from a healthy leaf collected from the same tree stand as control. After 2 days, oviposition and feeding by the bronze bug were assessed by counting eggs and fecal droplets on each leaf square.

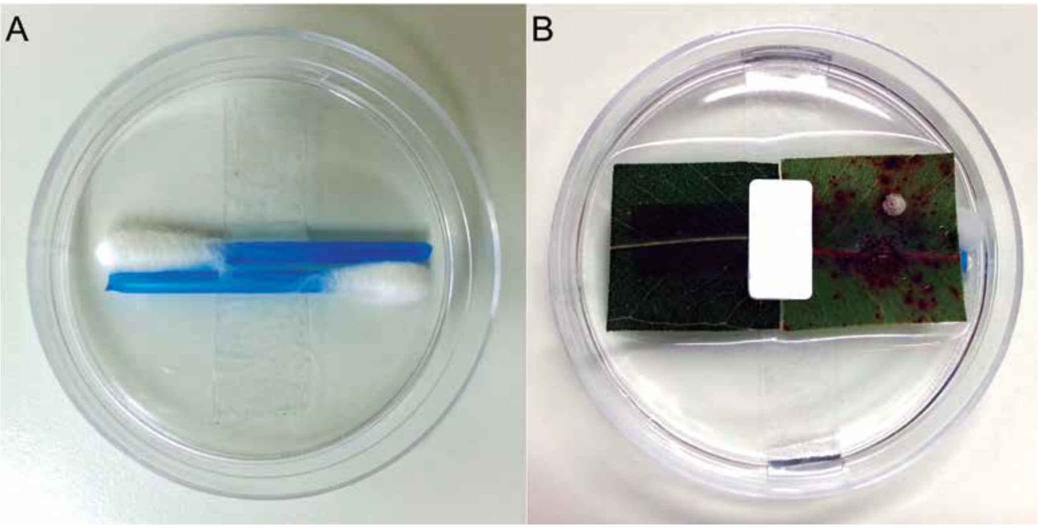


Figure 5.2. Experimental arena: (A) Cotton swab cut in halves and attached at the bottom of the Petri dish; (B) Arena ready for the bioassay with the two treatments and a paper label as neutral area.

Nymphal performance

Recently-laid bronze bug eggs were placed on a leaf square (3 x 3 cm) floating on water inside a Petri dish as explained above. Immediately after hatching, bronze bug neonates were individually transferred to leaves belonging to one of the following treatments: healthy *E.*

tereticornis leaves, leaves infested with 10-20 *G. brimblecombei* lerps, and leaves similarly infested after removing the lerps with a needle. The distal part of the leaves was previously cut to a length of 5 cm, and their petiole was enclosed in cotton and inserted in a test tube filled with water (Figure 5.3). The tubes were then incubated at 25 ± 5 °C, 60 ± 10% r. h. and L12:D12. As a measure of performance, nymphal survival was recorded daily until the nymphs had either molted or died. We chose to assess the performance during the first instar based on previous studies showing that mortality is highest during this period (Martínez et al., 2014, 2017). In addition, a shorter experimental time prevented potential artifacts derived from the degradation of leaf sections.

Data analysis

Female preference was analyzed by calculating the proportion of fecal drops and eggs in the tested treatment with respect to the total number of fecal drops and eggs (treatment + control), following Grostal & Dicke (1999). Hence, feeding and oviposition preference indices were established for each replicate, so that zero represents a complete preference for the control (*E. tereticornis* healthy adult leaves), 0.5 represents no preference, and 1 means a complete preference for the treatment under evaluation. The calculated indices were then transformed by the arcsine/square root transformation, and the resulting data were analyzed by a Student's t-test, under a null hypothesis of no preference.

For performance data, the duration of the first instar for each individual was compared among treatments by a one-factor ANOVA, after square root data transformation. Tukey's test was employed for *post hoc* comparisons among treatments, under a 95% family-wise confidence level. Survival curves of nymphal development were compared in pairs by using the log-rank test (Peto & Peto, 1972). All tests were run in R v. 3.4.0 (R Development Core Team, 2009).

Figure 5.3. Setup for *Thaumastocoris peregrinus* nymphal performance bioassays. A typical leaf infested with *Glycaspis brimblecombei* lerps is shown. The lighter spots correspond to either lerps on the other side of the leaf or former lerps on the same side.

Results

Preference

A total of 200 females were tested, equally divided over the oviposition and feeding preference tests. Females preferred to oviposit on lerp-carrying leaves over healthy leaves (t = -3.6935, df = 86, P = 0.0004, n = 87) but did not discriminate between these two treatments for feeding (t = -0.7804, df = 91, P = 0.43, n = 92) (Figure 5.4). Females that died or did not oviposit were not included in the analysis (13 and 8 for the oviposition and feeding preference tests, respectively). Females laid a total of 556 eggs, 374 on lerp-carrying leaves (4.1 ± 0.3 eggs/female), most of them adjacent to a lerp (all but 3 eggs). In contrast, only 182 eggs were laid on healthy leaves (2.0 ± 0.3 eggs/female). During the experiment, several females were observed feeding next to or directly on the lerps.

Nymphal performance

The duration of the first instar was different among treatments: Nymphs reared on healthy leaves molted earlier than those reared on leaves infested by *G. brimblecombei*. Between infested leaves, the first instar was longer when lerps had been previously removed (Figure 5.5). During the daily observations, nymphs reared on lerp-carrying leaves were frequently found on or adjacent to a lerp.

The survival rate on lerp-removed infested leaves (67%) was significantly lower than nymphal survival on healthy leaves (84%), while lerp-carrying leaves resulted in survival rates (76%) not different from either treatment (Table 5.1).

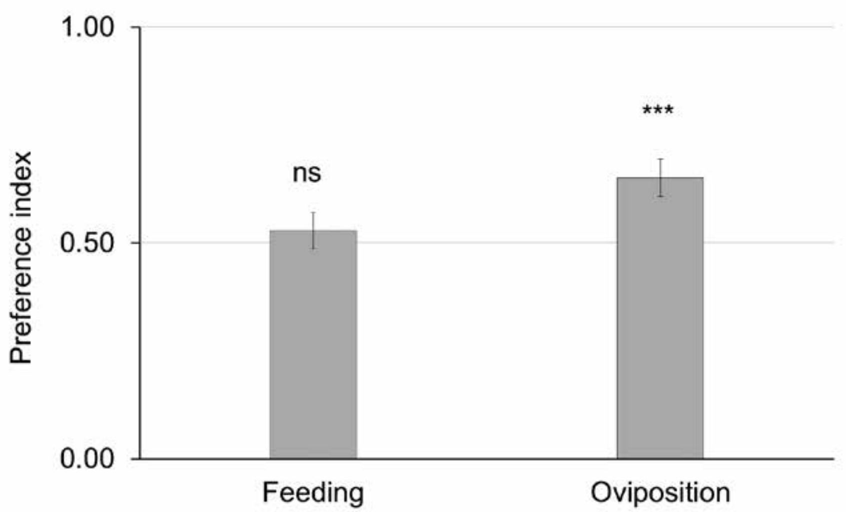


Figure 5.4. Average feeding (n = 92) and oviposition (n = 87) preference index of *Thaumastocoris peregrinus* females in dual choice bioassays with lerp-carrying vs. healthy *Eucalyptus tereticornis* leaf squares. An index above 0.5 indicates preference for the lerp-infested leaf square. Error bars represent SEM. ***: P < 0.001; ns: not significant.

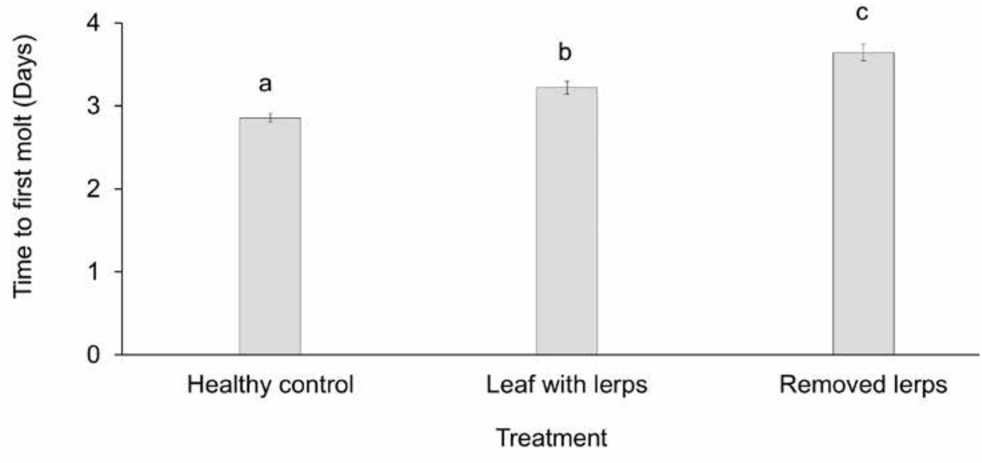
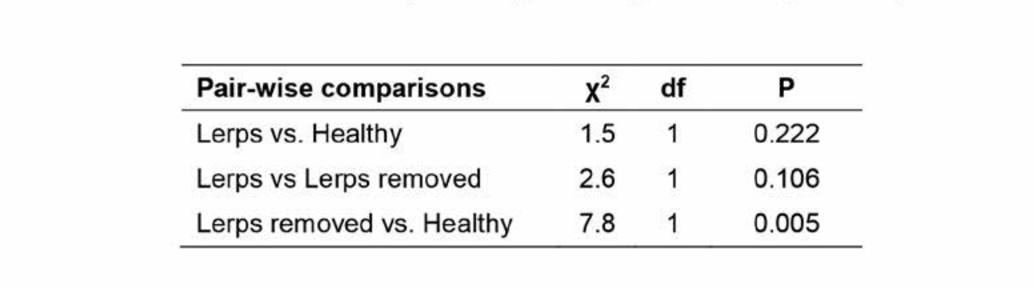


Figure 5.5. Duration of the first instar of *Thaumastocoris peregrinus* nymphs reared on leaves of *Eucalyptus tereticornis*. The leaves were either healthy (control), leaves with 10-20 lerps of *Glycaspis brimblecombei*, or leaves with lerps previously removed. Columns with different letters correspond to significant differences at a confidence level of 95%. Error bars represent SEM (n = 100 nymphs/treatment).

Table 5.1. Log rank test for survival rates of *Thaumastocoris peregrinus* first instar nymphs reared on healthy *Eucalyptus tereticornis* leaves, lerp-carrying leaves, and leaves whose lerps were previously removed (n =100).



Pair-wise comparisons	χ^2	df	P
Lerps vs. Healthy	1.5	1	0.222
Lerps vs Lerps removed	2.6	1	0.106
Lerps removed vs. Healthy	7.8	1	0.005

Discussion

We evaluated potential competitive interactions between two sucking phytophagous insect that occur on *Eucalyptus* trees. Our experimental results demonstrate an oviposition preference by the bronze bug for sites already colonized by the lerp psyllid, and a reduced performance of the nymphs on these sites, partially mitigated by the actual presence of lerps. The lower performance of nymphs as a result of lerp psyllid co-occurrence suggests some form of competition between the two species, most likely mediated by the plant. However, our results also show that the nymphs increase their survival rates and shorten their initial development in the presence of lerps. Together, these findings indicate that these two insects interact.

Bronze bug females laid more eggs on psyllid-infested leaves, but no such preference was observed for feeding. Among insects in which juveniles and adults share similar feeding habits, the selection of optimal oviposition sites may be difficult to take apart from the selection of optimal feeding sites (Scheirs & De Bruyn, 2002). In our study, we found a preference for lerp-carrying leaves over healthy leaves only regarding oviposition site selection. Similarly, oviposition but not feeding preference was previously found in this insect with respect to conspecific-infested plants (Martínez et al., 2017). This study confirms that *T. peregrinus* females also include the co-occurrence of *G. brimblecombei* as a criterion to discriminate between oviposition sites.

At first glance, the selection for lerp infested leaves as oviposition sites by female bronze bugs does not seem to result in an increased performance by their offspring. Indeed, nymphs feeding on healthy leaves developed faster than those feeding on leaves colonized by the lerp psyllid. The worst performance was observed on infested leaves from which all lerps had been removed, resulting in the slowest development and the lowest survival. A negative impact of *G. brimblecombei* co-occurrence may result from induced changes in plant chemistry or an overall decrease of resources available to the bronze bug as a result of competition. In fact, psyllids affect their host plants by removing sap and reducing photosynthesis as a consequence of both the construction of lerps and the excretion of honeydew that facilitates sooty mold growth (Hollis, 2004). Species within the genus *Glycaspis* have been reported as phloem feeders (Moore, 1964; Sharma et al., 2013). Feeding by the related genus *Cardiaspina* induces premature senescence in leaves of *Eucalyptus moluccana* Roxb. (Steinbauer et al., 2014). Likewise, in our study system we observed chlorotic spots on the leaf surface below the lerps, suggesting senescence induced by *Glycaspis* (Figure 3). All these changes may explain the poorer performance of the bronze bug nymphs on plants exposed to the lerp psyllid shown by our study.

The presence of lerps improves nymphal performance. Although nymphal duration was longer on leaves exposed to the lerp psyllid than on healthy leaves, we found no effect on survival if lerps were left on the leaves. In our experimental setup we used leaf sections containing lerps cut out from leaves with moderate to heavy infestation, so it may be assumed that induced plant responses to *G. brimblecombei* herbivory should be present in such treatment, regardless of lerp removal. A counteracting positive impact, however, may be in place given the rich sugar contents of the lerp. For instance, the use of lerps as food sources has been documented for birds (Oliver, 1998; Florence, 2005) and native Australian human populations (Turner et al., 1994), but we found no previous evidence in the literature for lerp feeding by insects. Nevertheless, we often observed bronze bug adults and nymphs feeding on lerps (figure 1A), a behavior that has also been observed in the field (Carlos Wilcken pers. comm.). Moreover, we recorded that eggs laid on lerp-carrying leaves were almost exclusively laid next to a lerp. So, it is likely that feeding on the lerps resulted in an improved performance of the nymphs in comparison with those reared on leaves attacked by *G. brimblecombei* but from which the lerps had been removed.

Considering seasonal dynamics may provide new insights to interpret the link between mother's preference and nymphal performance observed in our study. We conducted our experiments between December and February, after the population peak of *G. brimblecombei*, and before the population peak of *T. peregrinus*. We specifically avoided the end of summer (March) because this is the period in which the trees are exposed to most leaf-disease causing pathogens in Uruguay (Balmelli et al., 2013; Soria et al., 2014). Therefore, the performance of bronze bug nymphs was evaluated under two scenarios: healthy leaves as an ideal resource, and a potentially sub-optimal situation with the co-occurrence of another herbivore, the lerp psyllid. During the population peak at the end of summer, with a reduced leaf nutritional quality due to the co-occurrence of several pests and pathogens, the apparent contradiction between the mother's choice for oviposition sites and the performance of the nymphs may be resolved differently. In such scenario, the extra source of sugars provided by the lerps may be more important for nymphal performance. This hypothesis deserves further experimentation.

Leaf structure may be another factor contributing to the preference of bronze bug females for lerp-carrying leaves. Bronze bug females usually select for oviposition sites elevated from the leaf surface (Wilcken et al., 2010; Nadel & Noack, 2012), and lerps provide an elevated structure. In our experiment, 371 out of 556 eggs were laid in direct contact with the lerp, suggesting that the observed preference for lerp-carrying leaves may be, at least in part, explained by the choice of leaf structural features. Other species in the Heteroptera select plants for oviposition according to particular structural features (Lundgren, 2011; Martínez et al., 2013), but in general these preferences have been related to a better offspring performance due to bottom-up (Lundgren et al., 2008) or top-down effects (Udayagiri & Welter, 2000). Further studies are needed to assess the role of the lerp' physical attributes on oviposition site selection by the bronze bug.

In conclusion, our study provides evidence for interactions between the bronze bug and the lerp psyllid that not only play a role in the oviposition preference of *T. peregrinus*, but also affect the performance of the immature stages. Initially these results suggest a competitive interaction between these two species. A more realistic scenario with leaves affected by other pests and pathogens and including a longer observation time frame may shed light on the potential benefits of oviposition-site selection observed in the bronze bug, to assess whether "mother knows best".

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Chapter 6

Rearing and releasing the egg parasitoid *Cleruchoides noackae*, a biological control agent for the bronze bug in Uruguay

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Submitted for publication

Abstract

Biological control is a major tool for forest insect pest management. We initiated a biological control program for the *Eucalyptus* bronze bug, *Thaumastocoris peregrinus*, with the egg parasitoid *Cleruchoides noackae*. Parasitized eggs were imported from a mass rearing in Brazil, and a rearing colony was set up. The wasp was reared in plastic tubes each containing ten individuals that were offered 100 eggs of the bronze bug and a solution of water and honey. During 30 generations, rearing quality parameters were assessed and compared against expected quality standards set *a priori*, based on information from other rearing colonies. We also assessed the effect of temperature on developmental time, measured as the first day of emergence of adult parasitoids, as well as the relation between host-egg abortion and parasitization by *C. noackae*, and the sex ratio of the emergent wasps within a single generation. Field releases were made yearly during summers in commercial plantations, starting in March 2013. Survival and emergence rates were either stable or increased throughout 30 generations. Sex ratio was female-biased. Rearing at 18 ° C and 20 °C took longer than at 22 °C, but productivity was similar. Host-egg abortion was increased by parasitization. The proportion of females increased as the emergence period progressed. Field surveys in two sites revealed that the wasp survived for two years. Field-collected parasitoids exhibited better quality indices than that of the laboratory rearing. Results suggest that biological control of the bronze bug with *C. noackae* is feasible with this rearing and release protocol.

Keywords: *Thaumastocoris peregrinus*; *Eucalyptus*; inoculative release; Thaumastocoridae; tree plantation; Uruguay; rearing quality.

