TRI-TROPHIC INTERACTIONS IN SOYBEAN AGRO-ECOSYSTEMS: MECHANISMS, ECOLOGICAL RELEVANCE, AND APPLICATION POTENTIAL OF NATURAL ENEMIES OF KUDZU BUGS

by

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ABSTRACT

A series of studies were conducted to evaluate herbivore-herbivore and herbivore-parasitoid interactions that may impact the kudzu bug, *Megacopta cribraria* F. (Hemiptera: Plataspidae) population in soybean. Experiments on herbivore-herbivore interactions using kudzu bugs and either southern green stink bug, *Nezara viridula* L. or brown marmorated stink bug, *Halyomorpha halys* Stål aimed to examine the potential competition or facilitation among these herbivorous pests. Parasitoid-herbivore interactions were conducted using kudzu bug egg parasitoids, *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae) and *Paratelenomus saccharalis* Dodd (Hymenoptera: Platygastridae) to better understand the factors influencing parasitoid effectiveness and improve biological control strategies for kudzu bug management. To understand kudzu bug-stink bug interactions, the direct and indirect impacts of kudzu bug infestation on the behavior of two stink bug species were investigated. The brown marmorated stink bug preferred healthy soybean plants in the presence of kudzu bugs but shifted towards infested plants when kudzu bug adults were removed, while the southern green stink bug consistently preferred infested plants. Kudzu bug density had a more significant effect on brown marmorated stink bug landing preference than on southern green stink bug. Kudzu bug infestation altered responses in soybean, reducing interspecific interaction for brown marmorated stink bug but enhancing it for southern green stink bug. To further understand kudzu bug-parasitoid interactions, the effects of different adult parasitoid food sources, host eggs: adult parasitoid ratios, and host exposure times on the proportion and sex ratio of the parasitoid offspring were evaluated. The study found that honey as a food source, a 21:7 host: parasitoid ratio, and three-five days of exposure time resulted in the highest proportion of female-biased offspring for *O. nezarae*, while the same food source and host to parasitoid ratio at a one-day host exposure were optimal for *P. saccharalis* to maximize female offspring production. These findings provide new insights into the biology of these egg parasitoids. Building on this knowledge, the study also investigated parasitoids' olfactory responses influenced by their physiological state and learning status during host-foraging strategies. It was found that both parasitoid species innately responded to host-associated stimuli, regardless of their hunger or host experience. Female parasitoids used volatile cues from infested soybean plants with kudzu bug eggs and footprint trails left by adult kudzu bugs to locate their host. In a final study, interspecific interactions between *O. nezarae* and *P. saccharalis* were investigated. It was found that simultaneous release of both parasitoids led to higher host egg parasitism than sequential release. *Ooencyrtus nezarae* produced more total offspring but mostly male offspring. In aggressive behavior, *P. saccharalis* used head butting to fight *O. nezarae*. *Ooencyrtus nezarae* was able to develop in host eggs parasitized by *P. saccharalis*, acting as a superior larval competitor. The findings of this project provide valuable insights for enhancing biological control strategies against kudzu bugs by understanding kudzu bug-stink bug interactions, kudzu bug egg parasitoids’ requirements, behaviors, and their interspecific interactions.

Keywords: Kudzu bug, Parasitoids, Biological control programs, Olfactory behavior, Plasticity, Chemical footprints

Dedication

With the utmost respect and gratitude, I dedicate my PhD dissertation to my esteemed father, Gyas Ahamad, and my revered mother, Irfana Khatoon, for bestowing upon me the opportunity to pursue my dreams and the love that has made them tangible. Additionally, I wholeheartedly dedicate this work to my supportive siblings, Tanveer Gayas, Meer Gayas, Taukeer Gayas, Tauseef Gayas, Zeba Warsi, Sheeba Warsi, and Samra Warsi, as well as to all my sister-in-law. To my cherished nephews, Waqas Tanveer and Uzair Tanveer, and my beloved niece, Alfisha Tanveer, I extend my deepest appreciation for their love, faith, and encouragement.

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 OVERVIEW

The presence of invasive species can cause considerable damage to economically important crops each year (USDA FS 2020). The number of established invasive species is expected to increase by 36% by 2050 worldwide (Seebens et al. 2021). Federal agencies detailed spending more than $500 million in 1999 and 2000 for activities related to invasive species (U.S. GAO 2000); half of this expenditure was spent on control, and Florida alone spent $127.6 million on management of invasive species (U.S. GAO 2000). A 2005 study accounted that such biological invasion cost the U.S. economy over $120 billion/year (U.S. FWS 2012). Paini et al. (2016) conducted a study from data retrieved from CABI CPC (Center for Agriculture and Bioscience International Crop Protection Compendium) on agricultural invasive species globally. They found that the United States had the highest total invasion cost (over $70 million). A recent study reported that biological invasion has resulted in a minimum of $1.288 trillion in associated costs over the last four decades worldwide (1970-2017), of which North America incurred the highest recorded costs (average of $11 billion/year) (Diagne et al. 2021).

Invasive true bugs are among the most significant pests in North American agriculture, particularly for soybeans (*Glycine max* L.). These Hemipterans historically cause frequent losses to soybean; for instance, southern green stink bug (*Nezara viridula* L.) infested ~80% of soybean acreage and caused the loss of ~126,000 tons in 2009 (Musser et al. 2010). Brown marmorated stink bug (*Halyomorpha halys* Stal) caused approximately $37 million in losses to many row crops, including soybean, within the mid-Atlantic regions of the U.S. in 2010 (Leskey and Nielsen 2018). Over the recent decade, kudzu bug, *Megacopta cribraria* F. (Hemiptera: Plastaspidae), another important pest of soybean has been recorded in as many as 15 states.

1.1.1 Study species: kudzu bug origin and discovery in the United States

The kudzu bug is cosmopolitan in distribution and originated in China, Japan, India, and Australia (Eger et al. 2010, Boyette et al. 2014, Ademokoya et al. 2018). Before 2009, this insect's range was confined to the eastern hemisphere; but in October of that year, it was found in nine counties in northeast Georgia, U.S. (Dhammi et al. 2016). At first, no natural enemies were known to serve as biocontrol for the kudzu bug population. This allowed the rapid range expansion of kudzu bug from Georgia as far west as Louisiana, north to Delaware and in some parts of Florida. As kudzu bugs are attracted to white and tan surfaces, including painted walls and vehicles, they also become unpleasant to homeowners (Lahiri et al. 2015, Dhammi et al. 2016).

Phylogenetic analysis of mitochondrial DNA sequences revealed *M. cribraria* population that invaded North America is evolved from *M. punctatissima* population (Hosokawa et al. 2014). *Megacopta punctatissima* is restricted to the northern Japanese islands and the Korean peninsula, whereas *M. cribraria* is distributed in the southern Japanese islands, China, and Taiwan. *Megacopta punctatissima* is comparatively bigger in size and darker in color than *M. cribraria.* Despite the different morphological features in both species, some taxonomists believe that both are synonymous (Hosokawa et al. 2014).

1.1.2 Dormancy and dispersal of kudzu bug

The kudzu bug has a multivoltine (three generations/year) life cycle in Asian temperate regions, but in the invaded regions of the U.S., it has a bivoltine (two generations/year) life cycle (Del Pozo-Valdivia and Reisig 2013). Adults can be found to overwinter in a wide variety of habitats such as under tree bark or rocks, in leaf litter, and around residential structures that offer a protective warmer microclimate from fluctuations in temperature during colder months (Suiter et al. 2010, Huskisson et al. 2015). Kudzu bugs prefer to overwinter under leaflitter in small groups (< 10 individuals/group) (Lahiri et al. 2015, Grant and Lamp 2017). In southern Alabama, adults begin to emerge from overwintering sites in or near kudzu (*Pueraria montana*) patches in mid-March (Figure 1.1 personal observation).

The first-generation instars develop on the kudzu vine or on alternative host plants such as early planted/or early maturing soybean or wisteria such as *W. sinensis* (Sims). Adults can migrate into soybean fields or remain on kudzu to lay eggs that develop into the second-generation (Zhang et al. 2012, Golec and Hu 2015, Lahiri et al. 2015). In late fall, the second generation moves to overwintering sites. Kudzu bugs are active between 07:00-21:30 hr during their active season and have sex-specific flight capabilities, with females flying at an average speed of 2.74 km/h and males at 2.37 km/h (Merwin and Yilmaz 2018).

1.1.3 ***Biology of kudzu bug***

Newly emerged adults have a soft white cuticle that is hardened within a few hours and then turns brown. Adults are slightly round, with females being larger in size (4.47 mm long and 3.86 mm wide) than males (3.99 mm long and 3.54 mm wide). Male and female can be easily distinguished by the shape and color of their terminal abdominal sternites, these are V-shaped and lighter in color in females and rounded with dark coloration in males (Zhang et al. 2012, Murphy 2015). A study conducted in Athens, Georgia in 2011 demonstrated that the sex ratio of males to females was 1:1 throughout the year (Zhang et al. 2012).

Mated females deposit an egg mass (average 15.6 eggs/mass) consisting of two parallel rows on the underside of the leaves (Zhang et al. 2012, Dhammi et al. 2016). However, in our three-year study (2018-2020) (unpublished data) conducted in multiple soybean fields across central Alabama, the average eggs/mass was 21.97 ± 10.74 (SD). Laying eggs underneath the leaf is a strategy that may protect the eggs from abiotic factors such as direct sunlight, wind, and rain and can also protect from biotic factors such as parasitoids, insect predators, birds, and reptiles. Each egg is around 1 mm long, 0.5 mm wide, and oval. Freshly laid eggs are white, but very soon afterward, the eggs become off-white. The egg cap is round and encompassed by spine-like structures (Zhang et al. 2012).

Endosymbionts are important for the proper growth, development, and survivability of newly hatched nymphs of kudzu bugs. Bacterial endosymbionts are present inside the lumen of the midgut in females thereby the end section of their midgut is swollen (Fukatsu and Hosokawa 2008). This section is known as the crypt-bearing midgut (Hosokawa et al. 2006). In males, bacterial cells were detected in the posterior end of the midgut, but this swollen section was missing. Females vertically transmit these microbial endosymbionts in capsules under the egg mass. These symbiont capsules, which are small and brownish, are transferred to the nymphs through egg feeding (Fukatsu and Hosokawa 2002, 2008). Symbionts γ-Proteobacterium and *Candidatus Ishikawaella* capsulata help in the digestion of plant sap and act as a defense mechanism of kudzu bug. These bacteria also help in the synthesis of the essential amino acids and vitamins that are absent in the phloem sap (Hosokawa et al. 2005, 2006). Deprivation of capsules affects the growth, size, and color of the immatures. For instance, nymphs grew slowly on heat-treated capsules and were smaller than nymphs that grew on normal capsules. The heat-treated capsule treatment group of nymphs were pale yellow, while the normal group of nymphs was green in color (Fukatsu and Hosokawa 2008). Removal of the capsules also resulted in sterility in adults (Hosokawa et al. 2006). Phylogenetic analysis shows that these extracellular symbionts are closely related to the intracellular symbionts of other insects, such as aphids (Hosokawa et al. 2006).

Like other hemipterans, kudzu bugs are hemimetabolous and progress through five nymphal instars before molting into their adult form (Gardner et al. 2013a). Body length and width vary across the nymphal stages. Average body length and eye width are different among instars, with the latter being a primary feature to distinguish instar stages. The first instars are slightly reddish and turn brown after several hours (Zhang et al. 2012). Neonates aggregate around the egg mass, and this aggregation behavior might provide a defense mechanism against predators. The second instars disperse to search for food, and the final instar is characterized by wing buds with a greenish-brown coloration (Dhammi et al. 2016).

1.1.4 Kudzu bug host plant preference

The kudzu bug adults and instar stages are phloem feeders primarily on stems, vines, petioles, and leaf veins, but early instars are found to feed on the leaves as well (Zhang et al. 2012). The recorded host range of kudzu bug includes 17 leguminous plants such as kudzu, lablab, soybean, hairy bush clover, Chinese bush clover, wisteria, black-eyed pea, black locust, redbud, wild indigo, lima beans, mung beans, green beans, and kidney beans, and sweetgum, and 14 non-leguminous plants such as walnuts in the U.S.A. (Eger et al. 2010, Zhang et al. 2012, Gardner et al. 2013b, Medal et al. 2013, Lovejoy and Johnson 2014, Medal et al. 2017, Yang et al. 2017). Legumes appear to be the primary host of kudzu bugs and the presence of kudzu bug on non-leguminous plants is considered an accidental occurrence. Zhang et al. (2012) investigated the host preference of kudzu bug for oviposition and development in northeast Georgia. They observed that females laid eggs on kudzu vines (528.8) > soybeans (320.0) > hairy bush clover (122.2) > Chinese bush clover (108.4) > wisteria (18.8) > American yellowwood (5.0). Kudzu bugs prefer tender leaf sheaths compared to older leaves or stems/vines for oviposition. It is also reported that the kudzu bug only completed development from egg to adult on soybean and kudzu. In addition, the emergence of the kudzu bug was higher on soybean (~67%) as compared to lima beans (~58%), mung beans (~42%), black-eyed peas (~41%), green beans (~15%), or kidney beans (~14%) (Yang et al. 2018).

1.2 ECONOMIC IMPORTANCE OF KUDZU BUG

This species poses a new threat for soybean growers in agricultural areas, and there is likewise the risk of spreading their population in areas they have not previously inhabited. In 2016, the total annual economic losses (combined treatment expenses and product yield loss) caused by kudzu bugs were estimated to be more than $4 million (Murphy 2015). In addition to direct impacts of feeding, the kudzu bug can also cause additional stress on the plant, making it more susceptible to drought, pests, and disease. Soybean is an important commodity crop in the U.S.A generating approximately $46.1 billion in revenue in 2020, in which Alabama alone generated more than $86 million (ERS USDA 2021). The American soybean association (ASA) estimated that approximately 30 states and more than 500,000 growers are involved in soybean cultivation in the country (ASA 2021). In Alabama, soybean production employs over 10,000 people (Brandy 2021). This bug has been found in every county of Alabama, making kudzu bug management strategies, and minimizing the risk of soybean loss vital to the region.

1.3 MANAGEMENT OF KUDZU BUGS

1.3.1 ***Planting date and maturity groups***

The planting date and physiological stage of the host plant can influence the kudzu bug population composition. Interactions between planting date and maturity significantly affect soybean yield (Del Pozo-Valdivia et al. 2016). To reduce yield loss, it is recommended that soybean growers plant soybean 6-8 weeks late, so that kudzu bugs can find other hosts or perish before planting occurs. However, late soybean planting incurs additional risk and potential expenses for farmers as other damaging pests such as stink bug complex are active throughout this period. There is a knowledge gap regarding the interspecific interaction between kudzu bugs and late-season insect species, specifically, the stink bug complex comprised of *Halyomorpha halys* Stål, the brown marmorated stink bug, and *Nezara viridula* L., the southern green stink bug. The kudzu bugs migrate into soybean fields in late May from either kudzu vines or alternative host plants and can be present in the field until October (Seiter et al. 2013), whereas the above-mentioned stink bugs species invade soybean crops late in the summer (Seiter et al., 2013; Grant et al., 2014). The stink bug complex and kudzu bugs can likely exist simultaneously in the late season in the soybean field, but interactions or competition have not been studied. Like stink bug complex, kudzu bugs prefer the reproductive stages of soybean over vegetative stages, but kudzu bugs do not feed on the seeds or pods (Ruberson et al. 2011). Results of behavioral bioassays showed that kudzu bug females are highly attracted to the odor of the reproductive stage compared to the vegetative stages (Yang et al. 2017).

1.3.2 Host plant resistant varieties

Plant variety and its structural components, such as leaf size and trichome density, can influence kudzu bug damage in host plants (Lahiri et al. 2020). Of 30 soybean resistant accessions, few varieties were found to be effective against kudzu bugs. The resistant varieties against nematodes, stink bugs and defoliators affect the development and survival of kudzu bug stages differentially. For instance, the soybean genotype N7103, which is notable for having narrower leaves and smaller seeds, displayed higher resistance against kudzu bug (Fritz et al. 2016). Mortality of the first instar was around 2x higher and the emergence rate of adults was approximately ten times lower on PI 567598B and PI 567336A than on Benning, (Bray et al. 2016, Lahiri et al. 2020). PI 548409 and PI 71506 have been identified as varieties susceptible to kudzu bugs, even though the trichome density of resistant varieties was similar to the susceptible varieties (Lahiri et al. 2020). However, to-date no kudzu bug resistant soybean variety has been made commercially available.

1.3.3 Tillage practices

Tillage type can also affect the kudzu bug density in a field. For instance, results from Del Pozo-Valdivia et al. (2017) showed that the conventional tillage system had threefold more kudzu bug egg masses compared to reduced tillage. A potential explanation for having a higher kudzu bug population in a conventional tillage system may be related to the healthier and greater biomass of the plants due to the higher availability of nitrate (or nitrogen) in the soil (Jokela 2016).

1.3.4 Insecticides

Another current recommendation for kudzu bug management is the use of synthetic insecticides. Seiter et al. (2015) evaluated the efficacy of insecticides with different active ingredients to control kudzu bugs. For example, the pyrethroid bifenthrin caused the highest mortality with up to 0.934 ± 0.027 (±SE) and 0.828 ± 0.091 (±SE) at 2 to 6 and 7 to 12 days, respectively (Seiter et al. 2014). The organophosphate acephate was also toxic against kudzu bugs (Wilson et al. 2015). Sulfoxaflor, a sulfoximine class chemical, significantly reduced the populations of both nymph and adult stages of kudzu bug. In China, kudzu bug populations have been controlled using chlorpyrifos and deltamethrin (Li et al. 2001, Zhang and Yu 2005, Fritz 2015). Despite being effective against kudzu bugs, insecticides can be hazardous to natural enemies and pollinators (Gill and Garg 2014), and organic farmers have minimal options to manage kudzu bug populations. Roberson et al. (2013) reported that a heavy population of kudzu bugs had destroyed an organic crop of snap bean in northern Georgia; however, economic losses have not been calculated. Furthermore, the current legislation on the use of pesticides and demands for pesticide-residue-free foods by consumers, retailers, and food industries have highlighted the need for a safe, non-toxic, and sustainable insect pest control method implemented with integrated pest management (IPM).

1.3.5 Natural enemies of kudzu bug

Natural enemies of kudzu bugs include predators, entomopathogenic fungi, and parasitoids. Several predators have been reported to prey on the mobile stages (nymphs and adults) of kudzu bug, such as *Podisus maculiventris, Euthyrhynchus floridanus, Geocoris uliginosus, and Zelus* spp. (Ruberson et al. 2013). Most predators are generalist feeders and are not expected to broadly manage kudzu bug populations (Ruberson et al. 2013).

Entomopathogenic nematodes (Family Mermithidae) have the potential to control a broad range of arthropod pests including kudzu bugs. Terrestrial mermithids in the soil and after short migration can reach the plant stem from the soil (Stubnins et al. 2015). Free-living and infective juveniles (IJs) of mermithids enter the hemocoel of the immatures or adults through natural openings (mouth, anus, and spiracles/respiratory system) and infect them (Stubbins et al. 2015). Nematodes develop in the abdomen, and typically IJs emerge from the posterior end of the insect. Stubbins et al. (2015 and 2016) reported the pathogenicity of mermithids in field-collected kudzu bug adults and mentioned that out of 422 adults only 20 adults were parasitized by nematodes.

The kudzu bug may also be infected with entomopathogenic fungi such as *Beauveria bassiana* (Britt et al. 2016, Portilla et al. 2016). Investigation of three *B. bassiana* strains: NI8-Native Delta, Mississippi Strain (ARSEF 8889), GHA-Commercial strain and KUDSC-001-South Carolina Kudzu Strain (ARSEF 13136) demonstrated that young adult kudzu bugs are more susceptible than old adults to infection (Portilla et al. 2016). However, young adults were less susceptible to the GHA strain than the isolate NI8 strain with mortality 3-8x greater in the latter (Portilla et al. 2016). Specific requirements of entomopathogens in terms of temperature, relative humidity (RH) requirements, and UV/direct sunlight may affect their performance. So far there are records of two parasitoid species of kudzu bug eggs in the southern U.S. including the *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae) and *Paratelenomous saccharalis* Dodd (Hymenoptera: Platygastridae) (Ruberson et al. 2013, Ademokoya et al. 2018).

1.4 KNOWN BIOLOGY OF PARATELENOMUS SACCHARALIS AND OOENCYRTUS NEZARAE

1.4.1 Distribution and description of P. saccharalis and O. nezarae

The first report of the parasitic wasp, *Paratelenomous saccharalis* was in 2013 in Alabama and Georgia (U.S.). The range of this parasitic wasp was only known in the eastern hemisphere prior to this discovery (Johnson 1996). This species has also been collected in Australia, India, western and central Europe, Middle East, and Africa (Johnson 1996).

In 2016, *Ooencyrtus nezarae,* was found in kudzu bug eggs in Alabama and since then has been reported in Georgia, Florida, and most of the neighboring states. Documented distribution of *O. nezarae* was initially limited to China, Japan, Thailand, South Korea, and Brazil (Kobayashi and Cosenza 1987, Noyes 2003, Zhang and Huang 2005).

1.4.2 Ecology of P. saccharalis and O. nezarae

Paratelenomus saccharalis is a specialist and solitary endoparasitoid of the Plastaspid family (Johnson 1996). The only known host of *P. saccharalis* in the U.S is *M. cribraria* eggs. Other host species of *P. saccharalis* include *M. punctissimum* (in Japan), *Coptosoma cribrarium* (in China) and *Brachyplatys subaeneuus* (in China). The latter host species was recently reported in Miami, Florida in 2020 (Eger et al. 2020).

Ooencyrtus nezarae is a generalist egg parasitoid reported to attack *M. cribraria/punctissimum, Piezodorus hybneri, Dolycoris baccarum, Nezara antennata,* and *Eysarcoris guttiger* (Takasu and Hirose 1985, Hirose et al. 1996, Mizutani et al. 1997). *Ooencyrtus nezarae* is a gregarious parasitoid in eggs of bigger hosts but in eggs of *Megacopta* species, it is usually a solitary parasitoid (Takasu and Hirose 1991).

Paratelenomous saccharalis and *O. nezarae* were reported to parasitize a maximum of 100% and 76.9% of hosts, respectively, in their native range of southeast Asia (Takasu and

Hirose 1985, 1986). In Alabama, *P. saccharalis* and *O. nezarae* have been observed in the same soybean field attacking kudzu bug eggs with parasitism rates ranging from 42-95% and 82.8-100%, respectively (Ademokoya et al. 2018).

Seasonal parasitism of both wasps appears to be different in their native and introduced ranges. The parasitism rates of *P. saccharalis* dominate from May to June, while in *O. nezarae*, the peak parasitism rate occurs from July to August (Hoshino et al. 2017). However, both species emerge simultaneously in the U.S. (Figure 1; unpublished data). Both overwinter as adults.

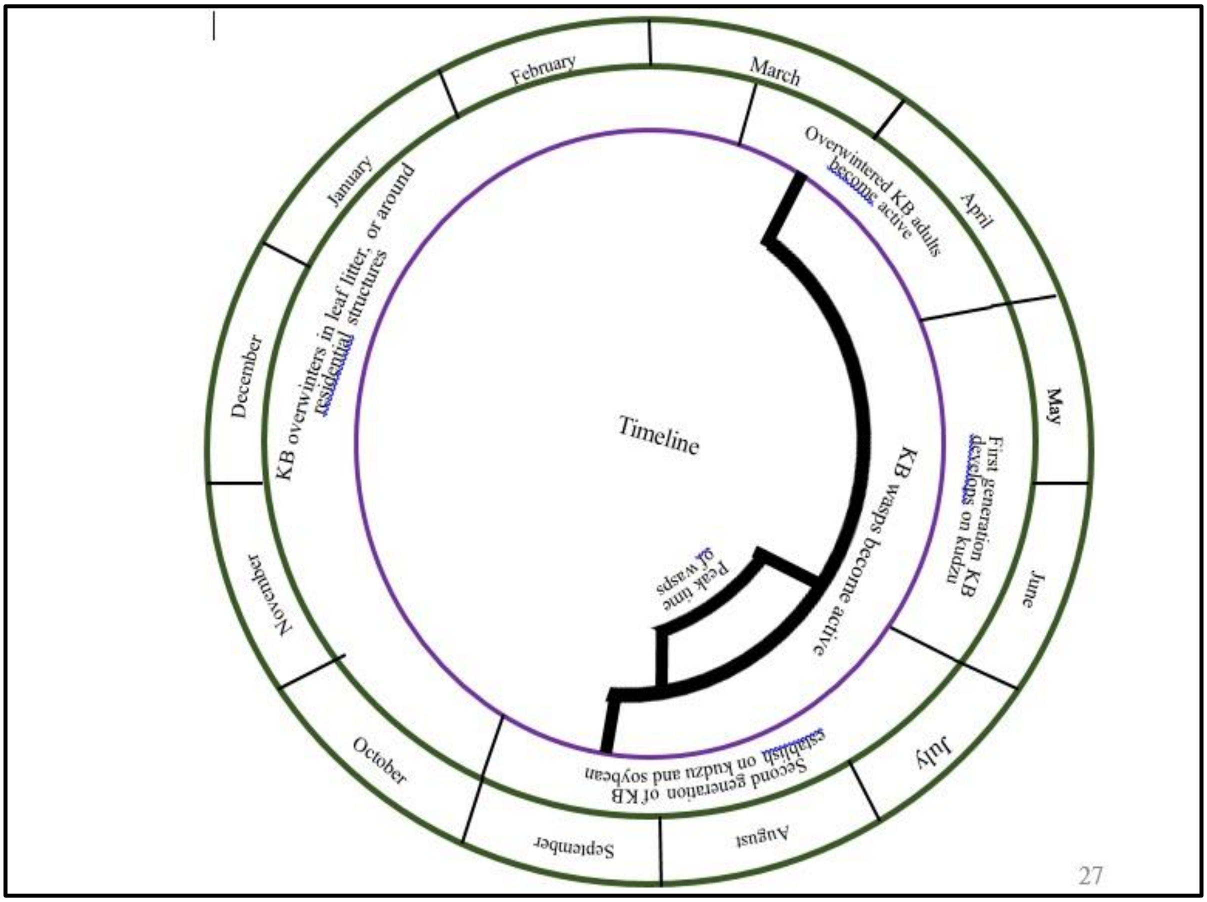

Figure 1.1Timeline of the emergence of kudzu bug (KB) and its parasitoids (*Ooencyrtus nezarae* and *Paratelenomus saccharalis* in Alabama, U.S. (unpublished study).

The average time it takes for the *P. saccharalis* population from Japan to develop from egg to adult is 11.7 ± 1.03 at 30 °C and 24.8 ± 0.66 at 20 °C. Furthermore, no development happens if the temperature falls below 11.8°C (Takagi and Murakami 1997). This species' development threshold temperature from egg to adult is 12 °C with a thermal constant of 208.3-degree days (Takagi and Murakami 1997). Based on this information, it is estimated that *P. saccharalis* has four generations from July 1 to August 31 in Japan (Takagi and Murakami 1997). Adults emerge within 11–13 days (Rajmohana et al. 2019). Males first emerge from the eggs, where they wait for the emergence of females with whom they can mate for 12-15 seconds (Rajmohana et al. 2019). In the native ranges, *P. saccharalis* females lay 25-40 eggs in their life (Takasu 2012). The peak time of their fecundity is on the second day after emergence (Takano and Takasu 2019). However, there is a conflict among research related to the longevity of *P. saccharalis*. According to Takasu 2012, *P. saccharalis* lives 5-9 days, but Takano and Takasu 2019 reported 40 days. The current study will present information on longevity and fecundity in its introduced range.

Paratelenomus saccharalis continues to produce offspring even in the absence of food. Food deprived wasps produced 21.2 ± 2.0 (± S.E.) offspring within two days after emergence. However, a starved female that lives for four days continues to lay eggs until she dies. This indicates that *P. saccharalis* seldom continues to mature eggs throughout her lifetime (Takano and Takasu 2019). Food deprivation affects the oviposition behavior in *P. saccharalis*; starved females lay most of their eggs on the first day after emergence, and honey-fed females lay a small number on the first day but continue to lay eggs for up to 8 days (Takano and Takasu 2019). It has also been reported that superparasitism is seldom observed in fed-females but frequently occurs in unfed females. Life history models predict that the propensity to superparasitize hosts increases due to a reduced life expectancy; thus, accepting lower-quality hosts may be less risky than searching for higher-quality hosts (Roitberg et al. 1992; Fletcher et al. 1994; Sirot et al. 1997).

Typically, *P. saccharalis* displays arrhenotokous reproduction, i. e., haploid males arise from unfertilized eggs, and diploid females or males arise from fertilized eggs in most of its native ranges. However, in Europe, it exhibits thelytoky sex determination in which diploid males arise from unfertilized eggs (Johnson 1996). The sex ratio of *P. saccharalis* is either equal or female-biased (range of bias); but older females typically produce a male-biased sex ratio of offspring (Takano and Takasu 2019).

The mean development time from egg to adult of *O. nezarae* is 10.0 ± 0.00 at 30 °C and 14.0 ± 0.30 at 25 °C (Numata 1993). This species requires 189 degree-days above 11.3 °C for complete development (Numata 1993). Female *O. nezarae* does not lay eggs at ≤15 °C. They can also live 40 days and reproduce up to 20 days of their life. The fecundity peaks five days after emergence. The lifetime reproduction of *O. nezarae* is ~75 offspring reared on *R. clavatus* eggs (Aung et al. 2012). However, reproduction of *O. nezarae* has not been investigated on the kudzu bug eggs.

Ooencyrtus nezarae is considered a concurrent host feeder that exploits nutritional resources before oviposition. This synovigenic species emerges with no mature eggs but matures two eggs after a single host feeding (Aung et al. 2009). Furthermore, when host feeding is coupled with a carbohydrate supplement, egg maturation is enhanced in *O. nezarae* as reported by Aung et al. 2010. They found that one-day honey-fed wasps (two-days old) matured a total of 11 eggs. The natural sugars (sucrose, glucose, galactose) and honey are equally acceptable by this wasp and resulted in no differences in wasp longevity. However, carbohydrate type had an effect on egg production. Egg production was higher on the honey diet (7-39), followed by sucrose (1-24), glucose (1-28), and galactose (1-21) (Teraoka and Numata 2000). The sex ratio of *O. nezarae* is female-biased in both native and non-native ranges: 1 male:2.5 females in the U.S. on *M. cribraria* and 1 male:3 females in Japan on *Reptortus pedestris* (Hirose et al. 1996, Aung et al. 2011a, b, Ademokoya et al. 2018).

Despite the importance of *P. saccharalis* and *O. nezarae* as biocontrol agents, studies regarding their biology have not been done in their invasive range. Therefore, this study aims to investigate the developmental biology of both wasps in different varying conditions.

1.4.3 Infochemicals use of P. saccharalis and O. nezarae

Only a few studies of the chemical ecology of both wasps are available. The role of host semiochemical in *P. saccharalis* was first identified in the U.S. by Ademokoya et al. (2017). This olfactometer study reported that *P. saccharalis* uses host-related chemical cues to forage for its host, *M. cribraria*. Similarly, *Ooencyrtus nezarae* females utilize host pheromone as kairomone to locate their hosts (Mizutani 2006). For instance, (E)-2-hexenyl (Z)-3-hexenoate (E2HZ3H), a male-specific feeding aggregation pheromone of *R. clavatus* attracts *O. nezarae*. Further investigation is needed to determine how *O. nezarae* females locate eggs laid on host plants through stimuli associated with the host females and their eggs. This present study will investigate all the possible outcomes for the host searching behavior of *O. nezarae* and *P. saccharalis*.

1.4.4 Interspecific interactions of P. saccharalis and O. nezarae

Paratelenomus saccharalis and *Ooencyrtus nezarae* coexist in the field and concurrently parasitize the same host egg mass. In Japan, Hoshino et al. (2017) noticed interspecific competitive interaction between these species in the field. They also observed that *P. saccharalis* displays aggressive behavior against its conspecific and heterospecific females to protect the host, whereas the ovipositing *O. nezarae* female does not exhibit aggressive behavior. It has also been observed in Takasu et al. (1998) that *O. nezarae* did not display aggressive behavior against *Gyron* spp. (*Gyron japonicum* and *G. nigricorne*) when both species simultaneously encountered *R. clavatus* eggs in the laboratory. However, indirect interspecific competition occurred between *O. nezarae* and *Gyron* spp. In that study, *O. nezarae* was successfully able to reduce reproduction of its competitor species. Similarly, there is possible direct and/or indirect interspecific interaction between *O. nezarae* and *P. saccharalis* as observed in their native range.

Over the last few years, there has been a sharp decline in *P. saccharalis* numbers in the U.S. Between 2018-2020, the number of *P. saccharalis* fell sharply and reached their lowest levels since population monitoring began in 2013 (unpublished data). Several hypotheses may explain this observed decline (Hoshino et al. 2017, Ademokoya et al. 2018, Merchant 2020). These ideas include: i) the arrival of the generalist species *O. nezarae* in 2016 (the interspecific competition between *O. nezarae* and *P. saccharalis* has not been studied in its invasive range), ii) the decline of the kudzu bug due to its natural predators and *B. bassiana*, and iii) the widespread use of insecticides to suppress kudzu bug population. However, there is no concrete evidence for or against any of these hypotheses.

This dissertation focused on the (i) preference of stink bugs for kudzu bug pre-infested soybean plant, (ii) the effect of sugar feeding, host eggs: parasitoid ratio, and host exposure time on the developmental biology of *P. saccharalis* and *O. nezarae*, (iii) host-seeking behavior in *P. saccharalis* and *O. nezarae*: insights from olfactometer and footprint bioassays, and (iv) interspecific competitive interactions between *Ooencyrtus nezarae* and *Paratelonomus saccharalis*. The overall goal of this study is to develop economically viable and environmentally-sound IPM strategies for kudzu bugs.

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Chapter 2

CHARACTERIZING DIRECT AND INDIRECT INTERACTIONS OF KUDZU BUG (*MEGACOPTA CRIBRARIA*) INFESTATION ON SOUTHERN GREEN STINK BUG (*NEZARA VIRIDULA*) AND BROWN MARMORATED STINK BUG (*HALYOMORPHA HALYS*) BEHAVIORS

2.1 INTRODUCTION

Soybean, *Glycine max* (L.) Merr., is one of the most economically important legumes in the United States (U.S.) (USDA-APHIS 2014). Pentatomidae members within Hemiptera pose a considerable challenge to soybean production in the U.S., by causing direct feeding damage to the crops in soybean-growing areas across the country (Kogan and Turnipseed 1987, Peiffer and Felton 2014). Stink bugs are a major concern in soybean production (Sosa-Gómez et al. 2020), and studies have estimated damages to soybean crops are approximately $324 million (Musser et al. 2022). The kudzu bug, another hemipteran pest first discovered in Alabama in 2012, is a significant threat to soybean production, causing up to 59% yield losses in extreme instances by feeding on plant phloem and disrupting nutrient distribution (Gardner et al. 2013, Seiter et al. 2013). This invasive species added a substantial burden to soybean farmers in seven southern states, with the combined economic impact of control measures and yield losses surpassing $4 million in 2016 (Musser et al. 2017). Stink bug species and the kudzu bug may interact with one another, because they often coexist in soybean fields mid to late season (McPherson and Bondari 1991, Rahman and Lim 2017). Their interaction can be mediated indirectly, by their shared host plant (Zhang et al. 2013a, b) or directly, driven by competition for similar food resources and space for reproduction (Inbar and Gerling 2008).

Two members of the stink bug complex that feed on soybean are the southern green stink bug (*Nezara viridula* L.) and the brown marmorated stink bug (*Halyomorpha halys* Stål), and they are of significant economic importance in soybean production across the southern United States (Ademokoya 2021). The southern green stink bug is native to equatorial regions and is hypothesized to originated from southern Asia as its origin (DeWitt and Godfrey 1972), or Ethiopia (Jones 1988, Panizzi 2008). The southern green stink bug has spread to Asia, the U.S., and Europe (Todd 1989). The first recorded appearance of this pest in the U.S. was documented by Distant (1880). The brown marmorated stink bug is native to China, and the first established U.S. population was reported in Allentown, Pennsylvania, in 1996 (Hoebeke and Carter 2003). Both stink bug species are generalist herbivores with a wide host range, including fruits, vegetables, and field crops but they often prefer soybean crops in southern regions (Haye et al. 2015, Oho & Kiritani 1960, Todd 1989, Panizzi 1997, Panizzi et al. 2000, Peiffer and Felton 2014, Ademokoya 2021). They have long been recognized as mid-to-late-season pests of soybean (Bundy and McPherson 2000), damaging petioles, stems, and pods (Mitchell et al. 2018). Their populations peak in late August through early October, primarily in mid-September in soybean fields (Reisig 2020).

In 2009, the kudzu bug (*Megacopta cribraria* F.) emerged as a new threat to U.S southern farmers (Dhammi et al. 2016). This species is a member of the family Plataspidae (Dhammi et al.

