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Competition between *Gonatocerus ashmeadi* and *G. triguttatus* for glassy winged sharpshooter (*Homalodisca coagulata*) egg masses

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Abstract

The introduction of a biological control agent can have significant effects on natural enemies that use the same host. Interspecific competition between natural enemies can impact the efficacy of control and, consequently, is the subject of increasing research scrutiny. Three experimental approaches were used to evaluate competitive outcomes between *Gonatocerus ashmeadi* and *G. triguttatus* parasitizing *Homalodisca coagulata* egg masses in the laboratory. (1) When both species were introduced to high densities of host eggs 1, 3 and 5 days of age, mean percentage offspring was significantly higher for *G. ashmeadi* offspring (23.2% greater than *G. triguttatus*). (2) When a female parasitoid of each species was offered a single egg mass, offspring production was statistically similar for the two species. *Gonatocerus triguttatus* showed aggressive behavior, although this only accounted for 0.8% of the female's total foraging time and did not lead to proportionately higher offspring production. (3) Regardless of order, more *G. triguttatus* offspring (up to 96%) emerged than *G. ashmeadi* offspring when one female was introduced sequentially to one egg mass. The relative success in offspring production was affected primarily by the sequence in which the parasitoids were introduced, and to a lesser extent by the interval between successive parasitoid introductions, and the age of the egg mass. These results illustrate the importance of experimental design in the assessment of competitive superiority between two species of parasitoids. Improper experimental design can lead to contradictory outcomes in laboratory-based competition studies due to the interplay of extrinsic and intrinsic competitive behavior. Biological control practitioners need to be aware of the complexity of competitive behavior when designing pre-introduction laboratory tests to determine *a priori* which natural enemy from several candidate species is likely to be the most effective agent at controlling the target.

Keywords: *Biological control, Cicadellidae, interspecific competition, Hemiptera, Hymenoptera, Mymaridae, natural enemy evaluations, oviposition preferences,* Homalodisca coagulata, *parasitoid, patch processing behavior, pre-introduction studies*

Introduction

Since its discovery in California, the population growth and spread of the glassy-winged sharpshooter [*Homalodisca coagulata* (Say)] (Hemiptera: Cicadellidae), an insect native to the southeastern areas of the USA and northeastern Mexico, has been

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remarkable (Purcell & Feil 2001). *Homalodisca coagulata* is a xylem feeder and vectors *Xylella fastidiosa*, a bacterium that causes scorch-like and stunting diseases in numerous crops including; grape, peach, plum, almond, alfalfa, citrus, and forest trees (Purcell 1997). Populations of *H. coagulata* have reached high densities in southern California, and in 2000, a classical biological control program was initiated to reduce the populations and progressive northward spread of *H. coagulata*, and to reduce the prevalence and severity of *Xylella*-related diseases vectored by this insect. The biological control program has focused exclusively on the use of hymenopterous mymarid parasitoids that oviposit into individual eggs of *H. coagulata* egg masses that are laid on the undersides of leaves. *Homalodisca coagulata* eggs are deposited in a slit cut with the ovipositor between the epidermis and parenchyma on the lower leaf surface (Irvin & Hoddle 2004). Of these parasitoids, *Gonatocerus ashmeadi* Girault has been resident in California since 1978 (Huber 1988). Genetic studies indicate it is native to the southeast USA and probably accompanied *H. coagulata* from its home range (Vickerman et al. 2004). Parasitism rates of up to 100% have been recorded for *G. ashmeadi* during certain times over summer in California, but *H. coagulata* populations remain high, in part because of poor control of the spring generation (Triapitsyn & Phillips 2000). Therefore, *G. triguttatus* Girault (Girault 1916; Huber 1988) was imported from eastern Texas and released in California in 2001 to assist in biological control of *H. coagulata.* Although over 150 recoveries of *G. triguttatus* have been made at release sites, widespread establishment has not yet been confirmed in California (CDFA 2004). Mass rearing and release of *G. ashmeadi* and *G. triguttatus* is ongoing (CDFA 2004).

Irvin and Hoddle (2005a) demonstrated in host utilization studies that *G. ashmeadi* and *G. triguttatus* prefer to parasitize eggs 3 and 4 days of age, respectively, and that each parasitoid species was able to utilize a range of egg ages around their most preferred age. This may lead to interspecific competition for egg masses under field conditions where a range of egg ages is present at one time. Understanding the dynamics of competitive interactions between parasitoid species may assist in the selection of effective agents for classical biological control (Mackauer 1990), and provide insight for predicting and interpreting field outcomes following natural enemy release and establishment (Murdoch & Briggs 1996). Should *G. triguttatus* establish widespread populations in California, it is unknown how this parasitoid will competitively interact in the field with the more common and abundant *G. ashmeadi*, how intense interspecific competition could become, or which parasitoid species would dominate should *H. coagulata* populations diminish to low densities as a result of natural enemy activity.

To understand the dynamics of resource acquisition and exploitation by *G. ashmeadi* and *G. triguttatus*, and the potential resulting competitive interactions between these parasitoids, information is required on extrinsic and intrinsic competition between these two species. Extrinsic competition refers to the ability of adult parasitoids to exploit the host population, and involves host searching efficiency, reproductive capacity, aggressive behavior between adults, and phenological synchronicity with the host (Zwolfer 1971; Hågvar 1989; Lewis et al. 1990; Tumlinson et al. 1993). Intrinsic competition refers to the interaction between immature parasitoids within the host (Collier et al. 2002). One species may intrinsically out perform competitors through larval combat or physiologically based mechanisms, and competition outcome can be affected by the developmental stage of host at time of

attack, sequence of oviposition (i.e., which species parasitizes first), and the time between oviposition events (van Strien-van Liempt 1983; Tillman & Powell 1992; DeMoraes et al. 1999; Collier et al. 2002).