Introduction

Sustainability of global *Eucalyptus* plantation forestry has been increasingly threatened in the last decades by invasions of Australian insects into newly planted areas, as a consequence of global trade and climate change (Wingfield et al., 2008; Paine et al., 2011). This trend requires the strengthening of current quarantine and pest management strategies of forest pests at a global scale (Wingfield et al., 2015). However, any management strategy involving the use of chemical pesticides is strongly discouraged within the framework of the eco-certification schemes such as the Forest Stewardship Council (FSC), due to the economic costs and the risks that pesticides pose to people and the environment (Willoughby et al., 2009). Non-chemical alternatives extensively utilized in plantation forestry include silvicultural methods (Klapwijk et al., 2016), the use of infochemicals (Nadel, Wingfield, Scholes, Lawson, & Slippers, 2012), and biological control. The latter represents the major contribution to pest management in forestry (Protasov et al., 2007; Garnas et al., 2012; Dittrich-Schröder et al., 2014; Slippers et al., 2015).

At the beginning of a biological control program, research efforts must focus on producing the biocontrol agent easily, effectively, efficiently, and in adequate quantities (Chambers, 1977; Van Lenteren, 2003). Developing reliable quality estimators is important for monitoring the status of a rearing colony and for dealing with eventual problems such as inbreeding depression or adaptation to rearing conditions (Chambers, 1977; Bigler, 1989; Van Lenteren, 2003). For instance, it is essential to monitor the sex ratio of the colony to prevent undesirable male-biased colonies, which can affect the sustainability of the rearing in the future and the efficacy of the control by the released agent (Heimpel & Lundgren, 2000). When the parasitized host (egg, larvae or pupae) is the stage to be released in the field, as opposed to adult individuals of the natural enemy, it is crucial to estimate the number of actual natural enemies that will be delivered (Van Lenteren, 2003).

One of the invasive pests that affects eucalypt plantations worldwide is the bronze bug *Thaumastocoris peregrinus* Carpintero et Dellape (Heteroptera: Thaumastocoridae). This is a small sucking insect that feeds on mature leaves within the genera *Eucalyptus* and *Corymbia*, causing leaf blight, increased stress and in severe cases defoliation or tree death (Nadel & Noack, 2012).

Cleruchoides noackae Lin Huber et La Salle (Hymenoptera: Mymaridae) is an egg parasitoid of *T. peregrinus* (Lin et al., 2007). This wasp is able to parasitize eggs of the bronze bug up to three-days-old (Mutitu et al., 2013). Adult lifespan of *C. noackae* extends between two and three days if fed on a honey solution (Mutitu et al., 2013; Souza et al., 2016). Given the potential of *C. noackae* as a biological control agent for the bronze bug, rearing colonies were set up in South Africa (Mutitu et al., 2013) and Brazil (Souza et al., 2016). Researchers in Chile also imported the parasitoid, reared it for two generations and released it in the field, this being the first release of *C. noackae* outside its natural range (Jaques, 2010). The optimal thermal range for development of *C. noackae* lies between 15 ºC and 25 ºC (Mutitu et al., 2013; Souza et al., 2016), but the duration of the immature stages at different temperatures has not been reported.

In order to start a biological control program for the bronze bug in Uruguay, a rearing colony of *C. noackae* was set up and the wasp was periodically released in the field. Here, we report the results after 4 years of continuous rearing of the parasitoid. We also report for the first time on the occurrence of parasitism in the field in Uruguay.

Material and Methods

First introduction and rearing setup

Cleruchoides noackae was introduced for the first time in Uruguay on February 22nd, 2013, from an *in vitro* rearing colony at EMBRAPA (Curitiba, Brazil). We imported a total of 24 tubes, each containing 100 bronze bug eggs exposed to ten *C. noackae* wasps belonging to the 8th generation emerged in Brazil after their introduction from Australia. We followed the official importation procedures, and obtained the rearing and release permits required by ISPM 3 and regional COSAVE standards (COSAVE; FAO, 2005). Half of the tubes were immediately released in two commercial eucalypt plantations (Sites 1 and 2, Table 6.1). The extant 12 tubes produced 180 females and 87 males of *C. noackae* (9th generation), which were the founders of the rearing colony. The parasitoid was reared on its natural host, bronze bug eggs, which were supplied from a permanent mass rearing at INIA Tacuarembó, Uruguay (Martínez et al. (2014). We selected eggs up to 48-h-old following information from previous studies on age-dependent parasitization (Mutitu et al., 2013).

This rearing colony of *C. noackae* (hereafter referred to as Colony 1) was maintained from March 2013 to November 2014. It comprised generations 10 to 40, following the counting started in Brazil. The wasps multiplied inside plastic tubes (3 cm diameter by 7 cm high, hereafter referred to as "rearing tubes") closed with a perforated plastic cap covered by voile mesh (Figure 6.1A). Each rearing tube was filled with 100 eggs of *T. peregrinus* and 10 adult individuals of *C. noackae*. A paper strip soaked with 50 % honey diluted in water was provided as a food source. The rearing tubes were placed in a rearing chamber (Daihan Wisd SWGC-450) at 22 ± 1 ºC, 65 ± 1 % relative humidity and 12:12 light: dark photoperiod). The tubes were opened 8 days later, nymphs were counted, and the wasps were sexed under a dissection microscope (Olympus SZH). After day 18, we inspected each rearing tube daily for wasp emergence until the occurrence of three consecutive days without emergence. Emerged wasps were counted and sexed, and assigned to new-generation rearing tubes.

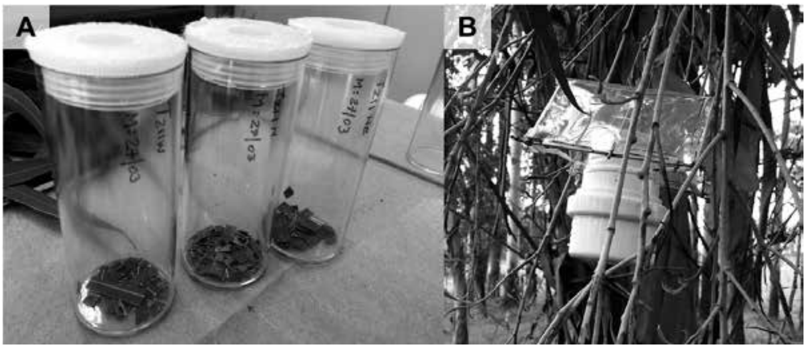

Figure 6.1. (A) Rearing tubes for *Cleruchoides noackae*; (B) Release device placed on a eucalypt tree within a plantation.

A new rearing colony was established in 2015 with 547 female and 83 male founder wasps that emerged in the laboratory from egg samples collected in the field (see below). We named the first generation of wasps that emerged from these founders generation zero. Here, we analyzed data from generations 0 to 31 of this rearing colony, hereafter referred to as Colony 2.

Temperature

C. noackae has been reared at 24 ± 2 ºC in South Africa (Mutitu et al., 2013) and at 22 ºC in Brazil (Leonardo Barbosa pers. comm.). We ran an experiment to check emergence at lower temperatures in order to facilitate winter rearing under Uruguayan conditions. Emerged wasps, not more than 24-h-old, were separated into groups of five couples and placed into new rearing tubes. The tubes were then randomly allocated to rearing chambers at 18 ± 1 ºC, 20 ± 1 ºC or 22 ± 1 ºC, at 65 ± 1 % r.h. and 12:12 L:D. On day 10, the tubes were opened and dead nymphs and wasps were removed. The tubes were then inspected daily for wasp emergence until three consecutive days were recorded without new emergent wasps. A total of 13,500 *T. peregrinus* eggs (4,500 eggs per treatment in 45 tubes) were used.

Basal egg hatching and parasitoid-induced egg abortion

Hatching rates of non-parasitized eggs of the bronze bug was monitored by randomly separating a set of 5 rearing tubes per generation, leaving them unexposed to the parasitoid. Such unexposed tubes were incubated in the same conditions as the extant tubes in the rearing from generations 30 to 40 of Colony 1. Hatching rate was calculated as the proportion of hatching nymphs relative to the total number of eggs in the tube, expressed as a percentage.

Egg parasitoids can cause host death without reproducing, which leads to an increased number of unhatched eggs or egg abortions relative to unexposed eggs (Abram et al., 2016). In order to investigate the effect of parasitization by *C.noackae* on egg mortality, we compared the abortion rate as the reciprocal of the hatching rate (i. e. the proportion of eggs producing neither nymphs nor wasps) in a set of rearing tubes containing 100 eggs and five couples of *C. noackae* (hereafter referred to as ‘exposed’) against the set of ‘unexposed’ tubes. In total, 5 tubes were prepared for each treatment (500 eggs). The experiment was replicated in generations 30, 35 and 40.

Sex ratio

In order to evaluate whether the availability of females remained constant during the emergence period, within and across generations, we divided the emergence period of each generation in half and we assessed the total number of females and males that emerged in each time period. In the case of an odd number of days within a generation, we included the middle day into the second half. The experiment was repeated 10 times in generations belonging to Colony 2.

Field release

In Uruguay, the bronze bug exhibits marked seasonal dynamics, with peaks usually occurring from the end of summer to the beginning of fall (March and April). After the first frosts there is a marked decrease in the populations that renders the insect undetectable until December (Martínez & Gómez, 2014; Martínez et al., 2014). Wasps from the rearing colony were periodically released in the field during summer, starting in March 2013. To select the release sites we used information from a national surveillance network consisting of yellow sticky cards that were counted on a monthly basis, with more than 50 monitoring nodes installed in commercial *Eucalyptus* stands (Martínez & Gómez, 2014). We checked the monitoring network for sites registering an increase in captures of the bronze bug, and double checked the level of infestation by surveying with local foresters.

Rearing tubes reaching day 19 (i.e. just before the onset of wasp emergence) were transported to the selected sites in a foam cooler. In the release site, we chose trees with fresh egg batches of the bronze bug. The parasitized eggs were transferred from the rearing tubes to a device consisting of a plastic chamber that serves as receptacle for the eggs, with an opening in the top covered with mesh to prevent ants and other predators from entering the chamber. A transparent plastic top protected the exit point from rain. Three release devices were hung on selected trees in each site (Figure 6.1B), and revisited the next year during summer. Bronze bug egg batches were collected from the trees hosting the release devices, and from adjacent trees if they were available. The egg samples were processed in the laboratory, where unhatched eggs were counted and enclosed into rearing tubes in the same conditions as used for the rearing. The tubes were checked daily for bronze bug hatching or wasp emergence, and discarded after a month.

Data analysis

To estimate the quality of the rearing we calculated for each generation three indices: the *percentage of emergence (*the number of emerged wasps relative to the total number of eggs offered for parasitization), the *percentage of survival* (the number of living individuals relative to the total parasitoid offspring) and the p*ercentage of females* (the number of emerged females relative to the total offspring). In Colony 1, emerging wasps were sexed in a randomly selected subset of the total rearing tubes (with a minimum of 30 tubes per generation). In Colony 2, and for practical purposes, all individuals that had emerged on each day were sexed at the end of the day (i.e. after collecting all the wasps that had emerged in all tubes).

We set *a priori* minimum quality standards of 20 % for emergence, 80% for survival and 50% for females, based on information from *in vitro* rearings in Brazil and Chile (Leonardo Barbosa, Cecilia Ruiz pers. comm.). We calculated the 95% confidence intervals for these three quality indices following the procedure recommended by Wajnberg (2003), against which we compared the quality goals. We also calculated these indices for the egg samples collected in the field, in the event of wasp emergence.

The first day of parasitoid emergence at 18° C, 20°C and 22°C was subjected to pairwise multiple comparisons with a Wilcoxon rank-sum test and p-adjusted values using the Bonferroni correction (R Development Core Team, 2009). Due to a scale factor (emergence time was computed in days, which resulted in underdispersion), data were not amenable to Poisson regression. To assess the differences in the obtained wasp harvest, we compared the proportion of live wasps, calculated over the number or parasitized host eggs, obtained at the three temperatures by multiple pairwise comparisons of the G-test of independence with adjusted p-values using the Bonferroni correction (MacDonald & Gardner, 2000).

The abortion rates in exposed vs. unexposed tubes, as well as the proportion of females in the first and the second half of the generation, were tabulated as 2x2 contingency tables and compared by means of a Cochran-Mantel-Haenszel X^2 test. We tested the null hypothesis that the two nominal variables (abortion vs exposition and sex vs period, respectively) were conditionally independent in each generation (Zhang & Boos, 1997). Homogeneity of the ratios in both cases was checked with a Breslow-Day test.

Results

Quality of the rearing colonies

The change in quality indices over generations in both rearing colonies is summarized in Figure 6.2. In general, the estimated parameters met the expected *a priori* set standard goals. Survival exhibited a considerable variation in Colony 1 but improved in Colony 2, showing values above 80% in all but one generation (Figure 6.2).

Emergence percentage remained between 20 and 30 % after generation 21 in Colony 1. In contrast, Colony 2 exhibited higher fluctuation in emergence percentage, although these were above 20% from generation18 onwards. In Colony 1, only 47 tubes out of 1793 exhibited emergence values above 50 wasps. Due to a limitation in the number of host eggs, and based on the fact that half of the eggs remained non-parasitized, we decreased the number of eggs from 100 to 50 per tube, beginning in generation 23 of Colony 2. As it did not affect the other indices, we decided to maintain this proportion in the subsequent generations.

Sex ratio was slightly biased towards females. The percentage of females remained significantly above 50 % in all but one generation (18) in Colony 1, and two generations (10, 15) in Colony 2.

Temperature

The first day of emergence of adult parasitoids was affected by temperature. Variation within each temperature setting was low. Emergence of parasitoids started on day 19 ± 0.9 (mean ± standard deviation) at 22 °C, on day 24 ± 0.9 at 20 °C, and on day 27 ± 0.9 at 18 °C (Wilcoxon rank sum test, corrected p-values in all pairwise comparisons < 0.001). No significant differences were observed in the percentage of live wasps harvested at the three different temperatures (G-test = 5.16, d.f. = 2, $p = 0.07$, Figure 6.3).

Basal hatching and parasitoid-induced abortion

Hatching rates of unexposed eggs remained above 80 % in all generations (Figure 6.4A). Abortion rates were significantly different between exposed and unexposed eggs (Cochran-Mantel-Haenszel $X^2 = 30.399$, d.f. = 1, $p<0.001$, Figure 6.4B). The ratio between treatments remained constant across generations (Figure 6.4B; Breslow-Day $X^2= 0.083$, d.f. = 2, p-value = 0.96).

Sex ratio

The proportion of females was higher during the second half of the emergence period within and across the generations observed (Cochran-Mantel-Haenszel $X^2 = 18.322$, d.f. = 1, $p<0.001$, Figure 6.5). The ratios remained homogeneous across generations (Breslow-Day $X^2= 3.0759$, d.f. = 2, p-value = 0.96).

Field release

Parasitized eggs were released in seven sites from 2013 to 2015 (Figure 6.6). The summer of 2014 was extremely rainy and stormy, which greatly affected the population of the bronze

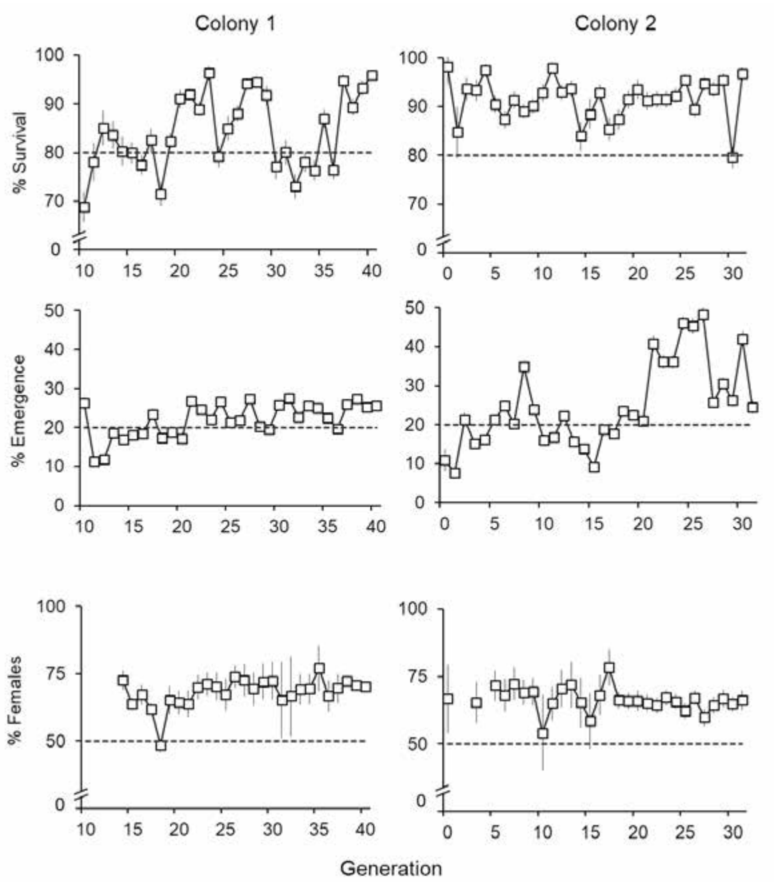

Figure 6.2. Percentage of living offspring, percentage of emergent wasps and percentage of female offspring (±95% confidence interval) per generation in the two rearing colonies of *Cleruchoides noackae*. Dotted lines represent a priori expected quality standards.

bug, as reflected in the national surveillance network (Martínez & Gómez, 2014). These conditions delayed the first monitoring campaign. During 2014, we sampled sites 1 to 4 in late March, and we made two new releases in April (sites 5 and 6). In May of the same year we revisited sites 2-4, due to the more favorable conditions and the remaining occurrence of the bronze bug in the field. During this sampling we recovered for the first time *C. noackae* from site 3 (Table 6.1). We revisited sites 1-6 in 2015 but could not find bronze bug eggs in most of the sites, so we did not make new releases that year. However, we managed to collect eggs from site 3, from which we recovered wasps again. In 2016, wasps were recovered again from site 3, and for the first time from site 4 in February and March, respectively. From March to May 2016 we also made releases in a new site (7).