2016). Kudzu bugs are a specialized herbivore primarily feeding on bean family plants (Fabaceae), including the kudzu vine (*Pueraria montana* var. lobata), with soybean crops being one of their preferred hosts (Dhammi et al. 2016). This plastaspid is originally from Asia and was first reported in the Georgia, U.S. (Eger et al. 2010, Boyette et al. 2014, Dhammi et al. 2016, Ademokoya et al. 2018). Kudzu bugs typically migrate into southeastern soybean fields in late May, originating from either kudzu vines (*Pueraria montana* (Lour) Merr.) or alternative host plants, and can persist in the field until October (Seiter et al. 2013). Kudzu bug population densities typically peak in early September in soybeans (McRight 2018). Unlike stink bugs, kudzu bug adults suck vascular fluids only from the stems and petioles (Tayutivutikul and Yano 1990, Xing et al. 2006, Suiter et al. 2010).

Studies have shown that herbivores indirectly impact the behavior of other herbivores by causing damage to the plant via feeding (Xue et al. 2010a, Zhang et al. 2013a, b, Tan et al. 2014). This damage can result in changes in nutrient levels or defensive compounds, and ultimately affect herbivore behavior such as their selection of plants for feeding (Howe and Jander 2008, de Vos and Jander 2010 Tan et al. 2014). Due to changes in plant chemistry (Howe and Jander 2008, de Vos and Jander 2010, Tan et al. 2014), herbivores may adjust their feeding preferences, selecting plants with higher nutrient levels and lower defensive compound concentrations. This adaptive response creates a dynamic interplay between herbivores and plants (Xue et al. 2010, Zhang et al. 2013a, b), with plants deterring herbivory through chemical defenses and nutrient modulation, and herbivores adapting their feeding behavior to overcome defenses and optimize nutrient intake. According to the Plant Stress Hypothesis, when a plant experiences damage it undergoes physiological stress making it more susceptible to herbivore attacks due to reduced protein synthesis and enhanced free amino acid content in tissue (White

1969). This susceptibility arises from resource redirection towards repair, regrowth, and recovery processes, which reduces protein synthesis due to trade-offs. Consequently, the limited resource allocation may cause an accumulation of free amino acids in plant tissues, as they are not utilized for new protein synthesis (White 1969). This increased amino acid creates more nutritious food for nitrogen-limited organisms, such as sap feeders (Sandström and Moran 1999). Prioritizing limited resources towards restoration in stressed plants also impacts their investment in defensive compounds, subsequently increasing their susceptibility to herbivore attacks (White 1969). The second hypothesis, the Pulsed Stress Hypothesis, predicts that sap feeders perform better on intermittently stressed plants, as pulsed stress events can temporarily alter the nutritional quality and defense mechanisms of plants, influencing herbivore preferences and feeding patterns (Mattson and Haack 1987, Heinrichs 1988, Horiuchi et al. 2003, Huberty and Denno 2004, Girling et al. 2011). Understanding these two hypotheses and their implications in plant-herbivore interactions is critical for effectively managing insect pests. Knowledge of these hypotheses may help in predicting insect pest population dynamics in soybeans if kudzu bug infestations alter host selection by stink bugs that infest plants later in the growing season.

In direct impacts, one herbivore species can directly influence another through physical interference to defend a feeding site on the shared plant (Howe and Jander 2008, de Vos and Jander 2010, Tan et al. 2014). One such strategy can be observed in specialist herbivores, which exhibit aggression to safeguard their specific food sources from generalist herbivores, as described by Schoener (1983). Specialists are more selective in their feeding habits, relying on a narrower range of plant species for sustenance (Bernays and Graham 1988, Poisot et al. 2011). Consequently, their survival is more closely tied to the availability of these specific resources, leading to aggressive behavior when faced with potential competitors (Denno et al. 1995, Agrawal et al. 2006). The intensity of aggression exhibited by specialist herbivores in defending limited food sources may be influenced by population density (Connel 1983). High population density in specialist herbivores can intensify competition for limited food sources, resulting in more frequent aggressive interactions as they defend their resources (Schoener 1983). Available information on plant-induced responses and direct competitiveness among herbivores rarely focuses on sap feeders, such as kudzu bugs or stink bug species.

No information is available on the responses of stink bugs to plants previously attacked by kudzu bugs, leaving a significant knowledge gap in understanding their interactions and potential consequences on shared host plants. To address the knowledge gap concerning the interspecific interactions between kudzu bugs and stink bugs, the present study aims to investigate the (i) effects of soybean responses induced by pre-infestation with kudzu bugs on the host selection behavior of the brown marmorated stink bug and southern green stink bug and (ii) effects of kudzu bug density on the behavior of the brown marmorated stink bug and southern green stink bug after feeding. To achieve these objectives, two hypotheses were tested: (i) in the absence of kudzu bugs, stink bugs will prefer soybean plants that have previously been infested with kudzu bugs, and conversely, (ii) when kudzu bugs are present, stink bug avoidance will increase as the density of kudzu bugs per plant increases.

2.2 MATERIALS AND METHODS

2.2.1 ***Plants***

Soybean seeds (var. Pioneer P49T97R-SA2P, Taleecon Farmers' Co. Notalsuga, AL, USA) were planted in seed trays under greenhouse conditions (26 ± 2°C and 55 ± 5% RH) at Auburn University (Ademokoya et al. 2017). Seeds were planted weekly, beginning on the second week of March. Approximately one week old, seedlings were transplanted into pots (12.7 cm diameter x 15 cm high) in Sunshine potting mixture #8 (SunGro Horticulture, WA). Plants were grown in the greenhouse at 26 ± 2°C and 55 ± 5% RH without pesticide application (Ademokoya et al. 2017). All plants were caged and maintained insect-free before use in experiments. Plants were watered daily (~200 mL per pot) and fertilized once a week according to the manufacturer's instructions (Scotts-Sierra Horticultural Product Company, Marysville, OH, USA). For each experiment, the flowering stage of the plant (R1-R2) was used because kudzu bugs tended to prefer early reproductive stages (Yang et al. 2017).

2.2.2 Insects

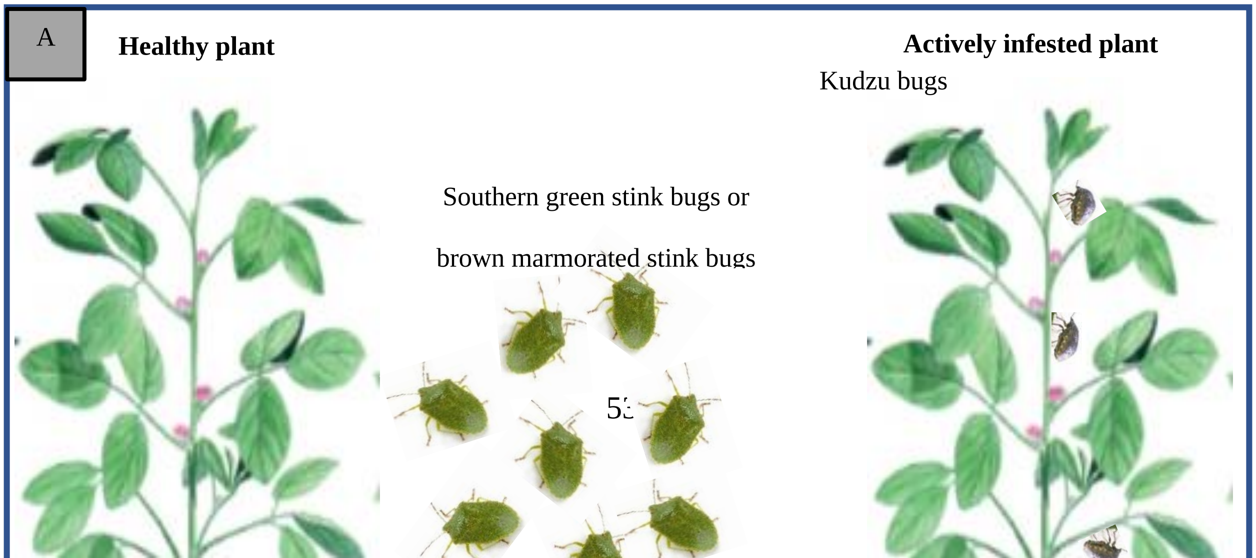
Kudzu bug adults of unknown age were collected from kudzu in Auburn, Alabama in early April 2021 and were maintained in ventilated rearing cages (30 cm x 30 cm x 30 cm) (BugDorm-1, Megaview Science Co., Ltd., Taichung, Taiwan) at 25 ± 1 °C, 14:10 h (L: D) photoperiod, and 50% relative humidity (RH), with approximately 4-week-old soybean plants provided as sustenance (Ademokoya et al. 2017). Soybean plants were replaced biweekly (Ademokoya et al. 2017). The sex of kudzu bugs was identified based on their terminal sternite morphology, which are V-shaped with a visible suture in females and rounded without a visible suture in males (Zhang et al. 2012).

Adults of southern green stink bugs and brown marmorated stink bugs were collected from soybean fields in Alabama in 2019. Following capture, they were reared separately in ventilated plastic cages similar to those used for kudzu bug rearing. The growth chamber conditions for stink bugs were 24 ± 2 °C, a 16:8 h (L: D), and 70 ± 5% RH (Cusumano et al. 2011, Boyle 2017). Stink bugs were fed a diet of organic green beans, shelled raw sunflower and buckwheat seeds 2-3 times per week (Cusumano et al. 2011, Herlihy et al. 2016, Boyle 2017). Distilled water was supplied on cotton balls. Stink bug eggs were collected and transferred to Petri dishes (10 cm diameter x 1.5 cm height, product no. P5856, petri dishes, polystyrene, Signa Aldrich Inc. St. Louis, MO, USA) with water supplied on cotton balls. Hatched nymphs were transferred to the rearing cages with food and water as described above. In the study, 1-2 days post-final molt, southern green stink bugs and brown marmorated stink bugs were used (Mitchell and Mau 1969, Funayama 2006). Only adult stink bugs were utilized in this study due to their high mobility and well-developed piercing-sucking mouthparts, which enable them to penetrate deep into plant tissue and cause significant feeding injury (Peiffer and Felton 2014). We included both male and female stink bugs in the present study to capture a more realistic and representative picture of their feeding behavior and potential impact on the host plant (Lara et al. 2018). This is consistent with the Panizzi et al. (1995) study, which has shown that both genders of stink bugs exhibit similar feeding behavior. Additionally, a mixed population of stink bugs consumed the host plant more extensively than either gender alone (Lara et al. 2018). Stink bug sex were distinguished by examining their genitalia located at the ventral tip of the abdomen. Males have two prongs or claspers on either side of the abdominal tip. Females have a rounded ventral surface at the tip of the abdomen (Acebes-Doria 2016). Stink bugs were starved for 24 hours before the use in the experiments.

2.3 EXPERIMENTAL DESIGN

The experimental design for both stink bug species was identical and consisted of two choices: an infested and a healthy plant (or control) (Figure 2.1). For the indirect impact experiment, the kudzu bugs fed on the plants for 24 hours and were then removed before introducing stink bugs. For the direct impact experiment, kudzu bugs remained on the infested plant when stink bugs were released.

In both direct and indirect impact experiments, stink bug species' responses were tested against three kudzu bug densities (1-2 days old) with equal males and females: 10 (5 pairs), 20 (10 pairs), or 30 (15 pairs). Densities were selected by observing adult kudzu bugs on plants in 2011, in northwest South Carolina and northeast Georgia, where the range spanned from 0 to over 50 kudzu bugs per plant (Suiter et al. 2010). This study included both male and female kudzu bugs, as it has previously shown that the combined feeding of both sexes of an insect can lead to increased green leaf volatile emissions from plants, compared to either sex alone (Li et al. 2020). Prior to the start of the experiment, kudzu bugs were starved by placing individuals in 3.8-liter containers with moistened cotton for 24 hours without any food source. Following this fasting period, a soybean plant was placed into a cage (3 feet x 3 feet x 3 feet) and kudzu bug adults were released to feed on the plant for 24 hours. After 24 hours, a healthy (control plant) soybean plant of the same age and stage was added to the cage. The plants were separated by a diagonal distance of 1.50 meters to minimize the movement of adult kudzu bugs between plants, as observed during the preliminary experiment. This experimental setup allowed for investigating stink bug species' responses to different kudzu bug densities while minimizing confounding factors such as kudzu bugs movement between plants. Once a healthy plant was placed in the cage, either southern green stink bugs (four pairs) or brown marmorated stink bugs (two pairs) were released in the center of the cage. The chosen number of stink bugs for each species was adapted from previous fruit exposure work (threshold of two pairs brown marmorated stink bugs/plant) (Lara et al. 2018) and yield loss in soybean (threshold of four pairs southern green stink bugs/plant), respectively (Suzuki et al. 1991). Twenty-four hours after the stink bug release, the location of each stink bug (i.e., on the infested plant, healthy plant, or not on a plant) was recorded. A preliminary experiment was conducted to determine the optimal exposure time for observing stink bugs' preferences for plants. This was done to ensure that the majority of stink bugs made a choice for the plants during the observation period. The selection of a plant is significant because it can provide insights into the insects' behavior such as feeding, mating, or oviposition. Results showed that a 24-hour exposure period significantly increased the stink bugs' tendency to land on plants (74% of the time) compared to shorter (6 or 12-hour) durations (<44%). This finding justified using a 24-hour exposure window for direct and indirect impact experiments to understand better the complex interactions between these herbivorous insects and their host plants. This study was replicated ten times with a factorial design: 2 experiments (direct and indirect) x 3 kudzu bug densities x 2 stink bug species x 10 replications = 120 cage trials.



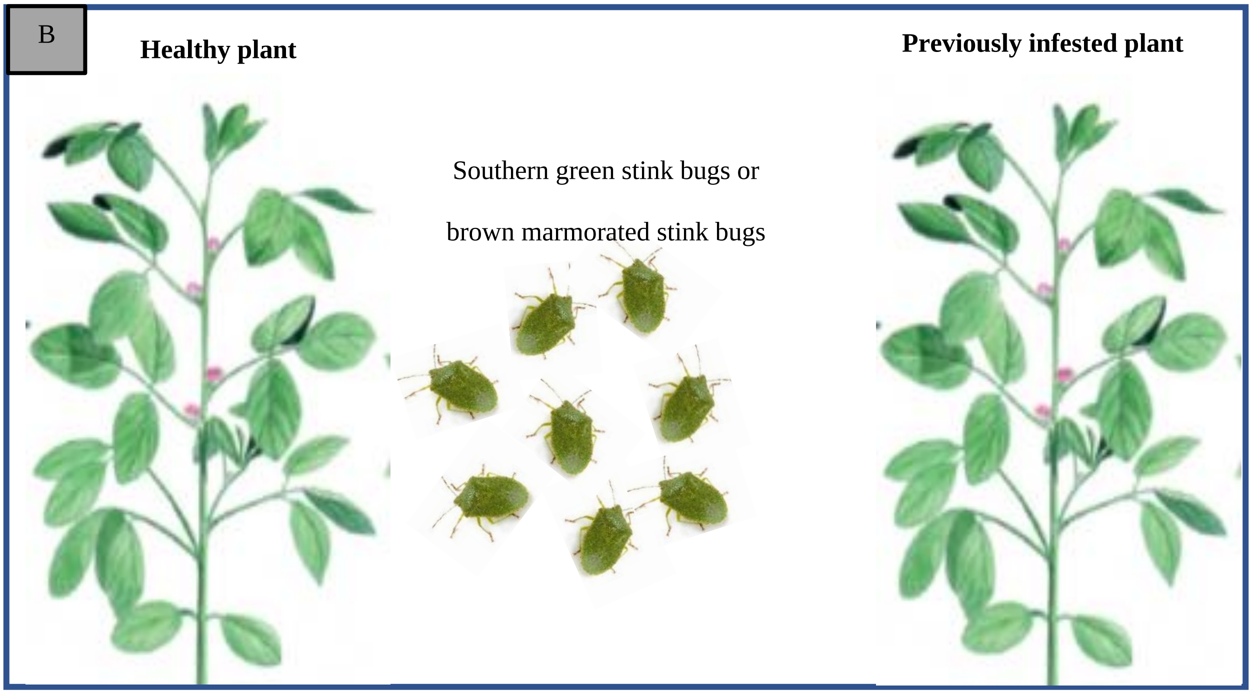


Figure 2.2 Diagrammatic representation of the experimental setup for southern green stink bug and brown marmorated stink bug in direct (A) and indirect (B) impact experiments.

2.4 STATISTICAL ANALYSIS

The predictor variable, kudzu bug density, was a categorical variable with three levels representing the density of kudzu bugs (10, 20, or 30) in direct and indirect impact experiments.

Figure 2.1 Diagrammatic representation of the experimental setup for southern green stink bug and brown marmorated stink bug in direct (A) and indirect (B) impact experiments.

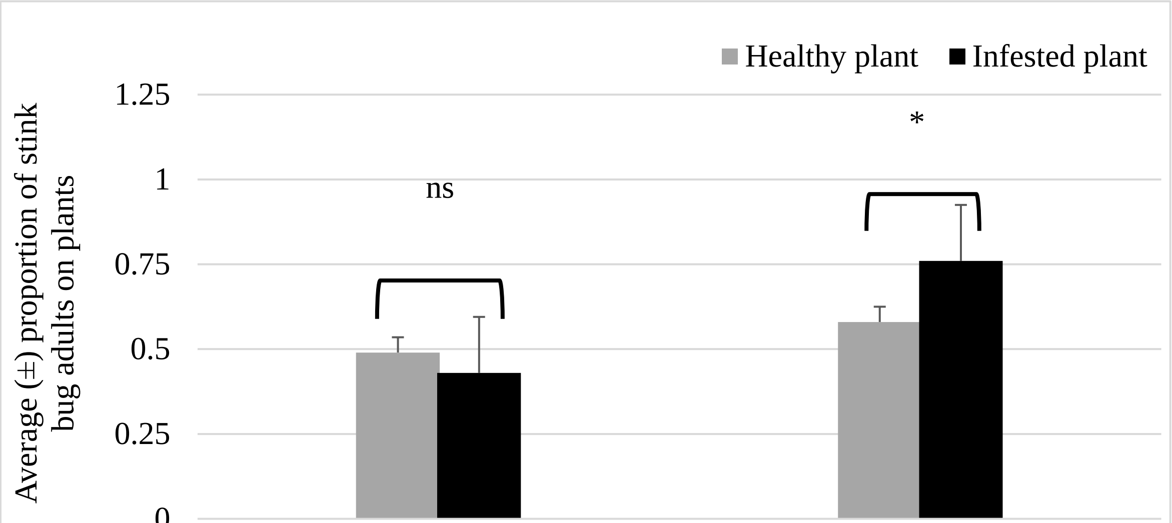
The response variable, stink bug proportion, indicated the proportion of stink bugs landing on healthy and infested plants. This proportion was calculated as the ratio of the stink bugs landing on the plant to the total number released in the cage. The proportion data were not normally distributed; therefore, it was subjected to arcsine-square-root transformation. The assumptions of normality and homoscedasticity were confirmed through Shapiro-Wilk and Lavene's tests, respectively. Logistic regression analysis compared the means of the transformed proportions of either stink bug species landing on healthy or pre-infested plants in direct and indirect impact experiments. Logistic regression analysis was also used to examine the effect of kudzu bug density on the selection of stink bugs for either healthy or infested plants to determine if there is a relationship between kudzu bug density and the likelihood of stink bugs selecting healthy or infested plants. When no significant effect of kudzu bug density was detected on the selection of stink bugs for healthy or infested plants, the densities were individually evaluated. To further investigate any differences between healthy and infested plants at each kudzu bug density level, a two-sided independent samples t-test was used. All statistical analyses were performed using SAS v9.2 (SAS Institute Inc., Cary, NC, USA), with a significance level at $p \leq 0.05$.

2.5 RESULTS

2.5.1 Direct impact experiments

In this experiment, there was a significant difference in the proportion of southern green stink bug adults landing on infested plants compared to healthy plants when kudzu bugs were also present on the infested plant (*p*=0.0003: Table 2.1). Approximately 1.31 times more southern green stink bug adults selected infested plants than healthy plants (Figure 2.1). The effect of kudzu bug density on plant selection by southern green stink bugs was not significant (Table 2.3). When comparing the preference of southern green stink bugs at each density level, between healthy and infested plants, their preference for infested plants over healthy plants was evident at all density levels with more than 70% of the stink bug population selecting infested plants (Figure 2.3A). The differences in proportions between healthy and infested plants were 0.15, 0.12, and 0.29 for 10, 20, and 30 kudzu bug adults, respectively (Figure 2.3A). As the density of kudzu bug adults increased from 10 to 30, the preference for pre-infested plants became more pronounced (Table 2. 4 and Figure 2.3A).

The proportion of brown marmorated stink bug adults that landed on the infested plants in the presence of the kudzu bugs was not significantly different from those on the healthy plants (Table 2.1 and Figure 2.1) indicating no strong preference for either type of plant. There was also no significant effect of kudzu bug density on the selection of healthy or infested plants by brown marmorated stink bugs (Table 2.1). The preference of brown marmorated stink bugs for infested plants over healthy plants was most pronounced at the lowest density (10 adults) with more than 50% of brown marmorated stink bugs selected the infested plants. However, as the kudzu bug's density increased from 20 to 30, their preference shifted, and more than 50% of their population tended to select the healthy plants (Table 2.4 and Figure 2.3B).



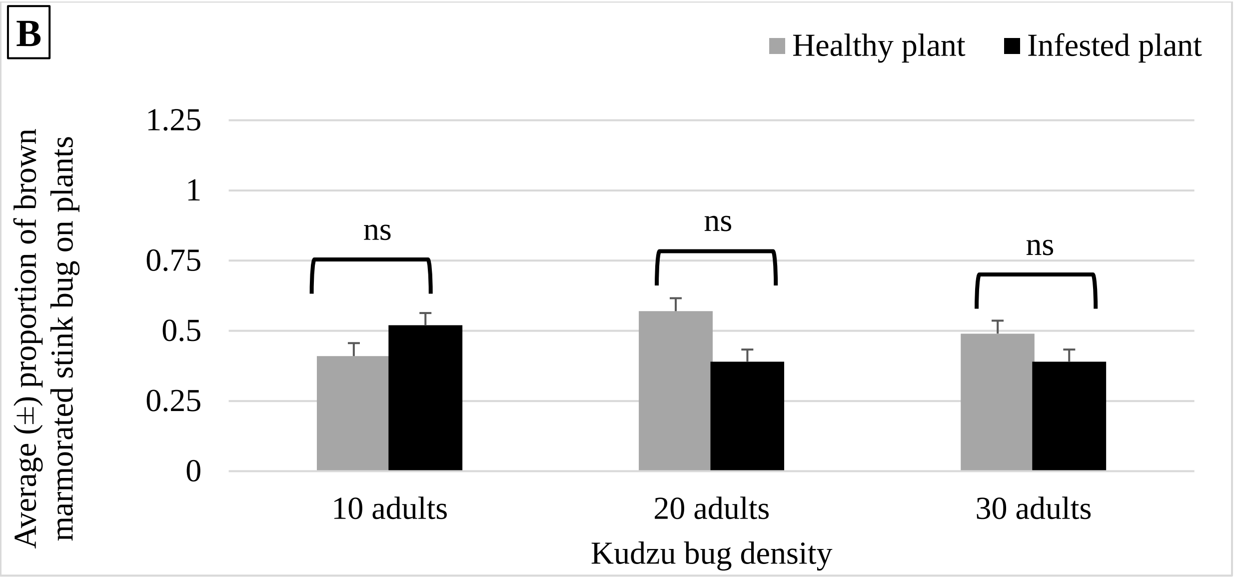


Figure 2.4 Average proportion (±S.E.) of (A) southern green stink bug and (B) brown marmorated stink bug adults on healthy and infested soybean plants with kudzu bugs density as direct impacts. Asterisk (*) indicates a significant difference between host selection preference in the healthy versus preinfested soybean plants (two-sided t-test, $p < 0.05$). ns. indicates no significant difference.

2.5.2 Indirect impact experiment

The proportion of green stink bug adults found on the infested plant significantly differed from the healthy plants ($p < 0.0001$; Table 2.1 and Figure 2.4). They were approximately 1.5 times more likely to select the infested plants than healthy plants, indicating a strong preference for infested plants (Figure 2.4). There was no significant relationship between kudzu bug density and southern green stink bugs selection of healthy or infested plants (Table 2.2). Adult green stink bugs consistently exhibited a high preference for infested plants over healthy plants at all densities (Table 2.3 and Figure 2.5A); at each density, more than 80% of stink bugs selected infested plants over healthy plants (Figure 2.5A). As the kudzu bug density increased from 10 to 30, the preference for infested plants over healthy ones became more evident, with an approximate difference of 0.28 (Figure 2.5A).

The proportion of brown marmorated stink bug adults found on the plants infested with kudzu bugs was significantly different than those not infested with kudzu bugs ($p=0.04$; Table 2.1 and Figure 2.4). Brown marmorated stink bugs showed a preference for infested plants over healthy plants, as they were more than twice as likely to land on infested plants than on healthy plants. There was a significant effect of kudzu bug density on the attraction of brown marmorated stink bugs to the healthy plants ($p = 0.04$) but no significant effect was observed for infested plants. When comparing the preference of brown marmorated stink bugs at each density of kudzu bugs, no significant differences were found, but there was a numerical trend showing fewer brown marmorated stink bugs on healthy plants (Figure 2.5B).

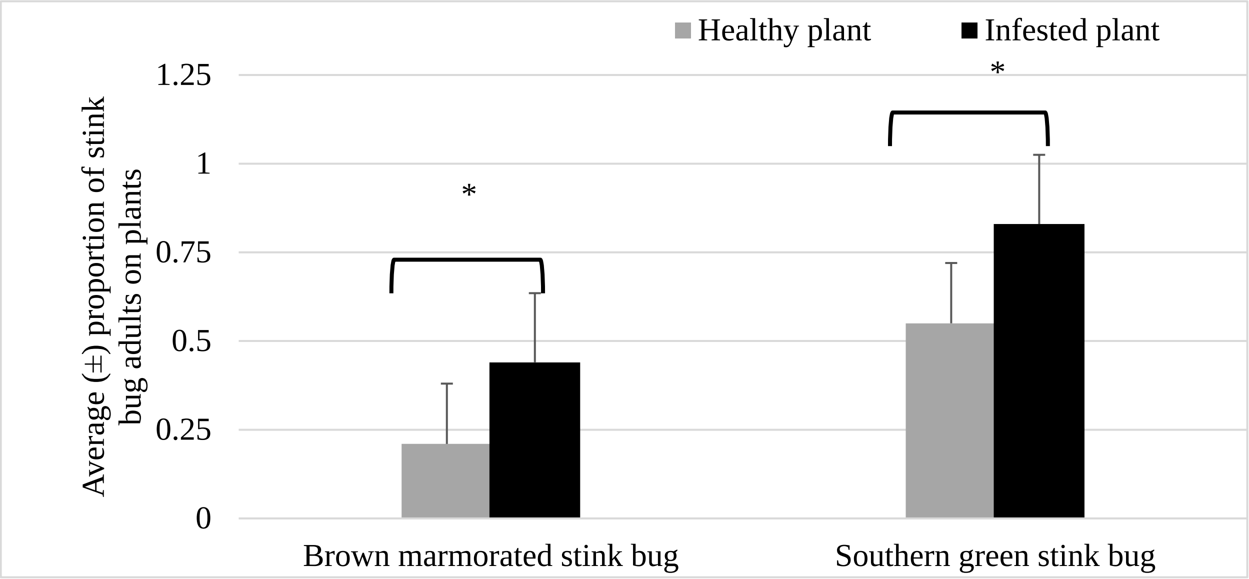
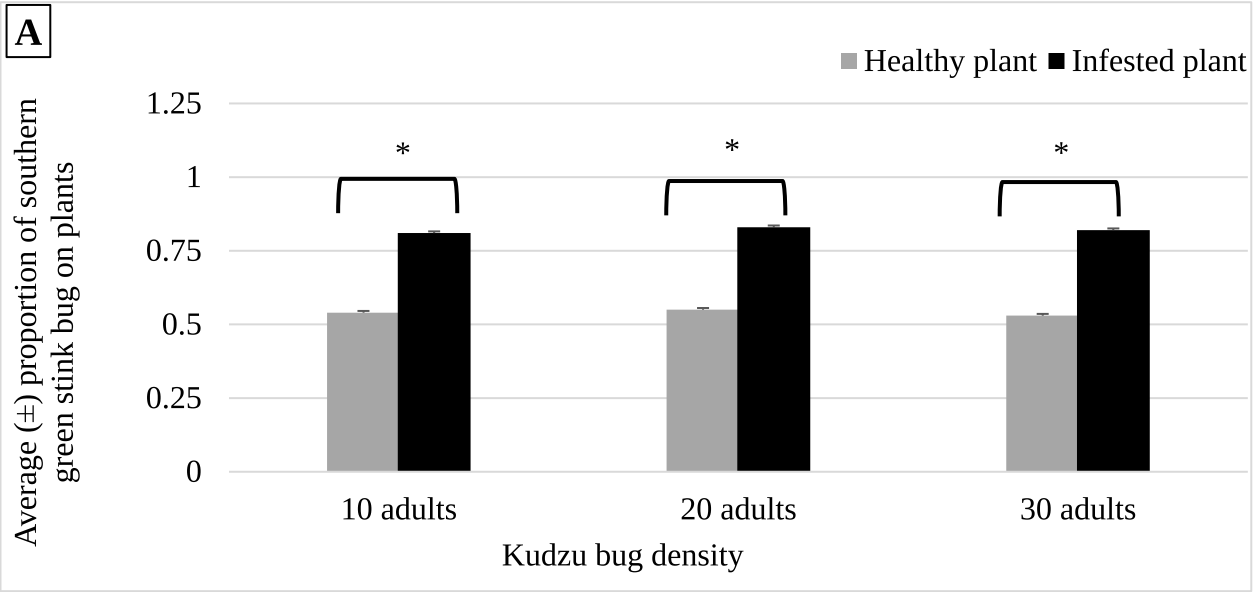


Figure 2.5 Average proportions (±S.E.) of southern green stink bug and brown marmorated stink bug adults on healthy and infested soybean plants when kudzu bugs were removed from the infested plant (indirect impacts). Asterisk (*) indicates a significant difference between host selection preference in the healthy versus preinfested soybean plants (χ2 goodness of fit test, *p* < 0.05). ns. indicates no significant difference.



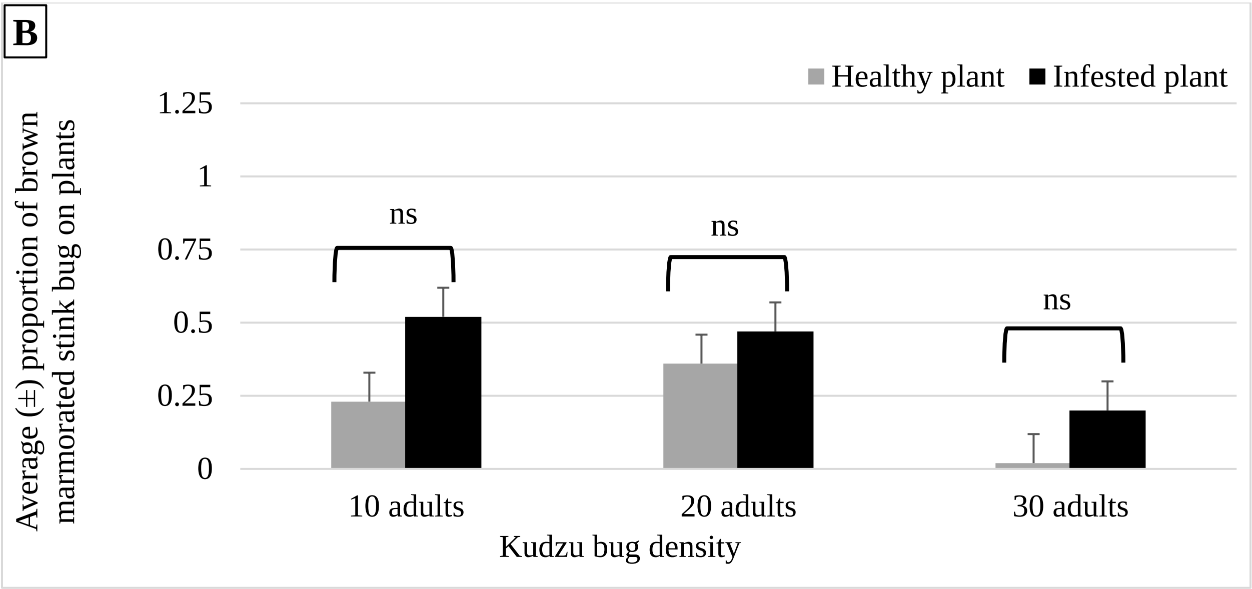


Figure 2.6 Average proportions (±S.E.) of (A) brown marmorated stink bug and (B) southern green stink bug adults on healthy and infested soybean plants with kudzu bugs density as indirect impacts. Asterisk (*) indicates a significant difference between host selection preference in the healthy versus preinfested soybean plants (two-sided t-test, *p* < 0.05). ns. indicates no significant difference.

Table 2.1 t-test results for brown marmorated stink bug and southern green stink bug adults landing on healthy plants and infested plants in direct and indirect impact experiments.

Experiment	**Stink bug species**	**t-value**	***p*-value**
Direct Impact	Brown marmorated stink bug	0.69	0.49
	Southern green stink bug	3.88	**0.0003**
Indirect Impact	Brown marmorated stink bug	2.10	**0.04**
	Southern green stink bug	5.79	**<0.0001**

Significant values ($p \leq 0.05$) are in bold font.

Table 2.2 Logistic regression results for the effect of kudzu bugs density (10, 20 and 30) on the attraction of brown marmorated stink bug and southern green stink bug adults to plants in direct and indirect impact experiments.

Experiment	Stink bug species	Plant type	$\chi 2$	*p*-value
Direct Impact	Brown marmorated stink bug	Healthy	0.93	0.62
		Infested	1.51	0.46
	Southern green stink bug	Healthy	0.56	0.75
		Infested	0.87	0.64
Indirect Impact	Brown marmorated stink bug	Healthy	6.37	**0.04**
		Infested	1.06	0.58
	Southern green stink bug	Healthy	1.49	0.47
		Infested	0.12	0.93

Significant values ($p \leq 0.05$) are in bold font.

Table 2.3 The table displays the t-values, and p-values for each comparison at different kudzu bug densities (10, 20 and 30).

Experiment	Stink bug species	Kudzu bug density	t-value	*p*-value
Direct Impact	Brown marmorated stink bug	10	0.67	0.51
		20	-0.96	0.35
		30	-0.97	0.34
	Southern green stink bug	10	2.44	**0.02**
		20	1.98	0.06
		30	2.47	**0.02**
Indirect Impact	Brown marmorated stink bug	10	13.06	0.21

		20	0.67	0.51
		30	1.63	0.11
	Southern green stink bug	10	3.51	**0.0025**
		20	3.41	**0.0031**
		30	2.47	**0.02**

Significant values ($p \leq 0.05$) are in bold font.

2.6 DISCUSSION

Interspecific interactions between herbivorous arthropods sharing the same host plants have been widely documented (Moran 1990, Panizzi et al. 1995, Anderson et al. 2009, Wise 2009, Kaplan et al. 2011, Sarmento et al. 2011). The present study focused on the interactions between kudzu bugs and two stink bug species (brown marmorated stink bug and southern green stink bug) on soybean plants. Our results demonstrated that both the brown marmorated and green stink bug responded differently to direct and indirect impacts of kudzu bug infestation. The density of kudzu bug infestations influenced both stink bug species' responses. It has been reported that phytophagous insects can positively or negatively impact each other's behavior through feeding-induced changes in the host plant's nutritional and allelochemical profile (Hunter 1992, Damman 1993, Denno et al. 1995). Sap feeders penetrate the host plant with their mouthparts and induce plant responses through plant hormone regulation, defensive protein expression, and secondary metabolite emission (Rahbe´ et al. 2003, Beale et al. 2006, Smith and Boyko 2007).

2.6.1 Direct interaction experiments

The direct interaction experiments showed that soybean plants infested with kudzu bugs attracted more southern green stink bugs than healthy plants, while brown marmorated stink bugs did not exhibit a preference (Figure 2.2). Several factors could contribute to the divergent responses observed in brown marmorated stink bug and southern stink bug species. Interspecific interactions could play a role, with brown marmorated stink bug and southern green stink bug responding to the physical contact of kudzu bugs (Xue et al. 2010, Erb et al. 2011) as potential competitors to directly compete for space and food resources, producing alarm pheromones when disturbed by competitors (Beale et al. 2006). Adult southern stink bug may be more tolerant of kudzu bug presence, while brown marmorated stink bug might prefer to avoid competition and settle on healthier plants.

In the direct impact experiments, kudzu bugs’ density on the infested soybean plants influenced brown marmorated stink bug and southern green stink bug differently. The preference of southern green stink bug for the infested plant was consistent across all kudzu bug density levels (Figure 2.3A). More than 70% of the southern green stink bug adults selected infested plants, with the preference for infested plants becoming more pronounced as kudzu bug density increased (Figure 2.3A). As the kudzu bug's density increased from 20 to 30 on the infested plants, brown marmorated stink bug adults' preference shifted, and they selected healthy plants (Figure 2.3A). This shift indicates that when kudzu bugs were present, the inhibitive effects on brown marmorated stink bug were enhanced with increasing kudzu bug density. The differential plant preferences observed in our study are consistent with patterns found in other insect species. For instance, the presence and feeding activity of mirid bugs, *Macrolophus pygmaeus* and *Dicyphus maroccanus* on tomato plants enhanced the attractiveness of the host plant to the leaf miner (*Tuta absoluta*), while neither attracting nor repelling the whitefly, *Bemisia tabaci* (Pérez-Hedo et al. 2018).