Within the context of the classical biological control program for *H. coagulata* we decided to investigate whether laboratory studies designed to investigate competitive interactions would consistently indicate whether *G. ashmeadi* or *G. triguttatus* would be the superior competitor and ultimately the most efficacious natural enemy of *H. coagulata*. Three competition studies were designed to investigate extrinsic and intrinsic competition between *G. ashmeadi* and *G. triguttatus*. Experiment one used high *H. coagulata* densities to simulate summer populations currently found in California, and investigated extrinsic competition by comparing overall progeny survival rates between species. Experiment two further investigated extrinsic competition between *G. ashmeadi* and *G. triguttatus* by recording observations on the aggressive behaviors of congeneric females concurrently competing for egg masses. Experiment three used low *H. coagulata* densities, thereby increasing interspecific competition to simulate low populations that may occur once biological control of *H. coagulata* progresses in California, and investigated both extrinsic and intrinsic competition when females of both species have access to parasitize the same egg mass. The developmental stage of the host (egg age), sequence of species introduction and time interval between potential oviposition events by each parasitoid species were varied to help broaden our understanding of parasitoid intrinsic competitive abilities. It is hypothesized that being first in the sequence of introduction, delaying the introduction of the second parasitoid, or offering a parasitoid species their most preferred egg age, may present a competitive advantage (DeMoraes et al. 1999). Finally, parasitoid larval morphology for each species was investigated using scanning electron microscopy (SEM) to assist with the interpretation of outcomes from possible congeneric larval intrinsic competition.

Methods

Plant and insect details, and laboratory conditions

Citrus limon (lemon) cv. 'Eureka' trees approximately 2 years of age and grafted to *Marcophylla* sp. rootstock were obtained from C & M Nurseries, Nipomo, California (CA) and maintained in greenhouses at University of California, Riverside. Trees were pruned to 60 cm in height, transplanted into 4-L containers, and fertilized every 2 weeks with Miracle-Gro (20 mL/3.5 L of water, Scotts Miracle-Gro Products Inc., Marysville, OH). 'Eureka' was chosen because it has been previously demonstrated that *H. coagulata* prefers this lemon variety for oviposition and parasitoid foraging is not adversely affected in comparison to other lemon cultivars (Irvin & Hoddle 2004). Field-collected *H. coagulata* were placed in cages ($50 \times 40 \times 40$ cm) containing two lemon trees and maintained in greenhouses. After 2–3 days, leaves bearing egg masses were collected and exposed to parasitoids in Petri dishes (9×1 cm, Becton-Dickinson Labware, Becton-Dickinson and Co., Franklin Lakes, NJ). Parasitoid colonies were maintained in controlled atmosphere rooms at the University of California, Riverside at $26 \pm 2^{\circ}$C and 30–40% RH under a L14:10D photoperiod, provisioned with honey–water solution (3 honey:1 water, Natural uncooked honey, Wild Mountain Brand, Oakland, CA) and checked daily for parasitoid emergence to assure uniform age for

experiments. All experiments were conducted in the laboratory at $26 \pm 2^{\circ}$C and 30–40% RH under light intensity of 1.2 ± 0.2 log lumens/m^2 and L14:D10 photoperiod.

Experiment 1: Simultaneous parasitoid exposure to H. coagulata *eggs – high density*

Stems of 4–6 'Eureka' leaves containing approximately 30 *H. coagulata* eggs in total ($\sim$1 day of age) were inserted through holes drilled in the lid of a 130-mL plastic vial filled with deionized water and 3 mL of antiseptic [(Listerine Antiseptic Mouthwash, Pfizer Inc., New York, NY) (to prevent bacterial rot)]. A second 130-mL plastic vial with ventilation [three 2-cm holes (one on the bottom, and one on each of two sides) covered with mesh netting (80 μm Jelliff Corporation, Southport, CT)] and a small drop of honey–water solution was inverted and attached to the lid of the vial holding the water and leaves. One newly emerged mated (females are immediately mated by sibling brothers that emerge from egg masses first) naïve female *G. ashmeadi* and *G. triguttatus* ($\sim$24 h old) were placed inside inverted vials that covered the test material and left for 24 h to forage and oviposit. This was replicated 15–20 times each for *H. coagulata* eggs 1, 3, and 5 days of age. This experimental design was successfully used by Irvin and Hoddle (2004, 2005a,b), and Hoddle and Pilkington (2004) have demonstrated that female *G. ashmeadi* mate and oviposit within 24 h of emergence.

After 24 h of exposure, parasitoids were removed and vials containing leaves with egg masses exposed to parasitoids were held for 3 weeks to allow parasitoids to emerge. Evaporated water was replaced with deionized water every second day to keep excised leaves turgid. The number of emerged *G. ashmeadi* and *G. triguttatus* adults were recorded, and eggs with no emergence were dissected and the number of identifiable *G. ashmeadi* and *G. triguttatus* offspring recorded (*G. ashmeadi* are black in color and *G. triguttatus* are yellow). The proportion of *G. ashmeadi* and *G. triguttatus* offspring produced by each vial was calculated as a function of total parasitism, e.g., the proportion of *G. ashmeadi* offspring = [the number of *G. ashmeadi* offspring/(the number of *G. ashmeadi* + the number of *G. triguttatus*)]. Since transformations failed to normalize data, the proportion of offspring produced per vial was compared between parasitoid species at each *H. coagulata* egg age, and among egg ages for each species using non-parametric Kruskal–Wallis χ^2 in SAS (SAS 1990). All results are presented as percentages.

Experiment 2: Simultaneous parasitoid exposure to H. coagulata *eggs – low density*

One *H. coagulata* egg mass (3–6 eggs per mass and 24–48 h of age) was placed into a 3.5-cm Petri dish (3.5×1 cm, Becton-Dickinson Labware, Becton-Dickinson and Co., Franklin Lakes, NJ) lined with moist filter paper (4.25 cm, Whatman Ltd. International, Maidstone, England), and exposed simultaneously to one newly emerged mated naïve female *G. ashmeadi* and *G. triguttatus* ($\sim$24 h of age) for 1 h. Visual observations were made for each female every 5 min (total of 12 observations for each Petri dish) for activity that was characterized as either resting/grooming (i.e., standing still or cleaning), searching leaf (antennating leaf surface), off leaf (walking on Petri dish or filter paper), egg inspection (egg mass being antennated), oviposition (insertion of ovipositor into an egg), and aggressive behavior (females on egg mass chasing each other). This experiment was replicated 21 times. Parasitoids were removed 1 h after introduction, and the Petri dishes containing the leaves with egg

masses were held for 3 weeks to allow parasitoids and *H. coagulata* nymphs to emerge. The percentage of *G. ashmeadi* and *G. triguttatus* offspring produced by each egg mass was calculated as previously described, and over all percentage data were compared between species using Friedman's χ^2 in SAS (1990). The proportion of time spent in each behavioral event was calculated for each species and compared between species, and within species, using Friedman's χ^2 and Kruskal–Wallis χ^2, respectively. Data were also grouped into oviposition (searching leaf, egg inspection, oviposition) and non-oviposition (resting/grooming, off leaf, chasing) behavior and compared between species using Kruskal–Wallis χ^2. Behavioral ethograms were constructed for each species by calculating the percentage occurrence for each behavioral path. It was hypothesized that competitive behavior may include aggression towards congenerics or patch defense strategies (Field 1998; Field et al. 1998).