Wasps emerging from the eggs collected in the field exhibited *in vitro* survival rates above 80% in all the samples except for the eggs collected from site 3 in 2014 (646 eggs in 7 tubes), The samples that were taken from site 3 in 2015 exhibited a higher emergence (51.7±1.8 %) than in previous field samples and rearing conditions. As mentioned earlier, we used these wasps to start Colony 2. Wasps emerging from the field collections had a 50% or slightly higher proportion of females (Table 6.1).

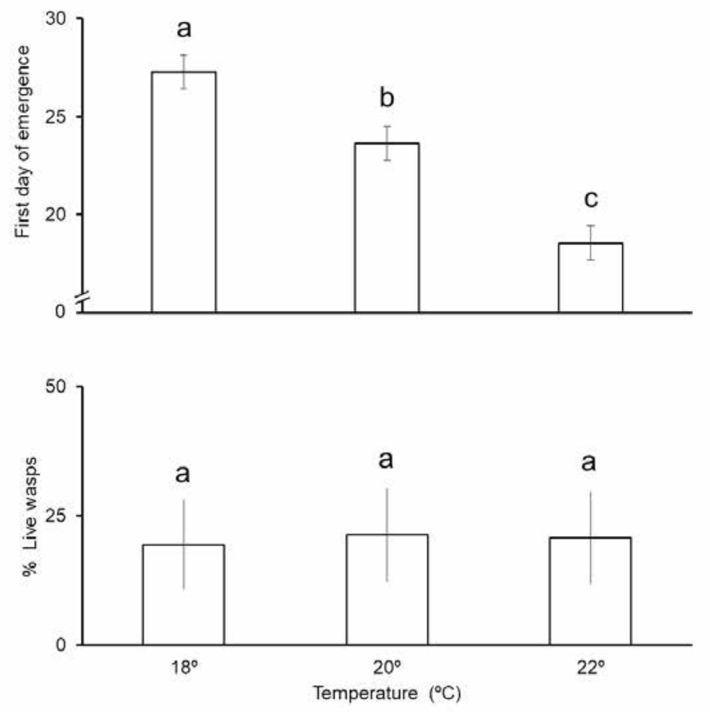

Figure 6.3. First day of emergence (mean ± standard deviation) and percentage of live *Cleruchoides noackae* wasps (±95% Confidence interval) at different temperatures. Different letters indicate significant differences between the treatments at α= 0.05 (Wilcoxon rank –sum test for first day, G-test of independence for % live wasps).

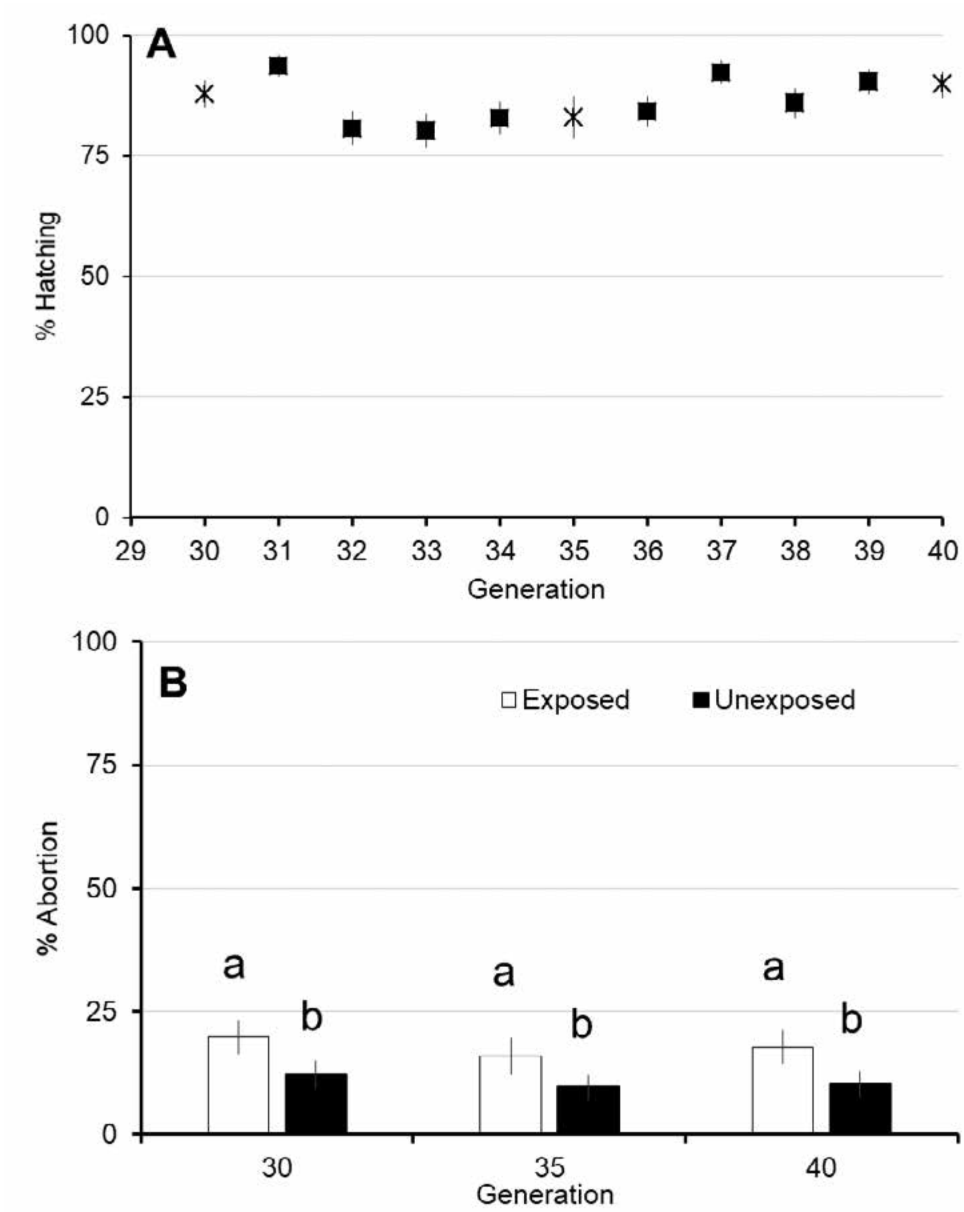

Figure 6.4. (A) Percentage of *Thaumastocoris peregrinus* egg hatching (± 95% confidence interval) in Colony 1 at different generations. Abortion rates of unexposed eggs were calculated by the generation marked with crosses; (B) Percentage of aborted eggs of *T. peregrinus* (±95% confidence interval) exposed or unexposed to parasitization by *Cleruchoides noackae*. Different letters indicate significant differences between treatments and across generations at $\alpha = 0.05$ (Cochran-Mantel-Haenszel X^2).

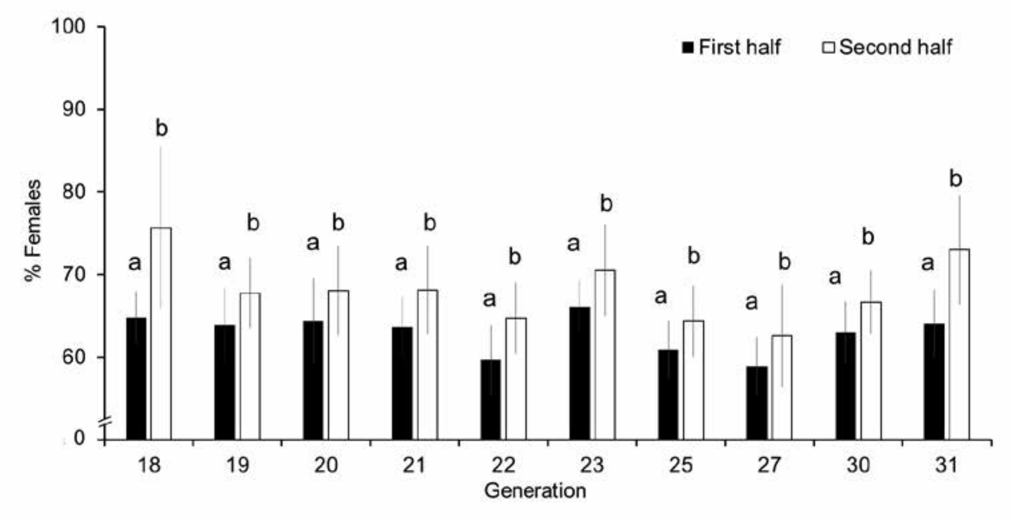

Figure 6.5. Percentage of females of *Clerucboides noackae* (± 95% confidence interval) for wasps that had emerged in the first half (black bars) and in the second half (white bars) of each generation. Different letters indicate significant differences between treatments and across generations at α= 0.05 (Cochran-Mantel-Haenszel X^2).

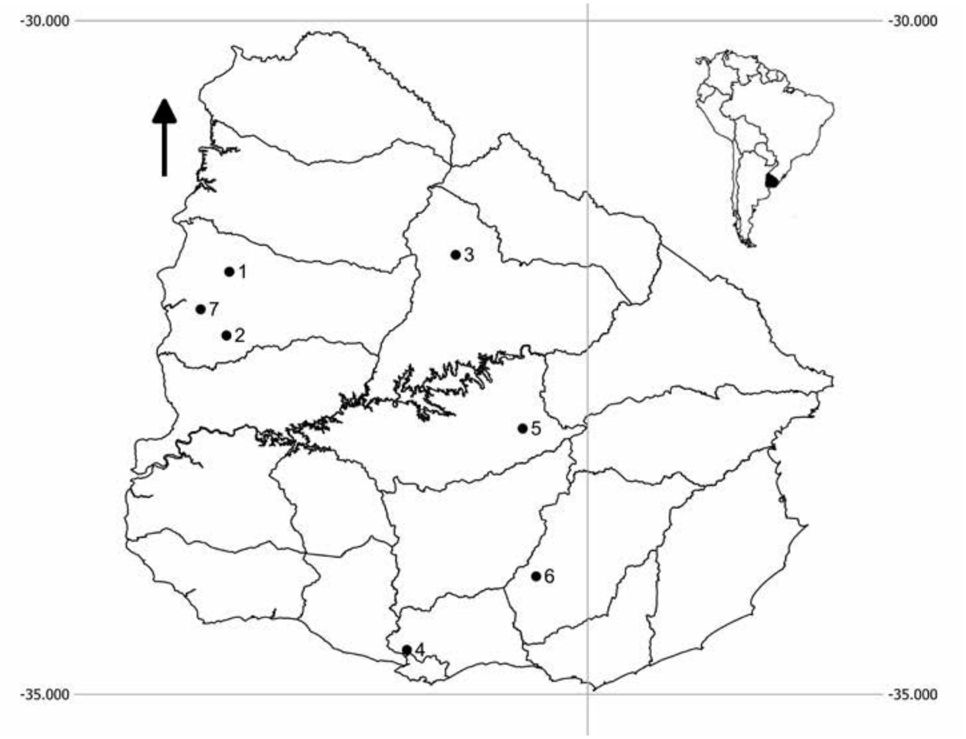

Figure 6.6. Map of Uruguay displaying release sites of *Clerucboides noackae*. Arrow indicates North.

Table 6.1. Summary data of field release sites for *Cleruchoides noackae* (Indices are given ± 95% confidence interval).

Date	**Site**	***Eucalyptus* species**	**Number of release event**	**Number of parasitized eggs released**	**Number of monitoring events**	**Number of *T.peregrinus* eggs collected (year)**	**Recovery**	**%Emergence (year)**	**% Females (year)**	**%Survival (year)**
03/2013	1	*E. dunnii*	2	500 500	3	250 (2014) Not found (2015)	No	-	-	-
03/2013	2	*E.benthamii*	2	500 500	3	Not found (2014) Not found (2015)	No	-	-	-
11/2013	3	*E. tereticornis* *E.maidenii*	2	1000 1000	2	646 (2014)	Yes	2.4±0.5 (2014)	73.8±9.5 (2014)	38.1±14.9 (2014)
02/2014	3	*E.tereticornis* *E.maidenii*	2	1000 1000 1000	2	2021 (2015) 335 (2016)	Yes	51.7±1.8 (2015) 35.5±11.7 (2016)	59.9±1.5 (2015) 58.8±15.0 (2016)	85.5±0.6 (2015) 100±19.6 (2016)
02/2014	4	*E. globulus*	2	1000 1000	3	Not found (2014) 1830 (2016)	Yes	26.1±2.6 (2016)	50.0±1.1 (2016)	80.5±2.8 (2016)
04/2014	5	*E. grandis*	1	1500	1	Not found (2015)	No	-	-	-
04/2014	6	*E. viminalis*	1	1200	1	Not found (2015)	No	-	-	-
03/2016	7	*E. benthamii*	3	1200 1200 1200	-	-	No	-	-	-

Discussion

The rearing protocol developed for *Cleruchoides noackae* and presented in this study allowed us to maintain a viable colony of the wasp for two years, without significant changes in quality parameters relative to standards based on rearings in Brazil and Chile. Wasps from the rearing were released periodically in the field and they were able to colonize at least two sites and reproduce in numbers that were high enough to initiate a new rearing exclusively from field-collected individuals. Together, these data demonstrate that it is feasible to maintain a rearing system of this parasitoid in the framework of a biological control program for the bronze bug.

Laboratory rearing

Based on the quality indices, preparation of 60 rearing tubes per generation would produce in the worst case scenario 570 live female wasps per 3000 eggs, which is sufficient to maintain the rearing during winter, when no releases are made. In summer, upscaling the rearing colony to 100 tubes would provide 960 female wasps per 5000 eggs, allowing for a field release. Daily tasks within the rearing facility involve the preparation of rearing units and counting of the emerged wasps. Two people working 30 h per week can take care of this colony during the emergence periods.

We set as target an emergence rate of 20% or more, at least 50% females, and more than 80 % survival based on the parameters estimated in a rearing set at EMBRAPA (Brazil) from where we obtained the founders of our first rearing colony. Survival was better in the second rearing colony starting from field-collected individuals. Better handling of the system due to experience may explain this, although adaptation of the wasps to the rearing conditions should not be discarded as an explanation (Sørensen et al., 2012).

According to our results, emergence of 20-30 wasps on average on a basis of 100 eggs implies that around half of the eggs remained unparasitized (if we consider 20 aborted eggs on average). The decision to reduce the number of eggs to 50 per tube allowed us to economize efforts without losing productivity. Indeed, the percentage of emergence doubled after this change (i.e. number of emerged wasps remained equal), whereas the extant indices either did not change or improved.

Although in some cases rearing conditions can affect parasitoid sex ratio within the Mymaridae (Heimpel & Lundgren, 2000), our results show a slightly female-biased sex ratio that remained consistent over generations. Data from our field collections show female proportions from ca 50% female to moderate female-biased. Female-biased populations have also been recorded for rearing colonies of other species of Mymaridae (Boivin, 1988; Chen et al., 2006), but Mutitu et al. (2013) reported a slightly male-biased sex ratio (although not significantly different from 50%) in a rearing colony of *C. noackae* in the only published study in this species. In our study, only one out of 60 generations exhibited a male-biased sex ratio, and no male-biased population was observed in the field. Given that *C. noackae* reproduces by arrhenotokous (haplodiploid) parthenogenesis (Mutitu et al., 2013), like other mymarid wasps, females are able to control the sex ratio of their progeny as a function of the quality of the colonized patches (Van Baaren et al., 1999).

Managing inbreeding depression is a major challenge when rearing insects, particularly at a medium scale population size (Charlesworth & Charlesworth, 1987). Our study of two separate rearings for more than 30 generations suggests that *C. noackae* exhibits a high tolerance to inbreeding depression in an *in vitro* rearing, a condition that has been observed in other wasp parasitoids (Quaglietti et al., 2017). In the case of *C. noackae* this can be due to the biology of this tiny wasp with limited dispersal capabilities, resulting in very fragmented small populations of the wasp with limited gene flow (Nadel, Wingfield, Scholes, Lawson, Noack, et al., 2012). Nevertheless, future studies should assess other life-history parameters and their changes during continuous rearing. Given our results, it is advisable to install a new rearing colony with fresh field individuals at least once every two years to avoid adaptation to the laboratory conditions (Sørensen et al., 2012).

Temperature

As expected, temperature affected the duration of the life cycle of *C. noackae*. Additionally, survival and emergence were not affected by rearing at lower temperatures. The optimal temperature range for *C. noackae* spans between 15 °C and 25 °C, according to previous studies (Souza et al., 2016), and rearing at 22 ° C allows for harvesting with 19 days intervals. Here, we checked two temperatures below the usual rearing temperature of 22°C, in order to optimize winter rearing. Based on our results we chose 18 °C as representative for winter rearing, which allows the harvest of a new generation every 27 days (monthly harvesting), without reduction in emergence or survival.

Basal hatching and parasitoid-induced abortion

Parasitoid-induced host egg abortion can be an additional biological control service provided by egg parasitoids (Abram et al., 2016). Our data show that under the rearing conditions as used here, on average 20% of the eggs laid by the bronze bug do not hatch. This is a percentage that is increased after exposure to *C. noackae* parasitization. Future studies should investigate whether non-hatching eggs of the bronze bug are the consequence of being unfertilized, or due to failures during embryonic development, or actually induced by *C. noackae*.

Sex ratio

In this study, we consistently observed an increase in the proportion of females in the second half of the generation, suggesting that males emerge earlier than females. When sibling mating or other non-panmictic mating is common, the resulting mating structure is known as local mate competition (Hamilton, 1967) which selects for a female-biased sex ratio, as it is observed here for *C. noackae*. Under local mate competition, early emergence may confer competitive advantages to the males in order to access a higher number of females (Hamilton, 1967; Werren, 1980), particularly in a species with a relatively short adult lifespan such as *C. noackae*. The observation of wasps copulating immediately after emerging, also reported by Mutitu et al. (2013), gives additional support to the local mate competition theory.

Field release

We report the successful recovery of field individuals of *C. noackae* in two field sites and repeatedly for three years. Based on these results we can confirm the establishment of a field population of *C. noackae* in Uruguay.