2.6.2 Indirect impact experiments

Our results showed that the indirect impacts of kudzu bug infestation were similar in brown marmorated stink bug and southern green stink bug. Both stink bug species demonstrated a preference for pre-infested plants over healthy plants (Figure 2.4). They were approximately 1.5 times more likely to land on pre-infested plants where kudzu bugs were absent than healthy plants. It appears that when the kudzu bugs were removed, the inhibitive effects on stink bugs were diminished.

In all kudzu bug density treatments, both stink bug species exhibited a preference for feeding on soybean plants pre-infested with kudzu bugs (Figure 2.5). Southern green stink bugs consistently displayed a significantly higher preference for pre-infested plants across all kudzu bug densities, without any major change in preference as density increased (Figure 2.5A). In contrast, brown marmorated stink bug demonstrated a greater preference for soybean plants pre-infested with the lowest kudzu bug density (10 adults), which gradually declined as the density increased (Figure 2.5B). Similar findings were found in a study by Tan et al. (2014), where whitefly showed a heightened preference for tomato plants pre-infested with lower densities (20 or 50 aphids/leaf) of green peach aphids.

2.7 CONCLUSIONS

In conclusion, our study reveals differential responses to kudzu bug infestation by brown marmorated stink bugs and southern green stink bugs. The findings of this paper can serve as baseline data regarding the direct and indirect impacts of kudzu bug infestations on the feeding preferences of brown marmorated stink bugs and southern green stink bug stink bugs. These results suggest that stink bug herbivores may be more attracted to plants previously infested with the kudzu bug, and future studies are needed to understand whether these results are consistent under field conditions and how they impact plant health and yield. Understanding these interactions may also help guide adjustments in scouting, studying spatial distributions, and management tactics of late-season stink bug pests.

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Chapter 3

DEVELOPMENTAL BIOLOGY OF KUDZU BUG (*MEGACOPTA* C*RIBRARIA*) EGG PARASITOIDS AS INFLUENCED BY THE AVAILABILITY OF FOOD SOURCES, HOST: PARASITOID RATIOS, AND HOST EXPOSURE TIMES

3.1 INTRODUCTION

The kudzu bug, *Megacopta cribraria* Fabricius (Hemiptera: Plastaspidae), is a new invasive pest of soybeans, *Glycine max* Linneanus, accidentally introduced in 2009 from Asia to the southern United States (USDA ERS 2020). At present, synthetic chemical insecticides are the primary control method for *M. cribraria* (Seiter et al. 2014). Repeated interventions of insecticides may cause insect resistance and eliminate beneficial species (Gill & Garg 2014). Parasitoids may be an ecologically benign, efficient, cost-effective alternative to regulate the *M. cribraria* population, but preserving parasitoid species richness and activity are crucial (Malik et al. 2007, Buchori and Sahari 2008). In the past decade, two egg parasitoids of *M. cribraria*, *Paratelenomus saccharalis* Dodd (Hymenoptera: Platygastridae) and *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae), were discovered in the United States (Gardner et al. 2013, Ademokoya et al. 2018). Although parasitism of *M. cribraria by O. nezarae* and *P. saccharalis* is known (Gardner et al. 2013, Ademokoya et al. 2018), there are gaps in knowledge about the biology of both parasitoids in the United States. Given the ubiquity of parasitoids and their importance in influencing the abundance and dynamics of their hosts (Klapwijk et al. 2010), it is essential to investigate the factors that affect their performance. This study aimed to investigate the effect of food sources, host: parasitoid ratio, and exposure time on the parasitism and reproductive efficacy of both parasitoids.

Several studies have shown that the presence of adult food sources maximizes parasitoid reproductive success by influencing emergence, female: male ratio, and longevity (Bezemer and Mills 2003). A carbohydrate-rich diet in animals can lead to the rise of glucose content in the follicular fluid which increases the number and size of ovarian follicles (Downing et al. 1995). Ingesting sugar may reduce the rate of lipid decline, thereby potentially saving reserves for oocyte maturation (Lee and Heimpel 2008). Additionally, the sugar that body store as trehalose or glycogen may be used in embryogenesis later (Yamada et al. 2019). Some studies suggested that diets rich in carbohydrates may decrease the mortality risk and, increase the residual lifespan of insects by inhibiting inflammatory pathways, thereby decreasing biological aging and oxidative stress (Partridge et al. 2011). Female parasitoid aging can affect the offspring sex ratio as sperm are generally depleted or the viability of sperm is reduced over time, which can increase the production of males (Onagbola et al. 2007, Benelli et al. 2017, Bezerra Da Silva et al. 2019, Liu et al. 2020). Control of sperm release from the spermatheca is generally weakened with wasp age due to physiological, physical defects or senescence in the maternal reproductive organs (Liu et al. 2020).

Host: parasitoid ratios and host exposure times can also alter the parasitoid offspring's fitness by influencing the series of host selection decisions made by adult parasitoids before oviposition (Chan & Godfray 1993). In general, female wasps should select a high-quality host because it provides sufficient food resources for developing embryos, increasing survivability (Bernal et al. 1999 King 2000). However, when high-quality hosts are limited, a parasitoid can accept a host that had already been parasitized by conspecific females (van Alphen and Vet

1986) even though sharing resources with competitors can decrease survival (Godfray 1994). When the parasitoids accept poor-quality hosts, they may allocate fewer resources to male offspring production, which can further bias the offspring sex ratio towards females (Charnov 1979).

This study on the availability of food sources, host: parasitoid ratio, and host exposure times was driven by three different goals: 1) to optimize the reproductive efficacy of parasitoids by investigating the factors affecting host selection, reproduction, and offspring survival; 2) better understand how the absence or presence of food alters offspring production and sex ratios, shedding light on parasitoid nutritional requirements for reproductive success and; 3) to examine how changes in parasitoid host alters reproductive attributes including offspring survival, development, and fitness, to deepen understanding of host-parasitoid interactions and their implications for ecological systems. The following hypotheses were tested: i) presence of food would enhance both parasitoid species' emergence and longevity; ii) wasps would produce a male-biased sex ratio later in their lifespan; iii) wasp emergence would be lower when low host: parasitoid ratio is low or with longer exposure periods; and iv) parasitoid offspring would be male-biased when female wasps are host-limited.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions

Soybean seeds (var. Pioneer P49T97R-SA2P) were planted into pots (15.24 cm diameter and 14.22 cm depth) in Sunshine potting mixture #8 (SunGro Horticulture, WA, U.S.) and grown in an incubator free of arthropods and pesticides at (26 ± 2 °C and 55 ± 5% RH) (Ademokoya et al. 2017). Plants were watered daily (~200 mL per pot) and fertilized (~15 gm per 3.78 L) according to the label (Scotts-Sierra Horticultural Product Company, Marysville, OH, U.S.) once a week until use for *M. cribraria* rearing.

3.3.1.1 Insect colony

A colony of adult kudzu bugs was established by collecting insects from kudzu, *Pueraria montana* (Lour) Merr., in Auburn, AL (32.5934° N, 85.4952° W) from early May to October 2022. They were reared in ventilated plastic cages (30 cm x 30 cm x 30 cm) (BugDorm-2, Megaview Science Education Services Co., Ltd., Taichung, Taiwan) at 28 ± 5°C, 16:8 h (L: D), and 50% RH in a growth chamber (Percival, Perry, Iowa, U.S.) and provided organic green beans and vegetative-stage (V2-V3) soybean plants. New soybean plants were provided every week (Ademokoya et al. 2017). Cages were checked every day for fresh eggs (≤ 24 h) that appeared milky white in appearance as compared to aged eggs (> 24 h) that were darker in color.

The parasitoid species used in the experiments originated from a soybean field in Auburn, AL. Soybean leaves with parasitized kudzu bug egg masses (grey color) (Britt 2016) were collected from the field. Each leaf was trimmed to keep only the sections containing the egg masses and were then placed into an individual 59.1 mL (top diameter: 6 cm, bottom diameter: 4.4 cm, and height: 2.8 cm) condiment cup with approximately 20 pin holes made on the rearing cup wall for aeration and to prevent condensation. All parasitoid colonies and experiments were conducted in these containers and incubated at 25 ± 1°C, 14:10 (L:D) h and 75 ± 5% RH photoperiod to maximize possible emergence (Ademokoya et al. 2017). Upon emergence, parasitoids were identified as *O. nezarae* or *P. saccharalis*, based on the characteristics provided by Gardner et al. (2013) and Ademokoya et al. (2018), and were separated into different rearing cups. Colonies of the wasps were reared by providing adults with a honey solution (70% honey to 30% water, v/v) and allowing them to oviposit into ≤ 24 h old kudzu bug eggs.

3.3 EXPERIMENTAL DESIGNS

3.3.1 Effect of sugar feeding on the reproductive fitness and longevity of Paratelonomus saccharalis and Ooencyrtus nezarae

Newly emerged (< 24 h) adult males and females of *P. saccharalis* or *O. nezarae* were allowed to mate for 24 h. From emergence through the mating period individuals did not have access to honey, water or hosts. On day two, wasps were individually placed into 59.1 mL condiment cups (dimension provided above). Nine adult food treatments were included in experiments following a completely randomized design (CRD). Individual adults of each wasp species were offered (i) water + hosts, (ii) honey + hosts, (iii) water + honey + hosts, (iv) no water, no honey + hosts, (v) water, (vi) honey (vii) water + honey, (viii) fasting (no water, no honey), and (ix) a control group of host eggs that were not exposed to parasitoid to assess natural host mortality. Treatments i-iv (i.e., host presence) were only tested for females since males of both wasp species do not exploit the hosts, but treatments v-viii (i.e., host absence) were tested for both males and females, separately. A total of 20 replicates of each treatment were conducted for each parasitoid species. Each treatment lasted for the entire life of the parasitoids.

Water was provided from a cotton string extending from a hole made at the bottom of a 0.5 mL Eppendorf microcentrifuge tube (Fisher Scientific, Waltham, MA, U.S.) filled with de-ionized water in the treatments (i, iii, v, and vii). To control for potential differences in the relative humidity among water-provided treatments (i, iii, v, and vii) and water-deprived (ii, iv, vi, and viii), a water source was provided across treatments. In water-deprived treatments moisture vapor was allowed to dissipate from the tube to prevent insect desiccation but insects could not access the water for consumption. The honey solution (70% v/v) was held in another

0.5 mL Eppendorf microcentrifuge tube with a hole in the bottom through which a cotton string was threaded in honey provision treatments (ii, iii, vi, and viii).

Each parasitoid was exposed to 24-30 host eggs that were less than 24 h old (treatments i-iv). The eggs were replaced with fresh host eggs after 24 h, and parasitoid survivability was recorded. The parasitoid-exposed host eggs were transferred to a new cup and maintained in the incubator. Each cup was monitored for 14-16 days until the emergence of wasps. The number of parasitized eggs, emerged kudzu bug nymphs, emerged wasps and their sex ratio (female/total emerged wasps) in treatments (i-iv) with host eggs were recorded. The survivability of adult individuals was monitored daily in treatments (v-ix) without host eggs.

3.3.2 Effect of host: parasitoid ratios and host exposure times on the fitness of Paratelenomus saccharalis and Ooencyrtus nezarae

In this objective, two experiments were conducted with adult female parasitoids that were mated and naïve (no prior oviposition experience) of each species. In one set of experiments data were collected on an individual wasp, and in the second experimental data were collected on seven female wasps released together. For both sets of experiments, combinations of two factors were studied and compared; wasps were released into experimental arenas with host egg densities of 21, 42, 84, or 168 (±7) and exposure times of 1, 3, or 5 days. Thus, the host: parasitoid ratio for the single and group wasp experiments were 21:1, 42:1, 84:1 or 168:1 and 21:7, 42:7, 84:7, or 168:7, respectively. A CRD was used and each host density x host exposure time was replicated 20 times for *O. nezarae* and 10 times for *P. saccharalis* due to availability of parasitoids. The maximum host density (168) was chosen based on a preliminary experiment in which the average wasp parasitized one egg/h in a high host density (150 eggs) environment over

6-24 h. Based on this, seven wasps parasitizing one egg/hour for 24 h could parasitize 168 eggs with little to no superparasitism. The exposure times were chosen based on a preliminary experiment that showed kudzu bug nymphs start to emerge on day seven. In all treatments, honey solution (70% v/v) was provided to the wasp throughout the experiment, as described above.

After the host exposure period ended, parasitized eggs were held for 14-17 days to allow time for *O. nezarae* and *P. saccharalis* adults to emerge (Goltz et al. 2020, Chicas-Mosier et al. 2022, personal observation). After the holding period, the final proportion of parasitized kudzu bug eggs, hatched host nymphs, wasp offspring, and wasp offspring sex ratio (female offspring/emerged wasps) were recorded.

3.4 STATISTICAL ANALYSIS

In the experiment examining the effects of food, adult survival was estimated using the Kaplan-Meier survival analysis. GraphPad Prism version 8.4.2 for Windows 10 (GraphPad Software, La Jolla, CA, USA) was used for survival analysis and generating survival curves. Survival curves (Figures 3.1-3.3) were compared using the log-rank (Mantel-Cox) test after confirmation of normality (Shapiro-Wilk, Cramer-von Mises, Anderson-Darling and Kolmogorov-Sminorv’s tests) and homoscedasticity (Barllet’s, Brown-Forsythe’s and Lavene’s test). Two-way ANOVA followed by Tukey’s test ($p \leq 0.05$) using SAS version 9.4. (SAS Institute, Cary, NC) was used to determine the effect of food source treatments on the proportion of parasitized host eggs (parasitized eggs/eggs in the patch; Figures 3.2A and 3.4A), the proportion of hatched host nymphs (nymphs hatched/eggs in the patch; Figures 3.2B and 3.4B), the proportion of wasp offspring (emerged wasps/eggs in the patch; Figures 3.2C and 3.4C) and the proportion of offspring sex ratio (female offspring/emerged wasps; Figures 3.2D and 3.4D).

GraphPad Prism software was used to generate graphs for parasitized host eggs, hatched host nymphs, wasp offspring, and offspring sex ratio. We also determined the effect of food sources on the average ± S.E. lifetime offspring production (sum of total emerged wasps per female; Table 3.1), and sex ratio (sum of total females /sum of total emerged wasps per female; Table 3.2) of the parasitoids by conducting one-way ANOVAs followed by Tukey's test ($p \leq 0.05$) using SAS version 9.4.

The interactive effects of host: parasitoid ratio and host exposure time factors on the parasitism and reproductive efficacy of individual wasps and groups of seven were analyzed as the proportion of parasitized host eggs (Figures 3.5A, 3.6A, 3.7A, and 3.8A; parasitized eggs/total eggs in the patch), the proportion of hatched host nymph (Figures 3.5B, 3.6B, 3.7B, and 8B; nymphs hatched/total eggs in the patch), the proportion of wasp offspring (Figures 3.5C, 3.6C, 3.7C, and 3.8C; emerged wasps/total eggs in the patch), and the proportion of wasp offspring sex ratio (Figures 3.5D, 3.6D, 3.7D, and 3.8D; females/total emerged wasps). In preliminary analyses using SAS version 9.4 (SAS Institute, Cary, NC) to transform data and test for normality of all datasets the proportions data from the host to parasitoid ratio and host exposure times experiment were not normally distributed. Different host: parasitoid ratio and host exposure times treatment groups had unequal variances (Barllet's, Brown-Forsythe's and Lavene's test). Therefore, nonparametric statistical analyses were performed using Software JASP version 0.16.4 (University of Amsterdam, Netherlands) to perform tests and generate graphs. A Kruskal–Wallis test, followed by Dunn's multiple comparisons tests, was used to test the effect of more than two treatments (i. e., 21, 42, 84, or 168 host densities and 1, 3 or 5 days host exposure times). For these statistical tests, the significance level ($p \leq 0.05$) was adjusted with the Bonferroni method by dividing the significance level by the number of comparisons being conducted.

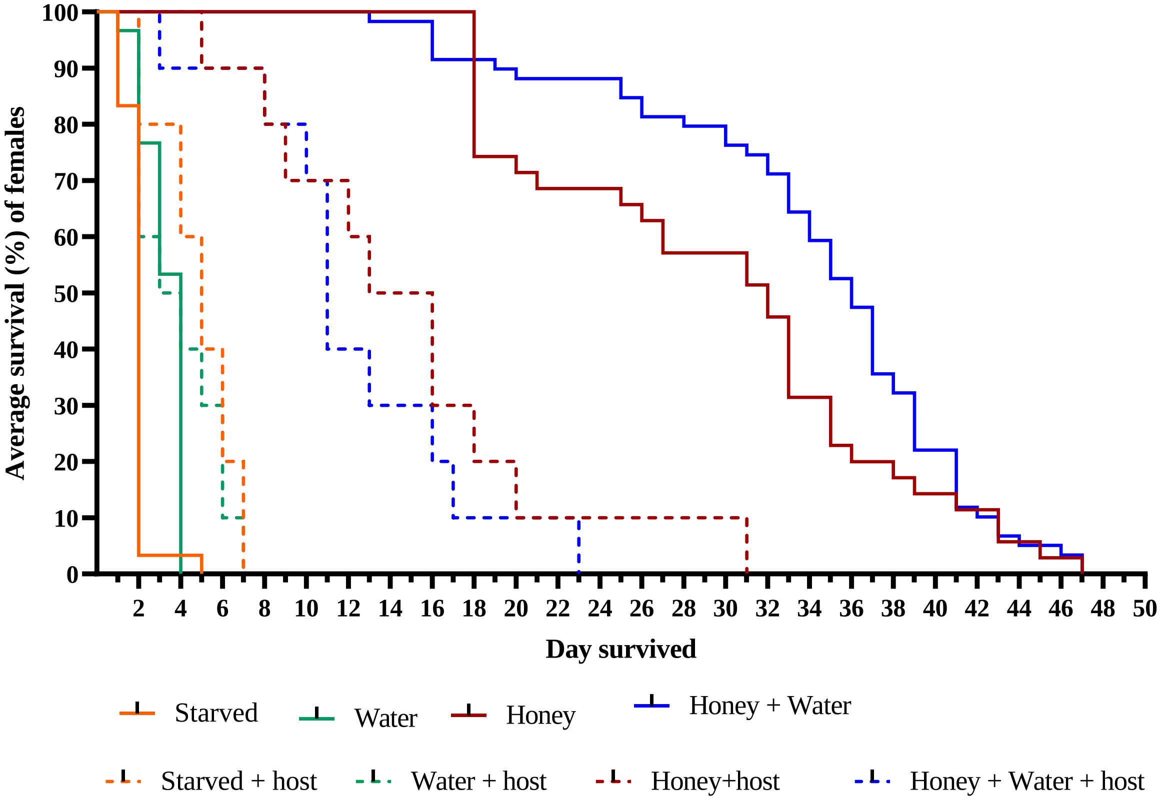
3.5 RESULTS

3.5.1 Effect of food availability on the life history traits of Ooencyrtus nezarae and Paratelenomus saccharalis

3.6.1.1 Life history traits of Ooencyrtus nezarae in the presence or absence of food sources

Survivorship curves for both sexes of *O. nezarae* are shown in Figure 3.1. The average longevity of *O. nezarae* adults at each diet, sex, and host combination is shown in Table 3.1. When *O. nezarae* females were provided with different diet regimens and placed in the presence of an oviposition host, their survival rate was significantly lower compared to when they were in the absence of an oviposition host (Figure 3.1A). This result suggests that even in the presence of food, female longevity is reduced following oviposition. Females with access to honey, regardless of the availability of water, lived longer than their honey-deprived counterparts in the presence or absence of host (Table 3.1). Males also had the longest average lifespan when kept with honey, independent of water (Table 3.1), indicating honey (or sugar source) maximizes the longevity of *O. nezarae* individuals (Figure 3.1B). However, the longevity of males was significantly lower than that of females. Female longevity was around two times higher than that of males in all comparative treatments.

(A) Females (with and without host)



(B) **Males**

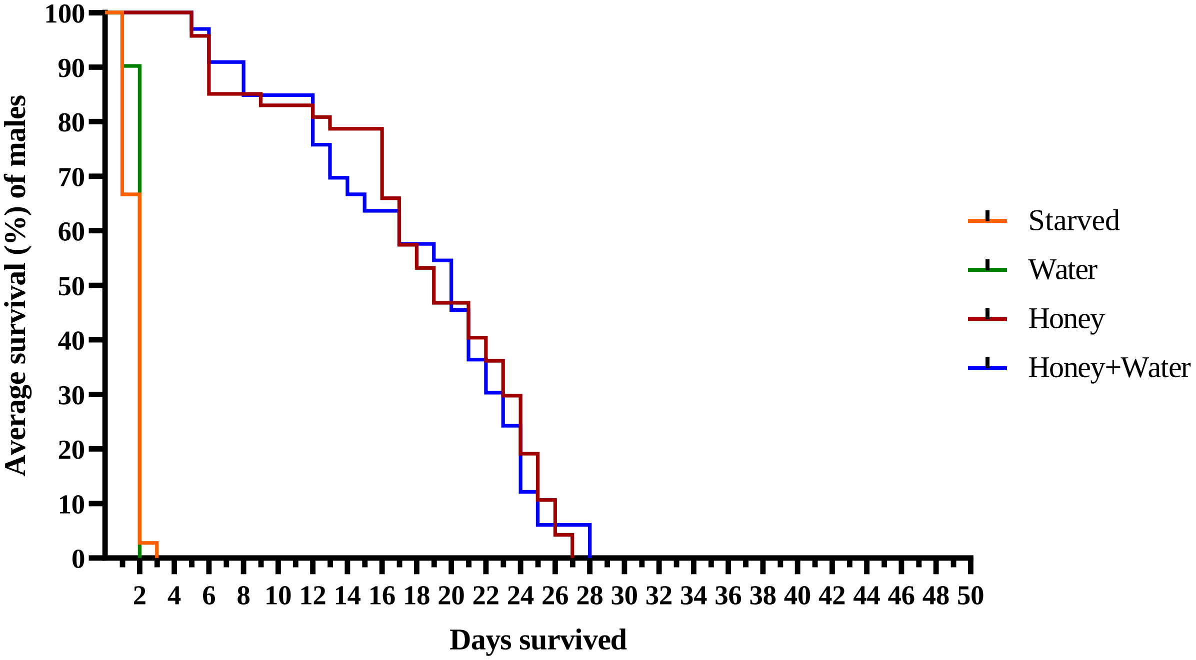


Figure 3.1Effects of the constant supply of water, honey, and/or eggs of kudzu bug on the survival curves of female (A) and male (B) adults of *Ooencyrtus nezarae*. Curves were

estimated according to the Kaplan-Meier method. The numbers of replicates individual wasps), respectively, for water, honey, water + honey, and starved (no water, no honey) were 30, 35, 59, and 30 for non-host provided females (A); and 10, 10, 10, and 10 for host-provided females (A); and 41, 47, 33, and 36 for males (B)

Table 3.1 The longevity (average ± S.E) of *Ooencyrtus nezarae* and *Paratelenomus saccharalis'* males, females, and females allowed access to oviposition on kudzu bug eggs that were either starved or provided access to a constant supply of water, honey, or honey +Water

		Males			**Females**			**Females + host eggs**		
Parasitoid species	Diet	Average ± S.E.	Range	Sample size	Average ± S.E.	Range	Sample size	Average ± S.E.	Range	Sample size
Ooencyrtus nezarae	Starved	1.69 ± 0.79b	1-3	36	1.93 ± 1.22c	1-5	30	4.80 ± 1.50b	2-7	10
	Water	1.90 ± 0.74b	1-2	41	3.26 ± 1.22c	1-4	30	3.90 ± 1.50b	2-7	10
	Honey	18.27 ± 0.69a	5-27	47	29.60 ± 1.13b	18-47	35	14.80 ±1.50a	5-31	10
	Honey + Water	17.93 ± 0.83a	5-28	33	34.06 ± 0.87a	13-47	59	12.30 ±0.83a	3-23	10
	One-way ANOVA values	df=3, 153; *F*=152.09; *p* < **0.0001**			df=3, 153; *F*=244.36; *p* < **0.0001**			df=3, 36; *F*=12.90; *p* < **0.0001**		
	Starved	2.31 ± 0.91b	2-3	29	2.92 ± 0.96b	2-5	28	2.62 ± 0.91b	2-3	8
	Water	3.50 ± 1.22b	2-6	16	2.74 ± 0.98b	1-5	27	2.62 ± 0.91b	2-4	8
	Honey	15.38 ± 1.36a	4-26	13	16.85 ± 0.98a	4-31	27	11.00 ± 0.91a	3-15	8

Paratelenomus saccharalis	Honey + Water	13.26 ± 0.89a	5-28	30	16.50 ± 0.93a	4-28	30	9.12 ± 0.91a	5-12	8
	One-way ANOVA values	df=3, 84; F=38.68; p **< 0.0001**			df=3, 108; F=68.23; p **< 0.0001**			df=3, 28; F=22.59; p **< 0.0001**		

In each column, the average followed by different letters is significantly different according to Tukey's test ($p < 0.05$). Significant values ($p \leq 0.05$) are in bold font.

Table 3.2 Parametric Two-Way ANOVA to evaluate the effects of supply of water and honey on life-history traits of *Ooencyrtus nezarae* reared on kudzu bug eggs, throughout the parasitoid's lifespan. *N* = (10) water, (10) honey, (10) water + honey, and (10) fasting. One mated individual wasp formed each replicate.

Life history trait	Source of variation	df	F	*p*-value
Parasitized host eggs	Diet	3, 253	16.37	**<0.0001**
	Age	29, 253	3.15	**<0.0001**
	Diet and age interaction	31, 253	1.12	0.315
Hatched host nymphs	Diet	3, 253	7.30	**0.0001**
	Age	29, 253	1.87	**0.0058**
	Diet and age interaction	31, 253	1.41	0.079
Wasp offspring	Diet	3, 253	13.44	**<0.0001**
	Age	29, 253	3.30	**<0.0001**
	Diet and age interaction	31, 253	0.96	0.528
Wasp offspring sex ratio	Diet	3, 253	7.17	**0.0001**
	Age	29, 253	2.40	**0.0002**
	Diet and age interaction	31, 253	0.66	0.917

Significant values ($p \leq 0.05$) are in bold font.

Ooencyrtus nezarae parasitism of *M. cribraria* eggs was significantly influenced by the diet and age of the parasitoid (see Table 3.2 for statistics). Honey provision increased the proportion and duration of parasitism by *O. nezarae* females compared to honey-deprived females, independent of water. Wasps with access to honey were able to parasitize up to 128 host eggs throughout their lifetime, nearly quadrupling the parasitism by wasps that did not have access to honey. The parasitism by honey-provisioned females was highest seven days post emergence (Figure 3.2A). The prevalence of parasitism consistently declined as parasitoids aged, regardless of the diet. A steeper decline was observed in the water and starved diets compared to the other treatments (Figure 3.2A). The parasitism capacity of *O. nezarae* was completely exhausted at ages ca. 6 (fasting), 6 (water), 16 (honey), and 19 (honey + water) days post-emergence. The likelihood that a wasp was found alive at each of those ages was 20, 10, 30, and 20%, respectively (Figure 3.1A).

In the control, wasp absence resulted in significantly greater proportion of hatched host nymphs than wasp presence (F= 15.82, df = 1, 339, $p < 0.0001$). In the absence of a parasitoid, the average proportion of nymphs eclosion was 0.86 ± 0.04. When wasps were present, around 90% of host eggs hatched into nymphs, and this was observed in starved and water treatments. The age of female wasps also factored into the proportion of successful host nymph eclosion (Table S1). More than 80% of the host eggs yielded nymphs after 18 days of wasp emergence, regardless of diet.

Diet and parasitoid age significantly influenced the proportion of parasitoid emergence (Table 3.2). Females with access to honey, independent of water, produced more offspring than honey-deprived females (Table 3.2). Honey-fed females showed the highest mean total lifetime offspring production, twice as high as that of females with access to only water and seven times as high as starved females (Table 3.2). Honey-provisioned females showed most offspring emergence in the first 13 days after adult emergence (Figure 3.2C). Females fed honey, honey + water, water alone, and starved (neither honey, nor water) showed the highest proportion of offspring emergence on day 6 (0.36), day 4 (0.32), day 3 (0.27), and day 2 (0.13), respectively (Figure 3.2C). The proportion of offspring emergence decreased with the increasing age in females, showing a clear effect of age on the emergence and viability of eggs. Fed females that lived up to 47 days did not produce offspring after 18 days of age.

The offspring sex ratio (female/total emerged wasps) was affected by diet and age (Table 3.2). There was a reduction in the proportion of female offspring, independent of diet, suggesting sperm depletion occurred over time (Figure 3.2D). This effect is more clearly observed in honey+ water-fed condition; the proportion of female offspring fell below 0.33 after 13 days (Figure 3.2D). When we considered the entire adulthood of *O. nezarae*, the sex ratio was strongly male-biased in honey-deprived wasps relative to their honey-fed counterparts (Table 3.3).

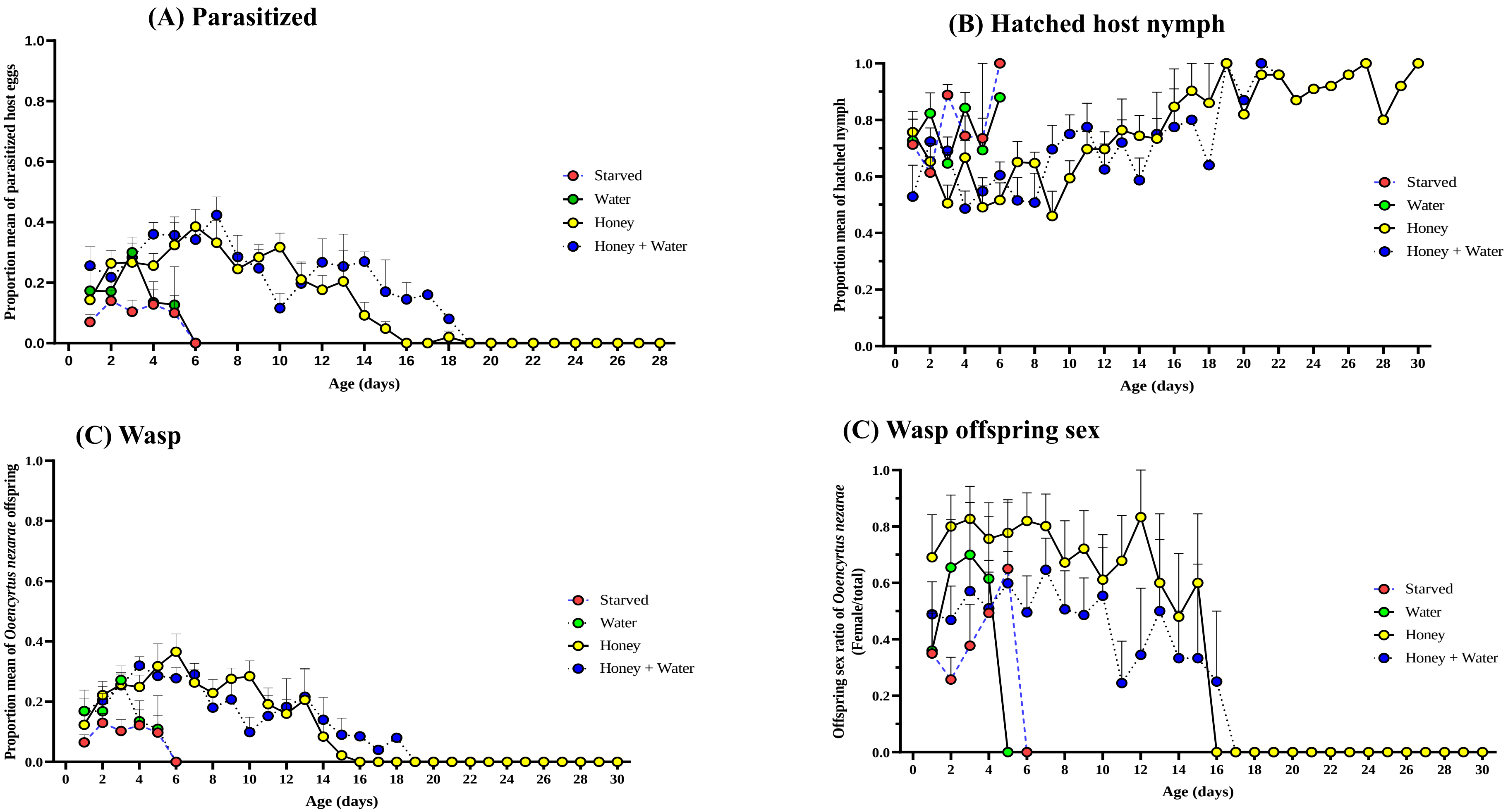


Figure 3.2 Effects of a constant supply of water and honey on life-history traits of mated female *Ooencyrtus nezarae* reared with access to eggs of the kudzu bug. The average (±S.E.) proportion of parasitized host eggs (A), the proportion of hatched host nymphs (B), the proportion of wasp offspring (C), and the offspring sex ratio (female/total) (D) were calculated and compared using two-way ANOVA followed by Tukey's test (*p* ≤ 0.05) n SAS. Graphs were prepared using GraphPad Prism.

Table 3.3 Lifetime offspring production (average ± S.E) of *Ooencyrtus nezarae* and *Paratelenomus saccharalis* females maintained with access to kudzu bug eggs that were either starved (no water, no honey), or fed water, honey, or honey + water.

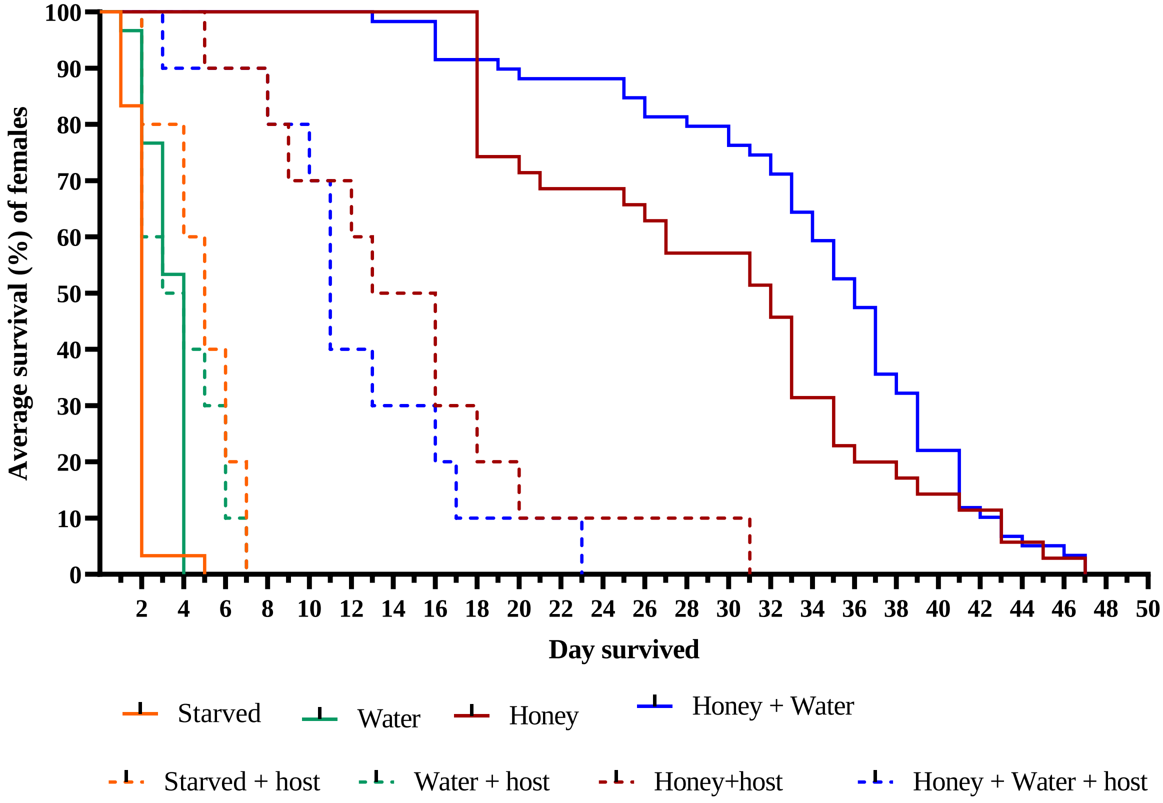
Parasitoid species	**Diet**	**Wasp offspring**			**Offspring sex ratio (female/total)**		
		Average ± S.E.	Range	Sample size	Average ± S.E.	Range	Sample size
Ooencyrtus nezarae	Starved	8.70 ± 7.27b	0-24	10	0.49 ± 0.10ab	0-11	10
	Water	15.70 ± 7.27b	0-68	10	0.32 ± 0.10b	0-24	10
	Honey	63.00 ± 7.27a	28-115	10	0.80 ± 0.10a	21-108	10
	Honey + Water	55.40 ± 7.27a	0-109	10	0.51 ± 0.10a	0-70	10
Paratelenomus saccharalis	Starved	21.50 ± 5.98b	17-24	8	0.50 ± 0.12ns	0-22	8
	Water	21.75 ± 5.98b	43-50	8	0.22 ± 0.12ns	0-31	8
	Honey	63.62 ± 5.98a	34-89	8	0.59 ± 0.12ns	6-59	8
	Honey + Water	65.50 ± 5.98a	32-92	8	0.54 ± 0.12ns	1-54	8

In each column, the average followed by different letters is significantly different according to Tukey's test ($P < 0.05$). NS= not significant effect.