Experiment 3: Sequential parasitoid exposure to H. coagulata *eggs – low density*

One *H. coagulata* egg mass (three to six eggs) either 1, 3, or 5 days of age was placed into a Petri dish lined with moist filter paper (see above), and exposed to one newly emerged mated naïve female (~24 h of age) of one parasitoid species for 1 h. After a specific time interval (see below), the same egg mass was exposed to one newly emerged mated naïve female of the second parasitoid species for 1 h. Egg number was restricted to three to six eggs to increase competition pressure and the chance of both females parasitizing the same host. The variables manipulated were *H. coagulata* egg age (1, 3, and 5 days of age), time interval between the introduction of the first and second species (1, 4, and 24 h), and sequence of species introduction [introducing *G. ashmeadi* to the egg mass first, followed by *G. triguttatus* (A-T), and introducing *G. triguttatus* first, then *G. ashmeadi* (T-A)]. Each 'egg age/species introduction sequence/intermission time' combination for a total of 18 treatments was replicated 10–20 times. Females were observed for insertion of their ovipositor into hosts every 5 min while offered egg masses in Petri dishes. Only those egg masses where both species were observed to insert their ovipositor into at least one egg within the egg mass were used for the analysis, and frequently ovipositor insertion was observed by both species in the same host. Petri dishes containing leaves with egg masses exposed to parasitoids were held for 3 weeks to allow parasitoids and *H. coagulata* nymphs to emerge. Those replicates where no parasitism occurred because of poor host survival were excluded from analyses.

The proportion of *G. ashmeadi* and *G. triguttatus* offspring produced by each Petri dish treatment was calculated as above. Transformations failed to fit proportion data to a normal distribution, therefore non-parametric tests were used for the statistical analyses. The overall percentages of *G. ashmeadi* and *G. triguttatus* offspring across all egg ages, intermission times, and introduction sequences was compared between species using Friedman's χ^2 (Iman & Davenport 1980). To investigate the effect of parasitoid species introduction sequence on offspring production, data were pooled over sequences and the percentage of *G. ashmeadi* and *G. triguttatus* offspring was compared between species and within species using Friedman's χ^2 and Kruskal–Wallis χ^2, respectively, in SAS (1990). For each 'egg age × intermission time × sequence' combination (data not pooled), two tests were conducted in SAS (1990): (1) offspring production was compared between species using Friedman's χ^2, and

(2) offspring production was compared between sequences, among intermission times, and among egg ages, within each species using Kruskal–Wallis χ^2.

Scanning electron microscopy of parasitoid larvae

Ten *G. ashmeadi* and *G. triguttatus* larvae were dissected from previously parasitized *H. coagulata* eggs 4 days after oviposition occurred. Larvae were fixed in 2% gluteraldahyde in 0.1 M phosphate buffer for 2 h, washed in buffer, and post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h. Parasitoid larvae were then washed in distilled water and dehydrated in an ethanol series and critical point dried. Larvae were mounted on SEM stubs and coated with gold-palladium. Photographs of larval morphology were taken at $\times 100-600$ magnification for each species and visually compared. SEM was used to determine if larvae of either species exhibited morphological characters (e.g., enlarged mandibles or spines) that could assist with interspecific competition within *H. coagulata* eggs.

Results

Experiment 1: Simultaneous parasitoid exposure to H. coagulata *eggs – high density*

Although there was no significant effect of species on offspring production when female parasitoids were offered *H. coagulata* eggs 5 days of age, *G. ashmeadi* offspring production was 32 and 27% greater than *G. triguttatus* when females were presented eggs 1 and 3 days of age, respectively (Figure 1). Similarly, the combined overall percentage across all egg ages was 23% higher for *G. ashmeadi* than for *G. triguttatus* (Figure 1). Differences in each species' offspring production did not significantly

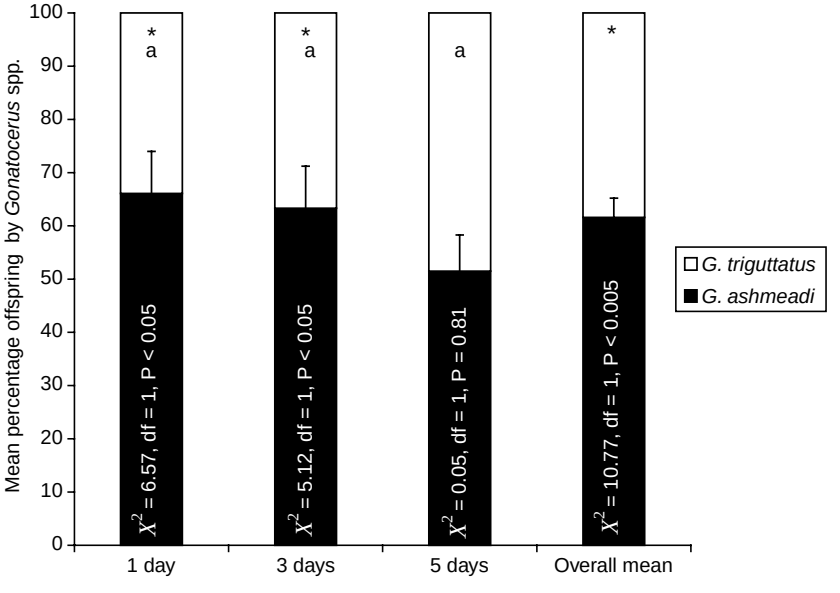

Figure 1. Percentage of *G. ashmeadi* and *G. triguttatus* offspring produced when *H. coagulata* eggs 1, 3 and 5 days of age were exposed to one female of each species [error bars indicate standard error of the means; different letters indicate significant ($P < 0.05$) differences among egg ages; asterisks and test statistics indicate significant differences between species].

differ among egg ages for both parasitoid species (Figure 1; $\chi^2 = 2.42$, df $= 2$, $P = 0.30$).