Releasing and monitoring of the parasitoids were greatly affected by adverse weather conditions. Two consecutive summer periods with events of heavy rain hampered bronze bug populations to a point that it was not possible to recover eggs from the majority of the field sites. Both sites that registered field parasitization were located near INIA stations, which allowed us to use mechanical elevators to collect eggs up to 20 m high. Thus, the lack of recovering populations in other sites may be due to a differential sampling effort. Moreover, we recovered *C. noackae* from site 4 two years after the releases, which implies that the wasps managed to maintain their population without further introductions from 2104 to 2016. With the sole exception of the first recovery in site 4 in 2014, survival of field populations remained above 80%, emergence above 20%, and more than half of the recovered individuals were females. Together, these results suggest that there are good opportunities for the establishment of field populations of this parasitoid.

Future prospects

After four years of rearing and releasing of *C. noackae,* we have set up a rearing procedure that allows for a stable production and field release of the parasitoids, and we have installed at least two separated field populations that assure the continuity of wasp production. The steps ahead involve the optimization of the release schemes and assessment of the efficacy of *C. noackae* as a biological control agent of the bronze bug in Uruguay. Improving both the rearing conditions and the success of field releases require additional studies on the mechanism of host-searching by this parasitoid.

Acknowledgements

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Chapter 7

Chemical cues exploited by *Cleruchoides noackae* to locate its host

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Submitted for publication

Abstract

Host selection by egg parasitoids involves solving the reliability-detectability dilemma regarding which cues to exploit. The aggregative behavior and the chemical apparency of many true bug species suggest that their egg parasitoids can resort to cues from nymphs and adults to locate their host's eggs. Here, we investigated the behavioral responses of the egg parasitoid *Cleruchoides noackae* (Hymenoptera: Mymaridae) to short-range cues from its host, the bronze bug *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae), including different host stages and their host plant. The response of recently emerged wasps to the different cues was assessed in dual-choice contact bioassays. Parasitoids were individually exposed for 5 minutes to a pair of treatments placed on an arena. We compared residence times in the treatment area, the control area and the remaining parts of the arena. The wasps exhibited longer residence time on arenas containing their host eggs, even if these had been removed, and by adult *T. peregrinus* cues and plant cues. Extracts of eggs and adult individuals of both sexes also elicited longer residence times by *C. noackae*. These results confirm that *C. noackae* exploits the infochemical detour to locate its host.

Introduction

Host selection by insect parasitoids comprises host-habitat location, host location, host recognition, and host acceptance (Vinson, 1976, 1998). Different cues may be exploited at each of these hierarchical steps (Fatouros et al., 2008). In searching for their hosts, parasitoids of insect herbivores are faced with a 'reliability-detectability dilemma': The most apparent cues, such as those originating from the host plant, are easy to detect but do not warrant the presence of the host, while the cues generated by the hosts are highly reliable but hard to detect from a distance (Vet & Dicke, 1992). This dilemma is particularly important in the case of egg parasitoids. Unlike other stages, eggs are inconspicuous, release few chemical cues, and tend to be present for short periods of time in the environment (Fatouros et al., 2008). These characteristics affect the foraging pattern of egg parasitoids, making them dependent on information from the first and the second trophic levels to locate their potential hosts (Rutledge, 1996; Venzon et al., 2001; Groot et al., 2003; Fatouros et al., 2012). Particularly, parasitoids may rely on chemical cues from other host stages such as juveniles and adults as an 'infochemical detour' to locate their egg hosts (Vet & Dicke, 1992; Fatouros et al., 2008).

Egg parasitoids of true bugs exhibit particular adaptations to their hosts. Heteropterans often produce a high diversity of volatile and non-volatile substances for both communication and defense (Aldrich, 1988). Egg parasitoids of true bugs can benefit from such 'chemical apparency' by eavesdropping on the chemical communication system of other stages of their hosts. For instance, there is evidence that parasitoids of true bugs exploit walking traces (Borges et al., 2003; Peri et al., 2006; Salerno et al., 2006; Colazza et al., 2009), defensive compounds (Mattiacci et al., 1993; Laumann et al., 2009), and adult pheromones (Leal et al., 1995; Mizutani et al., 1997; Bruni et al., 2000; Krupke & Brunner, 2003; Aldrich et al., 2006; Mizutani, 2006; Silva et al., 2006). The fact that many species of true bugs exhibit subsocial and aggregative behavior (Bongers, 1968; Groot et al., 2003; Blackmer et al., 2004), increases the probability of finding eggs in the vicinity of other host stages, and thus the reliability of this infochemical detour. On the other hand, information from host plants that may be reliable to locate eggs of monophagous herbivore species, such as the majority of the Lepidoptera, may not be useful to locate eggs of polyphagous species, which is a common trait within the Heteroptera. Nevertheless, many parasitoids of true bugs exhibit attraction toward feeding-induced and oviposition-induced plant volatiles (Colazza, Fucarino, et al., 2004; Colazza, McElfresh, et al., 2004; Moraes et al., 2005; Conti et al., 2006, 2008; Williams et al., 2008). Moreover, several egg parasitoids of true bugs are known to respond to cues from their host eggs, usually at a short-range (Conti et al., 1996, 1997, 2003, 2010; Takasu & Nordlund, 2001).

Here, we studied the host-location behavior of *Cleruchoides noackae* Lin and Huber, an egg parasitoid for the eucalypt bronze bug *Thaumastocoris perergrinus* Carpintero and Dellape. We aimed at determining the responses of the parasitoid to short-range infochemicals originating from the host eggs, other host stages and the host plant *Eucalyptus tereticornis*. Based on its small size (0.2 mm) and its short lifespan of 2-3 days (Lin et al., 2007; Mutitu et al., 2013), we hypothesized that *C. noackae* would benefit from the use of an infochemical detour. Thus, we expect it to be attracted not only to cues from eggs but also to respond to cues from host stages. We also expect to find attraction toward infested plants.

Material and methods

Insects and plants

Eucalyptus tereticornis branches containing fresh leaves were collected at INIA's experimental station in Tacuarembó, Uruguay (31°44'19.24"S; 55°58'43.75"W). Leaves from these branches were offered as oviposition substrates to adult *Thaumastocoris peregrinus* obtained from a permanent rearing (Martínez et al., 2014, Chapter 3 in this thesis), while the extant leaves constituted the source of undamaged leaves for the bioassays. Adult females of *Cleruchoides noackae* were obtained from a permanent rearing set at INIA (Chapter 6).

Contact bioassays

We used an arena consisting of a platform printed with polylactic acid (PLA) with an Ultimaker^2 3D-printer (Figure 7.1). This platform measures 2.5 cm long, 1 cm wide and 1 cm high. The central area (0.5 cm^2) was used as the release area for the focal wasp. Two square areas for the leaf sections (1 cm^2) were on each side of the release area. The platform was glued to a Petri dish (5.5 cm diameter) and surrounded by water to prevent the wasps from leaving the arena.

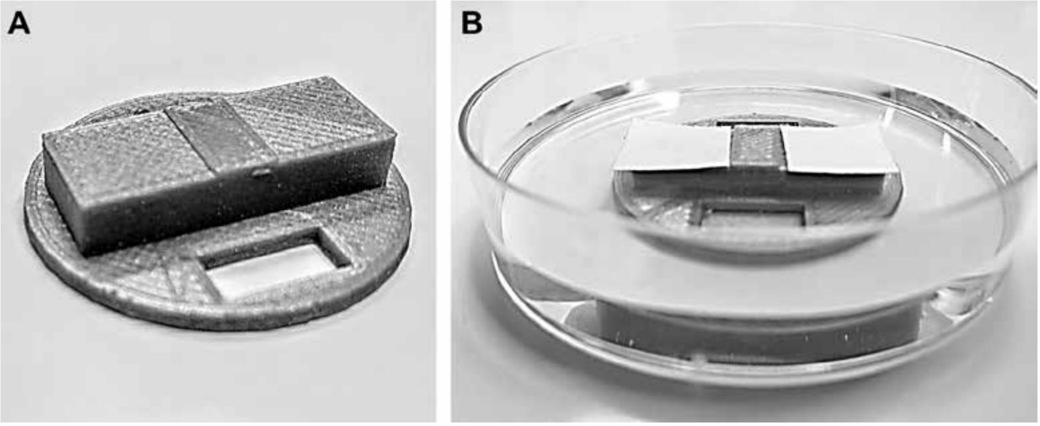

Figure 7.1. (A) arena printed with polylactic acid used in the contact bioassays with *Cleruchoides noackae* parasitoids; (B) Position of the arena in a Petri dish filled with water.

The two-choice bioassays were conducted from 13:00 to 18:00 h in an isolated laboratory at 20 ± 1 ºC and 60 ± 5 % RH. The arena was illuminated by uniform light (4.9 lux) and filmed with a webcam (Logitech™ C-190) for data collection. A single wasp, less than 24 hours old, was released in the center of the release area and allowed to move for 5 min. The residence time on both leaf squares was recorded as a measure of preference, and the time spent on the release area and on the borders of the arena was recorded as searching time. To avoid effects of unforeseen asymmetric conditions, the arena was rotated 180 degrees after each replicate, reused once and then replaced by a clean one. Each wasp was used only once, and each choice test was performed with fifty replicates. Wasps that remained motionless for more than 1 minute were considered non-responsive and were discarded.

The first set of bioassays explored the response of *C. noackae* to *T. peregrinus* eggs and egg-related cues. To this end, females of *C. noackae* were offered a leaf square of *E. tereticornis* containing 5 to 10 *T. peregrinus* eggs laid 48 h before the test, or a similar leaf square from which the eggs had been removed by gently detaching them with an insect pin. In both cases, a clean (i.e. unexposed to the bronze bug) leaf square of *E. tereticornis* from the same branch was offered as control.

In a second set of bioassays, we tested the response of the wasps to potential cues from two sources, i.e. the host itself in the adult stage, including cues left on the leaves, and the plant, including induced chemicals produced by the plant as a response to herbivory by the bronze bug, and cues left by the bronze bug on the leaves. To do this, female wasps were offered, in separate bioassays, a clean leaf square of *E. tereticornis* with one adult male or female of *T. peregrinus* (recently killed by freezing), or a leaf square of *E. tereticornis* previously exposed to 150 adult individuals of *T. peregrinus* for 48 h (simulating field densities). In both cases, a clean leaf square was used as control. Finally, we tested the basal response to a clean leaf square by comparing it against a square piece of filter paper.

We ran a third bioassay series, testing for the behavioral responses of *C. noackae* to extracts obtained from eggs and adult bugs. Egg extracts were made by rinsing 100 eggs of the bronze bug in 500 µL of hexane or methanol in 2-mL glass vials. The vial was shaken for one minute, left to rest for 30 minutes and shaken again for an additional minute. The liquid phase was transferred to a second vial and concentrated under a nitrogen stream to 100 µL. Additional egg extracts were made by extracting eggs first with methanol and then with hexane (denoted by 'sequential'). We also extracted 50 females or 50 males with hexane, using the same protocol as for the egg extracts. To test the different extracts, 10 µL were applied to a clean leaf square of *E. tereticornis*, hence applying 10 egg-equivalents or 5 insect-equivalents to the 1 cm^2 leaf square. In all cases, the control was a leaf square with 10 µl of the solvent (methanol or hexane). As a baseline control test, a similar two-choice bioassay was conducted with an untreated leaf square and one on which the solvent had been applied.

Chemical analysis

Hexane egg extracts were analyzed by GC-MS, and methanol extracts were analyzed by GC-MS, LC-MS and NMR. One hundred eggs (mass range: 1.77-2.25 mg) were extracted in hexane or methanol as detailed above, except for the sample for NMR analysis in which the eggs were soaked in 0.6 mL of 99.8% CD_3OD.

GC-MS analyses were performed on a QP-2010 Shimadzu GC-MS, equipped with a Stabil wax column (Restek, USA) (30 m × 0.25 mm i.d., 0.25 µm film thickness), operated with a constant He flow of 1 ml/min. The temperature of the GC oven was programmed from an initial temperature of 40 °C (1 min), then heated to 250 °C at 7 °C/min, and held for 1 min.

The injector temperature was 220 °C and the interphase temperature was 260 °C. Injection (1 µl) was in the splitless mode, and mass spectra were acquired from *m/z* 30 to 350 in the scan mode (70 eV). Mono- and bi-dimensional NMR spectra were obtained on a Bruker ASCEND 500 (500 MHz for ^{1}H NMR; 64 and 8 scans for ^{1}H and COSY, respectively). Direct infusion mass spectrometry was performed in a linear ion trap mass spectrometer LTQ XL from Thermo Scientific, with an ESI interface. Instrument control and data collection were done using Xcalibur software (v3.0.63). The ESI parameters were as follows: spray voltage 4.0 kV, capillary voltage 33 V, tube lens offset 250 V, sheath gas (nitrogen) flow rate 8 (arbitrary units, a.u.), no auxiliary gas, and capillary temperature 300 °C. The LTQ mass spectrometer was operated in positive mode, scanning from *m/z* 125 to 800. The egg extracts were infused in the ESI source using a syringe pump (flow rate 5 µl/min). Further analysis of the ions produced by ESI was performed by means of MS^2 experiments. To optimize the ESI-MS^2 conditions, the collision energy percentage was increased up to 30 to produce a whole fragmentation pattern showing an array of all possible fragments.

Data analysis

We compared residence time between treatment and control areas with a Wilcoxon signed-rank test. The time spent by wasps in areas other than the leaf squares (i.e.: no response) across pairs of treatments in each bioassay series were compared with a Wilcoxon rank-sum test and, if there were more than two treatments, by pairwise multiple comparisons and p-adjusted values using the Bonferroni correction. All tests were conducted in R, version 3.4.0 (R Development Core Team, 2009).

Results

Contact bioassays

We tested a total 815 wasps, obtaining 600 responsive wasps that completed the 5-minute test. Discarded replicates include 129 individuals that remained motionless for more than one minute, 76 wasps that drowned in the surrounding water, and 10 flying wasps. All wasps included in the analyses visited all three areas of the arena and entered the treatment and control areas several times, as opposed to staying in one area after a single visit.

Cleruchoides noackae wasps spent more time on leaf squares of *E. tereticornis* containing egg batches of *T. peregrinus* (Wilcoxon signed-rank test W= 1257, $p< 0.001$) than on clean leaves (Figure 7.2 A). Moreover, such a preference was also observed on leaves from which the eggs had been removed (W= 1221, $p< 0.001$) (Figure 7.2 A), although the time spent in other parts of the arena increased when eggs were not present (W = 844, $p = 0.005$) (Figure 7.2 B).

Wasps also spent more time on leaf squares exposed to feeding by *T. peregrinus*, as well as on leaf squares carrying dead adult insects (Figure 7.3 A). On the former, *C. noackae* females

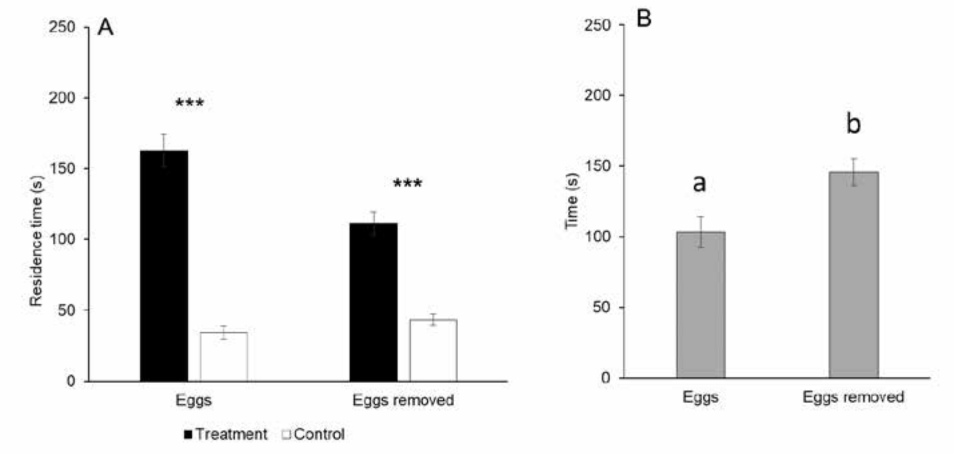

Figure 7.2. Response of *Cleruchoides noackae* females to egg cues in dual-choice contact bioassays. (A) Mean residence time in seconds (± SEM) on the treatment (egg-carrying leaf squares of *Eucalyptus tereticornis*) and control (clean *E. tereticornis* leaf squares) areas. Asterisks indicate significant differences in time spent on treatment vs control areas within the same bioassay (***: P <0.001, n = 50 Wilcoxon's signed-rank test); (B) Mean time spent (± SEM) in areas other than the leaf squares. Different letters indicate significant differences in time spent on the rest of the arena between different bioassays (Wilcoxon's rank-sum test, α=0.05, n= 50).

showed higher residence times than on the control leaf squares (W= 1076.5, p < 0.001). In turn, the wasps spent more time on the leaf squares containing a dead male (W= 1091, p< 0.001) or female of *T. peregrinus* (W= 1057, p< 0.001), than on the respective control leaf squares. The time spent on a clean leaf square of *E. tereticornis* was also longer when compared to a piece of filter paper (W= 1111.5, p<0.001) (Figure 3 7.A). Finally, the time spent outside the test and control areas was longer on leaves containing a dead male when compared with the rest of the bioassays in this experimental series (Bonferroni corrected p-value <0.005 in all cases) (Figure 7.3 B).