3.6.1.2 Life History Traits of Paratelenomus saccharalis in the Presence or Absence of Food Sources

Survivorship curves for both sexes of *P. saccharalis* are shown in Figure 3.3. Host presence resulted in a significantly lower survival curve of *P. saccharalis* females than host absence treatments. This result suggests that even in the presence of food, female longevity is reduced by the act of oviposition. The average longevity of *P. saccharalis* adults at each diet, sex, and host combination is shown in Table 3.1. Both in host presence and absence, females with access to honey, independent of water, had greater longevity than honey-deprived females (Table 3.1). Similar results were obtained for *P. saccharalis* males (Figure 3.3B). Honey-provisioned males also lived significantly longer than honey-deprived males (Table 3.1). Honey increased average longevity by approximately five times for females (without host) and males compared with individuals that did not have access to honey. There were no significant differences in the longevity of males and females without the host.

(A) Females (with and without host)



(B) Males

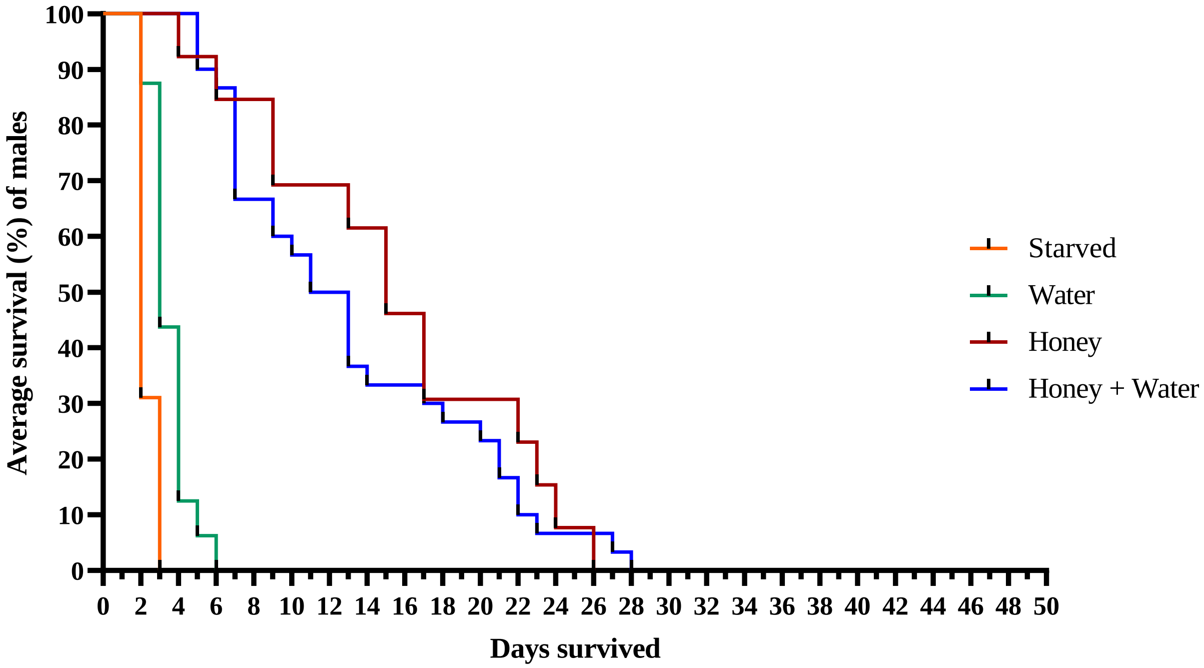


Figure 3.3 Effects of the constant supply of water, honey, and/or eggs of kudzu bug on the survival curves of mated female (A) and male (B) adults of *Paratelenomus saccharalis*. Curves were estimated according to the Kaplan-Meier method. The numbers of replicates, respectively, for water, honey, water + honey, and starved (no water, no honey) were 27, 27, 30, and 28 for non-host provided females (A) and 8, 8, 8, and 8 for host-provided females (A); and16, 13, 30, and 29 for males (B)

Host parasitism of *P. saccharalis* was not affected by diet (Table 3.4). The starved and water-provisioned female wasps parasitized fewer hosts than the wasps provided with honey or honey + water (Figure 3.4A). Honey-deprived wasps parasitized 11- 43 host eggs throughout their lifespan, whereas honey-provisioned wasps were able to parasitize up to 90 eggs throughout their lifespan. The proportion of parasitized host eggs by *P. saccharalis* consistently declined as female wasps aged. A sharp decline in parasitism was observed in starved and water-provisioned wasps just before death. The parasitism capacity of *P. saccharalis* was completely exhausted at ages ca. 3 (fasting), 4 (water), 14 (honey), and 9 (honey + water) days post-emergence. The likelihood of a wasp being found alive at each of those ages was 62.5, 25, 25, and 37.5%, respectively (Figure 3.3A).

A significantly greater proportion of hatched host nymphs was observed when *M. cribraria* eggs were exposed to a female wasp than the control (F = 31.75, df = 1, 193, $p <$ 0.0001). The proportion of hatched host nymphs was nearly double in the absence of a parasitoid (Figure 3.4B). Diet availability also influenced the proportion of host nymphs, and more host nymphs survived when female parasitoids were starved (Figure 3.4B). Female age significantly influenced the proportion of hatched host nymphs (Table 3.5). More than half of the host eggs yielded nymphs when exposed to female wasps over 3 days old, regardless of diet.

Table 3.4 Parametric Two-Way ANOVA to evaluate the effects of supply of water and honey on life-history traits of *Paratelenomus saccharalis* reared on kudzu bug eggs, throughout the parasitoid's lifespan. *N* = (8) water, (8) honey, (8) water + honey, and (8) fasting. One mated individual wasp formed each replicate.

Life history trait	**Source of variation**	**df**	**F**	***p*-value**
Parasitized host eggs	Diet	3, 141	2.12	0.1002
	Age	13, 141	17.80	**<0.0001**
	Diet and age interaction	13, 141	1.98	**0.026**
Hatched host nymphs	Diet	3, 141	2.34	0.0763
	Age	13, 141	10.84	**<0.0001**
	Diet and age interaction	13, 141	1.51	0.120
Wasp offspring	Diet	3, 141	1.91	0.1309
	Age	13, 141	16.28	**<0.0001**
	Diet and age interaction	13, 141	2.26	**0.0101**
Wasp offspring sex ratio	Diet	3, 141	2.44	0.066
	Age	13, 141	3.47	**0.0001**
	Diet and age interaction	13, 141	1.27	0.235

Significant values ($p \leq 0.05$) are in bold font.

The proportion of *P. saccharalis* offspring was not affected by diet (Table 3.5). Honey provisioned females had higher offspring production throughout their lifespan than honey-deprived females (Figure 3.4C). The mean lifetime number of offspring when wasps were fed honey was three times as high as females with access to water or starved females (Table 3.4). Females produced the highest average offspring in the first two days after adult emergence, independent of diet (Figure 3.4C). The proportion of offspring production in females declined as their age increased. Fed females that survived up to 30 days did not produce offspring after 10 days of age (Figure 3.4C).

Offspring sex ratio (female/total emerged wasps) was affected by the parasitoid's diet (Table 3.4). However, the average lifetime offspring sex ratio did not differ between starved and fed females (Table 3.3). The highest number of female offspring were observed on day one, independent of diet. Then sex ratio became male-biased with increasing maternal age (Figure 3.4D).

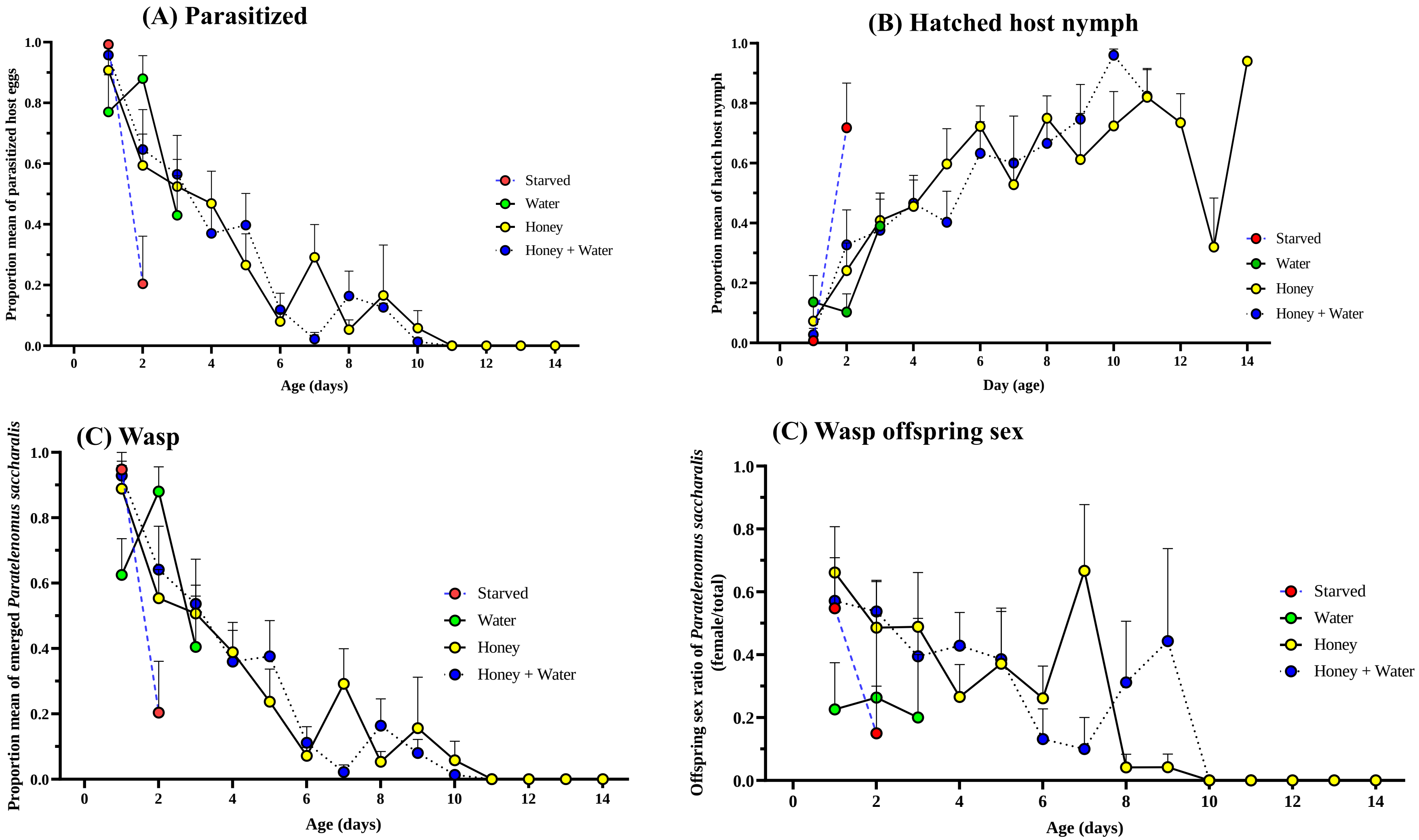


Figure 3.4 Effects of supply of water and honey on life-history traits of mated female *Paratelenomus saccharalis* reared on the kudzu bug eggs. The average (±S.E.) proportion of parasitized host eggs (A), the proportion of hatched host nymph (B), the proportion of wasp offspring (C), and the offspring sex ratio (female/total) (D) were calculated and compared using two-way ANOVA followed by Tukey's test (*p* ≤ 0.05) in SAS. Graphs were prepared using GraphPad Prism.

3.5.2 Effect of host: parasitoid ratios and host exposure times on the biology of Ooencyrtus nezarae and Paratelenomus saccharalis

3.6.2.1 Biology of Ooencyrtus nezarae under different combinations of host: parasitoid ratios and host exposure times

Host parasitism was influenced by host density and exposure time by parasitoids either foraging singly or in a group of seven individuals (for statistics, see Tables 3.3 and 3.4). When a wasp was foraging singly, the highest proportion of parasitized eggs (~0.70) was obtained at the lowest host density (21 eggs) over the higher exposure times (three to five days) (Figure 3.5A). However, at the higher host densities (48, 84, and 168), the proportion of parasitized eggs was less than 0.40, at all exposure times. When the number of wasps increased from one to seven, the proportion of parasitized eggs was consistently more than 0.65 at higher host egg exposure times (three to five days) at each host density (Figure 3.6A). Similarly, the proportion of hatched nymphs was significantly affected by host density (Table 3.3) and exposure time (Table 3.4). The release of multiple wasps (Figure 3.6B) significantly reduced eclosion of host nymphs compared to a single wasp release (Figure 3.5B).

Table 3.5 Results of a Kruskal–Wallis ANOVA with Dunn's post hoc test comparing kudzu bug eggs parasitized, hatched host nymph, wasp offspring, and offspring sex ratio (female/total) among host densities for *Ooencyrtus nezarae* and *Paratelenomus saccharalis*

Parasitoid species	Experiment	Host density comparison	Host eggs parasitized	Hatched host nymphs	Wasp offspring	Offspring sex ratio
Ooencyrtus nezarae	**Single wasp**	21 vs. 42	z =1.84, p =0.06	z =-1.74, p =0.08	z =1.40, p = 0.16	z =-1.43, p =0.15
		21 vs. 84	z =6.09, **p < 0.001**	z =-5.71, **p < 0.001**	z =5.71, **p < 0.001**	z =-1.56, p =0.11
		21 vs. 168	z =7.87, **p < 0.001**	z =-6.68, **p < 0.001**	z =7.94, **p < 0.001**	z =-0.22, p =0.82
		42 vs. 84	z =4.25, **p < 0.001**	z =-3.96, **p < 0.001**	z =4.30, **p < 0.001**	z =-0.13, p =0.89
		42 vs. 168	z =6.03, **p < 0.001**	z =-4.93, **p < 0.001**	z =6.54, **p < 0.001**	z =1.20, p =0.22
		84 vs. 168	z =1.77, p = 0.07	z =-0.97, p =0.33	z =2.23, **p = 0.02**	z =1.34, p =0.18
			(H = 80.18, df =3, **p < 0.001)**	(H = 60.71, df =3, **p < 0.001)**	(H = 82.10, df =3, **p < 0.001)**	(H = 3.90, df =3, p =0.27)
	Seven wasps	21 vs. 42	z =2.02, **p =0.04**	z =-1.65, p =0.09	z =-1.45, p = 0.14	z =-1.54, p = 0.12
		21 vs. 84	z =3.24, **p =0.001**	z =-3.94, **p < 0.001**	z =-1.91, **p = 0.05**	z =-2.04, p = 0.04
		21 vs. 168	z =7.78, **p < 0.001**	z =-8.82, **p < 0.001**	z =2.39, **p = 0.01**	z =-1.39, p = 0.16
		42 vs. 84	z =1.22, p =0.22	z =-2.29, **p = 0.02**	z =-0.46, p = 0.64	z =-0.50, p = 0.61
		42 vs. 168	z =5.76, **p < 0.001**	z =-7.17, **p < 0.001**	z =3.85, **p < 0.001**	z =0.14, p = 0.88
		84 vs. 168	z =4.53, **p < 0.001**	z =-4.88, **p < 0.001**	z =4.31, **p < 0.001**	z = 0.64, p = 0.51
			(H = 65.20, df =3, **p < 0.001)**	(H = 88.36, df =3, **p < 0.001)**	(H = 22.62, df =3, **p < 0.001)**	(H = 4.60, df =3, p =0.20)
Paratelenomus saccharalis	**Single wasp**	21 vs. 42	z =0.94, p = 0.34	z = -0.96, p =0.33	z =0.53, p = 0.59	z = -0.54, p =0.58
		21 vs. 84	z =4.29, **p < 0.001**	z = -4.15, **p < 0.001**	z =3.73, **p < 0.001**	z = 1.07, p =0.28
		21 vs. 168	z =6.78, **p < 0.001**	z = -7.10, **p < 0.001**	z =6.35, **p < 0.001**	z = -0.67, p =0.49
		42 vs. 84	z =3.34, **p < 0.001**	z = -3.17, **p = 0.001**	z =3.20, **p = 0.001**	z = 1.62, p =0.10
		42 vs. 168	z =5.84, **p < 0.001**	z = -6.14, **p < 0.001**	z =5.82, **p < 0.001**	z = -0.14, p =0.88
		84 vs. 168	z =2.58, **p = 0.01**	z = -3.04, **p = 0.002**	z =2.69, **p = 0.007**	z = -1.74, p =0.08

			($H = 58.24$, df =3, **$p < 0.001$**)	($H = 62.37$, df =3, **$p < 0.001$**)	($H = 52.60$, df =3, **$p < 0.001$**)	($H = 3.83$, df =3, $p = 0.28$)
	Seven wasps	21 vs. 42	$z =-0.48$, $p = 0.62$	$z = -1.63$, $p =0.10$	$z = -2.12$, **$p =0.03$**	$z = -3.59$, **$p <0.001$**
		21 vs. 84	$z =1.01$, $p = 0.30$	$z = -2.48$, **$p =0.01$**	$z = -0.85$, $p =0.39$	$z = -3.13$, **$p =0.002$**
		21 vs. 168	$z =2.85$, **$p = 0.004$**	$z = -4.97$, **$p < 0.001$**	$z =1.15$, $p = 0.24$	$z = -4.65$, **$p <0.001$**
		42 vs. 84	$z =1.50$, $p = 0.13$	$z = -0.84$, $p =0.39$	$z =1.27$, $p = 0.20$	$z =0.45$, $p = 0.64$
		42 vs. 168	$z =3.34$, **$p < 0.001$**	$z = -3.33$, **$p < 0.001$**	$z =3.28$, **$p = 0.001$**	$z = -1.06$, $p =0.28$
		84 vs. 168	$z =1.84$, $p = 0.06$	$z = -2.49$, **$p =0.01$**	$z =2.01$, **$p = 0.04$**	$z = -1.52$, $p =0.12$
			($H = 13.14$, df =3, **$p = 0.004$**)	($H = 25.84$, df =3, **$p < 0.001$**)	($H = 11.51$, df =3, **$p = 0.009$**)	($H = 24.01$, df =3, **$p < 0.001$**)

Significant values ($p \leq 0.05$) are in bold font.

Table 3.6 Results of a Kruskal–Wallis ANOVA with Dunn's post hoc test comparing kudzu bug eggs parasitized, hatched host nymphs, wasp offspring, and offspring sex ratio (female/total) among host exposure times (day) for *Ooencyrtus nezarae* and *Paratelenomous saccharalis*

Parasitoid species	Experiment	Host exposure time comparison	Host eggs parasitized	Hatched host nymphs	Wasp offspring	Offspring sex ratio
Ooencyrtus nezarae	Single wasp	1 vs. 3	$z = -6.19$, **$p < 0.001$**	$z = 4.81$, **$p < 0.001$**	$z = -5.88$, **$p < 0.001$**	$z = -2.65$, **$p = 0.008$**
		1 vs. 5	$z = -7.71$, **$p < 0.001$**	$z = 7.63$, **$p < 0.001$**	$z = -7.25$, **$p < 0.001$**	$z = -0.72$, $p = 0.46$
		3 vs. 5	$z = -1.51$, $p = 0.12$	$z = 2.81$, **$p = 0.005$**	$z = -1.36$, $p = 0.17$	$z = 1.92$, **$p = 0.05$**
			(H=66.76, df =2, **$p < 0.001$**)	(H=59.59, df =2, **$p < 0.001$**)	(H=59.48, df =2, **$p < 0.001$**)	(H=7.52, df =2, **$p = 0.02$**)
	Seven wasps	1 vs. 3	$z = -4.15$, **$p < 0.001$**	$z = 4.02$, **$p < 0.001$**	$z = -1.29$, $p = 0.19$	$z = -0.42$, $p = 0.67$
		1 vs. 5	$z = -5.78$, **$p < 0.001$**	$z = 5.38$, **$p < 0.001$**	$z = 0.80$, $p = 0.42$	$z = 0.38$, $p = 0.70$
		3 vs. 5	$z = -1.62$, $p = 0.10$	$z = 1.35$, $p = 0.17$	$z = 2.09$, $p = 0.03$	$z = 0.80$, $p = 0.42$
			(H=36.63, df =2, **$p < 0.001$**)	(H=31.32, df =2, **$p < 0.001$**)	(H=4.46, df =2, $p = 0.10$)	(H=0.64, df =2, $p = 0.72$)
Paratelenomus saccharalis	Single wasp	1 vs. 3	$z = -0.95$, $p = 0.34$	$z = 1.75$, $p = 0.07$	$z = -1.08$, $p = 0.28$	$z = 2.60$, **$p = 0.009$**
		1 vs. 5	$z = -1.17$, $p = 0.24$	$z = 1.20$, $p = 0.22$	$z = -0.87$, $p = 0.38$	$z = 0.39$, $p = 0.69$
		3 vs. 5	$z = -0.21$, $p = 0.82$	$z = -0.55$, $p = 0.58$	$z = 0.20$, $p = 0.83$	$z = -2.21$, **$p = 0.02$**
			(H = 1.55, df =2, $p = 0.46$)	(H = 3.23, df =2, $p = 0.19$)	(H = 1.32, df =2, $p = 0.51$)	(H = 7.89, df =2, **$p = 0.01$**)
	Seven wasps	1 vs. 3	$z = -0.97$, $p = 0.32$	$z = 0.53$, $p = 0.59$	$z = -1.07$, $p = 0.28$	$z = -0.27$, $p = 0.78$
		1 vs. 5	$z = 1.16$, $p = 0.24$	$z = -1.94$, **$p = 0.05$**	$z = 1.02$, $p = 0.30$	$z = 0.85$, $p = 0.39$
		3 vs. 5	$z = 2.14$, $p = 0.03$	$z = -2.47$, **$p = 0.01$**	$z = 2.10$, $p = 0.03$	$z = 1.12$, $p = 0.25$
			(H = 4.59, df =2, $p = 0.10$)	(H = 6.79, df =2, **$p = 0.03$**)	(H = 4.41, df =2, $p = 0.11$)	(H = 1.38, df =2, $p = 0.49$)

Significant values ($p \leq 0.05$) are in bold font.

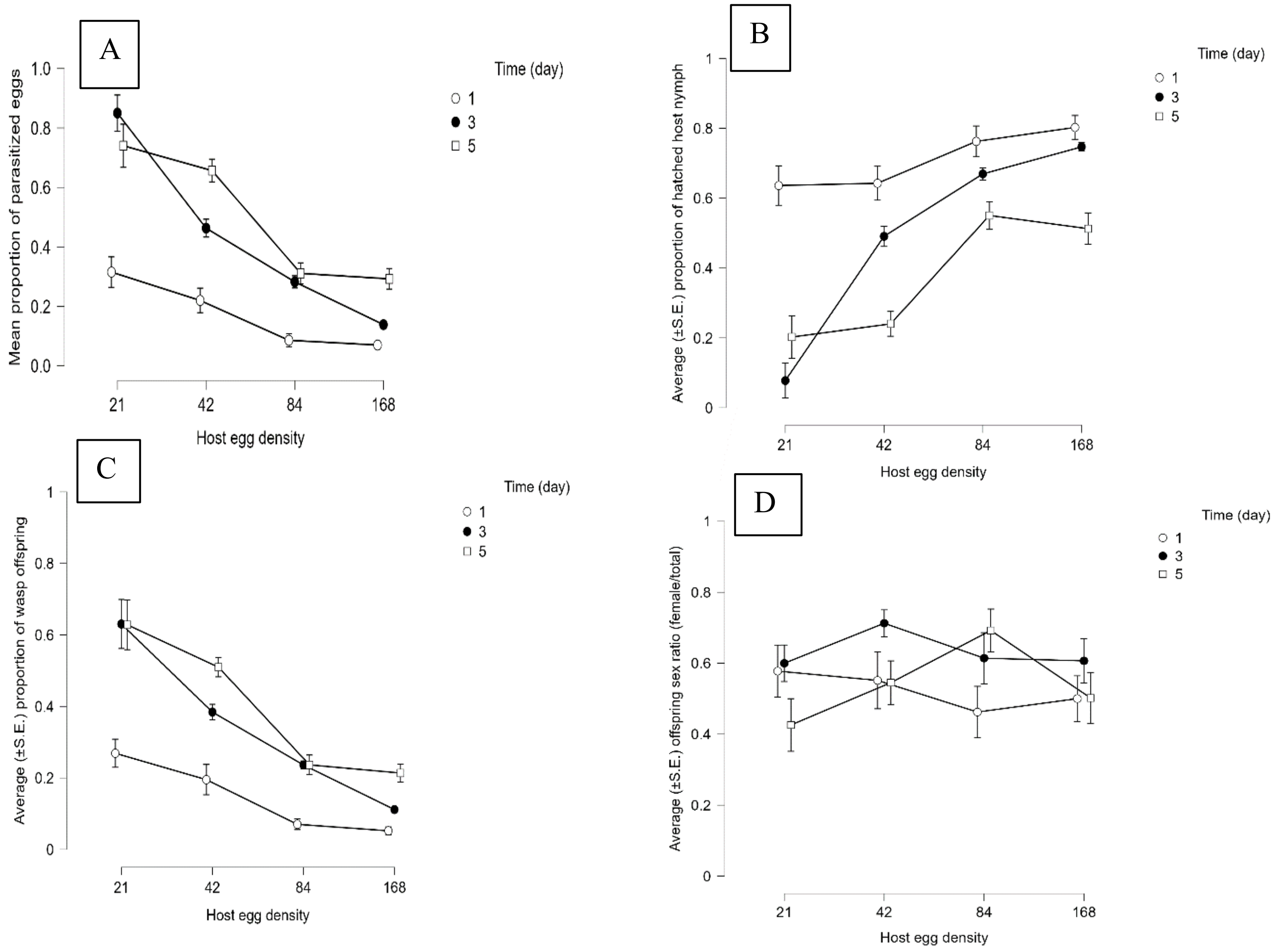


Figure 3.5 Effects of the host to parasitoid ratio and host exposure time on the average (±S.E.) (A) proportion of parasitized kudzu bug eggs, (B) proportion of hatched host nymphs (C) proportion of wasp offspring and offspring sex ratio (female/total) (D) for individual *Ooencyrtus nezarae* females.

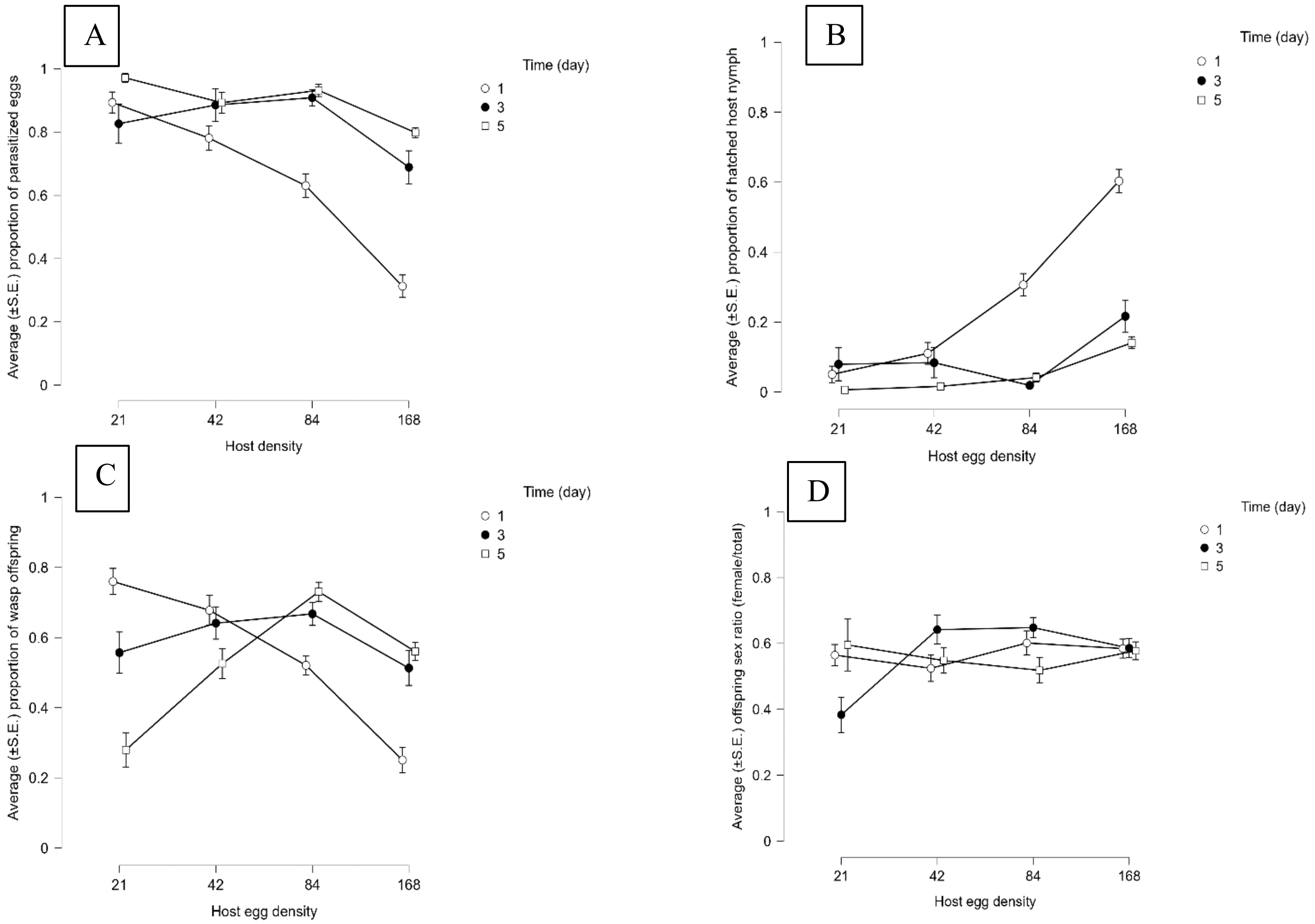
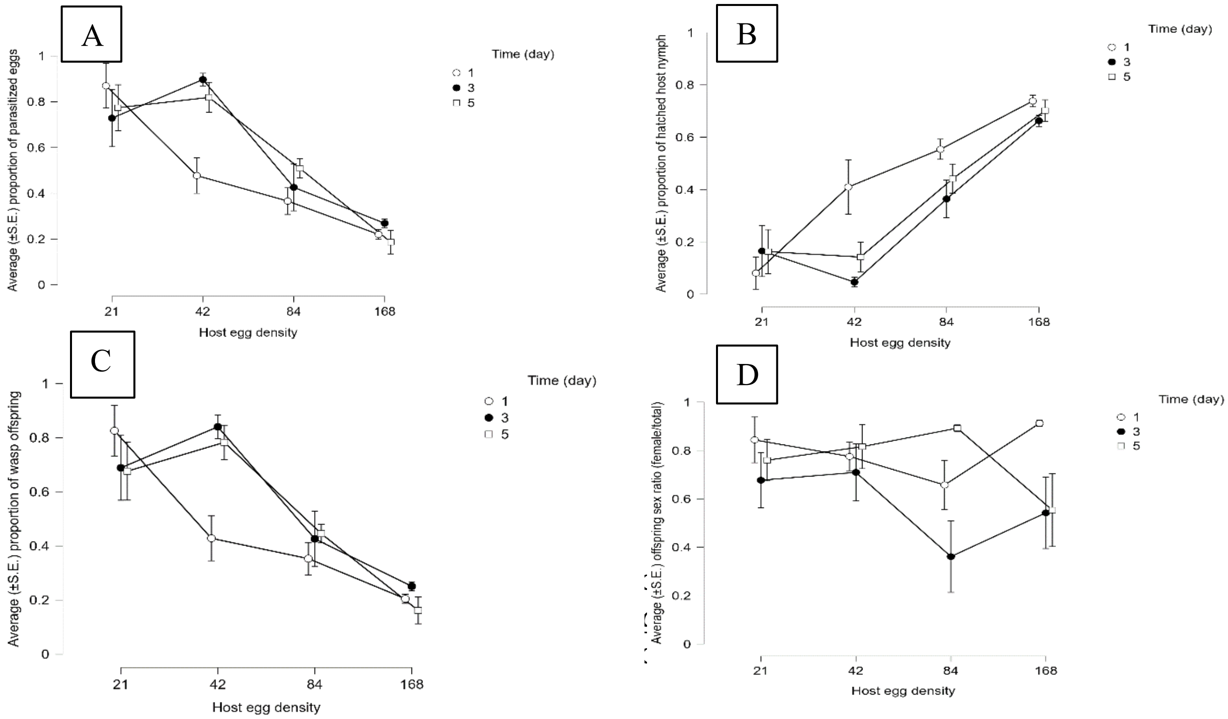


Figure 3.6 Effects of the host to parasitoid ratio and host exposure time on the average (±S.E.) (A) proportion of parasitized kudzu bug eggs, (B) proportion of hatched host nymphs (C) proportion of wasp offspring and (D) offspring sex ratio (female/total) for groups of seven *Ooencyrtus nezarae* females released together.

Wasp emergence was also influenced by host density in both single and multiple wasp release experiments (Table 3.3). Host exposure time did not affect the proportion of emerged wasps in the multiple wasp release experiment, but did affect the proportion emerged in the single wasp experiment (Table 3.4). In the single release wasp experiment, the proportion of parasitoids emerged increased as the host exposure time increased from one to five days at different host densities. A consistent declining trend of wasp emergence was observed as the host density increased (Figure 3.5C). The proportion of emerged wasps was up to 0.63 for 21 eggs, 0.50 for 48 eggs, 0.23 for 84 and 168 eggs (Figure 3.5C). The overall decline of 79% of the emergence of parasitoids was observed as host density increased in the single wasp release experiment. However, when wasps were released in a group of seven, the proportion of emerged wasps decreased as the host exposure time increased at low host densities (21 and 48 eggs; Figure 3.6C), opposite to the single wasp release experiment. In the treatment with a host density of 21 the proportion of wasps fell from 0.75 to 0.27 as the host exposure time increased from one to five days (Figure 3.6C), indicating there may be competition among conspecifics for limited host resources. The sex ratio of parasitoid offspring was almost equal (1:1) in all treatments in a single (Figure 3.5D) and, multiple (Figure 3.6D) wasp experiments.



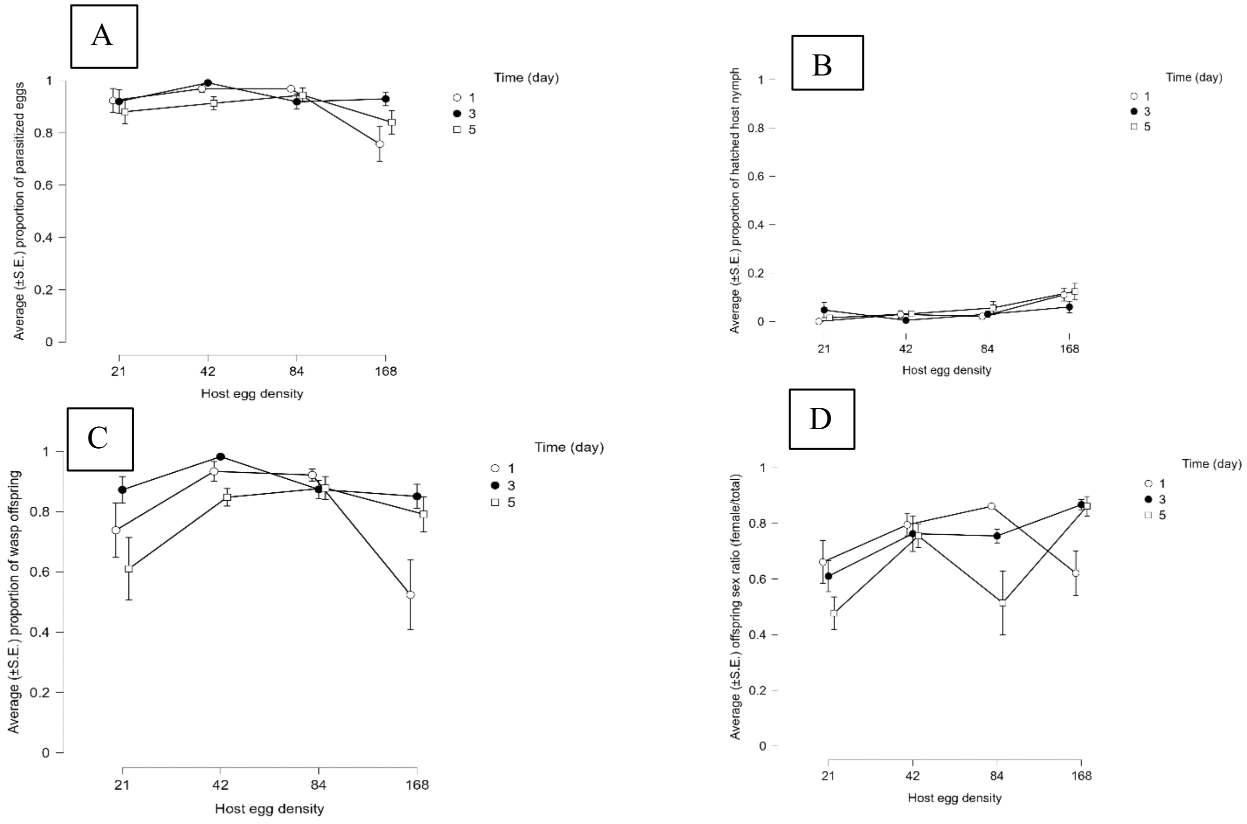


Figure 3.7 Effects of the host to parasitoid ratio and host exposure time on the average (±S.E.) (A) proportion of parasitized kudzu bug eggs, (B) proportion of hatched host nymphs (C) proportion of wasp offspring, and (D) offspring sex ratio (female/total) in group *Paratelenomus saccharalis* females releases.