Experiment 2: Simultaneous parasitoid exposure to H. coagulata *eggs – low density*

In this experiment, *G. triguttatus* produced 22% more offspring than *G. ashmeadi*, which is the opposite outcome obtained from the first experiment. However, this result was not significant (Figure 2; $\chi^2 = 1.04$, df $= 1$, $P = 0.31$). There was no significant effect of parasitoid species on the proportion of time spent in each behavioral event (Figure 2; $P > 0.05$). When data was grouped into oviposition (searching leaf, egg inspection, oviposition) and non-oviposition (resting/grooming, off leaf, chasing) behavior, *G. triguttatus* ($52 \pm 7\%$) allocated significantly more time to oviposition behavior compared with *G. ashmeadi* ($31\% \pm 7$; $\chi^2 = 4.49$, df $= 1$, $P < 0.05$).

Within each species, the proportion of time allocated to each behavior significantly differed among behavioral events for both *G. ashmeadi* and *G. triguttatus* (Figure 2). *Gonatocerus ashmeadi* allocated up to 58% more time to resting/grooming compared with all remaining behavioral events ($P < 0.005$). Time allocated by *G. ashmeadi* to oviposition, off the leaf, and searching the leaf were all statistically equivalent ($P > 0.05$), and no observations of *G. ashmeadi* aggressively chasing *G. triguttatus* off egg masses were recorded. *Gonatocerus triguttatus* allocated statistically equal time to resting/grooming and oviposition ($P > 0.05$), and these behaviors were up to 40% higher compared with all remaining behavioral events (Figure 2; $P < 0.01$). Female *G. triguttatus* spent 0.8% of time aggressively chasing *G. ashmeadi* off *H. coagulata* egg masses. This behavior involved running directly at *G. ashmeadi* and often concluded with physical contact. Although *G. triguttatus* displayed aggressive behavior towards

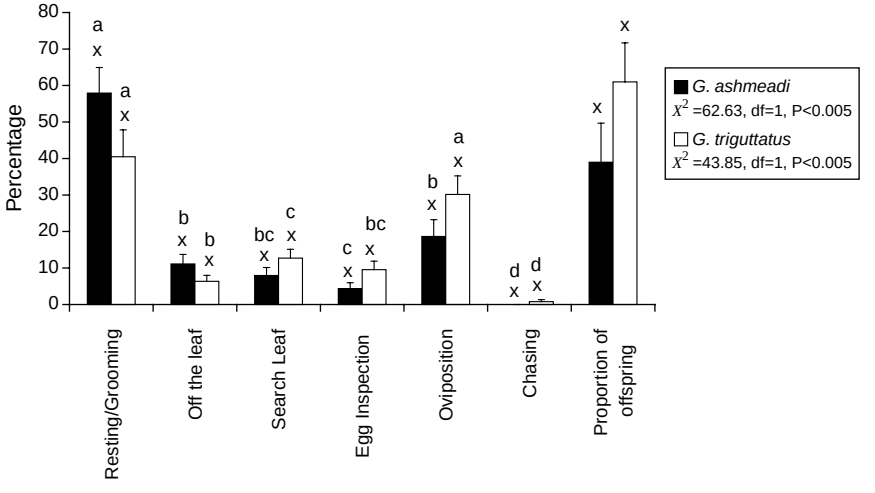

Figure 2. Percentage offspring production and the proportion of observations that female *G. ashmeadi* and *G. triguttatus* allocated to several behavior events during simultaneous exposure to one *H. coagulata* egg mass for 1 h in the laboratory [error bars indicate standard error of the means; different letters (x, y) indicate significant ($P < 0.05$) differences between species for each behavioral event; different letters (a, b) and test statistics indicate significant ($P < 0.05$) differences among behavioral events within species].

G. ashmeadi, eight accounts (6.5% of observed behavioral events) of simultaneous oviposition on the same egg mass by both species was observed in four Petri dishes.

A total of 90 and 121 behavioral transition events were recorded for *G. ashmeadi* and *G. triguttatus*, respectively. Female *G. ashmeadi* allocated most of their time transitioning from resting/grooming to moving off the leaf (12.2%), from being off the leaf to resting/grooming (10.0%), and from oviposition to resting/grooming (10.0%) (Figure 3). Aggressive chasing by *G. triguttatus* resulted in female *G. ashmeadi* engaging in resting/grooming activity (2.2%), and harassed female *G. ashmeadi* did