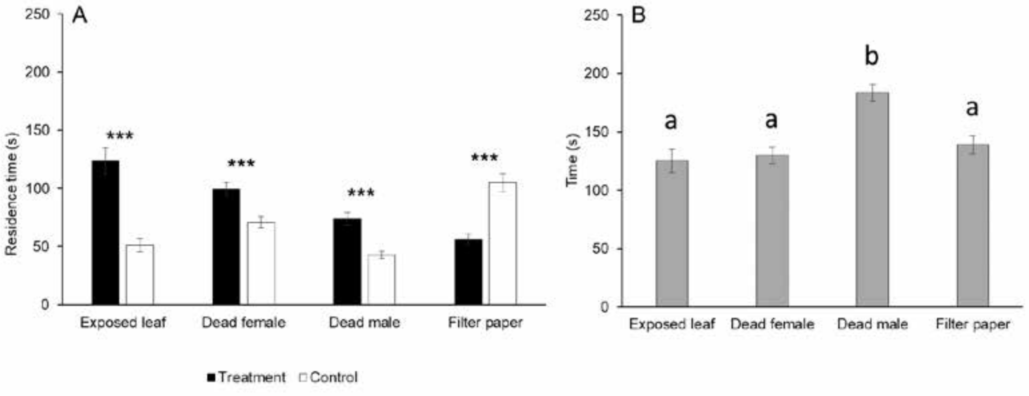

Figure 7.3. Response of *Cleruchoides noackae* females to cues from plant and adult bronze bugs in dual-choice contact bioassays. (A) Mean residence time in seconds (± SEM) on the treatment (exposed leaf squares, leaf squares with a dead male or female, and a filter paper square piece) and control (clean *Eucalyptus tereticornis* leaf squares). Asterisks indicate significant differences in time spent in treatment vs control areas within the same bioassay (***: P <0.001, n = 50 Wilcoxon's signed-rank test); (B) Mean time spent (±SEM) in areas other than the leaf squares. Different letters indicate significant differences in time spent on the rest of the arena between different bioassays (Wilcoxon's rank-sum test, α=0.05, n= 50).

Leaf squares containing egg extracts also triggered a behavioral response by the wasps (Figure 7.4). Both methanolic (W=1153, p< 0.001) and hexane egg extracts (W= 1092, p<0.001) resulted in longer residence times when applied to a leaf square against the corresponding solvent-treated control leaves (Figure 7.4A). However, testing sequential extracts showed that hexane egg extracts did not affect the parasitoid behavior if the eggs had been previously extracted with methanol (W= 544.5, p= 0.502). This result prompted us to further test the methanolic extract on filter paper, without leaf-related cues. We found that the wasps also exhibited longer residence times on filter paper treated with the methanolic egg extract against methanol-treated filter paper (W=1205.5, p< 0.001). Nonetheless, the use of paper instead of leaf squares significantly increased the time spent outside test and control areas (Figure 7.4B).

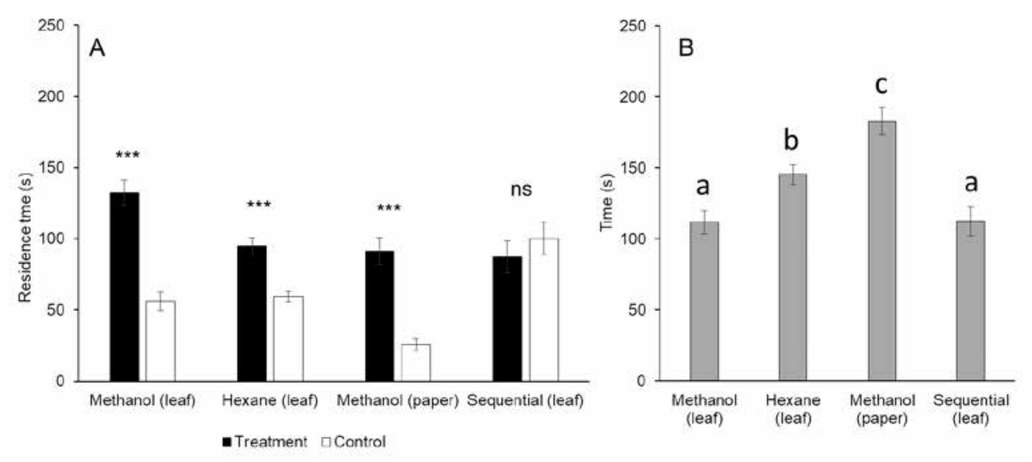

Figure 7.4. Response of *Cleruchoides noackae* females to egg extracts made with methanol, hexane or hexane after methanol extraction ('Sequential'), applied to either filter paper or leaf squares, in dual-choice contact bioassays. (A) Mean residence time in seconds (± SEM) on the treatment and control areas. Asterisks indicate significant differences in time spent on treatment vs control area within the same bioassay (***: P <0.001, n = 50 Wilcoxon's signed-rank test); (B) Mean time spent (± SEM) in areas other than the leaf squares. Different letters indicate significant differences in the time spent on the rest of the arena between different bioassays (Wilcoxon's rank-sum test, α= 0.05, n = 50).

The wasps also spent more time on leaf squares containing female (W= 928, p= 0.005) or male (W= 838, p= 0.025) hexane extracts, versus a control leaf square treated with hexane (Figure 7.5A). The time spent on other areas of the arena was more than half of the total experimental time, but it was not significantly different across bioassays (W= 1222, p= 0.85, Figure 7.5B).

Chemical analysis of the extracts

GC-MS analyses of hexane and methanolic surface extracts of *T. peregrinus* eggs showed trace amounts of several mono- and sesquiterpenes that, given their small amount, could not be identified. The main difference between methanolic and hexane egg extracts was that the former contained larger amounts of a compound related to hexadecanoic acid, possibly an ester that remains to be identified.

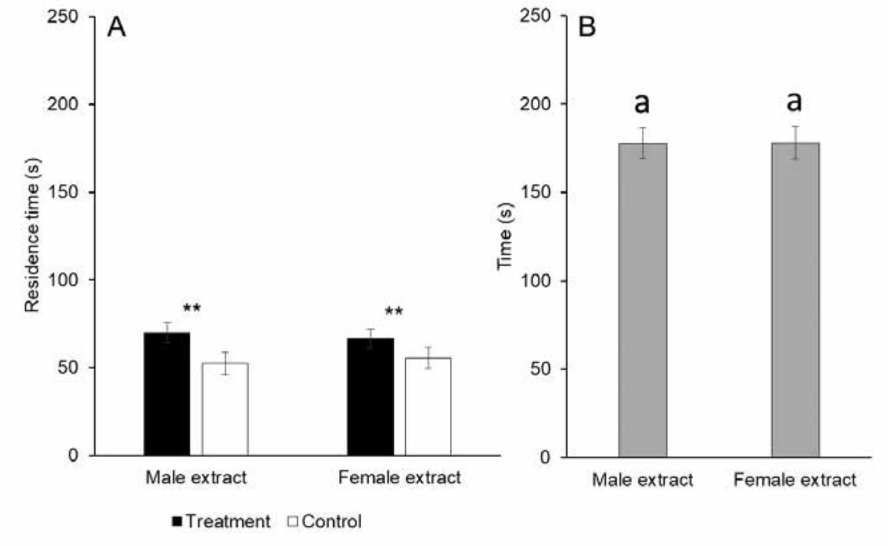

Figure 7.5. Response of *Cleruchoides noackae* females to hexanol bronze bug adult egg extracts applied to clean *Eucalyptus tereticornis* leaf squares in dual-choice contact bioassays. (A) Mean residence time in seconds (± SEM) on the treatment and control (hexanol treated *E. tereticornis* leaf square) areas. Asterisks indicate significant difference in time spent on treatment vs control area within the same bioassay (***: P <0.001, n=50 Wilcoxon's signed-rank test); (B) Mean time spent (±SEM) in areas other than the leaf squares. Different letters indicate significant differences in time spent on the rest of the arena between different bioassays (Wilcoxon's rank-sum test, α=0.05, n= 50).

NMR spectra did not show significant amounts of any discernable compound (Figure S1). The spectra are dominated by the solvent residual signals and water (between 3 and 5 ppm). Small signals in the aromatic (7.9 – 8.5 ppm) and aliphatic regions (0.9 – 2.0 ppm) are present, but they could not be assigned to candidate compounds due to their low intensities.

ESI-MS analyses of three independent egg extracts consistently showed a major ion at *m/z* 711 ($M + H^+$), among other less abundant ions that differed in 28 mass units (Figure S2, above). The ion at *m/z* 711 showed fragment ions in MS^2 that suggested the loss of several water molecules (Figure S2). The identification and significance of these compounds require further studies, including the chromatographic separation of the mixture in order to acquire the mass spectra of individual compounds.

Discussion

Our study shows that *C. noackae* responds to short-range cues from different sources, which may contribute to their location of *T. peregrinus* host eggs. In our dual-choice bioassays, we observed the wasp behavioral response as longer residence times on the treatment areas when compared to control areas. Thus, the wasps responded to egg-carrying leaves even when the eggs had been removed. Egg extracts obtained with polar and non-polar solvents also elicited a response. Additionally, *C. noackae* responded to dead bronze bug adults, both males and females, as well as to their extracts when applied to leaf squares. Finally, they also responded to leaf squares previously exposed to adult bronze bugs, and they spent more time on clean leaves of *E. tereticornis* than on filter paper. Collectively, these results suggest that in their host location process, *C. noackae* exploits information from the first and the second trophic level, including chemical cues present on the surface of its egg hosts, adult cues as infochemical detour, and plant cues.

Egg parasitoids generally disperse passively downwind due to their reduced size. In the present study we focused on short-range interactions, assuming likewise that due to their size (0.2 mm), *C. noackae* females would not actively fly towards a plant in an open system. The fact that in our open setup and at 20 °C we hardly observed flights by *C. noackae* (only 10 out of 815 individuals) gives additional support to this idea. Unfortunately, small-sized dark-brown colored wasps offer no contrast against an eucalypt leaf, which prevented us from using software for behavioral analysis and restricted our observation to direct observations.

Our results show that *C. noackae* exploits chemical contact cues from the egg surface. As expected, the longest residence times were observed on egg-carrying leaf squares, where all the cues assessed (egg chemical cues, plant cues, and adult cues) are likely to be present. We also obtained a response by *C. noackae* females to both hexane and methanol egg extracts, but the loss of the biological activity of the sequential hexane extract, obtained after extracting the same set of eggs first with methanol, suggests that these cues are more soluble in polar solvents such as methanol. Host egg extracts in polar solvents such as ethanol and water have triggered behavioral responses in *Trissolcus viktorovi* and *T. grandis* (Scelionidae), egg parasitoids of *Eurydema* spp. (Pentatomidae) and *Eurygaster integriceps* Put. (Scutelleridae), respectively (Buleza & Mikheev, 1978). Furthermore, water extracts from eggs of *Nezara viridula* (L.) (Pentatomidae) elicit host recognition behavior by its parasitoid, *T. basalis* (Woll.), and this has been linked to the adhesive secreted by the female (Bin et al., 1993). Egg extracts elicited a response even if tested in the absence of plant cues, as shown by our tests on filter paper as substrate. Further studies should address the chemical identity of these egg cues, and whether they are present on the egg surface, in the adhesive secretion, or both. Analysis of methanolic extracts by ESI-MS showed some consistent compounds that were not identified, but that represent a promising starting point. This technique provides more options for analyzing relatively polar, non-volatile compounds than GC-MS, hence potentially more appropriate for contact chemical cues.

The wasps spent more time on leaf squares containing dead *T. peregrinus* adults or cut out from leaves previously exposed to bronze bugs, than on clean leaf squares. They also responded to hexane extracts of both male and female adults, suggesting that *C. noackae* exploits chemical cues from adult *T. peregrinus*. Egg parasitoids of other species in the Heteroptera are known to rely on adult cues to locate their hosts. For instance, several species of egg parasitoids of stink bugs respond to chemical traces from adult stages of their hosts (Borges et al., 2003; Conti et al., 2004). Furthermore, the egg parasitoid *Trissolcus basalis* (Wollaston) responds to hexane extracts of cuticular lipids of the adult stage of its host, with a strong preference for extracts of females (Colazza et al., 2007). Such extracts contained a mixture of linear alkanes that differed quantitatively and qualitatively between sexes. Interestingly, compounds present on the exuviae of bronze bug nymphs include aldehydes, primary alcohols and fatty acids, which are commonly found in other true bugs as repellents or attractants (Martins et al., 2012). Also an aggregation pheromone, mostly produced by adult males, has been characterized (González et al., 2012) and found on the body surface of the male bugs (Martins et al., 2012).

While this compound is expected to be present in the male hexane extracts we have used, we did not find evidence of sexual discrimination by *C. noackae*, apart from an increased time outside the leaf squares when exposed to dead males. As many other true bugs, *T. peregrinus* exhibits gregarious behavior which implies the simultaneous occurrence of individuals of different age classes, including eggs, in a given location. Therefore, exploiting adult cues as infochemical detour to locate eggs of the bronze bug is a reliable strategy rendering this behavioral trait likely to be favored by natural selection. Future research should assess the potential effect of the bronze bug male pheromone on *C. noackae* searching behavior, given the role that this compound seems to play in mediating aggregative behavior.

Our results also show that plant-related cues may play a role in host location at a short-range by *C. noackae*. First, wasps spent more time examining a clean leaf square than a square piece of paper, suggesting a basal response towards *Eucalyptus* cues. Additionally, oviposition-induced or feeding-induced plant synomones may contribute to the response. For instance, *Trichogramma brassicae* Bezdeko exhibit arrestment in the vicinity of host eggs, likely to be due to oviposition-induced changes in leaf surface chemicals (Fatouros et al., 2005). Our results suggest a behavioral response closer to that observed in *Trissolcus brochymenae* Ashmead, an egg parasitoid of the Harlequin bug *Murgantia histrionica* Hahn. Indeed, this species exhibited increased residence times (but no arrestment) and a synergistic effect to oviposition, feeding damage and insect footprints (Conti et al., 2010). It is likely that also leaf epicuticular waxes mediate the behavioral responses by absorbing egg and adult kairomones (Colazza et al., 2009). Further research is required to elucidate the role of the plant in short-range foraging behavior of *C. noackae*.

Three main solutions have been proposed for the reliability-detectability dilemma of carnivorous arthropods: (1) to use an infochemical detour by exploiting information from stages of the host other than the target instar; (2) to rely on infochemicals that are a product of the interaction between the herbivore and the host plant, or (3) to link highly detectable plant cues to more reliable host cues through associative learning (Vet & Dicke, 1992). According to our results, and in concordance to our expectations, *C. noackae* exhibits a host selection strategy that relies strongly on the use of the infochemical detour by exploiting cues from other host stages. Biological constraints given by the wasp's size and short lifespan, along with the gregarious behavior of its host species, provide plausible explanations to the use of this strategy. Future studies should target the chemical basis of the observed responses, and on the role of the host plant in mediating host location by egg parasitoids at short distances.

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Supporting information

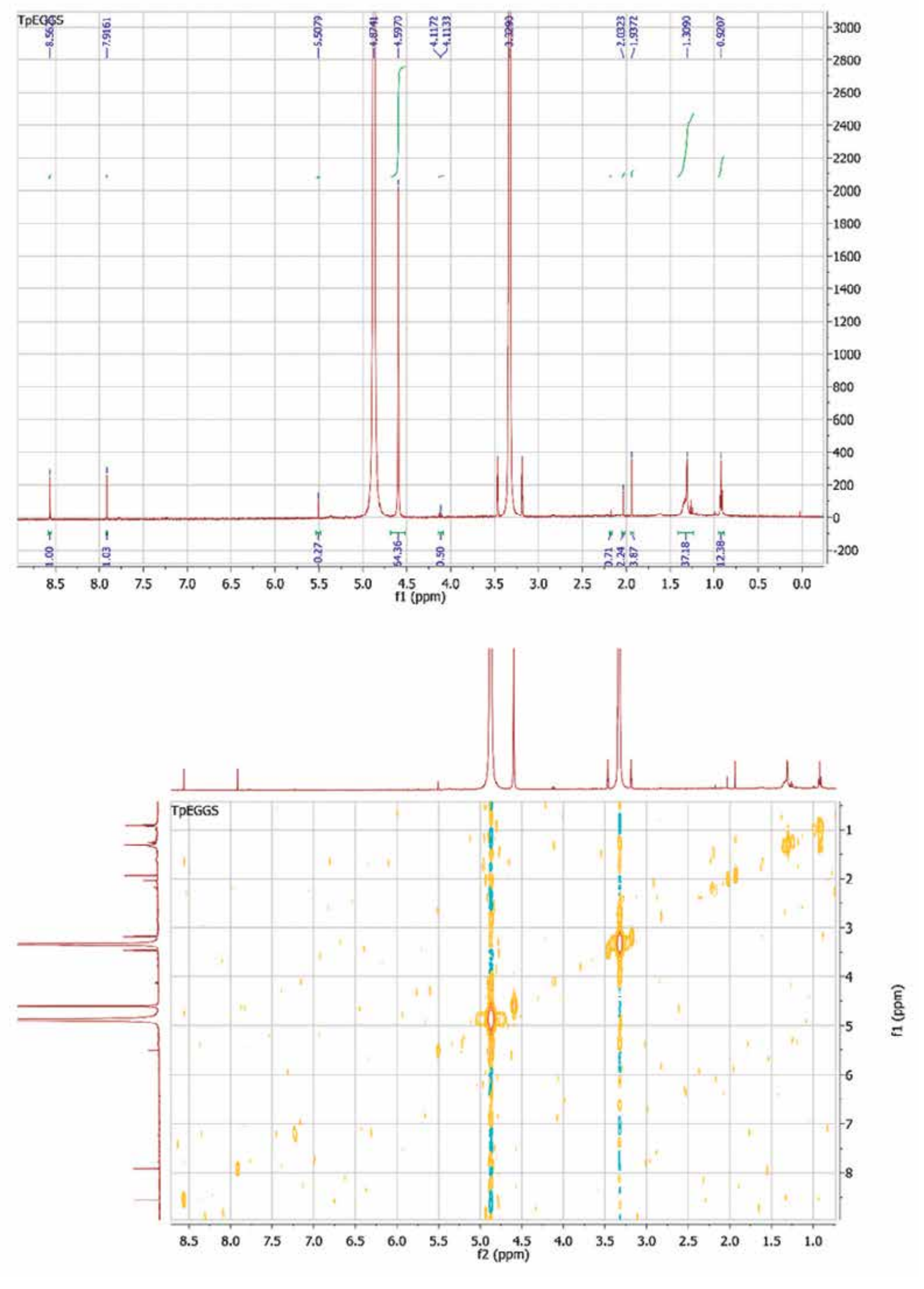

Figure S1: ^{1}H (above) and COSY (below) spectra of a methanolic (CD_3OD) surface extract of *Thaumastocoris peregrinus* eggs.