3.6.2.2 Biology of Paratelenomus saccharalis Under Different Combinations of host: Parasitoid Ratios and Host Exposure Times

Host parasitism was influenced by host density, not exposure times, in individual foraging wasp or groups of seven wasps (Tables 3.3 and Table 3.4). It indicates that the number of hosts is more important than the time available for parasitism. At each host exposure time, a lower proportion of *M. cribraria* eggs were parasitized in both experiments as the host density increased. In the single wasp release experiment, the proportion of parasitized eggs was more than 0.70 at lower host densities (21 and 48 eggs) over higher exposure times (three and five days) (Figure 3.7A). However, in multiple wasp releases, the proportion of parasitized eggs was 0.99 in most treatments (Figure 3.8A). More hatched nymphs were observed in the single wasp experiment (Figure 3.7B) compared to multiple wasp experiment (Figure 3.8B). The proportion of hatched nymphs in the single wasp release was noticeably higher at the density of 168 eggs at each host exposure time (Figure 3.8B).

Similarly, only host density affected the proportion of emerged wasps in either experiment (single or multiple wasp release, Table 3.3). As the host density increased, the proportion of emerged wasps decreased at each exposure time when a single wasp was exposed to *M. cribraria* eggs (Figure 3.7C). However, when multiple wasps were exposed, the results varied with the host density and exposure time (Figure 3.8C). The highest number of wasps emerged after three days of exposure at each host density (Figure 3.8C). The sex ratio of offspring was also affected by host density only (Table 3.3), not by host exposure time (Table 3.4). The proportion of parasitoid female offspring was not lower than 0.50 in most treatments, whether host eggs were exposed to a single wasp (Figure 3.3D) or multiple wasps (Figure 3.8D).

3.6 DISCUSSION

3.6.1 Effect of food availability on the life history traits of Ooencyrtus nezarae and Paratelenomus saccharalis

3.7.1.1 Survival curves

The lifespan of parasitoids can be determined by sex (Maceda et al. 2003), particularly when males and females allocate nutritional resources differently for survival and reproduction (Hoogendoorn et al. 2002). In this study, the impact of honey with/without a water supply on adult *O. nezarae* and *P. saccharalis* were similar between the sexes. When provided with honey and water, *O. nezarae* and *P. saccharalis* lived 28-47 (Figure 3.1), and 28 days (Figure 3.3), respectively. When provided with honey alone, *O. nezarae* and *P. saccharalis* could live 27-47 (Figure 3.1), and 26-31 days (Figure 3.3), respectively. However, individuals who were starved survived for 3-5 days in both wasp species (Figures 3.1 and 3.3). This observed survival period under starvation conditions is somewhat similar to that of *O. nezarae* and *P. saccharalis* populations found in Asia, which lived for two days (Aung et al. 2010) and four days (Takano and Takasu 2019), respectively. Females had similar or longer lifespans than males when food resources like water and honey were absent. Takasu and Hirose (1991) reported that adult *O. nezarae* females feed on host fluids before egg deposition, which may help to explain why females can live longer than males when resources are absent. Honey-deprived, oviposition host-provided females survived much longer than males (Figures 3.1 and 3.3). However, honey supply suppressed this longevity advantage, indicating that males rely more on alternative sugar sources such as extrafloral nectar and hemipteran honeydew (Wäckers et al. 2008). These results suggest, females have an advantage over males in the field during water and sugar shortages because they are less sensitive to the scarcity of these resources and can meet some of their nutritional needs through host feeding.

3.7.1.2 Host Parasitism

According to our data, female *O. nezarae* and *P. saccharalis* exhibited a high parasitism rate of *M. cribraria* eggs, but this varied w/diet (Figures 3.2A and 3.4A). A supply of honey led to the highest parasitism regardless of water availability showing that sugar plays an important role in both wasp species' total host-parasitism capacity over their adult lifespan. This sugar effect was not observed in newly emerged *P. saccharalis* females (1–2 days old) but became more apparent as they aged. This data suggests that as females age, they exhaust their nutritional reserves and depend more on sugars to compensate for their nutritional loss (Bezerra Da Silva et al. 2019).

3.7.1.3 Wasp Emergence

The emergence of *O. nezarae* and *P. saccharalis* offspring depended on the diet and age of females. Honey-fed wasps were more fecund over their lifespan than wasps in other treatments (Figures 3.2C and 3.4C). The highest emergence of offspring was primarily observed from eggs laid by young female parasitoids (Figures 3.2C and 3.4C). This indicates that the nutritional status and age of both parasitoid species affect their ability to produce offspring. The effect of nutrition and age of parasitoid have also been observed in other hymenopterans such as *Pachycrepoideus vindemmiae* (Bezerra Da Silva et al. 2019) on its host, *Drosophila suzukii.*

3.7.1.4 Offspring Sex Ratio

Maternal age and diet are known to affect the offspring sex ratios in parasitic wasps (King 1987), and our results provide more evidence that the offspring sex ratio of *O. nezarae* and *P. saccharalis* is related to maternal age and diet. The proportion of female offspring continually decreased as females aged. The decline was more substantial in honey-deprived wasps than in their honey-fed counterparts (Figures 3.2D and 3.4D). The females used in this study mated following their emergence, but after 24 h, they were separated. As females had no opportunity to re-mate, they could not replenish their sperm storage during egg-laying. These findings indicate that females of both parasitoids may need to mate multiple times throughout their lifespan to continue to produce female offspring.

3.6.2 Effect of host: parasitoid ratios and host exposure times on the biology of Ooencyrtus nezarae and Paratelenomus saccharalis

The number of host eggs and the time available to parasitize the eggs also affected offspring emergence and sex ratio. Stable parasitism (Figures 3.5A and 3.6A) and maximum yield of parasitoid offspring (Figures 3.5C and 3.6C) in *O. nezarae* occurred when a single or a group of female wasps was exposed to a low host density. The exposure time required to maximize parasitism depended upon the number of parasitoids. The highest parasitism with a female: male sex ratio of at least ~1:1 was achieved with a three- and five-day exposure duration for a single wasp, or a one day exposure time for a group of wasps. For *P. saccharalis*, the proportion of host eggs parasitized (Figures 3.7A and 3.8A) and emerged offspring (Figures 3.7C and 3.8C) reached a plateau at 48 host egg density over three days exposure time when hosts were provided to both single or groups of *P. saccharalis* females. In this study, host density affected both *O. nezarae* and *P. saccharalis* parasitism and reproductive performance differently depending on the exposure time.

In a study using a single *O. nezarae* or *P. saccharalis* females, parasitism decreased as the host: parasitoid ratio increased across all host exposure times (Figures 3.5A and 3.7A). When *O. nezarae* females were released individually or in a group, parasitism was lowest at one day of exposure, and highest at three and five days. This suggests that a one-day exposure time might not be enough time for the *O. nezarae* females to parasitize large host patches. When a single or a group of *P. saccharalis* females was exposed to host eggs, the level of egg parasitism for a one day exposure was almost the same as that for five days of exposure. It indicates that a one-day exposure time might be enough for the *P. saccharalis* females to parasitize up to a proportion of 0.90 available hosts in one day. A previous study found that host exposure time significantly impacted the parasitism rate of *Telenomus remus* Nixon, on *Spodoptera litura* Fabricius eggs with the highest parasitism rate observed after a one day exposure (Chen et al. 2021). Similarly, Wang et al. (1997) did not find a major difference between the host parasitism at two and three days of exposure using *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) eggs as host for *Trichogramma ostriniae* Peng & Chen (Hymenoptera: Trichogrammatidae). The possible explanation for the parasitism results is that the specialist, *P. saccharalis*, has better host-handling strategies (an average of 10.48 min) (Bernays 2001, Takano and Takasu 2019) than the generalist, *O. nezarae* (an average of 19.32 min) (Takasu and Hirose 1991).

There was a major reduction in the offspring of *O. nezarae* and *P. saccharalis* at the lower densities of *M. cribraria* eggs exposed to a group of female parasitoids for five days. Long exposure time (five days) may result in superparasitism due to a shortage of unparasitized hosts, decreasing the successful development and emergence of parasitoid offspring (Smith 1996).

Intraspecific competition for limited nutrients can also decrease wasp emergence. The nutrients within host eggs are usually enough for only one wasp larva to complete its development. This fierce competition for limited nutrients causes cannibalism and increases parasitoid larval mortality (Cave 2000). These results are consistent with a previous study that found a negative correlation between the quantity of emerged adults and host egg: parasitoid ratio at prolonged exposure times (Wang et al. 1997).

For *O. nezarae*, the proportion of female offspring followed an inverse relationship between emerged wasps and sex ratio (female/total offspring) in single (Figure 3.5D) and multiple (Figure 3.6D) wasp release experiments. When the host egg: parasitoid ratio increased in the five-day exposure treatment, the proportion of female offspring increased in single wasp release experiments (Figure 3.5D) but decreased in group wasp release experiments (Figure 3.6D). For *P. saccharalis*, the proportion of female offspring (Figures 3.7D and 3.8D) followed a similar pattern with the emergence of wasps in both single and multiple wasp release experiments. The lowest proportion of female offspring in both wasp species was observed at a low host eggs to parasitoid ratio (21:7) for prolonged exposure, possibly due to superparasitism, although our study did not provide direct evidence to confirm this phenomenon. This finding is aligned with Chen et al. (2021), where a low host eggs to parasitoid ratio (80:80) led to more male offspring in *T. remus*, with confirmed superparasitism.

3.7 CONCLUSIONS

The present study demonstrates that consistent supplies of water and honey increase the survival of male and female wasps, their parasitism capacity, and sex ratios of their offspring. It was also demonstrated that a relatively low host: parasitoid ratio and exposure time optimize the reproductive potential of *O. nezarae* and *P. saccharalis.* Both parasitic wasps are long-lived and can significantly contribute towards *M. cribraria* biocontrol through parasitism. These findings call for additional research to understand which flowering plants can serve as nectar sources for *O. nezarae* and *P. saccharalis* in the field and aid in conserving the biological control of *M. cribraria*.

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Chapter 4

EXPLORING HOST-SEEKING BEHAVIOR IN *PARATELENOMUS SACCHARALIS* AND *OOENCYRTUS NEZARAE*: INSIGHTS FROM OLFACTOMETER AND FOOTPRINT BIOASSAYS

4.1 INTRODUCTION

The kudzu bug, *Megacopta cribraria* Fabricius (Hemiptera: Plataspidae), is an invasive pest of soybeans, *Glycine max* (L.), in the southern United States (Ruberson et al. 2013). Currently, synthetic chemical insecticides are the primary control method for this pest, but repeated use of these chemicals can lead to insecticide resistance and harm beneficial species (Seiter et al. 2015). As a result, there is a growing interest in using parasitoids as an alternative control method (Grønvold et al. 1996). Two egg parasitoids of *M. cribraria*, *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae), a generalist, and *Paratelenomus saccharalis* Dodd (Hymenoptera: Platygastridae), a specialist, were discovered in the United States in 2016, and 2013, respectively (Gardner et al. 2013, Ademokoya et al. 2018). In their native Southeast Asia, *P. saccharalis* and *O. nezarae* parasitize *M. cribraria* eggs at rates of 100% and 76.9%, respectively (Takasu and Hirose 1985, 1986), while in Alabama's soybean fields, they coexist and exhibit parasitism rates ranging from 42-95% for *P. saccharalis* and 82.8-100% for *O. nezarae* (Ademokoya et al. 2018). The importance of these findings lies in the potential for biological control of *M. cribraria* in soybean crops. High parasitism rates of

O. nezarae and *P. saccharalis* on *M. cribraria* eggs suggests that these parasitoids could effectively regulate the pest population, reduce crop damage and improve farmers' yields. Understanding the the factors associated with parasitoid host foraging for *M. cribraria* is important for developing effective biological control strategies.

One challenge faced by female parasitoids, notably egg parasitoids, is the 'detectability' or 'reliability' problem during host foraging because the egg stage of their host is relatively inconspicuous (Fatouros et al. 2008). Insect eggs are small, sessile, and do not feed nor release feces; consequently, they lack many long-range cues that might be exploited by parasitoids that feed on immature or adult life stages (Fatouros et al. 2008). Egg parasitoids use strategies to detect volatile organic compounds (VOCs) from herbivore-host plant interactions and adult host-related cues to overcome host detection challenges (Fatouros et al. 2008). Herbivore-infested plants emit informative volatile cues that serve as long-range cues for parasitoids to find host habitat (Morawo and Fadamiro 2016), and may be easier to detect because plants have a larger biomass and produce more abundant volatiles than insect herbivores (Turlings et al. 1995). Upon reaching the host habitat, female parasitoids employ short-range, indirect host-related cues left by the adult stage of the host, such as their chemical footprints (Conti and Colazza 2012). These cues modify the searching intensity of the parasitoids, enabling them to better locate their hosts (Conti and Colazza 2012). Chemical traces left by pre-ovipositional host females can be reliable indicators for parasitoids before the host eggs are too old to be physiologically acceptable for oviposition (Peri et al. 2016). The use of host-associated chemical cues as a host-finding strategy can be influenced by the dietary specialization of egg parasitoids (Peri et al. 2013) as specialist species tend to rely on specific cues more often than generalist species (Vet and Dicke 1992, Steidle and Van Loon 2003).

Previous studies have shown that host-seeking behavior in generalist and specialist parasitoid species relies not only on chemical cues, but also on the information they obtained through experience in the adult stage (the chemical legacy hypothesis and the neo-Hopkins' principle) is crucial for maximizing their foraging success (Turlings et al. 1990, Giunti et al. 2015). However, parasitoids' innate responses to olfactory cues vary with their physiological state (Lewis and Takasu 1990). The nutritional status of parasitoids has been shown to affect their olfactory abilities to locate and identify their hosts for successful parasitism (Takasu and Lewis 1993, 1995, Damien et al. 2019). The nutritional status of the insects may also impact learning and memory processes because the brain requires a large amount of energy to build up memory and maintain neural tissue (Niven and Laughlin 2008). When energy resources are limited, insects may acquire energy from other important fitness-related characteristics, such as the maintenance of the nervous system (Burns et al. 2011). Such trade-off in energy allocation from neural to other tissues such as tracheal and excretory systems may affect the benefits of learning-based behavioral plasticity in insects (Chittka and Niven 2009, Burns et al. 2011).

No information is available on nutritional status and learning-based behavioral plasticity in *O. nezarae* and *P. saccharalis* to locate their host. This information could provide valuable insights into the factors influencing host location strategies in these species and help improve our understanding of host-parasitoid interactions. This study was conducted to examine the effect of nutritional status and ovipositional experience of *O. nezarae* and *P. saccharalis* females on their behavioral responses to host-associated olfactory cues (olfactometer bioassay) and non-host-associated contact chemical cues (footprints bioassay). Four hypotheses were tested in this study: (i) female parasitoids of both species that have been honey-fed and have ovipositional experience will choose herbivore-damaged plant VOCs more often than alternatives (i.e., healthy plant or host egg VOCs) when compared to starved (i.e., no honey, no water) and inexperienced (i.e., no prior ovipositional experience) females, (ii) *P. saccharalis* (specialist) will show greater attraction to the VOCs emitted from host-plant interactions than *O. nezarae* (generalist), (iii) *O. nezarae* and *P. saccharalis* will show attraction to host chemical footprints, and (iv) *P. saccharalis* will show greater attraction to host chemical footprints than *O. nezarae.* To test the first and second hypotheses, the Y-tube olfactometer was used to compare the attraction of parasitoids to stimuli. To test the third and fourth hypotheses, EthoVision XT® tracking software (Version 13; Noldus Information Technology Inc., Leesburg, VA, US) was used to compare the behavioral responses of *O. nezarae*, and *P. saccharalis* to *M. cribraria* adult chemical footprints in a Petri dish arena. The residency time of the parasitoids in different zones of the arena was used as a measure of attraction to investigate the responses and level of commitment to the host chemical footprints of parasitoids. Visuals such as heat maps and track paths were also provided to illustrate the results and provide a clear understanding of parasitoid behavior. This study highlights an interesting interaction in which semiochemicals attract an insect species. Further, the possible influence of host specialization on chemical-mediated attraction in parasitoids is discussed.

4.2 MATERIALS AND METHODS

4.2.1 Plant

Soybean seeds (var. Pioneer P49T97R-SA2P) were planted into 15.24 cm diameter and 14.22 cm depth pots in Sunshine potting mixture #8 (SunGro Horticulture, Bellevue, WA, USA). Plants were grown in a greenhouse free of pesticides at 26 ± 2 °C, 16:8 h (L/D) photoperiod, and 55 ± 5% relative humidity (Ademokoya et al. 2017). Plants were housed in ventilated rearing tents (60 cm x 60 cm x 60 cm) (BugDorm-2120, Megaview Science Co., Ltd., Taichung, Taiwan) to prevent unintended insect infestations. They were irrigated once a day (~200 mL per pot) and fertilized once a week according to the manufacturer's instructions (Scotts-Sierra Horticultural Product Company, Marysville, OH, USA). Soybeans at the vegetative (V3–V4) growth stage were used for the Y-tube olfactometer bioassays (Fehr et al. 1971).

4.2.2 Insects

Adult *M. cribraria* were collected from kudzu vines, *Pueraria montana* (Lour) Merr., in Auburn, Alabama (AL), United States, in 2020 and a colony was maintained in rearing tents (60 cm x 60 cm x 60 cm) (BugDorm-2120, Megaview Science Co., Ltd., Taichung, Taiwan) at 25 ± 1 °C, with a 14:10 h (L:D) photoperiod, and 75 ± 5% relative humidity in an incubator (Percival, Perry, IA, USA) (Ademokoya et al. 2017). The colony was offered soybean plants at the vegetative stage (V2-V3) weekly (Ademokoya et al. 2018).

Parasitized *M. cribraria* eggs (grey color) (Britt 2016**)** were collected from the soybean fields from Auburn Old Rotation (Alabama Agricultural Experiment Station), Auburn, AL and placed into an individual 59.1 mL (top diameter: 6 cm, bottom diameter: 4.4 cm, and height: 2.8 cm) condiment cup at 25 ± 1 °C, 14:10 (L:D) h and 75 ± 5% RH in an incubator (Percival, Perry, IA, USA) (Ademokoya et al. 2017). Approximately 20 pin holes were made on the rearing cup wall for aeration and to prevent condensation. These pin holes were too small for parasitoids to escape. Upon emergence, parasitoids were identified as *O. nezarae* or *P. saccharalis*, based on the identification key of Gardner et al. (2013) and Ademokoya et al. (2018), and were separated into different rearing cups. Colonies of the wasps were reared by providing adults with a honey solution (70% v/v) (Warsi et al. 2023) and ≤ 24 h old *M. cribraria* eggs. The age of tested female *P. saccharalis* and *O. nezarae* was 24 (Takano and Takasu 2019) and 96 h (Aung et al. 2010) old, respectively. This differential age range was chosen because the wasp's reproductive peaks were entirely dissimilar and times were chosen to represent the optimal reproductive age (Aung et al. 2010, Takano and Takasu 2019).

4.2.3 Physiological and learning status treatments

4.3.3.1 Experimental design

Upon emergence (egression of wasps from host eggs), females and males were placed into 59.1 mL condiment cups (top diameter: 6 cm, bottom diameter: 4.4 cm, and height: 2.8 cm) to ensure insemination. Female wasps were assigned to four groups to determine the odor response of the parasitoid, i.e., physiological and learning status. For experimental purposes, physiological or nutritional status is defined as hungry, or fed while learning status is defined as experienced or inexperienced with oviposition (Table 4.1). The first group of female wasps were provided with 70% honey solution (v/v) without host eggs (fed- no host eggs). In this group, female *P. saccharalis* was fed for 24 h, and *O. nezarae* was fed for 96 h according to their reproductive peaks. The second group had the same conditions as the first but was also given host eggs along with the honey solution (fed + host eggs). The third group consisted of hungry females without host eggs (hungry –no host eggs). The starvation methods differed for each species. Female *O. nezarae* were allowed to feed on honey for 72 h before being starved for the next 24 h, while *P. saccharalis* was completely deprived of the honey solution for 24 hours. Previous studies report that starved *P. saccharalis* could live up to 72 h (Takano and Takasu 2019), but when *O. nezarae* were not fed, they could live for only 48-72 h (Takasu and Hirose 1991). To be consistent with the *O. nezarae* peak reproductive age, the females were allowed to feed for 72 h (determined by preliminary experiment), and they were starved for the remaining 24 h as mentioned in Table 4.1. Females in the fourth group were starved and offered host eggs (hungry + host eggs). The honey solution was held in a 0.5 mL Eppendorf microcentrifuge tube with a hole in the bottom through which a cotton string was threaded. Each female in the second and fourth groups were provided 24-30 *M. cribraria* eggs (≤ 24 h old) as our preliminary study showed that honey-fed female wasps could parasitize at least 24 *M. cribraria* eggs within 24 h. Female *O. nezarae* received host eggs on day 4 and *P. saccharalis* on day 1 (Table 4.1). In the footprint's bioassay, only honey-fed and inexperienced (prior oviposition experience) parasitoids were used due to a limited number of available parasitoids.

Table 4.1 Summary of parasitoid physiological and learning status used in Y-tube olfactometer study

Parasitoid condition	***Paratelenomus saccharalis***	***Ooencyrtus nezarae***
Group 1: fed –no host eggs	Honey 24 h	Honey 96 h
Group 2: fed + host eggs	Honey + host eggs for 24 h	Honey 72 h followed by 24 h with host eggs and honey
Group 3: hungry - host eggs	Neither honey nor host eggs	Fed wasp for 72 h then deprived for the next 24 h
Group 4: hungry + host eggs	Host eggs for 24 h	The same conditions as described in group 3 but host eggs were given on day four for 24 h.

4.3.3.2 Olfactometer bioassays

Olfactory responses of each group of wasps had been tested separately in the Y-tube olfactometer for the following treatments (i) a healthy soybean plant (i.e., plant with no physical or herbivore damage), (ii) an infested soybean plant, (iii) *M. cribraria* eggs only**,** (iv) an infested plant plus *M. cribraria* eggs, and (v) control blank (i.e., clean air) (Table 4.2). Each soybean plant for treatments ii and iii were at vegetative growth stage V3–V4. Thirty nymphs, and 20 pairs of adult *M. cribraria* (10 males and 10 females) which had been starved for 24 hours, were introduced to a plant in a ventilated rearing tent (60 cm x 60 cm x 60 cm) (BugDorm-2120, Megaview Science Co., Ltd., Taichung, Taiwan) for 24 h (Ademokoya et al. 2017). Egg masses of *M. cribraria* (six to seven egg masses, ~ 150-170 eggs) for treatments iii and iv were collected from the cage liner (paper towel) or sides of the cage rather than leaves. This approach was taken because detaching leaves may cause stress to the plant, potentially altering its volatile composition. This could, in turn, affect the results of the olfactometer study. A preliminary study showed that ~ 50% of wasps were attracted to five egg masses containing ~ 130 to 150 eggs in a Y-tube olfactometer. Therefore, more than five egg masses were used to ensure that odorants from the egg masses reached the focal parasitoid. The whole egg mass was taken as to not affect VOC composition. In both sets (no-choice and choice) of olfactometer bioassay, ≤ 24 h old *M. cribraria* eggs were used.

In this bioassay, treatments were assessed in a completely randomized design (CRD). Olfactory responses of parasitoids to stimuli were evaluated in two sets of bioassays: no-choice and choice. In the no-choice olfactometer bioassay, all stimuli were tested against clean air. In the choice bioassay, all possible combinations of the stimuli were tested (Table 4.2).

The Y-tube olfactometer (Figure 4.1) setup was similar to that used by Ademokoya et al. (2017) with slight modifications. Briefly, the Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) consists of a central tube (10 mm diameter × 13.5 cm long) and two lateral arms (10 mm diameter × 6.5 cm long). Each arm is connected to an extending glass tube (8 mm diameter × 14cm long). A sieve inlayed in each extending glass tube prevented the escape of insects. Humidified and purified (charcoal-filtered) air from an air delivery system (Analytical Research Systems, Gainesville, FL) was passed through Teflon tubes at 300 mL/min into air-tight glass chambers (5000 mL) containing treatments. Each glass chamber also has an air outlet carrying odorous air into each arm of the olfactometer. Air was removed by suction from the central tube of the olfactometer at 600 mL/min to avoid mixing odors. Illumination was provided by a white light lamp (20 W, 250 Lux) suspended above the apparatus. Each plant pot with soil was wrapped in aluminum foil to reduce contamination. The control for the no-choice bioassays was a pot with soil wrapped in aluminum foil but without a plant. When the attraction of parasitoids to the egg mass only was tested, the eggs were placed on Whatman® No. 1 filter paper (Fisher Scientific, Hampton, NH) inside a small (400 mL) glass chamber (Morawo and Fadamiro 2016), whereas the control chamber contained only a filter paper. For the second bioassay, an undamaged plant was used as the control.

Each female parasitoid was allowed five minutes to choose one of the Y-olfactometer arms. A choice was considered to have been made when a wasp traveled halfway into any arm (Figure 4.1). If the wasp did not make a choice in this period, then the data were discarded. All wasps were removed and discarded after five minutes. Thirty females of each parasitoid species were tested for each treatment. The attraction of parasitoids was recorded in a binary model as 1 for "response" and 0 for "no response."

Table 4.2 Summary for the treatments of the Y-tube olfactometer bioassays.

	Choice A	Choice B
No- choice bioassay	Healthy soybean plant	Clean air
	Infested soybean plant	Clean air
	M. cribraria egg masses	Clean air
	Infested soybean plant plus *M. cribraria* egg masses	Clean air
Choice bioassay	Infested soybean plant	Healthy soybean plant
	M. cribraria egg masses	Healthy soybean plant
	Infested soybean plant plus *M. cribraria* egg masses	Healthy soybean plant
	Infested soybean plant	*M. cribraria* egg masses
	Infested soybean plant plus *M. cribraria* egg masses	*M. cribraria* egg masses
	Infested soybean plant	Infested soybean plant plus *M. cribraria* egg masses

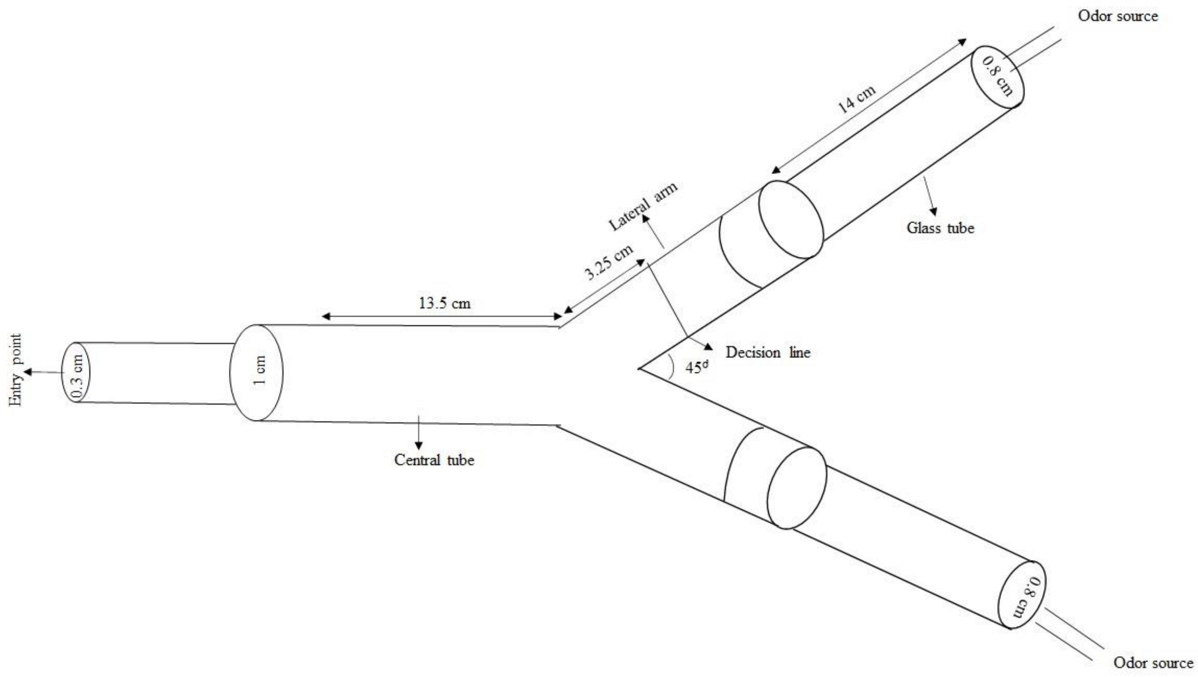


Figure 4.1 Schematic representation of the Y-tube olfactometer used to study the behavior of kudzu bug egg parasitoids, *Ooencyrtus nezarae* or *Paratelenomus saccharalis,* in response to host-associated olfactory cues. A single female parasitoid from either species was introduced into the arena via the entry point of the glass tube.

4.3.3.3 Footprint bioassays

Footprint bioassays followed a completely randomized design consisting of parasitoid species tested for their response to footprints of three adult *M. cribraria*. Treatments in this bioassay included groups of only male, only female, or both male and female *M. cribraria*. Both sexes were reproductively mature**,** approximately 30 days post-adult eclosion (Hosokawa and Suzuki 2001). During this period, host females were in a pre-ovipositional physiological state (Hosokawa and Suzuki 2001). Three adult *M. cribraria* were released in the footprints arena because personal observations indicated adults moved through a larger area within the arena with three or fewer adults present. The footprints arena can easily accommodate three adults, given their 3.5 to 6.0 mm size (Khadka et al. 2022). Adults were allowed to walk for one hour in the walking area to obtain their chemical footprints, as determined in preliminary experiment.

In this bioassay, responses of egg parasitoids to chemical host footprints were evaluated in two sets of bioassays: no-choice and choice. In the no-choice footprints bioassay, a petri dish with a 10 cm diameter and 1.5 cm height (product no. P5856, polystyrene petri dishes, Signa Aldrich Inc. St. Louis, MO, USA) was used to allow wasps to move freely (Figure 4.2A). A smaller petri dish with 3.5 cm diameter and 1 cm height (product no. P5112, polystyrene petri dishes, Signa Aldrich Inc. St. Louis, MO, USA) was placed at the center of the larger dish, designating it as the host footprint zone (Figure 4.2A). The area surrounding the smaller petri dish was considered as a non-footprint or control zone. *Megacopta cribraria* adults were positioned under the smaller petri dish, and the test surfaces (larger petri dish) were inverted to facilitate footprint collection on the petri dish, because *M. cribraria* (Figure 4.2A) has the tendency to move up. After footprints were collected over an hour, the larger petri dish was inverted and utilized as the experimental test arena.

In choice footprints bioassay, the petri dish (10 cm diameter x 1.5 cm height) was divided into two equal sections by marking a line with a permanent marker (Figure 4.2B). A plastic sheet (10 cm x 1.5 cm) was positioned in the petri dish's center along the marked line to facilitate footprint collection (Figure 4.2B). Consequently, randomly selected male *M. cribraria* were placed in one half of the divided zone, while females occupied the other half. Prior to introducing adults into the testing arena, each zone was labeled as female (F) or male (M) on the petri dish's underside to facilitate clear identification during video analysis. Accordingly, two treatments were tested in the separate zones: i) male footprints and ii) female footprints. For the bioassays, one female from either parasitoid species was introduced to the arena's center, devoid of any stimuli (marked by a line). Female parasitoids (either *O. nezarae* or *P. saccharalis*) were first placed in a separate petri dish inside the PhotoBox, allowing it to acclimate to the light conditions for five minutes before transfer to the assay arena. The randomly chosen females were then introduced very carefully into the petri dish, where the dividing line had been marked. Once introduced, parasitoid behavior was recorded for 10 minutes. Each parasitoid, host, and petri dish were used once and discarded. Experiments with each parasitoid species were conducted on separate days, between the hours of 0900 and 1700. Fifty percent of the wasps tested in each treatment were released inside and 50% outside of the footprint arena (control zone) to reduce experimental bias.

The movements of parasitoids were recorded using a Hero10 black GoPro digital camera with 5.3K60 ultra-HD 1080p video (GoPro® model Hero10 Black, Mag. 24.7X; San Mateo, CA, USA). A camera was positioned 15 cm above the arena within a professional PhotoBox (dimensions: 35.99 cm x 35.99 cm x 8 cm), sourced from the Puluz online store (vendor: Amazon.us). Lighting was provided by an inbuilt fluorescent lamp (24-26 lumens) within the PhotoBox and an additional LED lamp (approximately 800 lumens). The recording room was kept at 25 ± 1 °C and 75 ± 5% RH. A white PVC Backdrop was placed underneath the petri dish, to provide sufficient contrast for the insects.

Videos were analyzed using Ethovision® XT tracking software with dynamic subtraction (Azandémè-Hounmalon et al. 2016). Parasitoid responses were quantified by residence time (time in sec. spent in the male and female footprint-treated area or footprint/no-footprint areas). We opted to measure time only, excluding other metrics such as distance moved, velocity, and number of zone visits, as search time is a crucial investment for parasitoids during host location foraging. Consequently, the analysis focused on the total time spent in the control zone (without footprints) and the host footprint zones. Ethovision®, the software also analyzed insect tracks and heat maps. Track data depicted the actual walking paths of parasitoids, while heat maps illustrated the relative time spent in various zones within the arena.

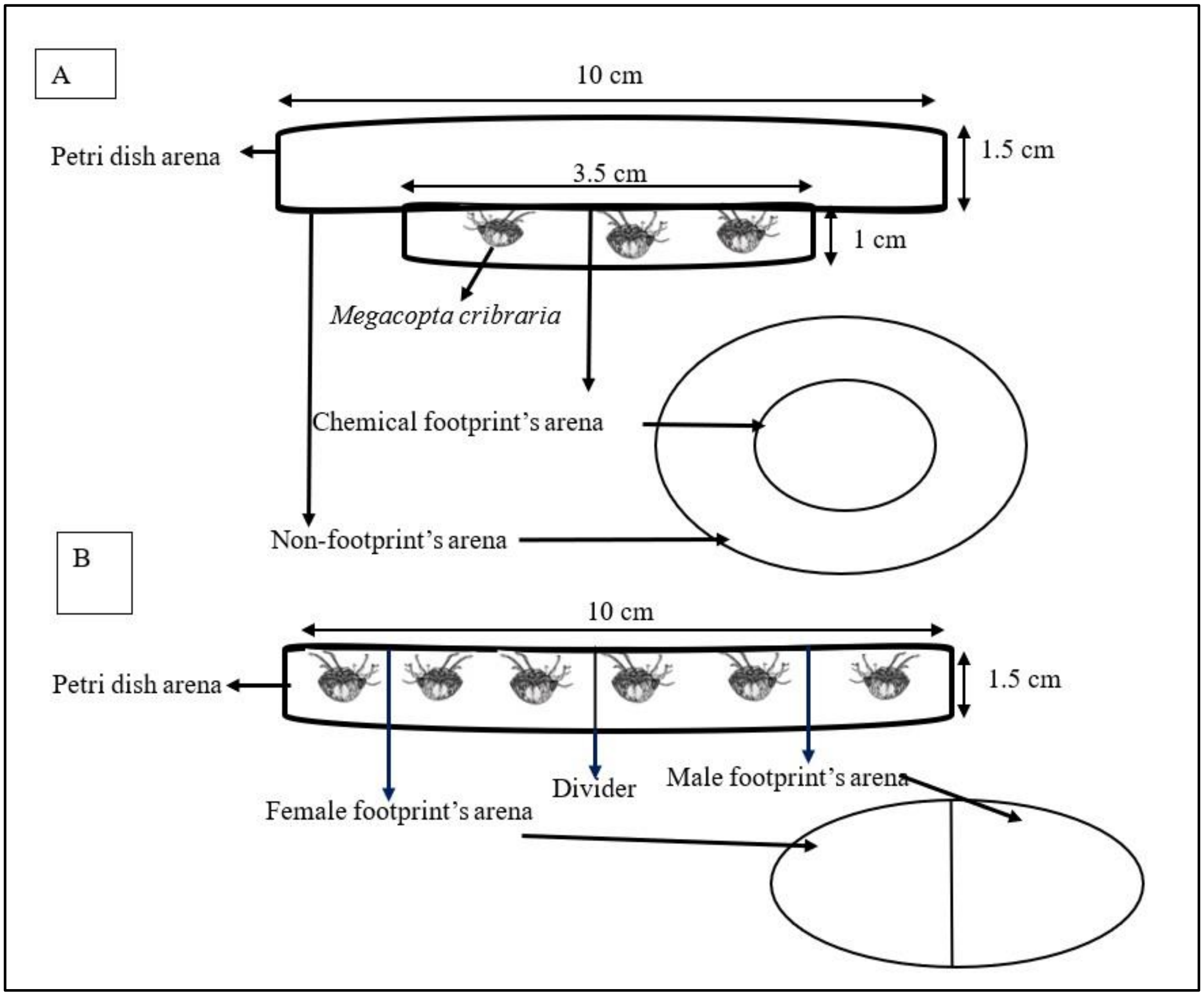


Figure 4.2 Schematic representation of the footprints bioassay procedure (A) no-choice and (B) choice.