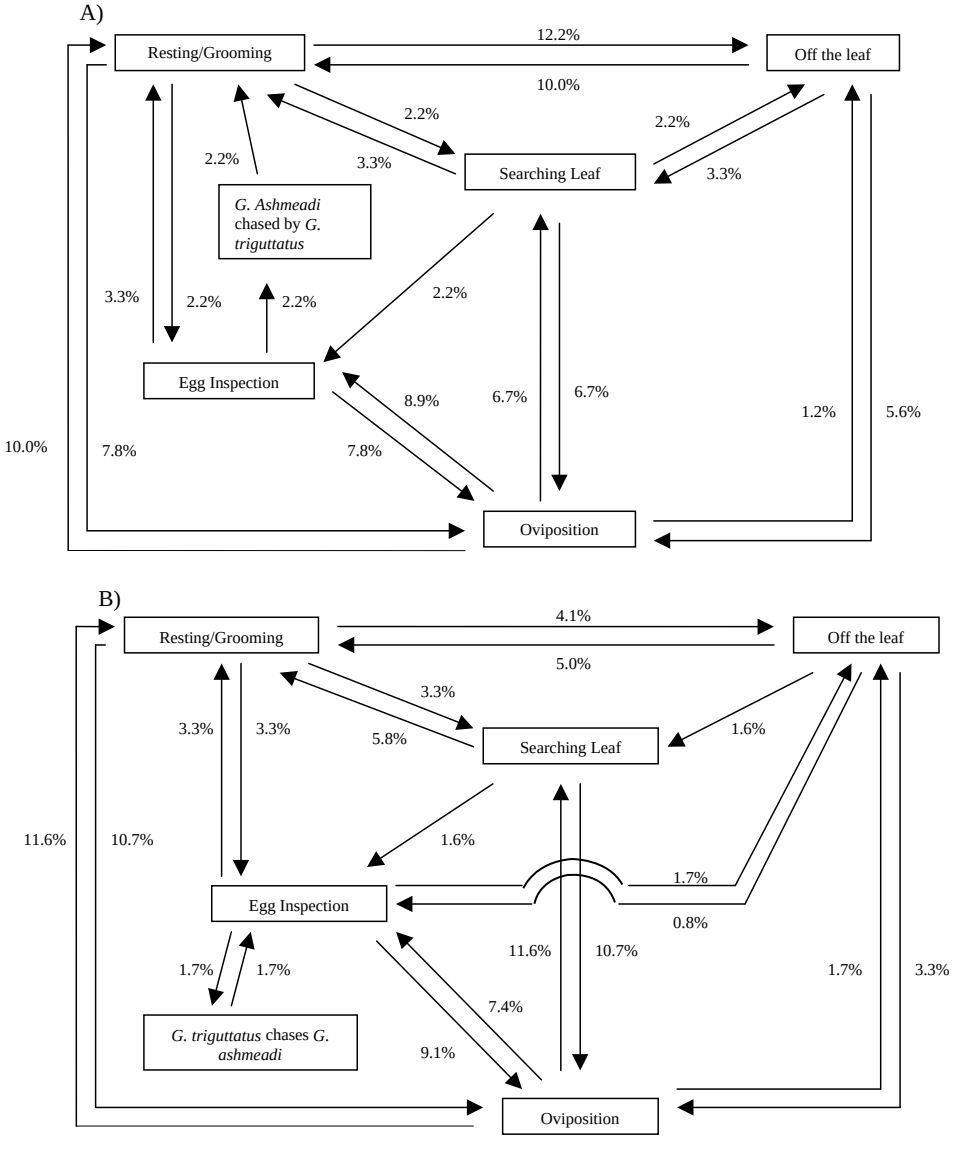

Figure 3. Ethograms for (a) *G. ashmeadi* and (b) *G. triguttatus* when one female of each species is exposed simultaneously to one *H. coagulata* egg mass for 1 h in the laboratory. Numbers adjacent to arrows are transition percentage calculated from total observed behavioral transition events.

not return to egg inspection, or searching (Figure 3). Female *G. triguttatus* allocated most of their time transitioning from resting/grooming to oviposition (10.7%), from oviposition to resting/grooming (11.6%), from searching the leaf to oviposition (10.7%), and from oviposition to searching the leaf (11.6%) (Figure 3). *Gonatocerus triguttatus* aggressively chased *G. ashmeadi* during egg inspection (1.7%) and subsequently always returned to inspecting the egg mass after an interspecific encounter (1.7%) (Figure 3).

Experiment 3: Sequential parasitoid exposure to H. coagulata *eggs – low density*

Pooling data over all egg ages, intermission times and both species introduction sequences showed that mean percentage offspring production was 28% higher for *G. triguttatus* ($64 \pm 3\%$) than *G. ashmeadi* ($36 \pm 3\%$; $\chi^2 = 42.26$, df $= 1$, $P < 0.005$). For sequence T-A, *G. triguttatus* percentage offspring production ($87 \pm 3\%$) was 74% higher compared with *G. ashmeadi* ($13 \pm 3\%$; $\chi^2 = 128.20$, df $= 1$, $P < 0.005$). For sequence A-T, the percentage of *G. ashmeadi* offspring produced ($58 \pm 4\%$) was significantly higher, but just 16% greater compared to *G. triguttatus* ($42 \pm 4\%$; $\chi^2 = 6.43$, df $= 1$, $P < 0.05$). Comparing percentage offspring production within species demonstrated that when *G. triguttatus* ($87 \pm 3\%$) and *G. ashmeadi* ($58 \pm 4\%$) were introduced first to an *H. coagulata* egg mass, total percentage offspring was over 2- and 4-fold higher, respectively, when compared to females being second in the oviposition sequence.

Gonatocerus triguttatus consistently produced more offspring up to 96% compared with *G. ashmeadi*, when *G. triguttatus* was introduced first onto an egg mass, regardless of egg age or intermission time (Figure 4; Table I). Additionally, *G. triguttatus* produced 76% more offspring compared with *G. ashmeadi*, when *G. triguttatus* was exposed to an egg mass 1 day of age, 1 h after *G. ashmeadi* had previously parasitized the same egg mass (Figure 4; $\chi^2 = 4.50$, df $= 1$, $P < 0.05$).

When *G. ashmeadi* was introduced to an egg mass first, the percentage of *G. ashmeadi* offspring was significantly higher (46%) than *G. triguttatus*, only when eggs were 3 days of age, and the time between introduction of *G. triguttatus* was extended to 24 h (Figure 4; $\chi^2 = 4.11$, df $= 1$, $P < 0.05$). All other comparisons between species were not significant (Figure 4; $P > 0.05$).

When eggs 1 day of age were exposed to *G. ashmeadi* first in an oviposition sequence, intermission time had a significant effect on the percentage of *G. ashmeadi* offspring produced (Figure 4; $\chi^2 = 7.17$, df $= 1$, $P < 0.05$). Percentage of *G. ashmeadi* offspring was up to 46% higher when the time between the introduction of *G. triguttatus* was 4 ($\chi^2 = 4.69$, df $= 1$, $P < 0.05$) and 24 h ($\chi^2 = 6.95$, df $= 1$, $P < 0.005$) after *G. ashmeadi*, compared with a 1-h intermission time, and *G. ashmeadi* percentage offspring production was equivalent between intermission times 4 and 24 h ($\chi^2 = 0.07$, df $= 1$, $P = 0.80$). Intermission time had no other significant effects on the percentage of *G. ashmeadi* and *G. triguttatus* produced for all other 'egg age by introduction sequence' combinations (Figure 4; $P > 0.05$).