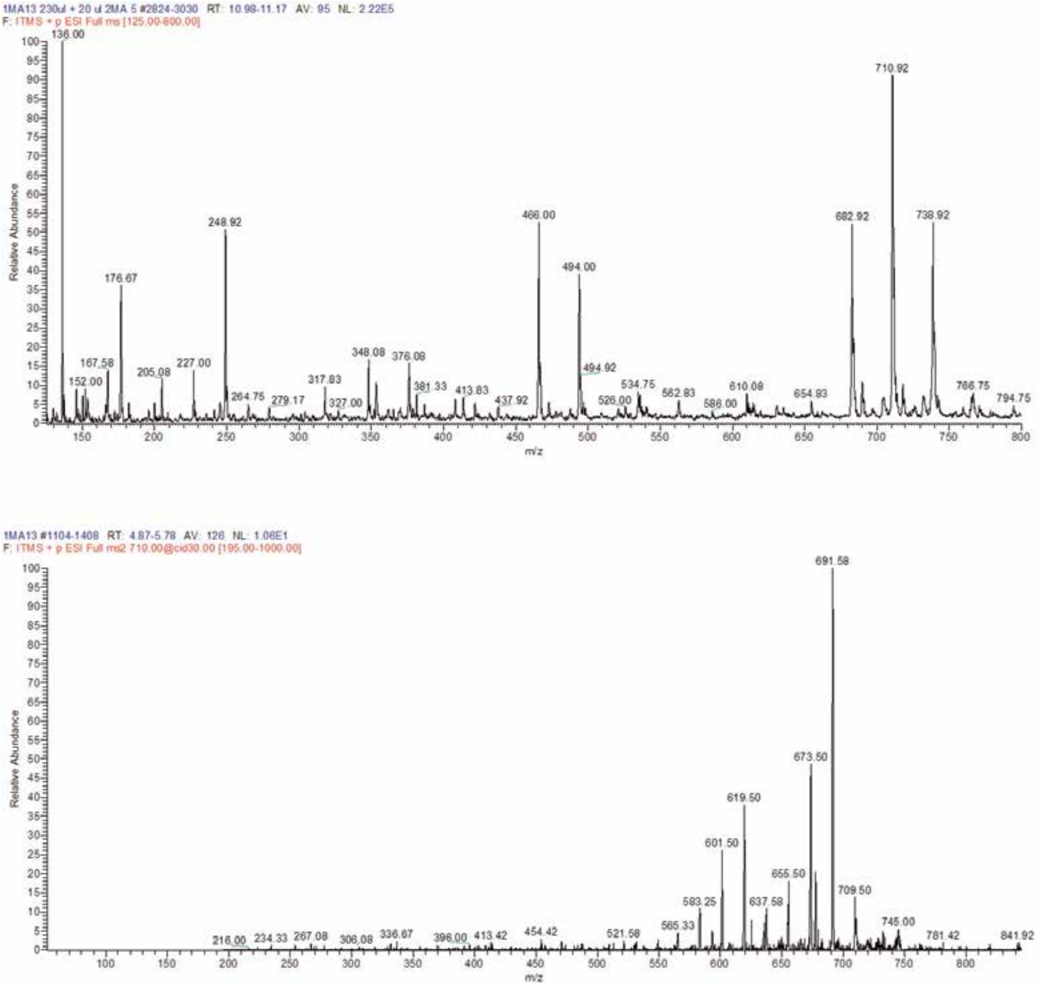

Figure S2. (above): Mass spectrum of a methanolic surface extract of *T. peregrinus* eggs in the ESI-MS positive scan mode. Below: Positive MS^2 spectrum of the precursor ion at *m/z* 710.

Chapter 8

General discussion: Integrating lessons from *Thaumastocoris peregrinus* oviposition preferences towards the improvement of pest management in *Eucalyptus* plantations

Forestry in the Southern hemisphere is characterized by the use of large plantations of exotic trees in single-species or monoclonal stands, aimed at obtaining timber or fiber for the paper industry (FAO, 2010). Managing forest pests to keep plantations in a healthy condition is a complex and expensive process (Ciesla, 2011). Trees are different from herbaceous plants: they are larger, older and exhibit a complex architectural pattern as a result of secondary growth, leading to the development of complex defense patterns (Eyles et al., 2010). Insects have evolved to counteract these defenses and exploit the diversity of niches offered by trees. When an exotic tree plantation is colonized by an insect from its area of origin, insect outbreaks and extensive damage are likely outcomes, given that the newly colonized area represents an unlimited supply of food, usually in an enemy-free environment (Ciesla, 2011). Thus, emerging pests are important threats to the productivity of the plantations.

Fundamental research on the biology of insect pests and how they integrate in a forest ecosystem is necessary since the beginning of forest pest management. I focused on one particular invasive pest attacking *Eucalyptus* tree plantations: the bronze bug *Thaumastocoris peregrinus*. This thesis is a first step towards understanding of the bronze bug in its environment. Current pest management strategies may include chemical control, tree breeding for resistance or tolerance, silvicultural tending operations (e.g. planting, cleaning, thining and/or pruning), and biological control. Chemical control has been used successfully on urban trees in Australia (Noack et al., 2009), although its use in large-scale plantation forestry is neither economically viable, nor feasible under the framework or the Forest Stewardship Council (FSC) certified plantations. In addition, tree breeding in search for resistant genotypes may not prove useful as a management strategy *per se*, given that *T. peregrinus* can develop on many species within the genus *Eucalyptus* (Soliman et al., 2012; Martínez et al., 2017 Chapter 4 in this thesis). So far, no silvicultural tending operations have been adapted to manage the bronze bug. In this context, many efforts have been made to develop a biological control program for the bronze bug by using parasitoids (Cross, 2011; Mutitu et al., 2013) or entomopathogenic fungi (Simeto et al., 2012). The main purpose of my research was to obtain basic information on the factors affecting oviposition in the bronze bug, within a system that included its host plant, a potential competitor and a parasitoid. In particular, I analyzed oviposition preferences of females and the performance of their offspring regarding different *Eucalyptus* species and leaf development, the co-occurrence of conspecifics (Chapter 4), and the previous infestation by a potential competitor: the lerp psyllid (Chapter 5). I also assessed the sources of short-range cues affecting host location by its main parasitoid (Chapter 7). Altogether, the thesis provided baseline information on the behavioral ecology of the bronze bug within its tritrophic environment. My aim in the following paragraphs is to discuss the main outcomes of this thesis within the context of the development of a sustainable integrated pest management for the bronze bug.

The plant: targeting oviposition in plant breeding and plantation design

In this thesis, I demonstrated that feeding preferences do not match oviposition preferences by the bronze bug. Thus, different *Eucalyptus* species may rank differently in terms of preference by the bronze bug, influenced by whether they are considered as sites for feeding or oviposition (Martínez et al., 2017 Chapter 4 in this thesis). I focused on three species within the genus *Eucalyptus*: the rose gum (*E. grandis*), the red gum (*E. tereticornis*) and the Dorrigo white gum (*E. benthamii*). The rose gum is one of the most commonly planted species in commercial plantations in Uruguay, due to its high productivity and tolerance to diseases. They are usually planted in large areas, comprised by several stands of approximately 25 hectares. A typical plantation in the North of Uruguay can comprise more than 1000 hectares. The Dorrigo white gum is also used in commercial forestry. Due to its tolerance to frost, stands of *E. benthamii* can be planted in lowlands inside a plantation, where other species cannot perform well. The red gums (*E. tereticornis* and *E. camaldulensis*) are widely planted as shelter forest in cattle ranches. These shelters comprise patches of around 1 hectare in the grasslands, providing shade to cows. In addition, red gums are commonly planted surrounding houses in rural areas, so they are frequently adjacent to larger *E. grandis* plantations. Hybridization of red gum and rose gum is increasingly used to obtain better timber quality.

How can we use the information obtained in this thesis to help protect *Eucalyptus* plantations against the bronze bug? First, the differential attraction to *Eucalyptus* species as oviposition sites can be exploited in plant breeding by selecting hybrids or clones that are less attractive to females. Currently, when selecting resistant plant material, breeders check for susceptibility to pests in terms of feeding damage. My results underline the importance of including oviposition preference when testing new plant materials, and the protocols to assess oviposition as developed in this thesis can be used to achieve this. The selection of *Eucalyptus* clones with delayed leaf change from juvenile to adult leaf morphology may be another approach in plant breeding to confer resistance to the bronze bug, given the poor performance of the nymphs on juvenile leaves. However, this strategy may prove not useful because juvenile leaves are more susceptible to fungal diseases like the myrtle rust *Puccinia psidii* and *Mycosphaerella* leaf disease (Coutinho et al., 1998; Pérez et al., 2009). The findings of this thesis can be also used to improve the efficacy of pest suppression techniques by considering the spatial distribution of tree species inside a plantation. For instance, the differential preference for some species as oviposition sites may result in stands that represent a source of nymphs (e.g. *E. tereticornis*, *E. benthamii*) and stands that will be preferred by adults for feeding (the most common *E. grandis*). In this example, the former are generally planted in fewer stands and usually confined to the margins. If we see the map of the plantation as a map of oviposition preference by the bronze bug, where some stands are more likely to have eggs than others, an interesting management prospect may be to concentrate management efforts to such stands that will carry more eggs and nymphs. Thus, a stand of *E. benthamii* or *E. tereticornis* in the border of a set of stands of *E. grandis* may be targeted for the release of *C. noackae* for biological control (Chapter 6). Other control practices that impact the eggs or the nymphal stages can also be applied in these stands. Such stand-centered management can render a sustainable control technique which would be impossible to apply in larger areas, increasing the probability of success.

The herbivore: the role of aggregative behavior

This thesis provides new evidence on the role of feeding aggregations in early stages in the bronze bug, and how it can be achieved by oviposition strategies. In Chapter 4, I demonstrated that bronze bug females prefer to oviposit on patches already colonized by conspecifics (Martínez et al., 2017). These results are in line with other studies on the Cimicomorpha, which showed an attraction of ovipositing females to cues induced by conspecifics in the host plants (Groot et al., 2003; Blackmer et al., 2004), thus adding support to the idea of a common strategy within the infraorder, as was discussed in Chapter 2. This preference to conspecific-related cues may explain the communal oviposition and the patchy distribution observed in the field, given that the presence of bronze bug individuals in a certain patch would increase the probability of additional females coming to oviposit in this patch. Such aggregated distribution has two consequences from the perspective of the forest manager: First, monitoring an insect with aggregated distribution requires higher sampling effort than for insects that exhibit other spatial distributions (Begon et al., 2006). Second, pest management strategies for insects with aggregated distributions tend to concentrate on suppressing the population within the aggregated patches (Rossi et al., 2009). Thus, a temporally and spatially restricted use of chemicals or biopesticides may be employed successfully within the patch, saving money and minimizing environmental risks. Likewise, the inoculative release of parasitoids has more probability of success when done directly in high-density patches. Research on the spatial and temporal dynamics of the aggregation by the bronze bug in the field is required to improve the accuracy of the monitoring and to adjust localized management practices

The potential competitor: combined pest management?

Oviposition-site selection by the bronze bug regarding plant and conspecifics as described in Chapter 4 matched the ‘mother knows best’ paradigm in general (Martínez et al., 2017). However, the assessment of the preference-performance relationship under the co-occurrence of a potential intraguild competitor exhibited a new, more complex scenario (Chapter 5). The plant-mediated response to the infestation by the lerp psyllid hampered bronze bug nymph development and survival, but this was probably partially counteracted by lerp feeding, which suggests an oviposition-site selection driven by the presence of the lerps. Thus, Chapter 5 introduces a novel angle to the discussion: While early infestation by the lerp psyllid could ‘vaccinate’ the plant against the bronze bug, as has been observed in other systems (Kessler & Baldwin, 2004), it may also introduce a potential new food source, directly produced by the competitor (i.e. the lerp), which may promote bronze bug development. Thus, this thesis contributes new elements to the discussion on plant-mediated interactions between same-guild herbivores that arrive at different times (Erb et al., 2011; Kroes et al., 2016). Given that both insect species are considered important invasive pests affecting *Eucalyptus* forestry at a global scale, these results may serve towards the improvement of combined pest management strategies for these insects. Particularly, our study suggests that an adequate and early management of the lerp psyllid population in *Eucalyptus* plantations may prove beneficial for reducing oviposition rates of the bronze bug later on in the season.

The parasitoid: Starting biological control

Biological control is defined as "The use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be" (Eilenberg et al., 2001). The use of natural enemies to suppress pest populations can be tracked as early as 304 CE, when Chinese farmers employed ants to control pests (Huang & Yang, 1987) but the milestone that marked the start of modem classical biological was the introduction from Australia to California in 1888 of the ladybug *Rodolia cardinalis* (Mulsant) to control the cottony cushion scale *Icerya purchasi* Maskell in citrus trees (Bentancourt & Scatoni, 2001). The great success of this introduction paved the way to the current use of biological control as a management tool. Biological control is the most important pest management strategy in forestry (Ciesla, 2011; Garnas et al., 2012). Uruguayan forestry has a long tradition of biological control starting in 1941 with the importation of *Anaphes nitens* Girault, a biological control agent of the *Eucalyptus* weevil, from South Africa (Morey & Porcile, 2002). The detection of new pests attacking plantations has usually triggered coordinated public and private initiatives towards the development of new biological control programs (Morey & Porcile, 2002; FAO-MGAP, 2006).

During the initial steps of a biological control program for the bronze bug with *C. noackae,* this thesis contributed by providing a rearing protocol for the host (Chapter 3) and a rearing and release protocol for the parasitoid, which resulted in a successful release and installation of field populations of the biological control agent within the first year (Chapter 6). Not only rearing is an essential initial step in biological control but also in this case provided a valuable opportunity for investigating key aspects of the biology of both the pest and the parasitoid, given that both species have only recently been discovered. Furthermore, Uruguay and other subtropical and temperate countries exhibit a seasonal dynamic of the bronze bug that implies an important reduction of the field populations during winter. Thus, rearing became an important element to maintain research during the year.

In Chapter 7, I assessed short-range cues involved in host-searching behavior by *C. noackae*. The wasp exhibited a behavior that can be described as the use of multiple cues, a strong response to egg cues, and an important role of the infochemical detour by exploiting adult cues. Particularly, the finding that *C. noackae* can react to healthy plant cues (Chapter 7) may be interesting for plant breeding. Indeed, to our knowledge current tree breeding programs do not consider effects of the plant's selected traits on the third trophic level, which should be assessed in light of these results.

Concluding remarks

The results of this thesis show that oviposition-site selection by the bronze bug is contingent on the multitrophic environment. Here, I demonstrate that in selecting where to oviposit, the bronze bug integrates cues from the first trophic level (plant species and developmental stage) and the second trophic level (co-occurrence of conspecifics and heterospecific competitors as well as plant-mediated cues). The data presented in this thesis also demonstrate that the egg parasitoid of the bronze bug locates the host by responding to cues from the first and the second trophic level. We previously discussed how the results of this thesis could be utilized in improving pest management strategies for this pest. Figure 8.1 summarizes the main aspects discussed here.

The study system explored in this thesis constitutes an interesting model for studying multitrophic interactions, provided it is comprised by Australian organisms established in an exotic ecosystem. Yet, as some of the constituent species are relatively new for science, more research is needed to better understand the ecological and evolutionary processes operating. For instance, it would be important to assess whether the presence of the bronze bug affects the behavior of the lerp psyllid. May an early colonization by the bronze bug affect the oviposition site selection by its competitor? Does the co-occurrence of both species affect the performance of the juveniles of the lerp psyllid? Similarly, the question remains as to whether the bronze bug responds to the presence of the parasitoid, and if so, which strategy it may deploy to minimize parasitization risks. Future research should also focus on characterizing the infochemicals triggering the responses observed in both the bronze bug and the parasitoid. Finally, the management strategies must be integrated and validated in field trials. In doing so, a fluent conversation between researchers and foresters is essential. This thesis opens the dialogue by providing a background for the inclusion of oviposition-site selection as a behavior to consider in integrated pest management.

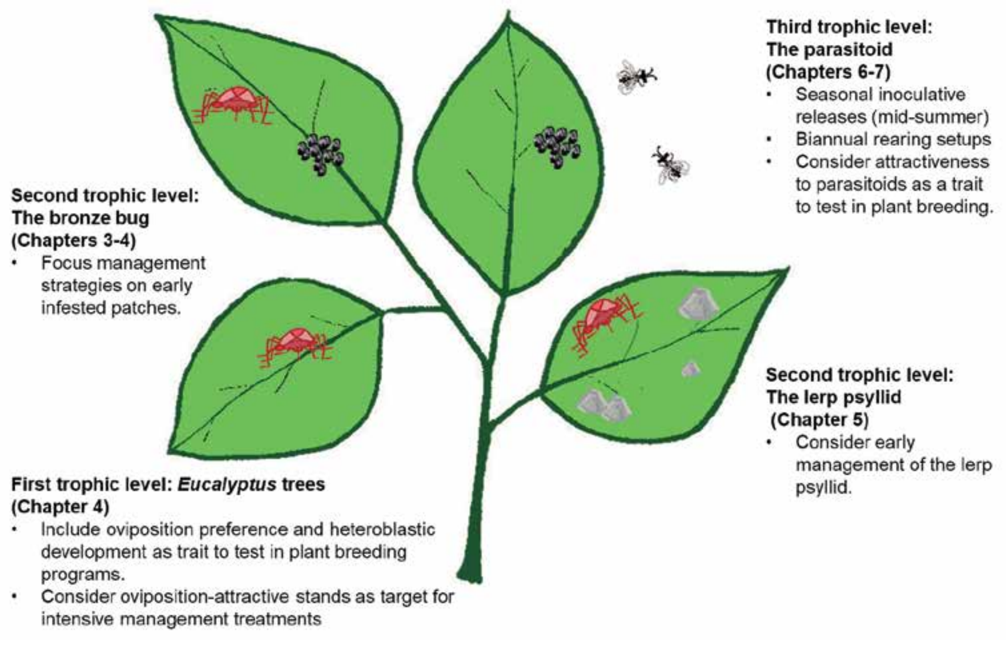

Figure 8.1 Potential application of the results of this thesis in management of the bronze bug in *Eucalyptus* plantations.