4.3 STATISTICAL ANALYSES

The attraction of *P. saccharalis* and *O. nezarae* to the four test stimuli in a Y-tube olfactometer was modeled as a binary response (stimulus = 1, control = 0) using logistic regression to assess potential interactions between their physiological and learning status. To confirm the model's validity, a likelihood ratio test was conducted. Model adequacy was confirmed for each experiment set. Physiological and learning status were individually evaluated when no significant interactions were detected. Analyzing factors individually when no interaction was found aided in understanding their separate effects on parasitoids' attraction to test stimuli. A Chi-square goodness-of-fit test was utilized to analyze the deviation of parasitoid responses from a 50:50 (stimulus: control) distribution. All analyses were performed with SAS v9.2 (SAS Institute Inc., Cary, NC, USA) with a significance level of $p = 0.05$.

In the footprint bioassay the behavioral responses of *P. saccharalis* and *O. nezarae* were characterized by residence time, representing the total time spent by females in various zones. Time data distribution failed to satisfy parametric test assumptions; thus, the data underwent the non-parametric tests. The Mann-Whitney U test was used to compare the total time spent by female parasitoids in the footprint zone to that spent in the control zone. Kruskal Wallis was used to compare the total time spent by each parasitoid species between male and female host footprint zones. A two-sided independent samples t-test was utilized to compare the total time *P. saccharalis* and *O. nezarae* spent in either female or male footprint zones. All analyses were conducted using SAS v9.2 (SAS Institute Inc., Cary, NC, USA) at $p \leq 0.05$.

4.4 RESULTS

4.4.1 Olfactometer bioassay

4.6.1.1 Effect of physiological and learning status of Paratelenomus saccharalis females on the attraction to test stimuli in the olfactometer bioassay

In the no-choice Y-tube olfactometer bioassay, there was no significant interaction between physiological and learning status for any of the four stimuli tested (healthy plant: *p* = 0.99, Figure 4.3A; infested plant: *p* = 0.94, Figure 4.3B; host eggs: *p* = 0.87, Figure 4.3C; infested plant plus host eggs: *p* = 0.50, Figure 4.3D; Logistic Regression). The *P. saccharalis* showed no preference for specific odors based on their hunger and host experience levels. However, fed females with ovipositional experience were significantly (70/30%) more attracted to host eggs compared to the control (Figure 4.3C, Table 4.3). This group of wasps also displayed significantly (83.33/16.67%) higher attraction to the combination of infested plants plus host eggs when compared to the control (Figure 4.3D, Table 4.3). Hungry females without ovipositional experience exhibited significant (70/30%) attraction to the combination of infested plants plus host eggs in comparison to the control (Figure 4.3D, Table 4.3). These findings suggest that fed *P. saccharalis* females with prior ovipositional experience were attracted towards damage plant in conjunction with kudzu bug eggs compared to hungry females without ovipositional experience.

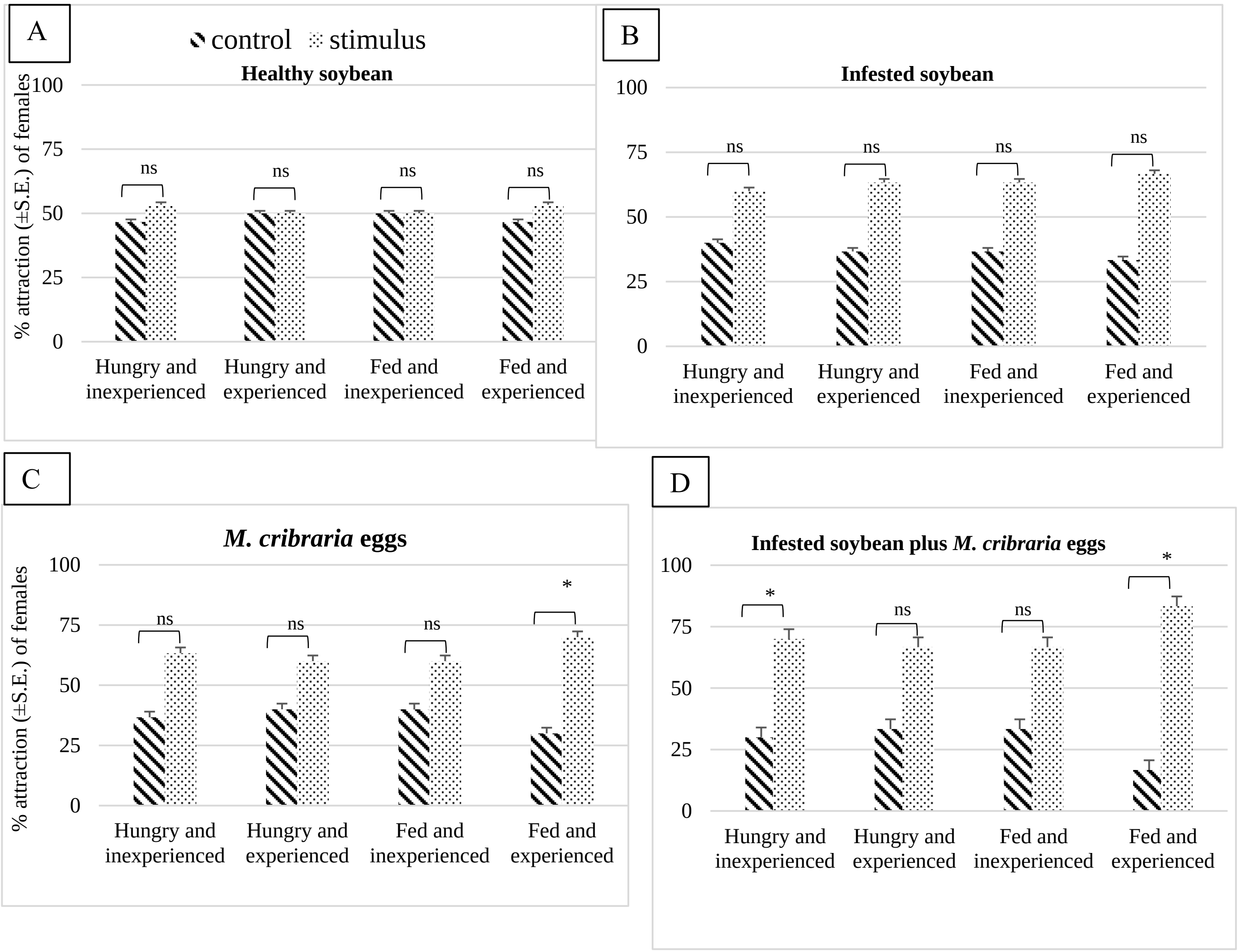


Figure 4.3 Effect of physiological state and learning status on attracting female *Paratelenomus saccharalis* to four odors in no choice Y-tube olfactometer bioassays. Bars represent the percentage of hungry and inexperienced, hungry and experienced, fed and inexperienced, or fed and experienced parasitoids when given a choice between control and (A) healthy soybean plant, (B) infested soybean plant, (C) *M. cribraria* eggs, and (D) infested soybean plant plus *M. cribraria* eggs. *Megacopta cribraria* nymphs (30) and adults (20 pairs) were allowed to infest soybean plants for 24 h before bioassays. Parasitoids were tested for each physiological states x choice treatment. Asterisk (*) indicates a significant

deviation of parasitoid responses from a 50:50 (stimulus: control) distribution ($\chi2$ goodness of fit test, *p* ≤ 0.05). ns = non-significant.

In a series of two-choice Y-tube olfactometer bioassays, the interaction between physiological and learning status of *P. saccharalis* females significantly influenced their preferences between infested plants plus host eggs vs. host eggs (*p* = 0.05, vs. *p* = 0.05) and infested plants with host eggs vs. infested plants (*p* = 0.05, vs. *p* = 0.05; Logistic Regression). For other combinations of stimuli, the interaction of physiological and learning status of *P. saccharalis* did not significantly affect their choice between infested plants vs. host eggs (*p* = 0.46, vs. *p* = 0.46), infested plants vs. healthy plants (*p* = 0.35, vs. *p* = 0.35), host eggs vs. healthy plants (*p* = 0.71, vs. *p* = 0.71), and infested plants with host eggs vs. healthy plants (*p* = 0.30, vs. *p* = 0.30; Logistic Regression). The preference of fed wasps for infested soybean plants with host eggs was significantly greater than for host egg alone (76.67/23.33%, Table 4.4). This group of wasps also showed preference for infested soybean plants when compared to host eggs alone (70.00/30.00%, Table 3). Fed parasitoids with (73.33/26.67%) and without (70.00/30.00%,) ovipositional experience, displayed a significant attraction to infested soybean plants compared to the healthy plant (Table 4.4). These findings indicate that the feeding status of *P. saccharalis* played a major role in their attraction to host-induced plant volatiles. Additionally, the learning status of *P. saccharalis* also influenced their attraction to specific stimuli but this effect seems to be more prominent for volatiles induced by feeding damage alone or when feeding damage occurred along with egg deposition.

Table 4.3 Effect of wasp condition (physiological state and learning status) of female *Paratelenomus saccharalis* on the attraction to four stimuli in two choice-Y-tube olfactometer bioassays.

Stimuli Pair (Stimulus 1 vs. Stimulus 2)	Wasp Condition	Attraction % (Stimulus 1/Stimulus 2)	χ2	df	*p*
Infested soybean plus host eggs vs. host eggs	Hungry & Inexperienced	43.33/56.67%	0.53	1	0.46
	Hungry & Experienced	50.00/50.00%	0.00	1	1.00
	Fed & Inexperienced	63.33/36.67%	2.13	1	0.14
	Fed & Experienced	76.67/23.33%	8.53	1	**0.0035**
Infested soybean vs. host eggs	Hungry & Inexperienced	50.00/50.00%	0.00	1	1.00
	Hungry & Experienced	56.67/43.33%	0.53	1	0.465
	Fed & Inexperienced	60.00/40.00%	1.20	1	0.273
	Fed & Experienced	70.00/30.00%	4.80	1	**0.028**
Infested plant vs. healthy plant	Hungry & Inexperienced	53.33/46.67%	0.13	1	0.71
	Hungry & Experienced	60.00/40.00%	1.20	1	0.273
	Fed & Inexperienced	70.00/30.00%	4.80	1	**0.028**
	Fed & Experienced	73.33/26.67%	6.53	1	**0.01**
Host eggs vs. healthy plant	Hungry & Inexperienced	56.67/43.33%	0.53	1	0.46
	Hungry & Experienced	53.33/46.67%	0.13	1	0.71
	Fed & Inexperienced	63.33/36.67%	2.13	1	0.14
	Fed & Experienced	66.67/33.33%	3.13	1	0.06
Infested plant plus host eggs vs. healthy plant	Hungry & Inexperienced	60.00/40.00%	1.20	1	0.273
	Hungry & Experienced	53.33/46.67%	0.13	1	0.71
	Fed & Inexperienced	63.33/36.67%	2.13	1	0.14

	Fed & Experienced	76.67/23.33%	8.53	1	**0.0035**
Infested plant plus host eggs vs. infested plants	Hungry & Inexperienced	53.33/46.67%	0.13	1	0.71
	Hungry & Experienced	50.00/50.00%	0.00	1	1.00
	Fed & Inexperienced	63.33/36.67%	2.13	1	0.14
	Fed & Experienced	76.67/23.33%	8.53	1	**0.0035**

Significant values ($p \leq 0.05$) are in bold font.

4.6.1.2 Effect of physiological and learning status of Ooencyrtus nezarae females on the attraction to test stimuli in Y-tube olfactometer bioassay.

In the no-choice Y-tube olfactometer bioassay, no significant interaction between physiological and learning factors was found for the four tested stimuli (healthy plant: $p = 0.85$, Figure 4.4A; infested plant: $p = 0.87$, Figure 4.4B; host eggs: $p = 0.74$, Figure 4.4C; infested plant plus host eggs: $p = 0.50$, Figure 4D; Logistic Regression). *O. nezarae* showed no preference for specific stimuli based on their hunger and host experience levels. However, fed *O. nezarae* with previous ovipositional experience demonstrated a significantly (73.33/26.67%) higher attraction to the infested plant plus host eggs than the control (Figure 4.4D, Table 4.4). Hungry females with or without previous ovipositional experience exhibited no significant attraction to any tested stimuli compared to the control (Figure 4, Table 4.4). These findings suggest that the attraction of *O. nezarae* to the infested plants with host eggs is dependent on their physiological state and learning status.

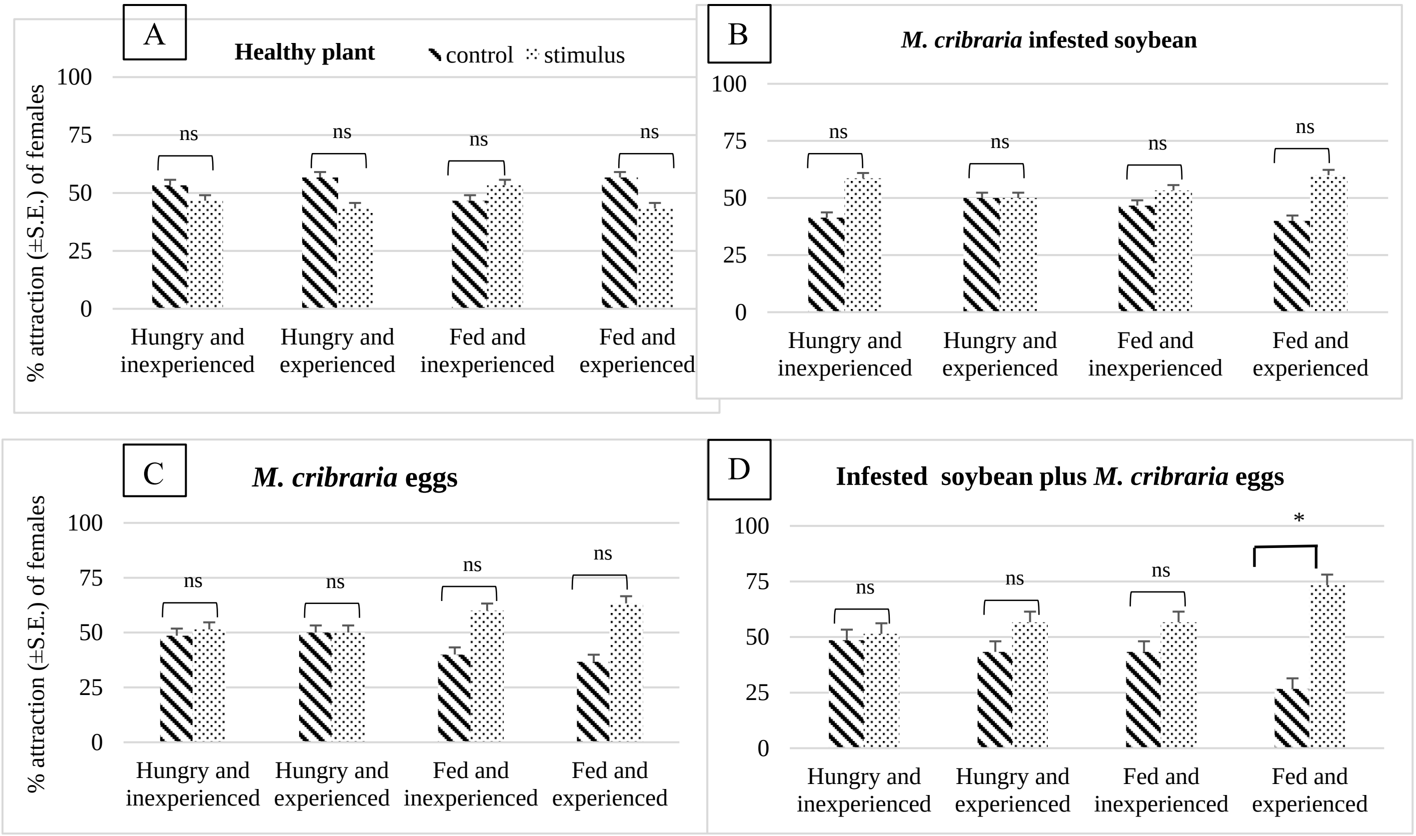


Figure 4.4 Effect of physiological state and learning status on attracting female *Ooencyrtus nezarae* to four odors in no-choice Y-tube olfactometer bioassays. Bars represent the percentage of hungry and inexperienced, hungry, and experienced, fed, and inexperienced, or fed and experienced parasitoids when given a choice between control and (A) healthy soybean plant, (B) infested soybean plant, (C) *M. cribraria* eggs, and (D) infested soybean plant plus *M. cribraria* eggs. *M. cribraria* nymphs (30) and adults (20 pairs) were allowed to infest soybean plants for 24 hours before bioassays. Parasitoids were tested for each physiological states x choice treatment. Asterisk (*) indicates a significant deviation of parasitoid responses from a 50:50 (stimulus: control) distribution (χ2 goodness of fit test, *p* ≤ 0.05). ns = non-significant.

In the two-choice olfactometer the attraction of *O. nezarae* females to any of the stimuli, was not significantly influenced by their physiological state and learning status for the following comparisons using logistic regression: infested plants with host eggs vs. host eggs (p = 0.20, vs. p = 0.20), infested plants vs. host eggs (p = 0.27, vs. p = 0.27), infested plants vs. healthy plants (p = 0.57, vs. p = 0.26), host eggs vs. healthy plants (p = 0.26, vs. p = 0.26), infested plants with host eggs vs. infested plants (p = 0.51, vs. p = 0.73); and infested plants with host eggs vs. healthy plants (p = 0.45, vs. p = 0.30). However, fed wasps that had ovipositional experience showed a significant preference for infested soybean plants over healthy plants (70.00/30.00%; Table 4.5). This group of wasps also favored infested soybean plants with host eggs rather than healthy plants (74.19/25.81%; Table 4.5). These findings suggest that the physiological and learning status of *O. nezarae* have little impact on their attraction to tested stimuli. Instead, *O. nezarae* females were attracted to induced volatiles by plants with feeding damage alone. Notably, their attraction was more pronounced when plant damage occurred along with egg deposition.

Table 4.4 Effect of wasp condition (physiological state and learning status) on attracting female *Ooencyrtus nezarae* to four stimuli in two choice Y-tube olfactometer bioassays.

Stimuli Pair (Stimulus 1 vs. Stimulus 2)	**Wasp Condition**	**Attraction % (Stimulus 1/Stimulus 2)**	**χ2**	**df**	***p***
Infested soybean plus host eggs vs. host eggs	Hungry & Inexperienced	53.33/46.67%	0.13	1	0.71
	Hungry & Experienced	56.67/43.33%	0.53	1	0.465
	Fed & Inexperienced	63.33/36.67%	2.133	1	0.144
	Fed & Experienced	74.19/25.81%	7.25	1	**0.007**
Infested soybean vs. host eggs	Hungry & Inexperienced	43.33/56.67%	0.53	1	0.46
	Hungry & Experienced	50.00/50.00%	0.00	1	1.00
	Fed & Inexperienced	60.00/40.00%	1.20	1	0.27
	Fed & Experienced	66.67/33.33%	3.33	1	0.06
Infested soybean vs. healthy plant	Hungry & Inexperienced	43.33/56.67%	0.53	1	0.46
	Hungry & Experienced	50.00/50.00%	0.00	1	1.00
	Fed & Inexperienced	60.00/40.00%	1.20	1	0.273
	Fed & Experienced	70.00/30.00%	4.80	1	**0.028**
Host eggs vs. healthy plant	Hungry & Inexperienced	43.33/56.67%	0.53	1	0.46
	Hungry & Experienced	53.33/46.67%	0.13	1	0.71
	Fed & Inexperienced	63.33/36.67%	2.133	1	0.144
	Fed & Experienced	66.67/33.33%	3.33	1	0.06
Infested plant plus host	Hungry & Inexperienced	53.33/46.67%	0.13	1	0.71

eggs vs. healthy plant	Hungry & Experienced	60.00/40.00%	1.20	1	0.273
	Fed & Inexperienced	63.33/36.67%	2.13	1	0.14
	Fed & Experienced	76.67/23.33%	8.53	1	**0.0035**
Infested plant plus host eggs vs. infested plants	Hungry & Inexperienced	53.33/46.67%	0.13	1	0.71
	Hungry & Experienced	53.33/46.67%	0.13	1	0.71
	Fed & Inexperienced	56.67/43.33%	0.53	1	0.465
	Fed & Experienced	70.00/30.00%	4.80	1	**0.028**

Significant values ($p \leq 0.05$) are in bold font.

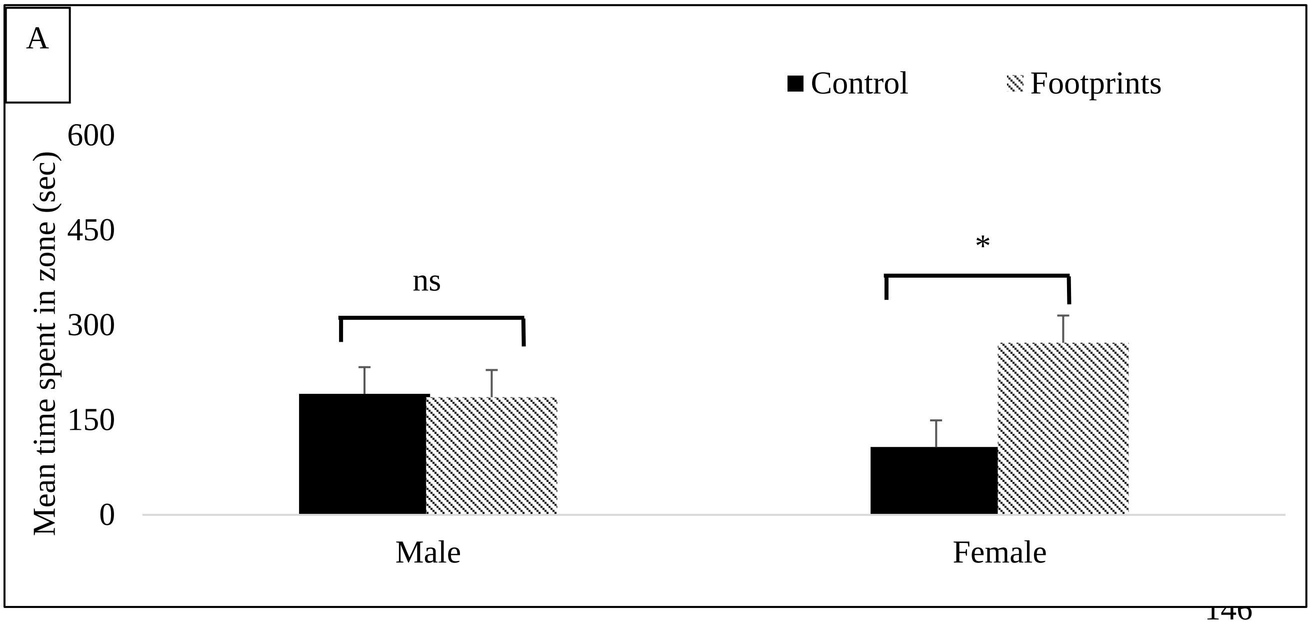
4.4.2 Footprints bioassay

4.6.2.1 Behavioral response of Paratelenomus saccharalis to Megacopta cribraria adult footprints bioassay

The response of *P. saccharalis* females to chemical footprints left on the petri dish by male or female adult *M. cribraria* is illustrated in Figure 4.5A. The *P. saccharalis* did not discriminate between the non-footprints control and host chemical footprints zone in female host footprints trial: $\chi 2 = 0.81$, $p = 0.36$, $N = 30$, and for male host footprints trial: $\chi 2 = 1.97$, $p = 0.16$, $N = 30$. These results were not impacted by the zone the female *P. saccharalis* wasp was introduced into, indicating that the parasitoid behavior was not influenced by positional effects. Female *P. saccharalis* spent significantly more time in the female kudzu bug footprint zone as compared to the control zone (W = 757.50, $p = 0.01$, $N = 30$), but female wasps did not spend significant amount of time in the male kudzu bug chemical footprint zone versus the control zone (W = 863.00, $p= 0.44$, $N = 30$). *Paratelenomus saccharalis* females were able to distinguish between the chemical traces left by host females and males, and results show a clear preference for female traces only (Figure 4.4) because they spent significantly more time in the zone marked by host females than males (W = 1673.0, *p* < 0.0001, *N* = 35).

4.6.2.2 Behavioral response of Ooencyrtus nezarae to Megacopta cribraria adult footprints bioassay

The female *O. nezarae* did not discriminate between the non-footprints control and footprints zone for the female host footprints trial: χ2 = 0.01, *p* = 0.90, *N* = 36, or for the male host footprints trial: χ2 = 1.82, *p* = 0.17, *N* = 32. There was no positional effect influencing parasitoid behavior regardless of which zone the parasitoid was introduced into first. Female *O. nezarae* spent a significantly longer time in the female footprint zone compared to control zone (W = 1002.00, *p* = 0.0004, *N* = 36; Figure 4.5B), but female wasps did not spend a significant amount time in the male footprint versus the control zone (W = 932.50, *p* = 0.146, *N* = 32; Figure 4.6). This suggests a high level of attraction of *O. nezarae* to *M. cribraria* female footprints. Similarly, no significant difference was observed between the total time spent by female *O. nezarae* in the zone marked with female host footprints zone versus male zones (W=1328.0, *p* = 0.87, *N* = 36; Figure 6).



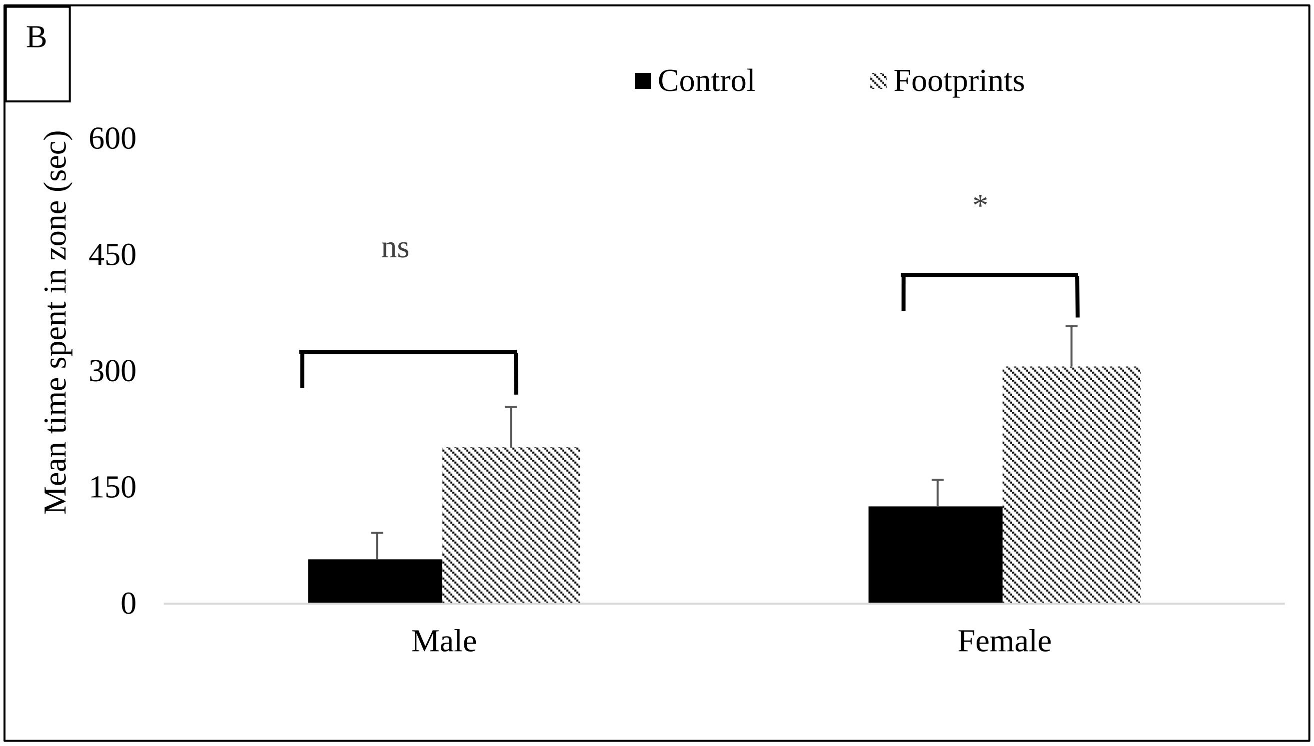


Figure 4.5 Residence times of female *Paratelenomus saccharalis* (A) and *Ooencyrtus nezarae* (B) in the control zone of a Petri dish arena relative to the chemical footprints of adult male or female kudzu bugs. Residence times of female *Paratelenomus saccharalis* (A) and *Ooencyrtus nezarae* (B) in the control zone of a Petri dish arena relative to the chemical footprints of adult male or female *M. cribraria*. Bars show the mean (± S.E.) of the total time spent (s) in zones by parasitoids. *N* = 30 per species. Asterisks (*) indicate a significant difference between residence time in the footprint zone versus control zones (zones without footprints) (p < 0.05; The Man-Whitney U test).). ns indicates no significant difference.

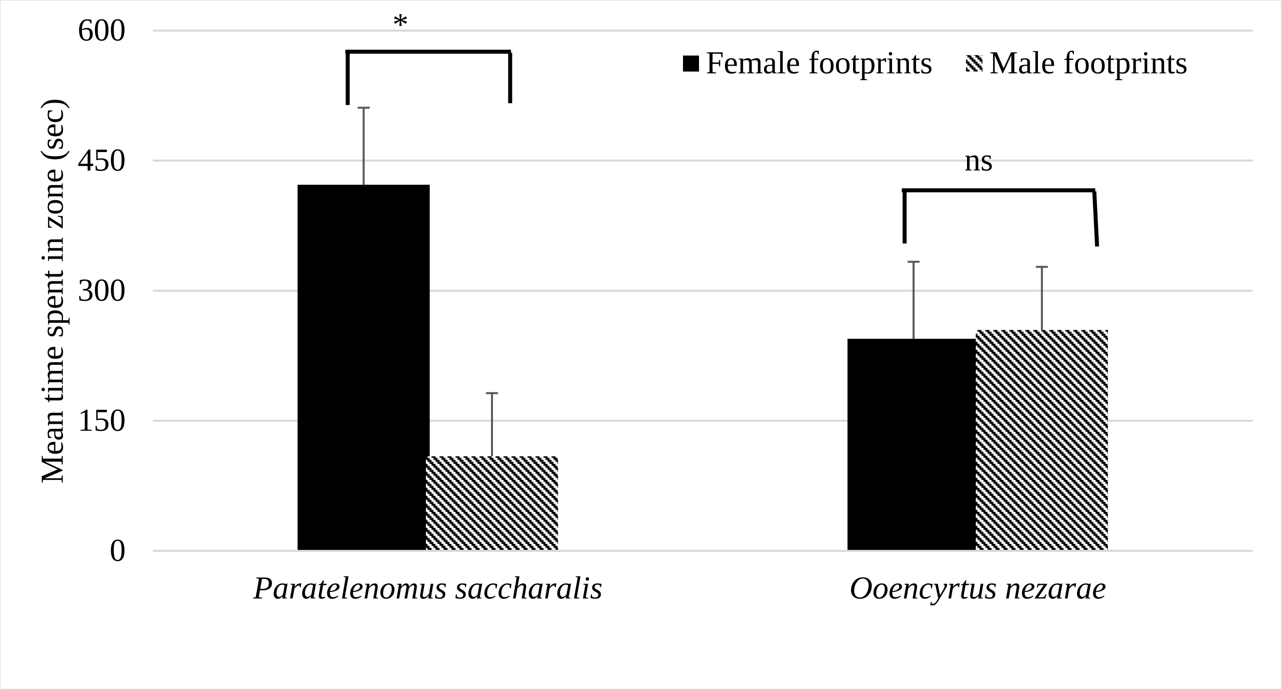


Figure 4.6 The residence time of females *Paratelenomus saccharalis* (*N*=35) and *Ooencyrtus nezarae* (*N*=36) exploring a petri dish contaminated with chemical footprints laid by three adult males and females of kudzu bugs. Bars show the mean (± S.E.) of the residence time of wasp females. Asterisks (*) indicate a significant difference between residence time in the female footprint zone versus the male footprint zone ($p < 0.05$; The Man-Whitney U test). ns. indicates no significant difference.

4.6.2.3 Comparing behavioral response of Paratelenomus saccharalis and Ooencyrtus nezarae to Megacopta cribraria adult footprints

The two parasitoid species showed key differences in their behavioral responses to male and female host footprints. Both parasitoid species exhibited different track patterns, with longer tracks observed for *P. saccharalis* (Figure 4.8A-D) and shorter tracks for *O. nezarae* (Figure 4.7E-H) when host footprints were compared with the control zone. Female *P. saccharalis* spent relatively more time in the zone marked with female host footprints (W=1653.0, $p < 0.0001$, $N = 35$; Figure 4.9). There was no significant difference in the time spent searching by both wasp species (W = 1006.5, $p = 0.38$; Figure 4.9) which suggests that both *O. nezarae* and *P. saccharalis* did not show a preference for spending more time in the male or female host footprint zone. Residence time results can be visualized from representative heat maps for *O. nezarae* (Figure 4.7I-J) and *P. saccharalis* (Figure 4.8K-L).

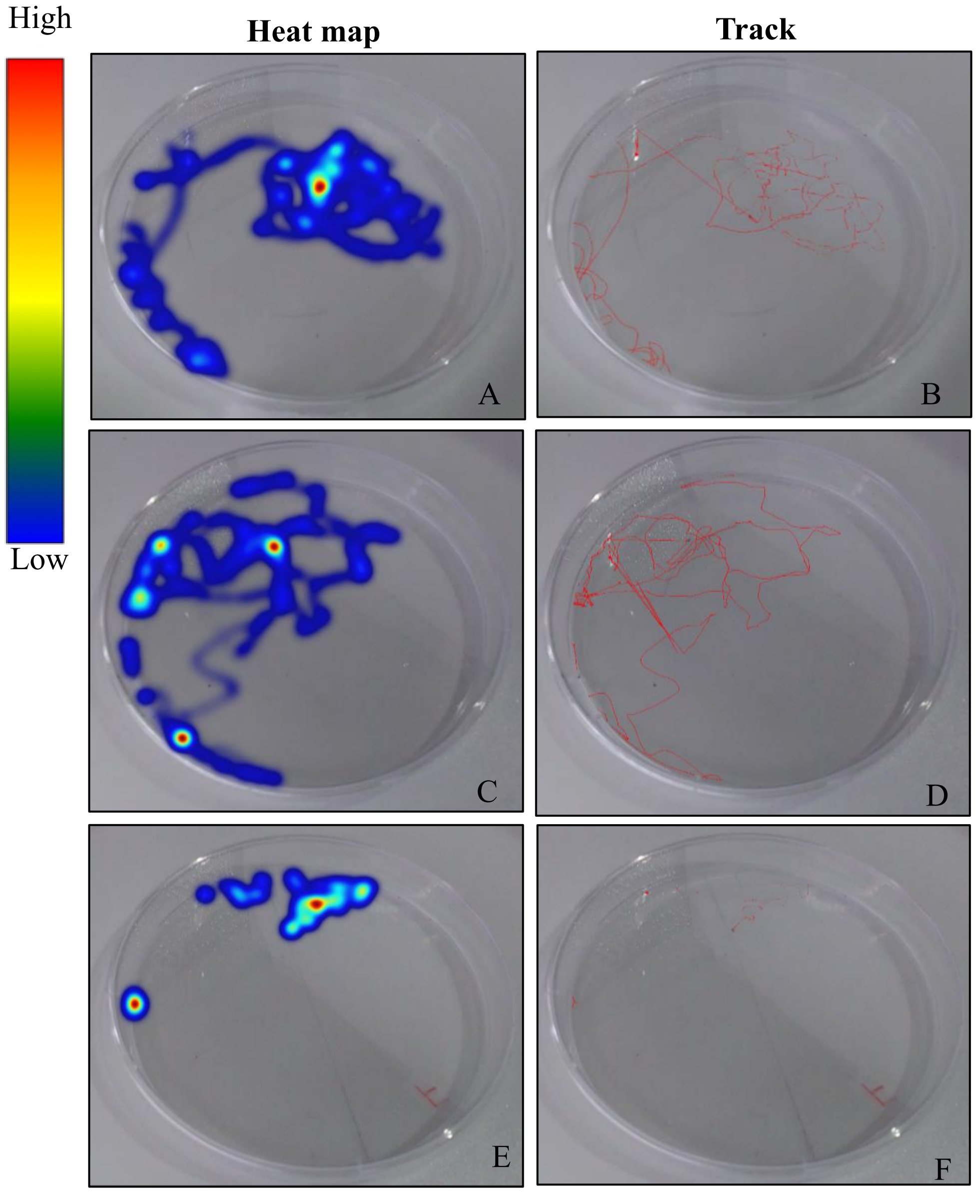


Figure 4.7 Representative heat maps and tracks of female *Ooencyrtus nezarae* in a Petri dish arena. Heat maps use a color gradient to show the relative amount of time spent in different arena locations while tracks represent the actual movement path of wasps. Female *O. nezarae* in the presence of only female host footprints (A-B)—footprints are in the inner zone and the outer zone is without host footprints; male host footprints (C-D)—footprints are in the inner zone and outer zone is without host footprints; both host male and female footprints (E-F) — female footprints are in the right side and male in the left side.