Egg age had a significant effect on the percentage of *G. ashmeadi* offspring produced when *G. ashmeadi* was first in an oviposition sequence and intermission time between species introductions was 1 h (Figure 4; $\chi^2 = 6.89$, df $= 2$, $P < 0.05$). For this combination, percentage of *G. ashmeadi* offspring was 59% higher when females were exposed to eggs 3 days of age, compared with 1 day of age ($\chi^2 = 6.78$, df $= 1$, $P <$

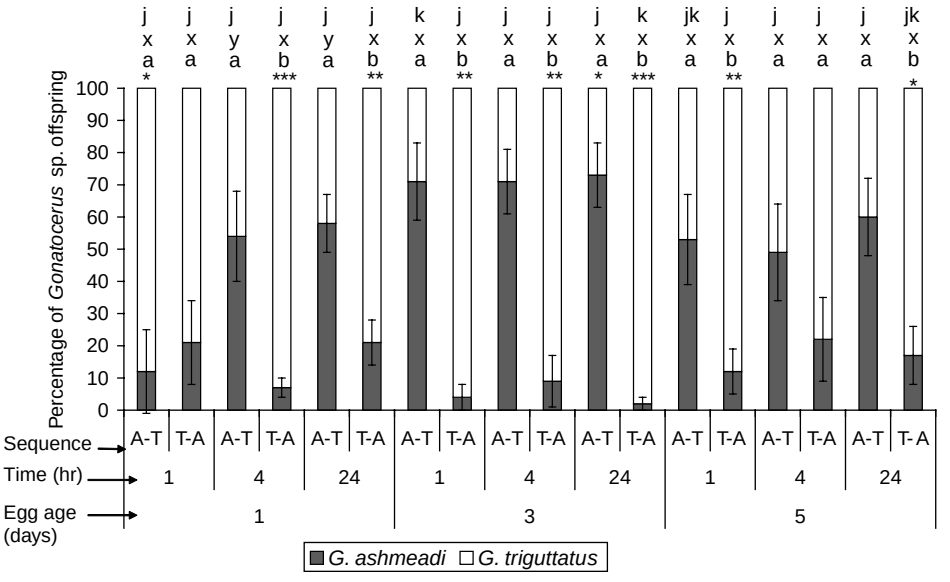

Figure 4. The effect of varying *H. coagulata* egg age, sequence of parasitoid species introduction and intermission time between introduction of the first and second parasitoid species on the proportions of *G. ashmeadi* and *G. triguttatus* offspring when one female of each species is introduced onto a *H. coagulata* egg mass for 1 h [error bars indicate standard error of the means; A-T = *G. ashmeadi* followed by *G. triguttatus*; T-A = *G. triguttatus* followed by *G. ashmeadi*; asterisks indicate significant difference between species (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.005$); different letters (a, b) indicate significant ($P < 0.05$) differences in proportion of *G. ashmeadi* and *G. triguttatus* between A-T and T-A within each egg age/intermission time combination; different letters (x, y) indicate significant ($P < 0.05$) differences in proportions of *G. ashmeadi* and *G. triguttatus* among intermission time categories within each egg age/sequence introduction combination; different letters (j, k) indicate significant ($P < 0.05$) differences in proportions of *G. ashmeadi* and *G. triguttatus* among egg age categories within each intermission time/sequence introduction combination].

0.01). There were no other significant differences in *G. ashmeadi* and *G. triguttatus* offspring between egg ages for this combination ($P > 0.05$). When *G. triguttatus* was first in an oviposition sequence and intermission time between species introductions was 24 h, egg age had a significant effect on *G. triguttatus* offspring production (Figure 4; $\chi^2 = 6.16$, df = 2, $P < 0.05$). For this combination, percentage of *G. triguttatus* offspring produced was 19% higher when females were exposed to eggs 3 days of age compared with 1 day of age ($\chi^2 = 6.37$, df = 1, $P < 0.05$). There were no other significant differences in *G. triguttatus* offspring among egg ages for this combination ($P > 0.05$). Furthermore, there were no additional significant differences

Table I. Table of statistical outcomes for each egg age and intermission time combination, when *G. triguttatus* was introduced to a *H. coagulata* egg mass prior to the introduction of *G. ashmeadi*.

Intermission time Egg age	1 h (χ^2, df, *P*)	4 h (χ^2, df, *P*)	24 h (χ^2, df, *P*)
1 day	3.35, 1, >0.05	11.32, 1, <0.005	8.51, 1, <0.005
3 days	8.36, 1, <0.005	8.25, 1, <0.005	14.75, 1, <0.005
5 days	8.80, 1, <0.005	3.27, 1, =0.07	6.40, 1, <0.05

in percentage parasitoid offspring production among egg ages for the remaining intermission time/sequence combinations (Figure 4; $P > 0.05$).

Scanning electron microscopy of parasitoid larvae

SEM revealed that *Gonatocerus ashmeadi and G. triguttatus* larvae are vermiform and possess tusk-like mandibles. Since immature *H. coagulata* eggs consist of cytoplasm which does not require chewing, mandibles could possibly be used for intra and interspecific larval combat should more than one larva be developing in an egg (Figure 5).

Discussion

The evaluation of putative biological control agents in the laboratory runs the risk of, not only ignoring critical factors associated with the organism's ecology, but also oversimplifying the factors that the researcher claims to be addressing. Within the context of the classical biological control program for *H. coagulata* we attempted to investigate competitive interactions between *G. ashmeadi* and *G. triguttatus*. The primary role of the study was to determine which species would be the superior natural enemy for *H. coagulata* suppression, and most likely dominate in the field. Two scenarios were employed; the first assuming high host densities such as those occurring during epidemic outbreaks, and the second assuming low densities that one might expect once *H. coagulata* populations diminish as a result of natural enemy activity. In experiment one, which involved exposing females to high *H. coagulata*

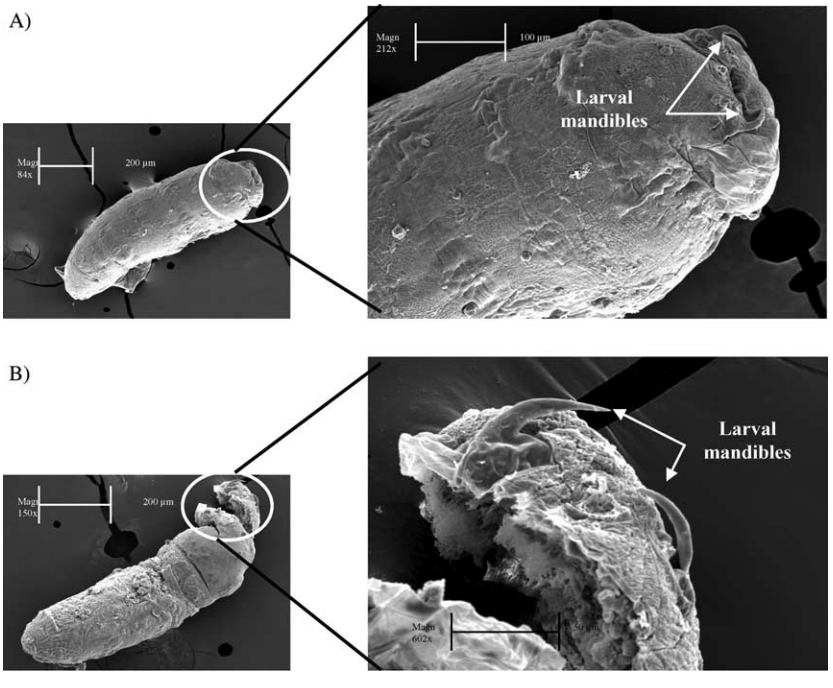

Figure 5. Scanning electron microscopy photographs showing larval mandibles of (A) *Gonatocerus ashmeadi* and (B) *G. triguttatus* dissected from *H. coagulata* eggs parasitized 4 days previously.