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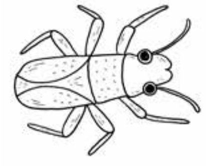


Summary

Plantation forestry has been increasingly affected in the last decades worldwide by invasions of exotic insects driven by climate change and global trade. The bronze bug, *Thaumastocoris peregrinus* is one of these invasive pests that has dispersed through the majority of the *Eucalyptus* production areas in the world in less than 15 years. Biological control represents one of the major tools for managing pests in tree plantations. In order to establish a successful biological control program, it is crucial to understand the biology of both the pest and the potential biocontrol agents, as well as to develop effective rearing systems for both. First, research efforts must be invested in understanding the process of plant selection by the insect pest, and how it is affected by other members of the multitrophic system. For instance, two major forces leading to plant selection by insect females are optimal foraging and optimal oviposition; the contribution of these forces to the host-selection process may vary, depending on the plant feeding habits of mothers and offspring. Additionally, oviposition site-selection may be greatly affected by competitive plant-mediated interactions between phytophagous insects. Finally, it is important to investigate the host searching behavior of the natural enemies of the pest. It is known that in searching for their hosts, parasitoids of phytophagous insects face a 'detectability-reliability dilemma': the most apparent cues, such as those originating from the host plant, are easy to detect but do not warrant the presence of the host, while the cues generated by the host are highly reliable but hard to detect from a distance. Thus, understanding how parasitoids solve this dilemma regarding which cues to exploit may be used as a tool to improve the biological control of the pest.

However, the knowledge on biology, host selection and multitrophic interactions of the bronze bug is scarce. No studies have been done on oviposition site-selection of the bronze bug. Furthermore, optimal oviposition, also known as the 'mother knows best' paradigm has been little studied in general for true bugs. Although *Eucalyptus* plantations are subjected to several pests and diseases, forest pests managers have focused on individual pests and interspecific interactions have been poorly investigated. Finally, several studies have analyzed the response of parasitoids to different cues but in general they have addressed volatile long-range cues.

This thesis explored the factors affecting the oviposition behavior of an herbivorous true bug (*Thaumastocoris peregrinus*), within a multitrophic system comprised of its host plant (*Eucalyptus* spp.), a common co-occurring sap-feeder (*Glycaspis brimblecombei*) and a specialist egg parasitoid (*Cleruchoides noackae*). Based on the information obtained, this study also pursued potential improvements in the management of *T. peregrinus* in *Eucalyptus* plantations, particularly in biological control with *C.noackae.*

At the beginning of this project, the literature on oviposition site-selection was reviewed with a focus on the 'mother knows best' paradigm and the particular aspect this theory shapes in true bugs. Oviposition decisions displayed by true bugs were compared to those of the well-studied order Lepidoptera. The review compared both taxa with regard to life-history characteristics and plant structural and induced defenses. The last part of the review addresses parasitoid foraging behavior and the strategies that have evolved in true bugs to deal with egg parasitization. I conclude that the aggregative behavior displayed by true bugs, the similar feeding habits of adults and juveniles and the dispersal abilities of the early instars may relax the assumptions of the 'mother knows best' paradigm, leading to a loose correlation between mother's choices and offspring performance. Also, the chemical apparency of the true bugs, a consequence of a plethora of chemical defenses and pheromones, together with their aggregative behavior allow for the use of an infochemical detour by egg parasitoids.

As a starting point for the research, in Chapter 3 life-history parameters of the bronze bug were assessed and a new mass rearing strategy developed and evaluated. This rearing strategy allowed for a successful production for the bronze bug and it proved useful to collect viable eggs within the time window for parasitization by *C. noackae*. Eggs and adult individuals of this rearing colony were utilized in the extant experiments of this thesis, and served as hosts for rearing the egg parasitoid *C. noackae*. First studies in this chapter showed differences in the oviposition rates by the bronze bug when reared in different *Eucalyptus* species. This topic was further investigated in the following chapter.

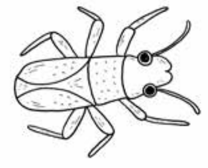
In Chapter 4, preference-performance correlations of the bronze bug were investigated. Feeding and oviposition preference of female bronze bugs were assessed between host species, developmental leaf stages, or prior plant exposure to conspecifics. The link between these preferences and the performance of the offspring was analyzed, by comparing survival and developmental time of nymphs reared on plants that underwent the various treatments. The results showed that bronze bug females can discriminate among *Eucalyptus* species and leaf developmental stages. However, female feeding preference differed from oviposition preference, indicating that they utilize different criteria for selecting a host plant for oviposition. In general, oviposition preference was linked to nymphal performance. Thus, the female's preference to oviposit on red gum (*E. tereticornis*) when compared to rose gum (*E. grandis*) even when they had preferred the latteras food, resulted in better performance of the offspring on *E. tereticornis* over *E. grandis*. The results also show a preference by females to both feed and oviposit on already colonized substrates but the effect of the oviposition preference on offspring performance was linked only to the first nymphal instar. This result when linked to field observations and previous data on other systems suggests that nymphs can modify the maternal decision and in the open field relocate to other patches. Finally, the study showed that feeding by the bronze bug induces changes in *Eucalyptus* leaf surface chemicals, opening the door to further investigations on the plant's induced responses to herbivory by this true bug.

Chapter 5 evaluated potential competitive interactions between two sucking insects that occur on *Eucalyptus* trees. The study focuses on the effect of the lerp psyllid (*Glycaspis brimblecombei*) on the selection of feeding and oviposition sites by the bronze bug. The lerp psyllid infests *Eucalyptus* trees earlier than the bronze bug and juveniles secrete a conical structure (the lerp) that is rich in carbohydrates. True bug nymphal performance was assessed by comparing developmental time and survival of the first instar reared on healthy *Eucalyptus tereticornis* leaves and on leaves infested with the lerp psyllid, either with or without lerps. The study demonstrates an oviposition preference by the bronze bug for sites already colonized by the lerp psyllid. However, feeding preference was similar between infested and healthy leaves, providing further evidence in support of the use of different criteria by bronze bug females when selecting sites for feeding or oviposition. Remarkably, nymphal performance was reduced on sites infested by *G. brimblecombei* when compared to healthy leaves, but the presence of lerps reverted the effect on survival and shortened the duration of the initial instar. This result suggests that the lerp may play a role as a food source in both the performance of the nymphs and in oviposition-site selection by the mothers. This chapter contributes to our understanding of the potential role of interspecific interactions on preference-performance correlations.

The last two chapters of the thesis introduce the third trophic level and the final actor of this story. In order to start a biological control program, the egg parasitoid *Cleruchoides noackae* was introduced for the first time in Uruguay. After approval of the rearing and release permits by the national and regional phytosanitary agencies, a rearing colony of the parasitoid was installed and supervised. In Chapter 6, the initial steps of the biological control program were described. A rearing protocol was developed and quality parameters were defined and assessed. The effect of temperature on developmental time was assessed in order to optimize a winter rearing. Field releases in commercial *Eucalyptus* plantations were made yearly during three summers. The rearing protocol allowed for the establishment of a viable colony of the parasitoid for two years without significant changes in quality. The study provided information for optimizing winter production at a lower temperature without losses in productivity. Interestingly, bronze bug egg abortion increased when eggs were exposed to the parasitoid, suggesting an additional non-reproductive effect of *C. noackae* on bronze bug egg mortality. As a remarkable result, two separate field populations of *C. noackae* were recorded as a result of field releases. The wasps survived for two years in the field and exhibited better quality indices than wasps from the laboratory rearing. A conclusion of Chapter 6 is that *C. noackae* is a promising biological control agent for the bronze bug that deserves further investigation. Host-searching behavior of *C. noackae* was assessed in the following chapter.

In Chapter 7, the behavioral responses of *C. noackae* to short-range cues were investigated. The study focused on comparing the responses of female *C. noackae* to cues from its host, the bronze bug, including different host stages, and their host plant. The results show that during the host-location process, *C. noackae* exploits information from the first and the second trophic level. The wasps responded to chemical contact cues from the egg surface, as shown by the behavioral responses elicited by the presence of eggs or egg extracts. Although the chemical identity of cues was not determined, the analysis of the egg extracts showed the presence of some consistent compounds that represent a promising starting point. Consistent with previous studies on egg parasitoids of true bugs, the results also show that *C. noackae* resorts to an infochemical detour to locate its host, by exploiting cues from adult bronze bugs. As many other true bug species, *T. peregrinus* exhibits gregarious behavior which implies the co-occurrence of individuals of different age classes, including eggs, in a given location. Therefore, exploiting adult cues as infochemical detour to locate eggs of the bronze bug seems a reliable strategy rendering this behavioral trait likely to be favored by natural selection. The fact that the wasp responds to both sexes opens the door to study the potential use of a male aggregation pheromone characterized in previous studies. Finally, plant cues alone or associated with host cues, also play a role in host location at a short-range by *C. noackae*. Chapter 7 contributes to expand our knowledge on egg parasitoid foraging behavior.

This thesis represents an initial effort to understand the intricate nature of forest health issues by considering the multitrophic system in the analysis of host-plant selection by an invasive pest. Integrating the lessons learned in the experimental chapters with my direct experience with foresters, I conclude discussing how the information presented in this thesis can be applied towards an integrated management of the bronze bug, within the context of Uruguayan plantation forestry. Current forestry activities, such as plant breeding or silvicultural tending operations, can be adjusted to take into account the bronze bug oviposition preferences. Similarly, both the interaction between the bronze bug and the lerp psyllid and the foraging behavior of *C. noackae* underline the importance of considering pests as components of systems instead of as isolated problems. Thus, a conversation between scientists and foresters may be an ultimate tool to keep and maintain healthy trees. The journey that started with an egg on a leaf ends up here, standing up in the woods.



Sumario

Sumario

Las plantaciones forestales a nivel mundial han sido afectadas en forma creciente en las últimas décadas por la invasión de insectos exóticos a consecuencia del cambio climático y el comercio global. La chinche de los eucaliptos, *Thaumastocoris peregrinus* es una de esas plagas invasoras que se ha dispersado por la mayoría de las áreas de producción de *Eucalyptus* en el mundo en menos de 15 años. El control biológico es una de las herramientas más importantes para el manejo de plagas en plantaciones forestales. A los efectos de establecer un programa de control biológico exitoso es crucial el conocimiento de la biología de tanto la plaga como el potencial agente de control biológico, así como el desarrollo de sistemas de cría eficaces para ambos. En primer lugar los esfuerzos de investigación deben enfocarse en la comprensión del proceso de selección de la planta por el insecto plaga y cómo este proceso es afectado por otros miembros del sistema multitrófico. Por ejemplo, dos fuerzas importantes que operan en la selección de la planta por parte de los insectos hembra son el forrajeo óptimo y la oviposición óptima. La contribución de esas fuerzas al proceso de selección de hospederos puede variar en función de los hábitos alimentarios de las madres y sus crías. La selección de sitios de oviposición puede ser afectada en forma importante además por interacciones competitivas entre insectos fitófagos, mediadas por la planta. Por último es importante investigar el comportamiento de búsqueda de hospederos de los enemigos naturales de la plaga. Se sabe que en la búsqueda de sus hospederos los parasitoides de insectos fitófagos se enfrentan a un dilema de detección *versus* fiabilidad: las claves más evidentes como aquéllas que tienen su origen en la planta hospedera son fáciles de detectar pero no garantizan la presencia del hospedero, mientras que las claves generadas en el propio hospedero son altamente fiables pero difíciles de detectar a la distancia. Comprender entonces cómo los parasitoides resuelven este dilema en lo que respecta al uso de claves puede ser útil como una herramienta para mejorar el control biológico de la plaga.

Sin embargo aún es escaso el conocimiento acerca de la biología, la selección de hospederos y las interacciones multitróficas de la chinche de los eucaliptos. No se han realizado estudios sobre la selección de sitios de oviposición de la chinche de los eucaliptos. Se ha estudiado muy poco cómo opera la oviposición óptima, también conocida como el paradigma de "las madres saben lo que es mejor" en chinches en general. A pesar de que las plantaciones de *Eucalyptus* están sometidas a una variedad de plagas y enfermedades, el manejo de plagas forestales tiende a enfocarse en una plaga a la vez, por lo que las interacciones interespecíficas han sido poco abordadas. Finalmente varios estudios han analizado la respuesta de los parasitoides a diferentes claves pero en general se han enfocado en claves volátiles de largo alcance.

Esta tesis explora los factores que afectan el comportamiento de oviposición de una chinche herbívora (*Thaumastocoris peregrinus*) en un sistema multitrófico compuesto por su planta hospedera (*Eucalyptus* spp.), otro picosuctor que comparte su hábitat (*Glycaspis brimblecombei*) y un parasitoide de huevo especialista (*Cleruchoides noackae*). En base a la información obtenida este estudio apunta a poder realizar mejoras en el manejo de *T. peregrinus* en plantaciones de *Eucalyptus*, particularmente en el control biológico con *C. noackae*.

Al inicio de este proyecto se revisó la bibliografía disponible sobre selección de sitios de ovi-

posición, con foco en el paradigma de "las madres saben lo que es mejor" y las formas particulares que esta teoría adquiere en lo que respecta a chinches. Las decisiones de oviposición exhibidas por las chinches se compararon con aquéllas tomada por los lepidópteros, un orden estudiado más extensamente. La revisión comparó ambos taxa con respecto a características de sus historias de vida y defensas estructurales e inducidas por las plantas. La parte final de la revisión bibliográfica aborda el comportamiento de forrajeo en parasitoides y las estrategias que han evolucionado en las chinches para lidiar con el parasitismo de huevos. Concluyo que el comportamiento gregario exhibido por las chinches, la concordancia de hábitos alimentarios entre adultos y juveniles y las habilidades de dispersión de los primeros instars pueden relajar los supuestos del paradigma de "las madres saben lo que es mejor", lo cual puede llevar a una correlación más laxa entre las elecciones maternas y el desempeño de las crías. Además la "notoriedad química" de las chinches, consecuencia de una plétora de defensas química y feromonas, así como su comportamiento gregario, permiten a los parasitoides de huevo usar un desvío infoquímico.

Como punto de partida para la investigación, en el Capítulo 3 se evaluaron parámetros de historia de vida de la chinche de los eucaliptos y se desarrolló y evaluó una nueva estrategia de cría masiva. Esta última permitió la producción exitosa de la chinche y resultó útil para colectar huevos viables dentro de la ventana de tiempo de parasitismo de *C. noackae*. Huevos e individuos adultos provenientes de esta cría fueron utilizados en los restantes experimentos de esta tesis y sirvieron como hospederos para la cría del parasitoide de huevos *C. noackae*. Estudios primarios en este capítulo mostraron diferencias en las tasas de oviposición de la chinche al ser criada con diferentes especies de *Eucalyptus*. Este tema fue investigado más a fondo en el capítulo siguiente.

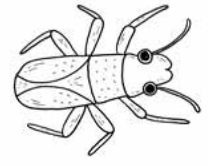
En el Capítulo 4 se investigaron correlaciones entre preferencia y desempeño de la chinche de los eucaliptos. Se evaluaron preferencias de alimentación y de oviposición de chinches hembras entre especies del hospedero, estados de desarrollo foliar o exposición previa a coespecíficos. Se analizó el vínculo entre esas preferencias y el desempeño de la cría comparando la sobrevivencia y el tiempo de desarrollo de ninfas criadas en plantas que fueron sometidas a los distintos tratamientos. Los resultados mostraron que las chinches hembras pueden discriminar entre especies de *Eucalyptus* y estados de desarrollo foliar. Sin embargo las preferencias de alimentación difirieron de las de oviposición lo cual indica que las hembras usan diferentes criterios para seleccionar una planta para oviposición. En general la preferencia de oviposición se vinculó con el desempeño de las ninfas. De esta manera la preferencia de las hembras para oviponer en eucaliptos colorados (*E. tereticornis*) en comparación con *E. grandis*, aun cuando hubieren preferido este último como alimento, resultaron en un mejor desempeño de la cría en *E. tereticornis* sobre *E. grandis*. Los resultados también evidenciaron una preferencia de las hembras tanto a alimentarse como a oviponer en sustratos ya colonizados pero el efecto de la preferencia de oviposición en el desempeño de la cría solo se vinculó al primer instar ninfal. Unido a observaciones de campo y a datos previos en otros sistemas este resultado sugiere que las ninfas pueden modificar la decisión materna y reubicarse en otros parches en situación de campo. Finalmente el estudio mostró que la alimentación de la chinche induce cambios químicos en la superficie foliar de *Eucalyptus*, dejando una puerta abierta para posteriores investigaciones sobre respuestas inducidas en la planta por herbivoría de esta chinche.