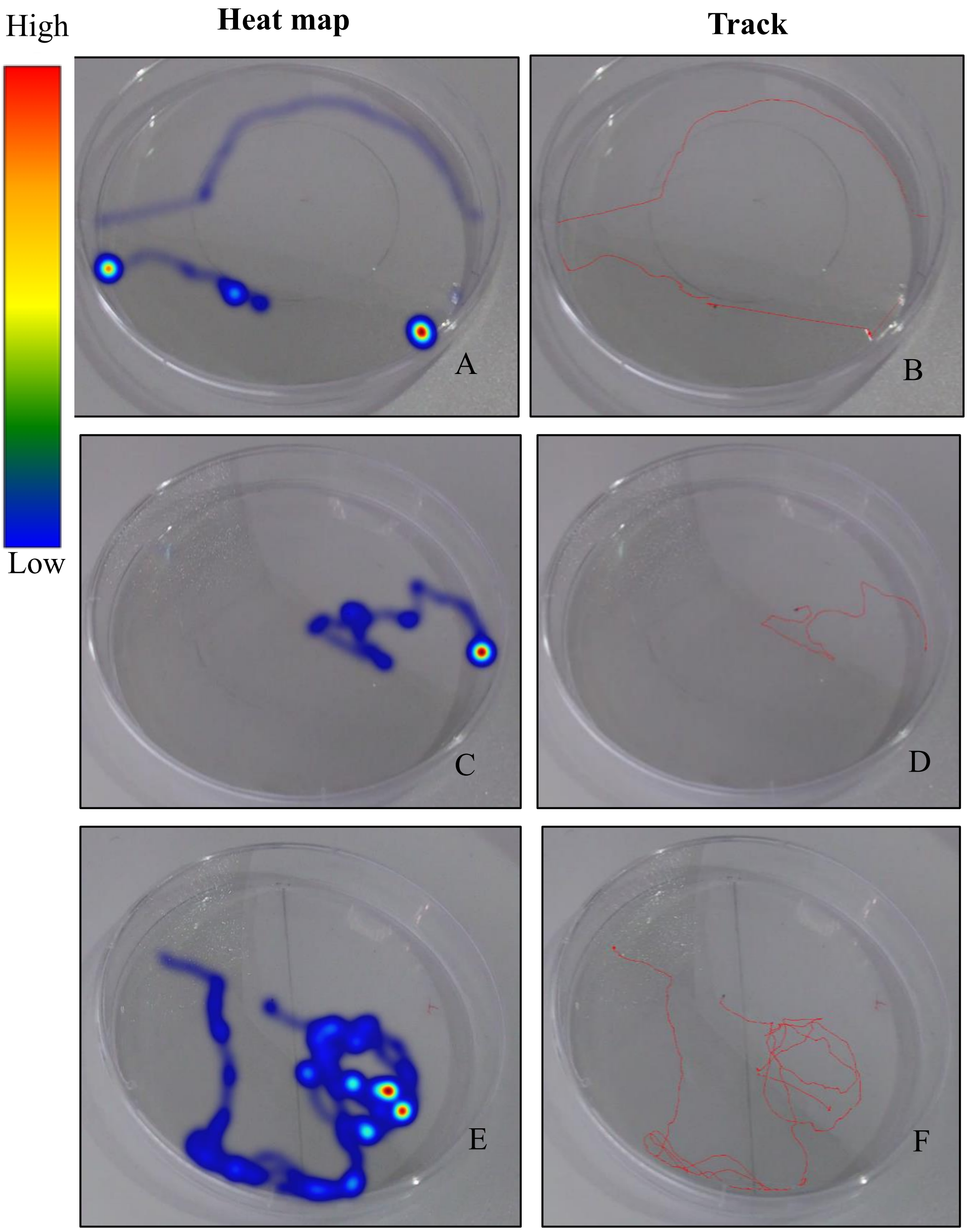


Figure 4.8 Representative heat maps and tracks of female *Paratelenomus saccharalis* in a Petri dish arena. Heat maps use a color gradient to show the relative amount of time spent in different arena locations while tracks represent the actual movement path of wasps. Female *P. saccharalis* in the presence of only female host footprints (A-B)—footprints are in the inner zone and the outer zone is without host footprints; male host footprints (C-D)—footprints are in the inner zone and outer zone is without host footprints; both host male and female footprints (E-F) — female footprints are in the right side and male in the left side.

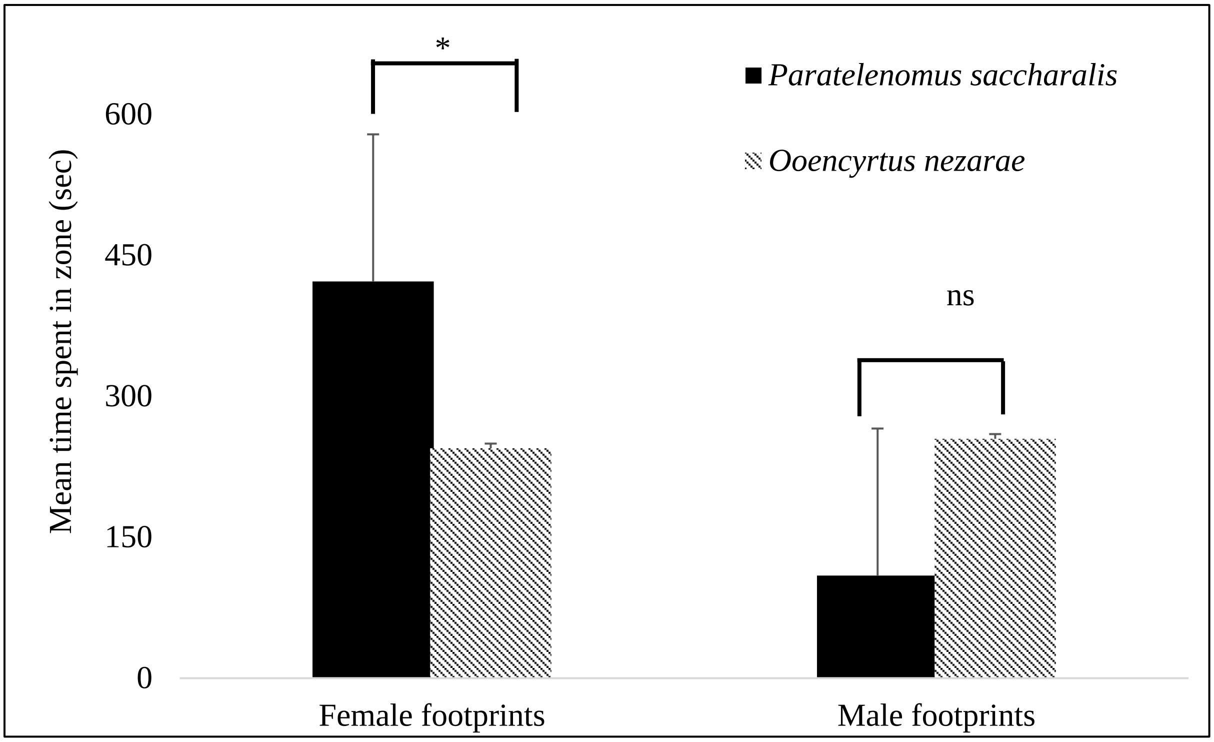


Figure 4.9 Residence times of female *Paratelenomus saccharalis* (sample size, *N*=35) and Ooencyrtus nezarae (sample size, *N*=36) in the petri dish arena marked with chemical footprints of *M. cribraria* adults. Bars show the mean (± S.E.) of the total time spent (sec) in zones by parasitoids. Asterisks (*) indicate a significant difference in the total amount of time spent in a zone by *P. saccharalis* versus *O. nezarae* ($p < 0.05$; Man-Whitney U test)

4.5 DISCUSSION

In this study, we investigated the host-seeking behavior and preferences of parasitoids using both olfactometer and footprint bioassay experiments. The olfactometer assays allowed us to examine the parasitoids' ability to detect and respond to host-related olfactory cues while the footprint bioassays provided insights into their movement patterns and interactions with male and female adults in a confined space. Our findings revealed important information about the parasitoids' host selection process, which has implications for understanding their ecological roles and potential applications in biological pest control. In the following discussion, we explored the significance of these results, compare them with previous research, and address the limitations and future directions of our study.

4.5.1 Y-tube olfactometer bioassay

The olfactory responses of parasitoids to odor cues during foraging can be influenced by a range of physiological factors, such as feeding and learning status. However, this study found that the olfactory responses of female *O. nezarae* and *P. saccharalis* were somewhat unaffected by their feeding or learning status when they were exposed to host-associated stimuli. Both well-fed and hungry parasitoids displayed almost similar levels of attraction to the test stimuli. Likewise, ovipositional experienced and inexperienced females showed little or no difference in their attraction to test stimuli (Figures 3 and 4). Although being well-fed may diminish the host-searching ability of female parasitoids in certain species, it does not necessarily result in a decline in odor perception across all species. Previous studies have indicated that relatively well-fed parasitoids may exhibit enhanced abilities to locate and parasitize suitable hosts, leading to increased reproductive success and overall fitness in comparison to their hungry counterparts (Lee and Heimpel 2008, Tunçbilek et al. 2012, Coskun and Emre 2015). This phenomenon could be partially attributed to the deterioration of some odorant processing apparatus or a reduction in energy levels among hungry parasitoids, which in turn may impact their foraging activities.

Studies also have indicated that the learning status of female parasitoids can influence their host foraging behavior and parasitization aptitude (Vet and Dicke 1992, Peri et al. 2006, Busto et al. 2010, Kruidhof et al. 2013, Bertoldi et al. 2021). According to this study, the learning status of both female parasitoid species did not play a major role in their olfactory responses to test stimuli because *O. nezarae* and *P. saccharalis* females, whether ovipositional experienced or inexperienced, were likely to utilize VOCs emitted by released by their host or their host food source. This finding is consistent with results obtained for the parasitoid, *Lariophagus distinguendus* (Först.) of the granary weevil, *Sitophilus granarius* (L.), which was attracted to host-associated chemical stimuli irrespective of its oviposition experience (Steidle and Schöller 1997).

Herbivore induced plant volatiles had significant effects on the parasitoids' attraction. It is well-documented that the feeding and oviposition activities of herbivorous insects trigger the release of volatiles that subsequently attract egg parasitoids (Colazza et al. 2004, 2007, Moraes et al. 2008). For instance, *Trissolcus basalis* responded to both synomones induced by feeding and egg deposition by *N. virudula* on fava and common beans (Colazza et al. 2004). Female *P. saccharalis* and *O. nezarae* showed increased attraction to infested soybean plants with host eggs (Tables 4.3 and 4.4). This finding suggests that the combination of plant volatiles released due to feeding damage and egg deposition are reliable cues for parasitoids to locate their hosts and essential components in the host-location process.

4.5.2 Footprints bioassay

Numerous studies have reported that several organisms have evolved adaptations to optimize the utilization of prey/host chemical footprints as odor cues for actively locating prey/host (Conti and Colazza 2012, Burrows et al. 2018, Cingolani et al. 2019). For instance, *Trissolcus basalis* females employ a sex-specific cuticular hydrocarbon from host *Nezarae viridula* males to distinguish between male or female bug residues (Peri et al. 2013). In this bioassay the behavioral responses of the parasitoids, *P. saccharalis* and *O. nezarae*, to the footprints of *M. cribraria* adults responsible for ovipositing their egg hosts, were tested in a Petri dish arena. Both parasitoid species displayed distinct track patterns when encountering female and/or male host chemical footprints. The results of the bioassay without a choice indicated that both parasitoid species are highly likely to pinpoint female host footprints upon entering the arena (Figure 4.5). The results of the bioassay with a choice demonstrated the innate ability of *P. saccharalis* to discriminate between male and female *M. cribraria* adults in which female host chemical footprints elicited a stronger attraction compared to male footprints (Figure 4.6A). Intriguingly, *O. nezarae* females did not discriminate between the footprints of either sex (Figure 4.6B).

The strength of female wasps' responses to the chemical traces left behind by their hosts may be seen as a component of the pairwise coevolution between insect hosts and their parasitoids, driven by natural selection (Godfray 1994, Vinson 998). Throughout all trophic levels, organisms do not evolve in isolation. Instead, they coevolve in either antagonistic or, less commonly, mutualistic ways, leading to varying advantages and fitness compromises (Loxdale et al. 2011). In the context of foraging parasitoids, one could hypothesize co-evolutionary interplay between the host and the parasitoid. For instance, wasps selectively search for hosts in high-quality areas, prompting hosts to lay eggs in less favorable locations as a co-evolutionary countermeasure (Schreiber et al. 2002). The capacity of *P. saccharalis* and *O. nezarae* to distinguish between male and female host footprints might be a crucial tactic for locating the egg clusters of *M. cribraria*, since this pest typically deposits eggs at considerable distances from where adults feed and mate (Ritchey 2019).

Comparing the behavioral responses of *P. saccharalis* (specialist) and *O. nezarae* (generalist) parasitoid species, *P. saccharalis* (Figure 4.8) exhibited a stronger attraction towards chemical traces left by host females than *O. nezarae* (Figure 4.7). This finding aligns with the hypothesis that specialist parasitoid species have an evolutionary advantage over generalist species when to use specific cues (Vet and Dicke 1992, Steidle and Van Loon 2003). when utilizing the chemical footprints of female host footprints (Peri et al. 2013). Stronger attraction towards female host traces of *P. saccharalis* may have evolved a more precise strategy to optimize its reproductive success within its limited host range.

4.6 CONCLUSIONS

To our knowledge, this is the first study to compare the responses of *P. saccharalis* (specialist) and *O. nezarae* (generalist) parasitoids to *M. cribraria-associated* chemical volatiles. In conclusion, Y-tube olfactometer study findings suggest that physiological and learning status do not play a major role in modulating olfactory responses of *P. saccharalis* and *O. nezarae* to host-related odors. Instead, the plasticity of olfactory response may be limited in parasitoids due to a strong innate sensitivity to host-related odor cues. This may have an impact on their potential as biological control agents. Other physiological factors, such as age and mating status may also significantly affect olfactory plasticity in parasitoids. This creates an opportunity for the augmentation of parasitoids after field releases. Future studies, especially in the field, should investigate the effect of other physiological conditions that may affect the plasticity of behavioral response to host-related odors in natural enemies.

The footprints study reported a chemical-mediated interaction in which two parasitoid species showed varying degrees of attraction to host-associated chemicals. Evolution might favor specialist over generalist parasitoid species in using host-associated volatiles as cues during the host foraging process to facilitate host location. Such an adaptive strategy favors specialists to efficiently exploit their host's chemical cues, leading to more effective host detection in their specific ecological niche. Future studies should investigate the mechanism(s) behind the allomone-kairomone function of *M. cribraria* regurgitant and should consider the responses to host-associated chemicals on the natural substrate such as leaf, stem, and petiole, especially in the context of their natural environment. This study highlights the possible impact of host specialization on the ability of parasitoids to forage host, with implications for the evolution of parasitoid-host interactions.

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Chapter 5

DIRECT AND INDIRECT COMPETITIVE INTERACTIONS BETWEEN *OOENCYRTUS NEZARAE* AND *PARATELENOMUS SACCHARALIS* PARASITIZING *MEGACOPTA CRIBRARIA* EGG PATCHES

5.1 INTRODUCTION

Adult female parasitoid wasps deposit their eggs in or on the body surface of other arthropods, which act as the host for developing larvae (Harvey et al. 2013). In general, parasitoids oviposit on an immature stage of the host, e.g., egg(s), larva(e), or pupa(e). The host is then consumed by the developing parasitoid larva(e). Parasitoid species may rely on shared common resources (e.g., host species); thus, interactions among these parasitoid species can occur frequently (Price, 1972; Bogran et al. 2002; Polis and Strong; 1996; Harvey et al. 2009; Brodeur and Rosenheim, 2000). Researchers have debated whether the occurrence of multiple parasitoids has a beneficial or antagonistic effect on pest suppression in agricultural systems. According to some studies, multiple parasitoids might work in tandem to greatly reduce pest populations through direct competition (Cusumano et al. 2012; Mohammadpour et al. 2014; Frago, 2016). Other research has shown that competition among parasitoids may impair pest suppression and disrupt biological control through indirect competition or intraguild predation of larvae (Veen et al. 2001). Understanding the interspecific competition between multiple parasitoids in relation to their shared host(s) is critical to determining the efficacy of biological control programs (Hassell and Varley, 1969; Hassell, 1971).

Parasitoids do not immediately remove prey from the habitat like predators, and intact hosts can still be exploited by conspecific or interspecific competitors which may result in indirect competition (Boivin and Brodeur, 2006). The outcome of parasitoid competition can be characterized as direct or indirect (Zwolfer, 1971). Direct competition involves interaction between two or more individuals that utilize the same resource. Direct competition can occur when adult parasitoids search and compete for the same host resource or among immatures that develop on/in the same host (i. e. superparasitism or multiparasitism) (Harvey et al 2013; Boivin and Brodeur, 2006; Zwolfer, 1971; Rosenheim et al. 1985; Moraes et al. 1999). An adult female's direct competition with a conspecific/heterospecific female can be lethal if she engages in fighting behaviors for egg mass (patch) ownership (Cusumano et al. 2016). The intensity of agonistic behaviors has been shown to escalate in specialist parasitoids because resources are limited as compared to generalists (Overington et al. 2008). For immature parasitoids, the presence of more than one larva in a host may alter host quality, quantity, and modify the successful emergence rate of the wasp by leading to the death of the weaker competitor or increase variability in body size among competitors (Boivin and Brodeu, 2006). As a result, the deposition of eggs in or on a parasitized host generally reduces the fecundity of ensuing adults. Dorn and Beckage (2007) reported that the number of emerging adults decreases as the number of immature individuals in the host increases. This phenomenon occurs commonly in solitary parasitoids where only one larva can develop inside the host (Gandon et al. 2006). Despite this, it has been observed that parasitoids with a broad host range (i.e., generalist species) are typically more likely to exploit already parasitized hosts (Broduer, 2000; Hunter and Woolley, 2001). Host quality can also impact sex ratios. According to local mate competition (LMC) theory, when high-quality hosts are limited, female wasps prefer to oviposit male eggs on poor-quality hosts.

In the haplodiploid mating system of parasitoids, female parasitoids produce more males if poor-quality hosts predominate, so multiparasitism and the prospects of poor host quality may result in male-biased sex ratios.

Adult parasitoid competitive success depends on patch allocation time and the decision to invest in the defense of an exploited patch or seek out an unparasitized patch (Cusumano et al. 2016). Many studies have shown that the first parasitoid that oviposits generally outcompetes later individuals of competitor species (Moraes et al. 1999; Tillman and Powell, 1992). There are two possible explanations in this case: the established larvae would have (i) consumed most of the nutrition of the host, or (ii) killed eggs or larvae and monopolized the host (Harvey and Porlman, 2013). Parasitoid larvae may eliminate competitors through physical attack or physiological suppression (Moraes et al. 1999; Uka et al. 2006). The first instar larvae of many parasitoids possess large mandibles which may provide them with a competitive advantage over first instars that possess small mandibles (Laing and Corrigan; 1987; Kfir and Hamburg,1988). Physiological suppression includes toxin secretion, asphyxiation, deprivation of nutrients, and hormonal interference (Wang and Messing, 2003). The order of oviposition and time lag between oviposition events can also influence the competition’s outcome (Moraes et al. 1999; Tillman and Powell, 1992).

Kudzu bugs, *Megacopta cribraria* Fabricius (Hemiptera: Plataspidae), and their egg parasitoids can be used as a model host-parasitoid system to evaluate the outcome of interspecific competition. Two parasitoids, a generalist *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae), and a specialist *Paratelenomus saccharalis* Dodd (Hymenoptera: Platygastridae), co-occur and parasitize kudzu bug eggs in Southeast Asia and the southeastern United States

(Takasu and Hirose, 1985, 1986; Hoshino et al. 1985). Immatures of both parasitoids feed and develop inside of the host eggs (Alim, M.A.; Lim, 2014; Knight et al. 2017).

Paratelenomus saccharalis is a primary and solitary endoparasitoid of the Plastaspid family (Johnson, 1996). To date, host data of *P. saccharalis* in the United States has only been reported from *M. cribraria e*ggs. Another potential host of *P. saccharalis* is *Brachyplatys subaeneuus* Westwood (Hemiptera: Plataspidae), which was reported in Miami, Florida in 2020 (Eger et al., 2020). *Ooencyrtus nezarae* is a generalist egg parasitoid reported to attack *M. cribraria*, and pentatomids that occur in Asia, Africa, Australia, and Europe (Hirose, 1996; Mizutani, 1997; Takasu and Hirose, 1985), and in 2016 was first reported in Alabama (Ademokoya et al. 2018). *Ooencyrtus nezarae* is a gregarious parasitoid in eggs of larger hosts, but in eggs of *Megacopta spp.*, it is usually a solitary parasitoid (Takasu and Hirose, 1991). In their native range of southeast Asia, *P. saccharalis* and *O. nezarae* have been reported to parasitize *M. cribraria* eggs at rates of 100% and 76.9%, respectively (Takasu, K.; Hirose, 1985; 1986). In Alabama, *P. saccharalis* and *O. nezarae* have been observed parasitizing kudzu bug eggs in the same soybean field with rates ranging from 42–95% and 82.8–100%, respectively (Ademokoya et al. 2018), but competition between *O. nezarae* and *P. saccharalis* has not been studied in this region. During a three-year field study (2018–2020) in central Alabama, the number of *P. saccharalis* fell sharply. The population of *P. saccharalis* reached its lowest level near zero throughout the collection period since population monitoring began in 2013 (Chicas-Mosier et al. 2022). A decline of *P. saccharalis* (specialist species) is concerning because this may disrupt the biocontrol of *M. cribraria*. One potential explanation is the arrival of *O. nezarae* in 2016 in Alabama (Ademokoya et al. 2018). Research is needed to understand how competitive interaction between these two parasitoid species impacts host suppression, and whether competitive interference may disrupt biocontrol.

The present study investigates competitive interaction through a series of experiments. Experiments were conducted to evaluate (i) the outcome of multiparasitism when *O. nezarae* and *P. saccharalis* are released simultaneously or sequentially (i.e., emerged wasp vs. host nymph), (ii) larval competition between *O. nezarae* and *P. saccharalis*, (iii) behavior of *P. saccharalis* adults when they directly interact with adult *O. nezarae* on host patches, and (iv) the outcome and consequences of intraguild predation of larvae. It is hypothesized that multiparasitism will increase larval mortality because the quality of host tissues is reduced in parasitized hosts, and will result in a decrease in fitness of surviving larvae (Potting et al. 1997). We also hypothesized that the specialist *P. saccharalis* adults will win direct competitions against *O. nezarae* as either the prior owner of the host or the intruder because specialists are more efficient in resource utilization (Singer and Stireman, 2003) and more likely to be aggressive over limited and valuable resources (Dubois, 2003; Benelli, 2015; Christopher, 2020). *Paratelenomus saccharalis* was also expected to outcompete *O. nezarae* during larval competition due to first instar *O. nezarae* larvae possessing small mandibles and remaining attached to their respiratory stalks within a host egg, which limits their mobility and ability to defend themselves against Platygastrid larvae that usually have large and sickle-shaped mandibles (Mohammadpour et al. 2014; Takasu, K.; Hirose, 1989; Cusumano et al. 2011).

5.2 MATERIALS AND METHODS

5.2.1 Plants

Soybean seeds (var. Pioneer P49T97R-SA2P) were planted into pots (15.24 cm diameter and 14.22 cm depth) in Sunshine potting mixture #8 (SunGro Horticulture, WA, USA) and grown in an incubator free of pests and pesticides at (26 ± 2 °C and 55 ± 5% RH) [30]. Plants were watered daily (~200 mL per pot) and fertilized according to the manufacturer's instructions (Scotts-Sierra Horticultural Product Company, Marysville, OH, USA) once a week until use for *M. cribraria* rearing.

5.2.2 Insect rearing

A colony of adult kudzu bugs was established by collecting insects from kudzu, *Pueraria montana* (Lour) Merr., in Auburn, AL (32.5934° N, 85.4952° W) from late March to May 2021. They were reared in ventilated plastic cages (30 cm × 30 cm × 30 cm) (BugDorm-2, Megaview Science Education Services Co., Ltd., Taichung, Taiwan) at 25 ± 1°C, 14:10 h (L: D), and 75 ± 5% RH [30] in a growth chamber (Percival, Perry, IA, USA.) and provided organic green beans and a vegetative-stage (V2-V3) soybean plant, approximately18 to 27 cm tall, that could easily fit into the cage. New soybean plants were provided every week (Ademokoya et al. 2017). Cages were checked every day for fresh eggs (≤24 h) that appeared milky white in appearance as compared to aged eggs (>24 h) that were darker in color. Each experiment used ≤24 h old kudzu bug eggs (24–30 eggs).

Parasitoid species used in these experiments originated from the same kudzu patches in Auburn, AL, as described above. Collected egg masses with parasitized kudzu bug eggs (grey color) [48] were kept in a 59.1 mL portion cup (Dart container corporation, Mason, MI, USA) at

25 ± 1 °C, 14:10 (L:D) h and 75 ± 5% RH (Ademokoya et al. 2017) until emergence. Parasitoids were identified as *O. nezarae* or *P. saccharalis*, based on details given in Takasu and Hirose (1991) and Gardner et al. (2013). Both species were separated into different rearing cups (59.1 mL), and colonies were reared by providing adults with a honey solution (70% *v*/*v*) and allowing them to oviposit into fresh kudzu bug eggs (≤24 h old). The honey solution was held in a 0.5 mL Eppendorf microcentrifuge tube with a hole in the bottom through which a cotton string was threaded. Another microcentrifuge tube filled with water was placed to control relative humidity in each cup. This tube was perforated above the water line to dissipate moisture vapor throughout the tube to prevent insect desiccation. Approximately 20 holes were made on the cup wall with a pin for aeration and to prevent condensation. Parasitoid rearing cups were maintained at 25 ± 1°C, 14L:10D h, and 75 ± 5% RH in an incubator (Percival, Perry, IA, USA). These same conditions were used for experiments described below.

5.2.3 Physiological condition of insects

In all experiments, one- and four-day-old *P. saccharalis* and *O. nezarae* adult, respectively, were used (Aung et al. 2010; Takano and Takasu; 2019). This differential age range was chosen to coincide with each wasp's reproductive peaks so that optimum reproduction is represented in these experiments. Within one day of emergence (egression of wasps from host eggs), individual *O. nezarae* wasp females and males were held together for 96 h to ensure mating occurred quickly after emergence (Ganjisaffar and Perring ; 2020). Only female *P. saccharalis* have emerged from field-collected *M. cribraria* eggs. We also observed that these emerged *P. saccharalis* females were able to make offspring without mating. Therefore, in all experiments, we used unmated *P. saccharalis* females. Both species were fed 70% honey solution and were naïve (never had oviposition experience) before use in the experiments.

5.2.4 Experiment I: Timing of adult arrival at the competition

This objective investigated the role of species oviposition order, and the time interval between oviposition of heterospecific females, on the outcome of parasitoid emergence. The following three combinations of wasp species introductions were tested at four-time intervals (12, 24, 48, or 72 h) each: (1) Simultaneous release: a *P. saccharalis* female and an *O. nezarae* female were released together for the total time interval (*P. saccharalis* + *O. nezarae*); (2) Sequential release i: one female *P. saccharalis* was released first for half of the total time trial, then it was removed and an *O. nezarae* female was introduced for the remaining half of the time (*P. saccharalis → O. nezarae)*; and (3) Sequential release ii: one female *O. nezarae* was released first for half of the total time trial, then it was removed, and a *P. saccharalis* female was introduced for the remaining half of the time (*O. nezarae → P. saccharalis*). Wasps from all treatments were removed from the experimental arenas (59.1 mL cup with dimensions of top diameter: 6 cm, bottom diameter: 4.4 cm, and height: 2.8 cm) after the time interval was completed. Host eggs exposed to wasps were incubated until offspring emerged as adults. The total number of live offspring was counted for each species, and unhatched or unparasitized eggs of *M. cribraria* were counted as ‘unascribed’.

For each treatment (order of oviposition × time interval), 20 replications were performed. The minimum exposure (12 h) and host egg density (24–30 kudzu bug eggs per replicate) were chosen based on the previous studies for *P. saccharalis* (Takano and Takasu; 2019) and preliminary studies for *O. nezarae* (unpublished data) that demonstrated both species alone can parasitize at least one egg/h. Increasing host-limitation over extended periods (e.g., 72 h and 24 eggs) would increase the likelihood of interspecific competition.

5.2.5 Experiment II: Characterizing aggressive behavior of parasitoids

The purpose of this experiment was to record any aggressive behavior of *P. saccharalis* or *O. nezarae* when encountering a heterospecific on the same host patch of kudzu bug eggs. One female *P. saccharalis* and *O. nezarae* were simultaneously released into a Petri dish (60 mm × 15 mm) with kudzu bug eggs. A total of 20 replications were conducted for this experiment. Their behavior was recorded for 1 h after their release in the experimental arena. This is the time (1 h) necessary for oviposition. However, these behaviors typically begin within the first 4 min (personal observation), and a female was discarded from the trial if she did not show oviposition behavior within 4 min. A handheld video camera mounted on a tripod stand was used to record the parasitoid behavior through a microscope eyepiece (Model no. SZ2-ILST, Olympus, Tokyo, Japan, magnification 5.6×). The number of confrontations were recorded and included wing waving, chasing, head butting, and boxing with forelegs (Benelli and Canale; 2016).

5.2.6 Experiment III: Intraguild predation of parasitoid larvae

Intraguild predation of *O. nezarae* on *P. saccharalis* was assessed. It was a unidirectional experiment in which *O. nezarae* was released after *P. saccharalis* because the former is a generalist species, and it has been observed that species with a broad host range generally act as hyperparasitoids that feed and oviposit on hosts and primary parasitoids [54]. A naïve female *P. saccharalis* was offered kudzu bug eggs for 24 h, and then removed. After removing *P.*

saccharalis, host eggs were held for 0, 24, 48, 72, or 96 h before exposing them to a mated, naïve female *O. nezarae* for 24 h to determine larval and pupal predation on *P. saccharalis*. Eggs exposed to *P. saccharalis* were held for a maximum of 96 h because it takes 168–192 h for kudzu bug eggs to hatch, the eggs were approximately 168 h old after exposure to *O. nezarae*. After exposing *O. nezarae*, host eggs were incubated until the emergence of *P. saccharalis* and *O. nezarae* offspring. Twenty replicates were conducted for this experiment.

5.3 STATISTICAL ANALYSIS

All statistical analyses were conducted with SAS (Ver. 9.4, SAS Institute, Cary, NC, USA). Interspecific competitive interaction results from Experiment I were analyzed using a generalized linear mixed model (GLIMMIX) with a normal distribution. The mean proportion of parasitism (parasitized eggs/total eggs), the proportion of kudzu bug nymphs (nymphs/total host eggs), the proportion of wasp offspring (total wasp offspring/total host eggs), and the sex ratio of wasps that emerged from eggs (females/total wasps) were compared among treatments. A two-way ANOVA using a simulated test for mean comparisons was used to analyze the independent variables host exposure time (12, 24, 48, and 72 h) and order of oviposition (*O. nezarae* + *P. saccharalis*, *O. nezarae* → *P. saccharalis*, and *P. saccharalis* → *O. nezarae*).

In Experiment II, the frequency of confrontation was calculated as the number of encounters divided by the length of the period (1 h) per replicate. All frequencies were summed together and then divided by sample size (*n* = 20) to calculate the average confrontation frequency. We also reported characterized aggressive behaviors including wing waving, chasing, head butting, and boxing with forelegs.

The same parasitism metrics were collected for Experiment III as defined for Experiment I. The independent variables of this experiment were time intervals (0, 24, 48, 72, or 96 h), wasp species (*O. nezarae* and *P. saccharalis*), and their interaction. Data were analyzed using a one way-ANOVA in Proc GLIMMIX with a normal distribution and a post hoc mean comparison was performed using the simulated test.

5.4 RESULTS

5.4.1 Experiment I: The timing of adult arrival at the competition

Summary statistics by release order of parasitoids are provided in Table 5.1. Order of release influenced the host parasitism and strength of interspecific competition, represented by the wasp emergence. The proportion of parasitized eggs was the highest when *P. saccharalis* arrived earlier or together with *O. nezarae*. However, the proportion of emerged wasps from parasitized eggs was significantly higher when both competitors arrived simultaneously at the host patch, intermediate when *P. saccharalis* arrived first, and lowest when *O. nezarae* arrived before *P. saccharalis* (for statistics, see Table 5.1).

Table 5.1 The proportion of parasitized host eggs and emerged wasps averaged across treatments in experiments conducted to examine how the order of Ooencyrtus nezarae and/or Paratelenomus saccharalis adult arrival at a Megacopta cribraria egg patch influences competition.

	(Proportion ± SE)	
Treatment	*M. cribraria* Parasitized Eggs	Emerged Wasp
Simultaneous release	0.50 ± 0.025 ^{a}	0.43 ± 0.02 ^{a}
Sequential release with *O. nezarae* first	0.24 ± 0.025 ^{b}	0.15 ± 0.02 ^{c}
Sequential release with *P. saccharalis* first	0.47 ± 0.025 ^{a}	0.34 ± 0.02 ^{b}
ANOVA for the main effect	F = 19.91; df = 2, 237;	F = 32.32; df = 2, 237;

	$p < 0.0001$	$p < 0.0001$

LS-means within a column followed by the same letter are not significantly different at $p < 0.05$. Data were presented with one-way ANOVA using simulated multiple comparison test.

Out of 6147 *M. cribraria* eggs, a total of 2501 was parasitized. The proportion of parasitized host eggs was affected by the order of the two competitors' release and the period after they first oviposited (Figure 5.1). Over the entire experiment, the distribution of parasitoid attack was generally higher with increasing host exposure time, with the exception of *O. nezarae* to *P. saccharalis,* indicating that prolonged exposure time provides parasitoids an advantage in parasitizing more host eggs (Figure 5.1). The increase in parasitism also resulted in a decrease in kudzu bug nymph survival (Figure 5.2). Interestingly, the host eggs where *P. saccharalis* first oviposited and were followed by *O. nezarae* showed higher mortality with longer exposure times (Figure 5.1).