densities (30 eggs per vial), *G. ashmeadi* was significantly more successful at parasitizing their host than *G. triguttatus*. However, at low host densities (three to six eggs; experiment two), the outcome was reversed. *Gonatocerus triguttatus* out competed *G. ashmeadi* in both sequential parasitism studies (experiment three) and in simultaneous parasitism studies (experiment two), although the latter was not significant. The simplistic identification of an agent as competitively inferior or superior evidently does not hold here. Determination of competitive outcome is strongly affected by experimental conditions, and raises the question as to whether such labels hold consistently with any biological control agent, especially when host density and environmental conditions regularly change under field conditions.

It has been argued that it is better to determine *a priori* which natural enemy shows the most potential as a biological control agent, and efforts should focus on establishing the most efficient species (Turnbull & Chant 1961; Watt 1965; Turnbull 1967; Ehler & Hall 1982; van den Bosch 1968; Huffaker 1971; Briggs 1993). Although investigating parasitism and progeny outcomes from interspecific competition studies in the laboratory can be valuable in determining which biological control agent has the potential to be the most efficacious, our work indicates that experimental design can produce results that favor different parasitoid species. Results suggest that a range of laboratory-based experimental designs need to be considered, however funding for rearing of biological control agents is limited, and testing for the best parasitoid species from a suite of candidates is costly and time consuming. So, how do we decide, from results of laboratory-based studies such as those outlined here, which parasitoid species from a suite of candidates shows the most potential as a biological control agent?

Experimental designs should realistically reflect prevailing and potential future field situations where competition between natural enemies for resources would occur. Experiment one (30 eggs/vial) is representative of high *H. coagulata* egg densities that occur in Californian citrus orchards over summer (Blua et al. 2001; Blua & Morgan 2003). Under these conditions our results suggest that *G. ashmeadi* should be the most effective natural enemy, which supports data from Irvin and Hoddle (2005b). Although parasitism by *G. ashmeadi* can reach up to 100% in summer for short periods (Triapitsyn & Phillips 2000), results presented here indicate that the intrinsic competitive ability and aggressiveness of female *G. triguttatus* may allow this species to successfully compete with *G. ashmeadi* and accomplish widespread establishment in California. To date, over 150 egg masses parasitized by *G. triguttatus* have been recovered in the field, and two recoveries were from locations that had not received *G. triguttatus* releases (Morgan, unpubl.) thereby supporting results shown here.

Experiments two and three (three to six eggs/Petri dish) are representative of low *H. coagulata* egg mass densities that occur in spring (Blua et al. 2001; Blua & Morgan 2003), and could potentially occur year round as a result of successful biological control that causes the target population to decline in abundance. Experimental designs that simulate low *H. coagulata* densities indicate that *G. triguttatus* may have a competitive advantage over *G. ashmeadi* at low *H. coagulata* egg densities. If biological control of *H. coagulata* California is successful, there may be a shift in current species dominance from the self-introduced *G. ashmeadi* to the deliberately released *G. triguttatus* as *H. coagulata* populations diminish. This may, in conjunction with climatic differences, partly explain the occurrences currently seen in parts of eastern Texas where *G. triguttatus* is the dominant parasitoid species in areas where

H. coagulata is extremely rare (Triapitsyn & Phillips 2000). In Florida, where *H. coagulata* is more common, *G. ashmeadi* is dominant and *G. triguttatus* is rare (Triapitsyn & Phillips 2000). However, large-scale field-based competition studies investigating searching efficiency, density responsiveness, and synchrony with the host, on a variety of *H. coagulata* host plants, are necessary to aid further speculation regarding the biological control potential and competitive ability of *H. coagulata* parasitoids.

One of the main objectives of this study was to investigate intrinsic competition between *G. ashmeadi* and *G. triguttatus* when females simultaneously foraged on the same *H. coagulata* egg mass, and to determine whether the sequence of introduction to an egg mass, supplying females with their most preferred egg age, or a delay in the introduction of the second parasitoid may present a competitive advantage. Results clearly showed that parasitoid introduction sequence had a dramatic effect on the proportion of offspring produced by *G. ashmeadi* and *G. triguttatus,* and that when a female parasitoid of either species oviposited in an unparasitized egg mass first, the chance for her offspring to emerge increased considerably. When *G. triguttatus* and *G. ashmeadi* were introduced first to an *H. coagulata* egg mass, total percentage offspring was over 2- and 4-fold higher, respectively, when compared to females of the same species being second in the oviposition sequence. This effect of parasitization sequence was most pronounced for *G. ashmeadi*, and suggests that it may be more important for this species to locate and oviposit in unparasitized hosts before *G. triguttatus* to ensure maximum progeny production when both species are competing for egg masses. This may become more important as biological control of *H. coagulata* progresses in California and target populations diminish.

When *G. ashmeadi* was introduced to an egg mass 1 h before *G. triguttatus*, percentage of *G. ashmeadi* offspring was 59% higher when females were exposed to eggs 3 days of age, compared with 1 day of age. *Gonatocerus ashmeadi* prefer to parasitize 3-day-old hosts (Irvin & Hoddle 2005a), consequently, results from the current study may suggest that the competitive ability of *G. ashmeadi* was increased when oviposition into *H. coagulata* eggs of the most preferred age occurred first. Similarly, when *G. triguttatus* was introduced to an egg mass 24 h before *G. ashmeadi*, percentage *G. triguttatus* offspring production was 19% higher when females were exposed to eggs 3 days of age compared with 1 day of age. Three-day-old hosts are readily utilized by *G. triguttatus* because they may provide a more favorable environment for developing *G. triguttatus* larvae (Irvin & Hoddle 2005a), thereby enhancing their competitive ability in the current study.