El Capítulo 5 evalúa potenciales interacciones competitivas entre dos insectos picosuctores que se dan en *Eucalyptus*. El estudio se enfoca en el efecto del psílido de escudo (*Glycaspis brimblecombei*) en la selección de sitios de alimentación y oviposición de la chinche de los eucaliptos. El psílido de escudo ataca árboles de *Eucalyptus* en forma más temprana en la estación que la chinche y los juveniles secretan una estructura cónica (el lerp o escudo) que es rica en carbohidratos. Se evaluó el desempeño ninfal de la chinche de los eucaliptos a través de la comparación del tiempo de desarrollo y la supervivencia de ninfas de primer instar criadas en hojas sanas de *Eucalyptus tereticornis* y en hojas infestadas con el psílido de escudo, con o sin lerps. El estudio demostró una preferencia de oviposición de la chinche por sitios ya colonizados por el psílido. Sin embargo la preferencia de alimentación fue similar entre hojas sanas e infestadas, lo cual aportó nueva evidencia al uso de diferentes criterios por las hembras de la chinche de los eucaliptos en la selección de sitios de alimentación u oviposición. Como elemento a destacar, el desempeño ninfal disminuyó en sitios infestados con *G. brimblecombei*, en comparación a hojas sanas, pero la presencia de lerps revirtió el efecto en la supervivencia y acortó la duración del primer instar. Este resultado sugiere que el lerp podría desempeñar un rol como fuente de alimentación tanto en el desempeño de la ninfa como en la selección de sitios de oviposición de la madre. Este capítulo contribuye a nuestro entendimiento del rol potencial de las interacciones interespecíficas en las correlaciones de preferencia-desempeño.

Los últimos dos capítulos de la tesis presentan el tercer nivel trófico y el actor final de esta historia. A los efectos de iniciar un programa de control biológico se introdujo por primera vez al Uruguay el parasitoide de huevos *Cleruchoides noackae*. Después de obtener los permisos de cría y liberación en las agencias sanitarias nacionales y regionales correspondientes, se instaló y supervisó una colonia de cría del parasitoide. En el Capítulo 6 se describieron los pasos iniciales del programa de control biológico. Se desarrolló un protocolo de cría y se definieron y cuantificaron parámetros de calidad. Se evaluó el efecto de la temperatura en el tiempo de desarrollo, a los efectos de optimizar una cría invernal. Se realizaron liberaciones a campo anuales en plantaciones comerciales de *Eucalyptus* durante tres veranos. El protocolo de cría permitió el establecimiento de una colonia viable del parasitoides durante dos años sin cambios significativos en calidad. El estudio proporcionó información para optimizar la producción de invierno a menor temperatura, sin pérdidas en la productividad. Un hallazgo interesante fue que el aborto en huevos de la chinche de los eucaliptos aumentó cuando éstos fueron expuestos al parasitoide, lo cual sugiere un efecto adicional, no reproductivo, de *C. noackae* sobre la mortalidad de huevos de la chinche. Como resultado a destacar se registraron dos poblaciones separadas de campo de *C. noackae,* resultantes de las liberaciones. Las avispas sobrevivieron por dos años en el campo y exhibieron índices de calidad mejores que las avispas de laboratorio. Una conclusión del Capítulo 6 es que *C. noackae* es un promisorio agente de control biológico para la chinche de los eucaliptos que merece una investigación más a fondo. El comportamiento de búsqueda de hospedero de *C. noackae* fue abordado en el siguiente capítulo.

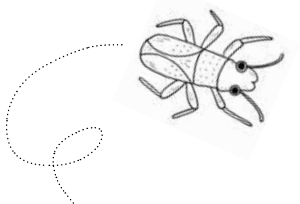
En el Capítulo 7 se investigaron las respuestas comportamentales de *C. noackae* a claves de corto alcance. El estudio se enfocó en la comparación de las respuestas de hembras de *C. noackae* a claves de su hospedero, la chinche de los eucaliptos, con la inclusión de diferentes estados del hospedero y su planta hospedera. Los resultados mostraron que *C. noackae* utiliza información del primer y el segundo nivel trófico durante el proceso de localización de su hospedero. Las avispas respondieron a claves químicas de contacto de la superficie del huevo, lo cual fue evidenciado por las respuestas comportamentales provocadas por la presencia de los huevos o extractos de los mismos. Aunque la identidad química de estas claves no fue determinada, el análisis de los extractos de los huevos mostró la presencia de algunos compuestos consistentes que representan un punto de partida alentador. En consonancia con estudios previos en parasitoides de huevos, los resultados también muestran que *C. noackae* recurre a un desvío infoquímico para localizar a sus hospederos, mediante la utilización de claves provenientes de adultos de la chinche de los eucaliptos. Tal como sucede en muchas otras especies de chinches *T. peregrinus* exhibe un comportamiento gregario, lo cual implica la presencia simultánea de individuos de diferentes clases de edades, huevos incluidos, en un lugar dado. Por lo tanto utilizar claves de los adultos como desvío infoquímico para ubicar los huevos de la chinche de los eucaliptos parece una estrategia fiable que vuelve este carácter comportamental pasible de ser favorecido por selección natural. El hecho de que la avispa responda a ambos sexos abre la puerta al estudio del uso potencial de una feromona de agregación del macho que fue caracterizada en estudios previos. Finalmente, claves provenientes de la planta, solas o en asociación con claves del hospedero, también juegan un rol en la localización a corto alcance del hospedero por *C. noackae*. El Capítulo 7 contribuye a expandir nuestro conocimiento sobre el comportamiento de forrajeo de parasitoides de huevo.

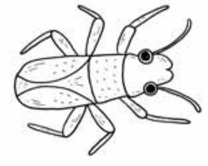
Esta tesis representa un esfuerzo inicial para comprender la naturaleza intrincada de los problemas sanitarios forestales, mediante la consideración del sistema multitrófico en el análisis de la selección de planta hospedera por parte de una plaga invasora. En un ejercicio de integración de las lecciones aprendidas en los capítulos experimentales con mi experiencia directa con productores forestales, concluyo con una discusión sobre cómo la información presentada en esta tesis puede ser aplicada de cara al manejo integrado de la chinche de los eucaliptos, en el contexto de la forestación uruguaya. Actividades forestales habituales tales como el mejoramiento o las operaciones silvícolas pueden ser ajustadas para tener en cuenta las preferencias de oviposición de la chinche de los eucaliptos. En forma similar, tanto la interacción entre la chinche de los eucaliptos y el psílido de escudo como el comportamiento de forrajeo de *C. noackae* subrayan la importancia de considerar a las plagas como componentes de sistemas, en lugar de problemas aislados. Asimismo una conversación entre científicos y productores forestales podría ser la herramienta definitiva para mantener los árboles sanos. El viaje que comenzó con un huevo en una hoja termina aquí, parado en el bosque.



About the author

Gonzalo Martínez Crosa was born in San José de Mayo, Uruguay. He grew up in a family with tight roots with the popular culture and music of rural Uruguay. Early on he developed a keen interest for the natural world, especially for small animals and plants. In 1992 he moved to Montevideo to study Biology at the Universidad de la República, with a specialization in entomology. During his BSc, he worked on a protocol for recovering and preparing plant histological samples from grasshopper feces for dietary studies. He was awarded a scholarship from the Program for the Development of Basic Sciences (PEDECIBA) to start his MSc under supervision of Dr. Carlos Carbonell. During his MSc he deepened his interest in the study of insect-plant interactions, working with the structure, species composition and food habits of grasshopper communities in two microhabitats commonly found in rural Uruguay, shrubland and grassland. His MSc thesis was presented in 2004, with the title 'Community parameters and feeding habits of a grasshopper (Orthoptera; Acridoidea) assemblage in a natural grassland'. From 2004 to 2007 he worked as a consultant for the Ministry of Housing, Territorial Planning and Environment of Uruguay. During this period, he integrated the team responsible for the elaboration of biosafety guidelines, an official document published under the title "Proposal for a National Biosafety Framework for Uruguay". During this period, he also collaborated in editing the Third National Report for the Convention on Biological Diversity. In 2008, he obtained a position as an Assistant Researcher at the Forestry Research Program of the National Agricultural Research Institute (INIA) in Tacuarembó, Uruguay. He then became more interested in insect-plant relations within the context of forest ecosystems, particularly toward the development of environmentally friendly management strategies. To pursue these interests, in 2009 he started collaborative work with the Laboratory of Chemical Ecology of Universidad de la República in Montevideo, and later he joined the Laboratory of Entomology of Wageningen University as a PhD student. Here he was able to improve his knowledge of the behavioral ecology of insect plant interactions. He currently holds a permanent position as an Associate Researcher at the Forestry Research Program at INIA in Tacuarembó, Uruguay.





Publications

Peer-reviewed journals

Martínez G, Finozzi MV, Cantero G, Soler R, Dicke M & González A (2017) Oviposition preference but not adult feeding preference matches with offspring performance in the bronze bug *Thaumastocoris peregrinus*. Entomologia Experimentalis et Applicata 163:101–111. **(Chapter 4 in this thesis)**.

Gómez D, Hirigoyen A, Balmelli G, Viera C & **Martínez G** (2017) Patterns in flight phenologies of bark beetles (Coleoptera Scolytinae) in commercial pine tree plantations in Uruguay. Bosque 38:47–53.

Jorge C, **Martínez G**, Gómez D & Bollazzi M (2016) First record of the eucalypt gall-wasp *Leptocybe invasa* (Hymenoptera: Eulophidae) from Uruguay. Bosque 37:631–636.

Martínez G, Gómez D & Taylor GS (2014) First record of the Australian psyllid *Blastopsylla occidentalis* Taylor (Hemiptera, Psylloidea) from Uruguay. Transactions of the Royal Society of South Australia 138:231–236.

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Gómez D & **Martínez G** (2013) Bark beetles in pine tree plantations in Uruguay: First record of *Orthotomicus erosus* Wollaston (Coleoptera: Curculionidae: Scolytinae). The Coleopterists Bulletin 67:470–472.

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González A, Calvo MV, Cal V, Hernández V, Doño F, Alves L, Gamenara D, Rossini C & **Martínez G** (2012) A male aggregation pheromone in the bronze bug, *Thaumastocoris peregrinus* (Thaumastocoridae). Psyche: A Journal of Entomology 2012:1–7.

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Martínez G (2007) Proposal for a National Biosafety Framework for Uruguay. Final report. Project URU-04-009 UNEP-GEF for the Development of a National Biosafety Framework in Uruguay. UNEP- DINAMA-GEF, Montevideo. http://staging.unep.org/biosafety/Documents/Uruguay_NBF.pdf

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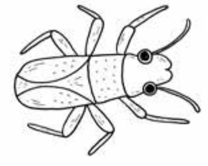


Training and education statement

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Behavioral ecology of oviposition-site selection in herbivorous true bugs (2013)

Writing of project proposal (4.5 ECTS)

- Multitrophic interactions affecting oviposition behaviour in the bronze bug *Thaumastocoris peregrinus* Carpintero & Dellappe (Heteroptera: Thaumastocoridae) a common pest of *Eucalyptus* plantations in Uruguay

Post-graduate courses (4.4 ECTS)

- Linear models; PE&RC (2011)
- Generalised linear models; PE&RC (2011)
- Congreso Argentino de Entomología; SAE; Bariloche, Argentina (2012)
- Seminário Internacional sobre Pragas Quarentenáris Florestais; EMBRAPA; Curitiba, Brazil (2012)
- Design of experiments; WIAS (2017)

Laboratory training and working visits (4 ECTS)

- Rearing of parasitoid wasps; EMBRAPA Florestas, Curitiba, Brazil (2012-2013)

Invited review of (unpublished) journal manuscript (2.5 ECTS)

- International Journal of Pest Management: current understanding of *Thaumastocoris peregrinus* in a quest for its management and control (2011)
- Ciência Rural: record of T. *peregrinus* in Goiás State (Brazil) (2012)
- Journal of Pest Science: population dynamics of *T. peregrinus* (2013)

Deficiency, refresh, brush-up courses (6 ECTS)

- Insect-plant interactions; Laboratory of Entomology WUR (2011)

Competence strengthening / skills courses (1.5 ECTS)

- Competence assessment; WGS (2011)
- Workshop TIMBO: use of national scientific literature repository; TIMBO, National Agency for Research and Innovation; Uruguay (2015)
- Leadership workshop "Los 4 Trabajos del Jefe" (The 4 Tasks of a Boss); Xn Partners; Uruguay (2016).

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

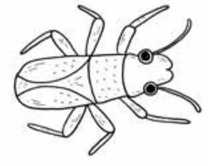
- PE&RC Weekend first year (2011)
- PE&RC Weekend last year (2017)

Discussion groups / local seminars / other scientific meetings (4.7 ECTS)

- Plant-insect interactions meetings (2011, 2012, 2017)
- LEQ Meetings; Laboratory of Chemical Ecology, Uruguay (2012-2015)

International symposia, workshops and conferences (15.6 ECTS)

- 14th Symposium on Insect-Plant Interactions (SIP); Wageningen, the Netherlands (2011)
- IUFRO Joint Meeting: "Pathogens, insects, and their associations affecting forestry worldwide"; Colonia, Uruguay (2011)
- 3rd International Entomophagous Insects Conference (IEIC3); Magog, QC, Canada (2013)
- XXIV IUFRO World Congress; Salt Lake City, USA (2014)
- XXV International Congress of Entomology; Orlando, USA (2016)
- 16th Symposium on Insect-Plant Interactions (SIP); Tours, France (2017)



Acknowledgements

I remember very well my first trip to Wageningen. It was the 1st of January 2011 and I had two connections, in São Paulo and Frankfurt. Guarulhos airport was absolutely deserted, like in a futurist dystopia, and a new president was giving her inauguration speech. Frankfurt was also desolate, and I remember myself thinking "that's it; you are starting a path on your own this time". What a trip it took from that insecure excited PhD student to who I am now! So many good stories, liters of coffee, unfortunate accidents, meetings, successful setups, late-night supportive conversations with friends and family. It is true, ultimately a PhD is the beginning of a journey on your own, but it could not be possible without the contribution of people, many people. I will try to address in these lines the most important people and institutions

I would like to start by saying thanks to my supervisors. Marcel, it has been a great honor to do my PhD under your supervision. When a research group is not only highly productive in scientific terms but also successful in making science a nice, friendly and joyful human experience, it tells a lot about the commitment and the value of the leader. Even being abroad most of the period, your support was always there, with sharp comments, with brilliant suggestions but also with nice words in the hard moments. I only regret not having spent more time in Wageningen to join you in the choir. Andrés, you are greatly responsible for keeping me into science. During these PhD years, I have learned a lot from your relentless commitment to your group and to research, against all odds. A relation that initially grew up based on admiration and respect has evolved into friendship. I hope this to be not the end of a journey but the beginning of new scientific adventures together. I would like to express my gratitude also to Roxina Soler who introduced me to the Laboratory of Entomology in Wageningen and who was a great support during the initial years of the PhD.

To INIA, my home institution, thanks for granting me the possibility to develop this PhD. I am looking forward to starting this new phase to give back to the institution all the time and effort invested in this adventure. An institution is not an abstract category but is made by its people. I would like to say thanks to all the people that have been there for helping me with the administrative tasks of the institute, reviewing the numbers of my project, giving me advice, or simply making a phone call. To the effortless workers from the experimental station in Tacuarembó, 'muchas gracias'. They have suffered from me showing up at unexpected times and in weekends. The weekend guards: Maicol, Julio, Daniel and Briam, represent very well the general support: they silently witnessed most of my advances and setbacks in our brief talks every weekend for the last three years.

Thanks to my home research group at INIA. Thanks to Roberto for letting me fly, to Gustavo for your sharp remarks, to Sofía for your insights and mental support, to Demian and Andrés for the suggestions and the countless 'ladies nights'. Thanks to the field team: to Pablo, to Federico and to Wilfredo, always devoted and efficient. Finally, my total gratitude and admiration to the lab team: Analía, Mariela, Juan Pablo and especially Gissel, my partner in crime. This PhD would have been impossible without their hard and constant work, the hours spent on the rearing, always smiling, always willing, always committed to the results. If I managed to sail through the storm, it was because I had the best sailors.

One of the coolest things of doing a sandwich PhD is that you expand your scientific family. In my case, I was lucky to join two new houses: The Laboratory of Entomology in Wageningen and the Laboratory of Chemical Ecology (LEQ) at the Universidad de la República in Montevideo. I want to thank both groups for making me feel at home. Thanks for the inspiring conversations, the support and the fun. They merged with my old tribe of friends that have been always around: the SPC, my ex classmates at the Faculty of Sciences in Montevideo, and my oldest friends from San José. All of them made a dense network of support that enlightens my life experience. Thanks.

To my paranymphs. Dani, my friend, thanks for the incredible support and generosity. Thanks Jeroen, the mighty fly, meeting you during this last period was the final nerdy cool final touch of this journey.

Embarking on a biological control program with an exotic parasitoid requires not only extensive research but also the development of a network of partners from both the public and the private sector, nationwide and internationally. I would like to acknowledge the support from the former Director of the department of agricultural protection, Inés Ares, who helped me and taught me a lot regarding international regulations. I also want to thank my international partners at EMBRAPA, Leonardo Barbosa and Edson Iede who helped me with the importation procedure, and generously offered to train me and my team in the handling of the parasitoid. We built up a professional relation that I am sure will bring more collaboration in the future. Last by not least, I express my gratitude to the foresters, the ultimate reason I started this journey. I want to say thanks to the Sociedad de Productores Forestales for granting access to their plantations, providing logistic resources, and feedback on our research. You provided the impulse for our research.

Finally, I want to express my gratitude to my family back in Uruguay. To my parents that taught me the importance of education. To my brothers and nephews and to the myriad of cousins, uncles and aunts. This thesis belongs to you.

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Propositions

1. True bug females rely on different criteria to select plants for either food or for oviposition sites.
(this thesis)

2. Using an infochemical detour is the main strategy employed by egg parasitoids of true bugs to solve the reliability-detectability dilemma.
(this thesis)

3. CRISPR/Cas9 and other emerging gene editing technologies require the development of a clear and consensual ethical framework.

4. Ignoring molecular data in taxonomy is nonsense.

5. The quantification of scientific productivity in terms of bibliometric indices should lie with non-profit organizations.

6. Whether one faces novelty with curiosity or fear defines one's life experience.

Propositions belonging to the thesis entitled
'Mothers in the woods: Multitrophic interactions and oviposition preference in the bronze bug *Thaumastocoris peregrinus*, a pest of *Eucalyptus*'.

Gonzalo Martínez
Wageningen, 17 October 2017