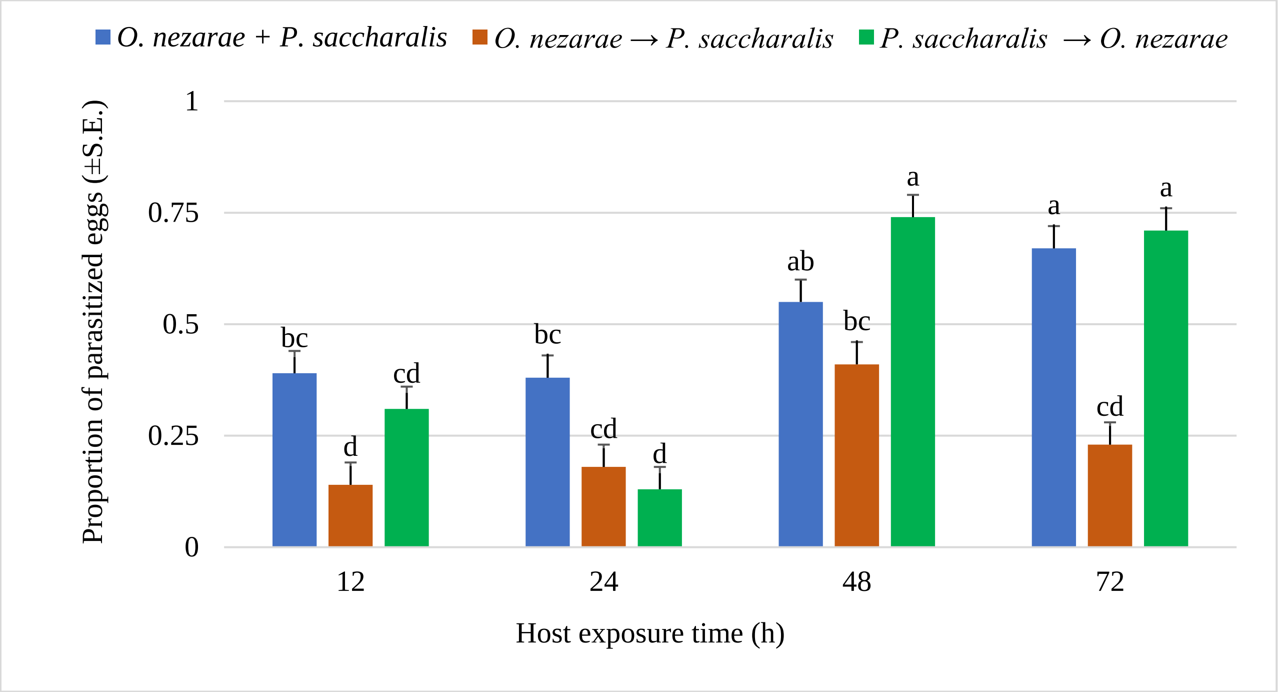

Figure 5.1 LS-mean (± S.E) proportion of parasitized *Megacopta cribraria* eggs under different parasitoid timing x host exposure time conditions. Scenarios examined included simultaneous release of *Ooencyrtus nezarae* and *Paratelenomus saccharalis* (*O. nezarae* + *P.*

***saccharalis*), sequential release in which *O. nezarae* was allowed to exploit the host patch first (*O. nezarae* → *P. saccharalis*), or sequential release in which *P. saccharalis* was released first (*P. saccharalis* → *O. nezarae*). Data were graphed with two-way ANOVA using simulated multiple comparison test: Release order (F = 31.45, df = 2, 228, *p* < 0.0001), host exposure time (F = 35.06, df = 3, 228, *p* < 0.0001) and their interaction (F = 6.86, df = 6, 228, *p* < 0.0001). Different letters indicate a significant difference at *p* < 0.05 for the interaction term. (Sample size, *n* = 20).**

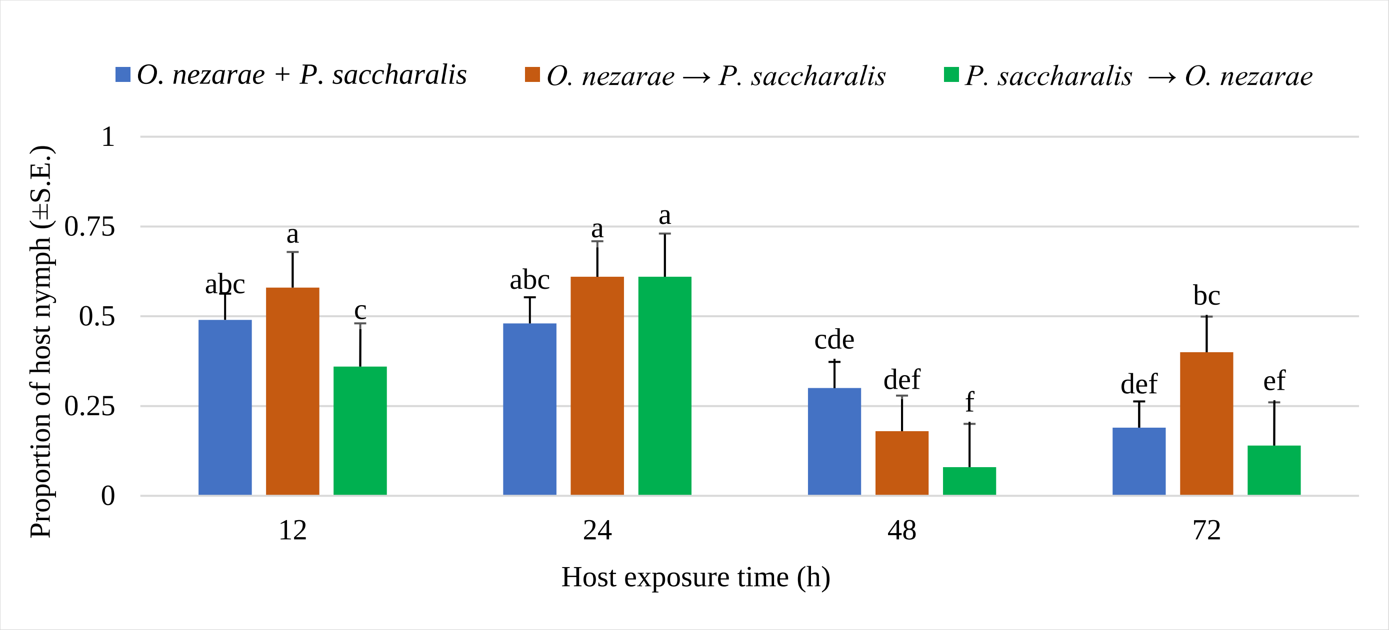

Figure 5.2 LS- mean (± S.E) proportion of *Megacopta cribraria* nymph hatching from eggs in the timing of adult arrival at the competition experiment. Scenarios examined included simultaneous release of *Ooencyrtus nezarae* and *Paratelenomus saccharalis* (*O. nezarae* + *P. saccharalis*), sequential release in which *O. nezarae* was allowed to exploit the host patch first (*O. nezarae* → *P. saccharalis*), or sequential release in which *P. saccharalis* was released first (*P. saccharalis* → *O. nezarae*). Data were graphed with two-way ANOVA using simulated multiple comparison test: host exposure time (F = 51.35, df = 3, 228, *p* < 0.0001), release order (F = 11.24, df = 2, 228, *p* < 0.0001), and their interaction (F = 4.96, df = 6, 228, *p* < 0.0001). Different letters indicate a significant difference at *p* < 0.05 for the interaction term. (Sample size, *n* = 20).

Ooencyrtus nezarae showed differences in its emergence when competing against *P. saccharalis*. From a total of 1893 parasitoids, *O. nezarae* yielded an average of 0.74 offspring.

The emergence of *O. nezarae* offspring was higher when their oviposition was simultaneous with *P. saccharalis* (Figure 5.3). It is noteworthy that *O. nezarae* emergence was relatively higher when the host eggs were exposed to both parasitoids for a prolonged period of 48–72 h (Figure 5.3). In addition, the proportion of *O. nezarae* offspring was around one-fold higher when *O. nezarae* arrived second to the host and when there was a delay of more than 12 h between oviposition of *P. saccharalis* and *O. nezarae*. It indicated that *O. nezarae* won almost all competitive events in the parasitized hosts.

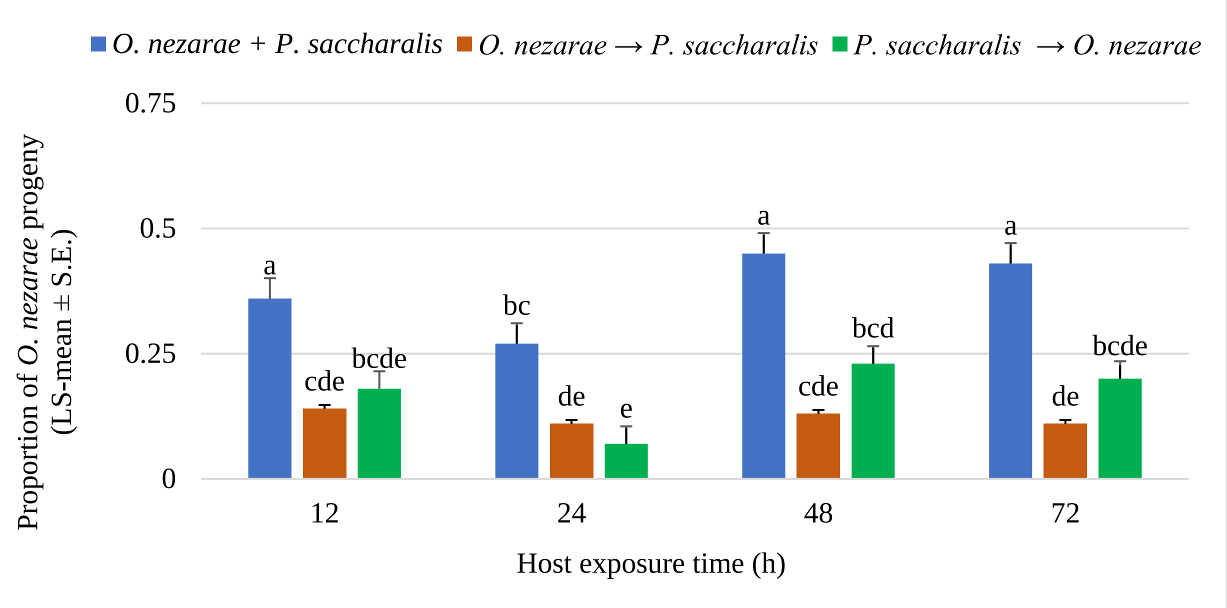

Figure 5.3 LS-mean (± S.E) of *Ooencyrtus nezarae* progeny that emerged from *Megacopta cribraria* eggs (Sample size, *n* = 20) in the timing of adult arrival at the competition experiment. Scenarios examined included simultaneous release of *O. nezarae* and *Paratelenomus saccharalis* (*O. nezarae* + *P. saccharalis*), sequential release in which *O. nezarae* was allowed to exploit the host patch first (*O. nezarae* → *P. saccharalis*), or sequential release in which *P. saccharalis* was released first (*P. saccharalis* → *O. nezarae*). Data were graphed with two-way ANOVA using simulated multiple comparison test: Release order (F = 63.78, df = 2, 228, p < 0.0001), host exposure time (F = 7.51, df = 3, 228, p < 0.0001) and their interaction (F = 1.77, df = 6, 228, p < 0.0001). Different letters indicate a significant difference at p < 0.05 for the interaction term.

The adult emergence for *P. saccharalis* was different from *O. nezarae*. A proportion of 0.26 *P. saccharalis* emerged from 1893 parasitized host eggs. The interaction between *O. nezarae* and *P. saccharalis* was not favorable for *P. saccharalis* offspring when *P. saccharalis* arrived at the host patch later or simultaneous with *O. nezarae*. In most cases, *P. saccharalis* emergence was highly affected by interference competition when it was introduced later, resulting in *P. saccharalis* having fewer offspring emergence. It indicates that *P. saccharalis* accepted a lower number of host eggs previously exposed to *O. nezarae* at all time points (Figure 5.4).

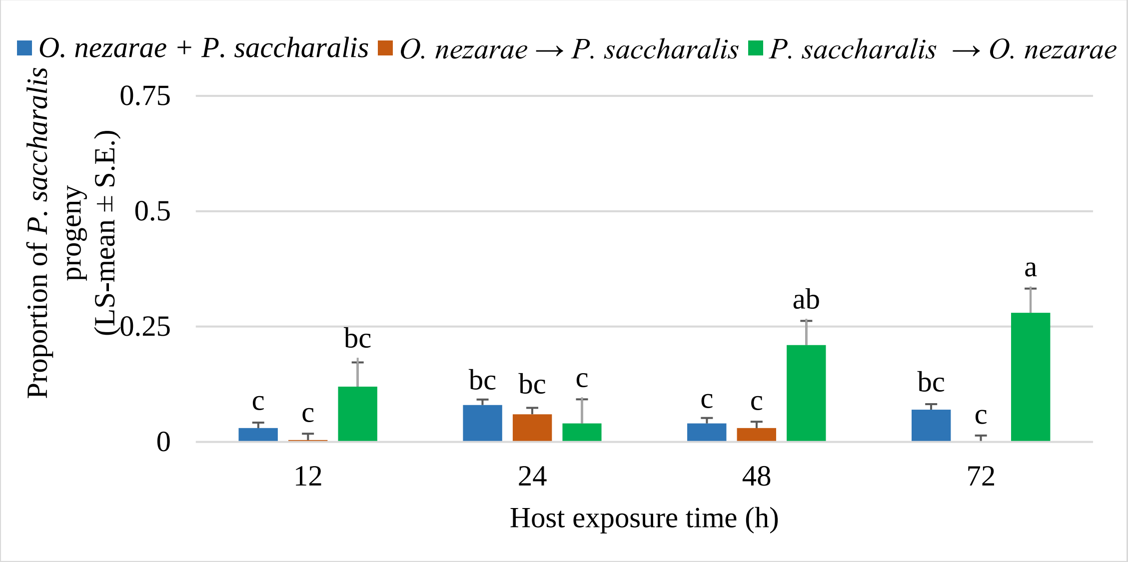

Figure 5.4 LS- mean (± S.E.) sex ratio (females/total progeny) of *Ooencyrtus nezarae* (Sample size, *n* = 20) on *Megacopta cribraria* eggs in the timing of adult arrival at the competition experiment. Scenarios examined included simultaneous release of *O. nezarae* and *Paratelenomus saccharalis* (*O. nezarae* + *P. saccharalis*), sequential release in which *O. nezarae* was allowed to exploit the host patch first (*O. nezarae* → *P. saccharalis*), or sequential release in which *P. saccharalis* was released first (*P. saccharalis* → *O. nezarae*). Data were graphed with two-way ANOVA using simulated multiple comparison test: Release order (F = 1.82, df = 2, 228, *p* = 0.16), host exposure time (F = 3.61, df = 3, 228, *p* = 0.01) and their interaction (F = 4.86, df = 6, 228, *p* = 0.0001). Different letters indicate a significant difference at *p* < 0.05 for the interaction term.

Emergence of fewer *P. saccharalis* offspring suggested that *P. saccharalis* has a competitive disadvantage when it does not have an opportunity to develop to the first instar larva prior to multiparasitism by *O. nezarae*. A proportion of 0.20 kudzu bug eggs remained unhatched when *P. saccharalis* and *O. nezarae* arrived at the host patch together. However, a higher proportion of eggs remained unhatched when competitors arrived in sequence. *Ooencyrtus nezarae's* arrival at the host patch first resulted in proportions of unhatched eggs exceeding 0.40 (Table 5.2), indicating potential feeding behavior on the host.

Table 5.2 The proportion of unascribed eggs (unhatched or unparasitized) averaged across treatments in experiments conducted to examine how the order of *Ooencyrtus nezarae* and/or *Paratelenomus saccharalis* adult arrival at a Megacopta cribraria egg patch influence competition.

	(Proportion ± SE)		
Time interval	***O. nezarae* + *P. saccharalis***	***O. nezarae* → *P. saccharalis***	***P. saccharalis* → *O. nezarae***
12-h	0.13 ± 0.04^{d}	0.27 ± 0.04^{cd}	0.33 ± 0.04^{bcd}
24-h	0.16 ± 0.04^{d}	0.21 ± 0.04^{cd}	0.27 ± 0.04^{cd}
48-h	0.20 ± 0.04^{cd}	0.64 ± 0.04^{a}	0.47 ± 0.04^{ab}
72-h	0.29 ± 0.04^{bcd}	0.47 ± 0.04^{ab}	0.38 ± 0.04^{bc}

	ANOVA table
Treatment	F = 26.40; df = 2, 228; p = <0.0001
time interval	F = 19.57; df = 3, 228; p = <0.0001
Treatment x time interval	F = 4.74; df = 6, 228; p = 0.0001

LS-means within a column followed by the same letter are not significantly different at $P < 0.05$. Data were presented with two-way ANOVA using a simulated multiple comparison test.

The proportion of females in *O. nezarae* offspring emerging from parasitized eggs was correlated with the interaction of the order of parasitism and time interval (for statistics, see below Figure 5.5). *Ooencyrtus nezarae* females appeared to adjust the sex allocation of their progeny in response to competition. In most cases, multiparasitism corresponded to the *O. nezarae* population having a male-biased sex ratio (Figure 5.5). Even though *P. saccharalis* only produced female offspring from the field, the number of females in *P. saccharalis* offspring was higher when *P. saccharalis* had the opportunity to arrive at the host patch earlier than their competitors (Figure 5.4).

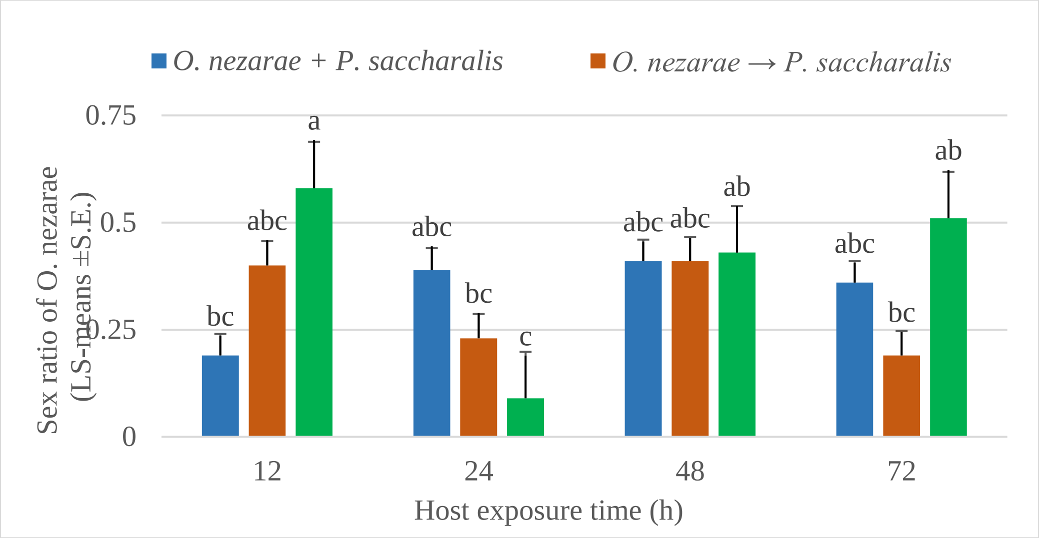

Figure 5.5 . LS- mean (± S.E.) sex ratio (females/total progeny) of *Ooencyrtus nezarae* (Sample size, *n* = 20) on *Megacopta cribraria* eggs in the timing of adult arrival at the competition experiment. Scenarios examined included simultaneous release of *O. nezarae* and *Paratelenomus saccharalis* (*O. nezarae* + *P. saccharalis*), sequential release in which *O. nezarae* was allowed to exploit the host patch first (*O. nezarae* → *P. saccharalis*), or sequential release in which *P. saccharalis* was released first (*P. saccharalis* → *O. nezarae*). Data were graphed with two-way ANOVA using simulated multiple comparison test: Release order (F = 1.82, df = 2, 228, *p* = 0.16), host exposure time (F = 3.61, df = 3, 228, *p* = 0.01) and their interaction (F = 4.86, df = 6, 228, *p* = 0.0001). Different letters indicate a significant difference at *p* < 0.05 for the interaction term.

5.4.2 Experiment II: Characterizing aggressive behavior

Ooencyrtus nezarae and *P. saccharalis* showed differences in their behavior when competing against each other. When both wasp species were using the host egg patch at the same time, interspecific aggressive behavior occurred between the two species. As soon as *P. saccharalis* encountered or noticed *O. nezarae* on the egg mass, they displayed aggressive behaviors, which occurred at a frequency of 1.85 ± 0.25 h^{-1}. The *P. saccharalis* employed head butting (37 times in 20 replications) to fight the *O. nezarae* in all their confrontations, and it occasionally flapped its wings (2 times in 20 replications), appearing ready to strike, then charged its competitor, causing it to leave the egg mass. No aggressive behavior was observed by *O. nezarae*. Female *O. nezarae* displayed two behaviors on approaching or encountering *P. saccharalis:* running, in which the *O. nezarae* female walked away from the *P. saccharalis*, and left the host patch, and avoiding, in which the *O. nezarae* avoided physical contact with the approaching *P. saccharalis* by changing her searching direction.

5.4.3 Experiment III: Intraguild predation of parasitoid larvae

The summary statistics of intraguild interactions between *O. nezarae* and *P. saccharalis* are summarized in Table 5.2. Intraguild predation of *O. nezarae* larvae often did not result in a decrease in the host population. *A* higher impact on the host was mostly achieved when *O. nezarae* was released right after the *P. saccharalis* was released. However, the proportion of host parasitism dropped from 0.23 to 0.19 in eggs that were exposed to *P. saccharalis* prior to being exposed to *O. nezarae*, 72 h or more before (Table 5.3).

Table 5.3 The proportion of parasitized host eggs and emerged wasps averaged across treatments in an experiment examining intraguild predation of larvae experiment

	(Proportion ± S.E)	
Host Exposure Time (h)	*M. cribraria* Parasitized Eggs	Emerged Wasp
0	0.38 ± 0.07 ^{ab}	0.35 ± 0.06 ^{ab}
24	0.35 ± 0.07 ^{ab}	0.23 ± 0.06 ^{b}
48	0.59 ± 0.07 ^{a}	0.58 ± 0.06 ^{a}
72	0.23 ± 0.07 ^{b}	0.20 ± 0.06 ^{b}
96	0.19 ± 0.07 ^{b}	0.16 ± 0.06 ^{b}

ANOVA for main effect	F = 5.21; df = 4, 95; p= 0.0008	F = 7.90; df = 4, 95; $p < 0.0001$

LS-means within a column followed by the same letter are not significantly different at $p < 0.05$. Data were presented with one-way ANOVA using simulated multiple comparison test.

The proportion of wasp emergence was also affected by intraguild predation of *O. nezarae* larvae (Table 5.3). The wasp emergence decreased from the parasitized eggs as the differences in the timing of oviposition between both parasitoids increased except for the time delay of 24 h. *Ooencyrtus nezarae* accepted all host eggs that had been exposed to *P. saccharalis* from 0–96 h before. The offspring of *O. nezarae* was even able to develop in host eggs parasitized by *P. saccharalis* 96 h earlier (Figure 5.6).

In most of the situations, the sex ratio of *O. nezarae* was male-biased and there was a trend towards longer duration experiments showing less favorable effects on female emergence of *O. nezarae* (Figure 5.7).

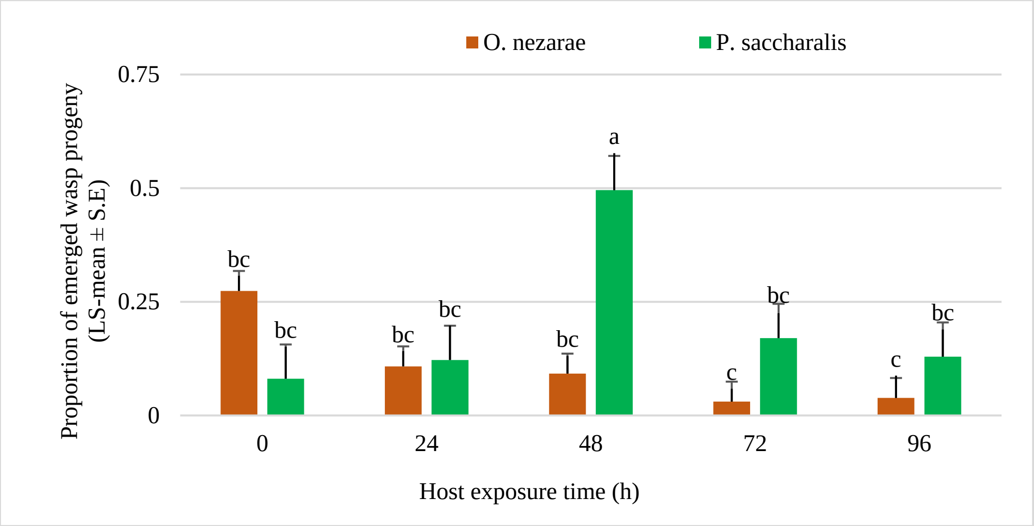

Figure 5.6 LS-mean (± SE) of *Ooencyrtus nezarae* and *Paratelenomus saccharalis* progeny that emerged from host eggs among treatments. Data were graphed with one-way ANOVA using simulated multiple comparison test (F =10.91, df =4, 95, $p < 0.0001$ for the main effect i. e., host exposure time). Different letters indicate a significant difference at $p < 0.05$. (Sample size, $n = 20$).

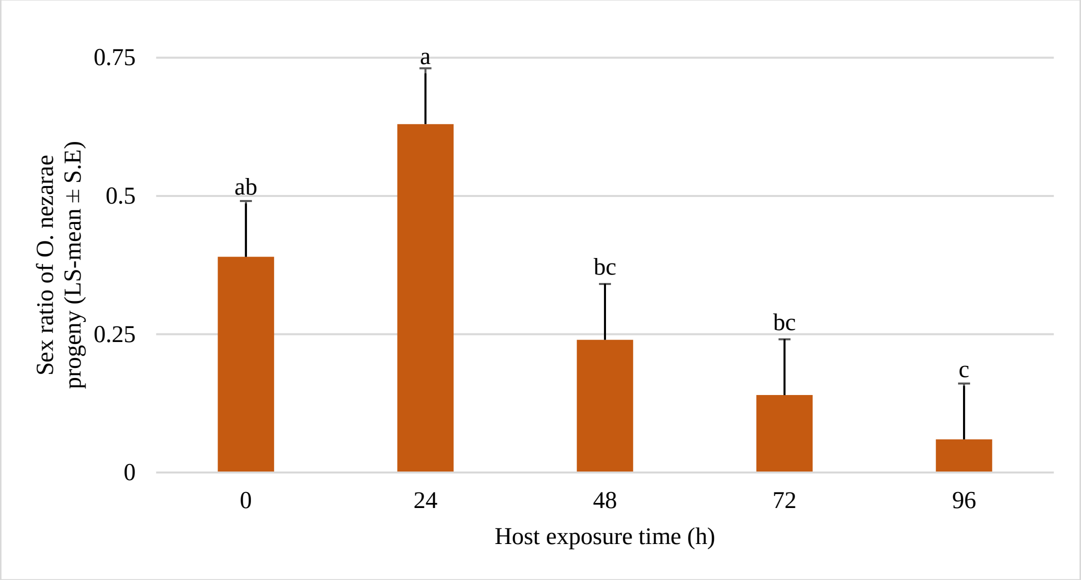

Figure 5.7 LS-mean (± S.E.) sex ratio (females/total progeny) of *Ooencyrtus nezarae* (Sample size, *n* = 20) on *Megacopta cribraria* eggs intraguild predation experiment. Data were graphed with one-way ANOVA using simulated multiple comparison test (F = 10.91, df = 4, 95, $p < 0.0001$ for the main effect i. e., host exposure time). Different letters indicate a significant difference at $p < 0.05$.

5.5 DISCUSSION

We investigated both the direct and indirect interspecific interference competition between *P. saccharalis* and *O. nezarae* by comparing the proportion of parasitized host eggs, host nymphs, and emerged parasitoids in a sequential or simultaneous release of both species. Both wasp species showed differences in host parasitism and emergence when competing against each other. *Ooencyrtus nezarae* acted as a superior larval competitor in almost all competitive events. *Paratelenomus saccharalis* adults showed agonistic behavior against its heterospecific female to defend the host patch. The outcomes of such competitive situations are discussed below.

5.5.1 Experiment I: The timing of adult arrival at the competition

The direct interference results showed that competition occurs between both parasitoid species for the common host, and the order of release, i.e., the timing of the wasp's arrival at the host patch, also influenced the outcome. When specialist *P. saccharalis* arrived earlier or together with generalist *O. nezarae*, egg parasitism rates were the highest. However, when *O. nezarae* exploited the host patch first, the overall parasitism was lower. Our results are consistent with those of (Tian et al. 2008) showing that specialist (*Microplitis mediator* Haliday) had more impact on the parasitism of *Helicoverpa armigera* Hübner larvae when it was released prior to a generalist (*Campoletis chlorideae* Uchida) (Tian et al. 2008). The possible explanation for these results is that the specialist species has better host handling strategies due to specificity and is much more efficient in host utilization compared to a generalist (Bernays, 2001; Takano and Takasu, 2019). The host handling time of female *P. saccharalis is much shorter* (an average of 10.48 min) (Takano and Takasu, 2019) than *O. nezarae* (an average of 19.32 min) (Takasu and Hirose, 1986).

The seasonal arrival time of *P. saccharalis* is quite different in nature. In the native range (Japan), *P. saccharalis* arrives in May and *O. nezarae* in June. In the United States, *O. nezarae* arrives earlier in May, whereas *P. saccharalis* first appears in July, and both species overlap from July to October in soybean fields (personal observation). *Ooencyrtus nezarae* host feeding behavior may also influence parasitism. When *O. nezarae* was released first, regardless of host exposure time, the highest proportion of kudzu bug eggs were neither hatched nor parasitized (i.e., unascribed eggs) by wasps. The proportion of unascribed eggs was equal to or more than that of parasitized eggs.

Parasitism and emergence were highest when *O. nezarae* and *P. saccharalis* arrived at the host patch simultaneously, and only a small fraction of parasitized eggs did not produce wasps in this treatment. When *O. nezarae* arrived later than *P. saccharalis*, the proportion of parasitized host eggs was higher, but fewer wasps emerged from parasitized eggs. More than half of the parasitized eggs did not yield parasitoids and were non-viable (Table 5.4).

Table 5.4 The proportion of unascribed eggs (unhatched or unparasitized) averaged across treatments in experiments conducted to examine how the order of Ooencyrtus nezarae and/or Paratelenomus saccharalis adult arrival at a Megacopta cribraria egg patch influences competition.

	(Proportion± SE)
Treatment	***M. cribraria* unascribed eggs**
O. nezarae + P. saccharalis	0.20 ± 0.024^{b}
O. nezarae → P. saccharalis	0.40 ± 0.025^{a}
P. saccharalis → O. nezarae	0.36 ± 0.025^{a}
ANOVA for the main effect	F = 19.86; df = 2, 237; *p* = <0.0001

LS-means within a column followed by the same letter are not significantly different at $P < 0.05$. Data were presented with one-way ANOVA using simulated multiple comparison test.

A similar result was found when *O. nezarae* arrived earlier than *P. saccharalis* at the host patch; the highest proportion of parasitized eggs that did not produce wasps was observed when the wasp was given a longer time to exploit the host. These results might be related to the preference of *O. nezarae* for parasitized eggs over unparasitized eggs. *Ooencyrtus nezarae* female prefers parasitized host eggs to save their energy and time in host drilling (Takasu and Hirose, 1991). In fact, the handling time of *O. nezarae* on a parasitized host is an average of 17.23 min, which is considerably shorter than an unparasitized host, taking only 19.32 min in handling (Takasu and Hirose, 1991). It also suggests that *O. nezarae* is superior in interspecific larval competition (intrinsic competition).

Experiment II: Characterizing Aggressive Behavior

When *P. saccharalis* and *O. nezarae* arrive at the host patch together, *P. saccharalis* exhibits aggressive behavior; *O. nezarae* did not show any distinct behavior toward *P. saccharalis* to defend the kudzu bug egg patch. It was observed that *O. nezarae* reached the host eggs earlier than *P. saccharalis* and took possession of the eggs. Then, *P. saccharalis* females that arrived later would fight with *O. nezarae* to access the host. *Paratelenomus saccharalis* exhibited continuous head striking of *O. nezarae* that generally caused *O. nezarae* to leave the host patch. Such aggressive tactics were also observed in other Platygastrid egg parasitoids such as *Trissolcus basalis* Wollaston against *O. telenomicida* Vassiliev to utilize *Nezara viridula* Linnaeus eggs (Potting et al. 1997). In Japan, *P. saccharalis* females were also observed to aggressively exclude females of *O. nezarae* from utilizing the host patch (Hoshino et al. 2017). Since *O. nezarae* can utilize both unparasitized and parasitized hosts, aggressive behavior from *O. nezarae* towards *P. saccharalis* is not advantageous, therefore leaving the host patch and searching out additional patches without a *P. saccharalis* female present would be expedient for them. The lack of aggressive behavior in *O. nezarae* females may also be related to their broad host range in the United States (Ademokoya et al. 2018), or due to its smaller size (max. overall body length = 0.77 mm) in comparison to *P. saccharalis* females (max. overall body length 0.83 mm) (Ademokoya et al. 2017; Johnson, 1996).

5.5.2 Experiment III: Intraguild predation of larvae

Intraguild predation data reaffirms the vulnerability of *P. saccharalis* immatures (72–96 h), as *O. nezarae* successfully emerges from multiparasitized eggs, speculating the adaptive outcome of the temporal trophic shift from *M. cribraria* eggs to its primary parasitoid, *P. saccharalis*. It increased the window of opportunity to exploit host eggs and allowed them to evade exclusion by the more fecund wasp *species*. However, this trophic shift ended with a detrimental outcome for both species, as non-reproductive mortality increased significantly (0.84 at 96 h). Our result was consistent with Cusumano et al. (2017), who observed high levels of dead host and parasitoid mortality with later-stage multiparasitism by *O. telenomicida*.

It has been observed that *Ooencyrtus* spp. with a broad host range generally shift their trophic position when hosts become scarce (Brodeur, 2000; Hunter and Woolly, 2001; Kidd and Amarasekare, 2012). Mohammadpour et al. (2014) investigated competition between *O. pityocampi* Mercet and *Trissolcus agriope* Kozlov and Le on the host eggs of *Brachynema signatum* Jakovlev. *Ooencyrtus pityocampi* was able to develop as a superior larval competitor or could be as a facultative hyperparasitoid on the latter species (Mohammadpour et al. 2014). This is presumably through direct physical attack between larvae (Mohammadpour et al. 2014). Studies also show that embryological differences between the species can be one possible reason to outcompete the competitor by hatching earlier (Harvey et al. 2013). However, the developmental biology of *P. saccharalis* and *O. nezarae* have yet to be investigated.

5.6 CONCLUSIONS

Both parasitoid species differed in terms of their host utilization and competitive interference strength. *Paratelenomus saccharalis* was the species that had the greatest ability to exploit the resource, while *O. nezarae* was the strongest species in the direct and indirect competition. Our work has shown that *O. nezarae* has the potential to impact the population dynamics of *P. saccharalis*, which could be detrimental to biological control programs of *M. cribraria*. Additional field studies, however, are needed to determine interspecific competition under natural conditions.

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Chapter 6

SUMMARY AND FUTURE STUDIES

6.1 SUMMARY AND FUTURE STUDIES

This project investigated aspects of the community ecology, developmental biology, and chemical ecology of kudzu bug-stink bug interactions and kudzu bug-parasitoid interactions that were underexplored. The first study using kudzu bugs and either southern green stink bug, or brown marmorated stink bug examined the direct and indirect interactions of the kudzu bug on stink bug host selection behavior. This study revealed that soybean responses altered by pre-infestations with kudzu bugs influenced stink bug species host selection process. Specifically, brown marmorated stink bugs initially preferred healthy soybean plants but switched to pre-infested plants once kudzu bugs were removed, while southern green stink bugs consistently favored infested and pre-infested plants, regardless of kudzu bug presence. The specific mechanisms behind these altered soybean responses and their effects on stink bug host selection were not investigated in this study. This observation can still provide valuable insights and serve as a starting point for future research to explore the underlying mechanisms and their implications for pest management in soybean crops. These findings underscore the complexity and interconnectivity of plant-herbivore relationships, illustrating how the actions of one species can have cascading effects on others within the ecosystem. This complexity stems from plants undergoing various chemical and physiological changes in response to herbivore attacks, subsequently influencing the behavior, feeding, or oviposition preferences of other herbivores. This emphasizes the need to consider the complexity of plant-herbivore interactions, as they are affected by various factors such as co-occurring herbivores and their densities. Future studies should focus on identifying and characterizing the specific induced compounds in soybean plants that influence stink bug preferences and understanding how these compounds may affect stink bugs feeding and oviposition behavior.

The second study focused on kudzu bug egg parasitoids, *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae) and *Paratelenomus saccharalis* Dodd (Hymenoptera: Platygastridae) and identified key factors such as parasitoid food sources, host eggs: parasitoid ratios, and host exposure times, that influence parasitoid effectiveness and their potential for biocontrol of kudzu bug populations. It was found that constant supplies of water and honey, a low host-to-parasitoid ratio, and limited exposure time optimized the reproductive potential of both parasitoids. Providing constant supplies of water and honey allows parasitoids to maintain optimal health, as water is essential for hydration and physiological processes, while honey serves as an energy source. A well-nourished parasitoid is more likely to exhibit higher reproductive potential, as nutrition impacts its health, energy reserves, and reproductive processes like egg production (Wäckers et al. (2005). Furthermore, adequate nutrition can increase lifespan, extending the time available for reproduction and enhancing overall reproductive success (Benelli et al. 2017). Additionally, a low host-to-parasitoid ratio can ensure ample access to host resources, reducing competition, and enhancing reproductive success by increasing the likelihood of successful parasitism. Lastly, limited exposure time to hosts can increase the efficiency of host-searching and utilization, leading to a more focused and effective parasitoid effort. These findings highlight the importance of understanding and providing ideal living conditions for beneficial insects to enhance their effectiveness in controlling pests. Implementing such knowledge in conservation efforts can lead to more sustainable and eco-friendly pest management strategies in agriculture and other settings. Future research could focus on identifying flowering plants as natural nectar sources for parasitoids, improving our understanding of their feeding preferences and the potential benefits of these plants in supporting parasitoid populations.

The third study aimed to determine the host foraging strategies that *P. saccharalis* and *O. nezarae* use to overcome challenges during host foraging, specifically the 'detectability' or 'reliability' problem, as the egg stage of their host is inconspicuous. Additionally, the study investigated whether parasitoids' hunger and host experience conditions influence their host-searching strategies. It was found that these species innately respond to chemical cues to their host-associated stimuli, irrespective of their hunger or experience. Both species were attracted to infested plants with their target host eggs and tracked chemical trails left by adult kudzu bugs to find their host. By detecting volatile organic compounds produced during herbivore-host plant interactions and adult host-associated cues, these parasitoid wasps can effectively overcome host detection challenges and successfully parasitize their targets. Future studies should focus on identifying the chemical cues left by female kudzu bugs that attract parasitoids and determining the molecular and olfactory mechanisms involved in the parasitoids' olfactory responses. Understanding the molecular mechanisms involved in olfactory responses can provide a better understanding of host-parasitoid interactions at the molecular level. This knowledge can contribute to our overall understanding of the ecology and biology of these species. By understanding the chemical cues and olfactory mechanisms that guide parasitoids to their hosts, a more targeted and efficient biological control strategies can be developed. For example, synthetic versions of these chemical cues could be developed and used as attractants in traps or as part of augmentative biological control approaches, increasing parasitoid effectiveness in controlling kudzu bug populations.

The final study focused on the direct and indirect competitive interactions between *P. saccharalis* and *O. nezarae*. This study results revealed that interspecific competition reduced *P. saccharalis* emergence in the presence of *O. nezarae.* Although adult *P. saccharalis* aggressively competed for shared hosts, *O. nezarae* larvae proved more successful in multiparasitized eggs. These results indicate that *O. nezarae* has the potential to negatively impact the population dynamics of *P. saccharalis*, which may subsequently affect the success of biological control programs targeting the kudzu bug. To address this issue, future research could focus on enhancing the aggressive behavior of *P. saccharalis* to better compete with *O. nezarae*. The first step toward achieving this goal would be to identify biomolecules that are elevated in *P. saccharalis* during aggressive behavior in response to chemical and visual cues from *O. nezarae*. The next step would involve identifying crop varieties or companion plants that promote the production of specific biomolecules in *P. saccharalis*. This could potentially improve the wasp's ability to compete with *O. nezarae* and enhance its efficacy as a biocontrol agent for kudzu bugs.

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