Increasing the time interval length between species introductions to hosts can affect parasitoid competitive outcomes because parasitoid larvae may hatch first and kill congenerics or physiologically change the host to inhibit congeneric oviposition or development (Salt 1961; Chow & Mackauer 1984; Chow & Mackauer 1986; Laing & Corrigan 1987; Tilman & Powell 1992). Results from the current study showed that when eggs 1 day of age were exposed to *G. ashmeadi* first in an oviposition sequence, up to 46% more *G. ashmeadi* offspring was produced when the time between the introduction of *G. triguttatus* was 4 and 24 h after *G. ashmeadi*, compared with a 1-h intermission time. However, intermission time did not have a consistently significant effect on parasitoid offspring production over all 'egg age, sequence, and intermission time' combinations.

For experiment three, the over all proportion of *G. triguttatus* offspring was 28% higher compared with *G. ashmeadi*, and when offspring production was compared between species at each 'egg age, intermission time, sequence combination', results showed that *G. triguttatus* consistently produced up to 96% more percentage offspring compared with *G. ashmeadi*, when *G. triguttatus* was introduced first onto an egg mass, regardless of egg age or intermission time. Additionally, *G. triguttatus* produced 76% more offspring compared with *G. ashmeadi*, when *G. triguttatus* was exposed to an egg mass 1 day of age, 1 h after *G. ashmeadi* had previously parasitized the same egg mass. In contrast, when *G. ashmeadi* was introduced to an egg mass first, *G. ashmeadi* offspring production was significantly higher (46%) than *G. triguttatus* only when eggs were 3 days of age (most preferred egg age), and the time between the introduction of *G. triguttatus* was extended to 24 h. Consequently, *G. triguttatus* may be intrinsically superior to *G. ashmeadi.* This experiment was purposely designed to use low host densities to maximize the strength of interference competition between *G. ashmeadi* and *G. triguttatus* [i.e., 3–6 eggs were available for parasitism which is well within the maximum daily oviposition rate for these parasitoids (Hoddle & Pilkington 2004)]. It has been demonstrated that multiparasitism occurs at low host densities and naïve female parasitoids, such as those that were used here, may multiparasitize due to an inability to recognize parasitism by another species (Godfray 1994). Consequently, the combination of low host density and female naivety most likely maximized the likelihood of intrinsic competition occurring between *G. ashmeadi* and *G. triguttatus* larvae within *H. coagulata* eggs.

Several mechanisms could be responsible for the intrinsic superiority of *G. triguttatus* demonstrated in the current study. First, *G. triguttatus* larvae may hatch more quickly and grow more rapidly than immature *G. ashmeadi* (Campbell & Mackauer 1973; DeMoraes et al. 1999). Second, *G. triguttatus* larvae may utilize specialized structures (e.g., enlarged mandibles) to physically eliminate competitors (Salt 1961; Chow & Mackauer 1986; Lawrence 1988; Mackauer 1990; Tillman & Powell 1992). Results from SEMs of 4-day-old larvae showed that both *G. ashmeadi* and *G. triguttatus* have enlarged mandibles possibly indicating that larvae of these species fight within hosts for resource procurement. Third, *G. triguttatus* may kill competitors via starvation (Simmonds 1943; Wylie 1971; Lawrence 1988), or fourth, by physiologically suppressing the growth of competitors (Fisher 1961; Salt 1961; Hofsvang & Hågvar 1983; Wylie 1983; Lawrence 1988; Mackauer 1990). Fifth, ovicide, the deliberating killing of a competitor's eggs by a parasitizing female with her ovipositior, may be employed by *G. triguttatus* (Mayhew 1997; Netting & Hunter 2000), or sixth, differences in behavior may exist between species, e.g., *G. ashmeadi* may prefer to parasitize larger egg masses, or lay one egg per host egg mass and then disperse.

When the introduction of female *G. ashmeadi* and *G. triguttatus* to an egg mass was staggered (experiment three), *G. triguttatus* showed significant intrinsic superiority by producing 28% more offspring production compared with *G. ashmeadi*. However, when females were introduced to an egg mass simultaneously (experiment two) percentage offspring production was not significantly different between species, suggesting that the competitive superiority of *G. triguttatus* may have been reduced. Decreased progeny production by *G. triguttatus* when *G. ashmeadi* was simultaneously competing for eggs may be attributable to competitive interference between adults. The presence of congenerics initiated patch defense behavior in *G. triguttatus*, and

direct contact between parasitoids may have caused a reduction in searching efficiency and time allocated to oviposition (Hassell & Varley 1969). Ethogram results showed that *G. triguttatus* allocated approximately 2% of its behavior to aggressively chasing *G. ashmeadi* off a contested egg mass and this behavior always interrupted egg inspection. This interruption may have caused a delay in oviposition, thereby contributing to the decrease in offspring production by *G. triguttatus*. However, *G. ashmeadi* harassed in this manner never returned to a contested egg mass, which almost certainly reduced its reproductive potential.

Although *G. triguttatus* showed aggressive behavior towards *G. ashmeadi*, eight accounts of simultaneous oviposition by both species was recorded on the same egg mass during observation experiments, which is similar to Irvin and Hoddle (2005b). These results demonstrate that congeneric females can co-exploit patches and encounter one another without initiating aggression. Reduction of aggressiveness allowing patch co-exploitation may occur if females are of similar size (Petersen & Hardy 1996).

In the current study, *G. triguttatus* allocated a statistically equal amount of time to resting/grooming and oviposition, and ethogram results demonstrated that there was a repetitive sequence consisting of transitioning from oviposition to resting/grooming (11.6%), and then returning to oviposition (10.7%). The oviposition–resting–oviposition cycle demonstrated by *G. triguttatus* may be a form of patch defense. Resting or stationary behavior in the egg parasitoid *Trissolcus basalis* (Wollaston) (Scelionidae) was a component of antagonistic behavior, and functioned as pre-emptive patch defense (Field 1998; Field et al. 1998). Females may sit stationary after oviposition to increase their sensitivity to movements, allowing a competitor to approach the patch more closely, thereby increasing the probability of detection (Field et al. 1998). An increase in stationary behavior by *G. triguttatus* as a patch defense strategy may have contributed to lower total offspring production by this species, when compared with staggered female introduction (experiment three). It would have been beneficial to investigate whether the presence of a competitor directly affected parasitoid behavior by including a non-competitor treatment, and comparing data between Petri dishes containing a competitor, and those without.

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