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Differing Success of Defense Strategies in Two Parasitoid Wasps in Protecting Their Pupae Against a Secondary Hyperparasitoid

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ABSTRACT During their larval development, endoparasitoids are known to dispose of host resources in several different ways. Some parasitoid wasps consume most or all tissues of the host, whereas others consume a small fraction of host resources and either ensure that the host moves away from the pupation site or allow the host to remain close to the parasitoid cocoon(s). Using a single host species, *Mythimna separata* Walker (Lepidoptera: Noctuidae), this study compares the success of the two pupation strategies in the solitary parasitoids *Microplitis* sp. and *Meteorus pulchricornis* Wesmael (Hymenoptera: Braconidae) against attack from a secondary hyperparasitoid, *Gelis agilis* F. (Hymenoptera: Ichneumonidae). The caudal appendages of *M. separata* caterpillars parasitized by *Microplitis* sp. remain physically attached to parasitoid cocoons and the caterpillars behave aggressively when disturbed. However, after *Me. pulchricornis* larvae emerge from caterpillars of their host, *M. separata*, the parasitoid larvae pupate in cocoons that are suspended by a single thick thread that hangs 1–2 cm from under a leaf. In choice tests conducted in petri dishes, significantly fewer cocoons of *Microplitis* sp. attended by caterpillars than unattended cocoons were hyperparasitized by *G. agilis*. By contrast, *Me. pulchricornis* cocoons that were hanging from corn, *Zea mays* L., plants were hyperparasitized as frequently as those which were attached to leaves. We discuss the potentially different selection pressures generated among natural enemies such as predators and hyperparasitoids in determining optimal pupal defense strategies in primary parasitoids.

KEY WORDS brood guarding, *Gelis agilis*, host manipulation, *Meteorus pulchricornis*, *Microplitis* sp.

Parasitoid wasps are insects that develop in, or on, the body of other arthropods, whereas the adults are free-living (Godfray 1994). Interactions between parasitoids and their hosts are characterized by a high degree of physiological intimacy, and many endoparasitoids are known to regulate the internal host environment in ways that optimize the utilization of host resources and the survival of their progeny (Vinson and Iwantsch 1980, Fleming 1992, Harvey 2005, Pennacchio and Strand 2006). However, during their development inside or on the host body, immature parasitoid stages are effectively defenseless against many extrinsic threats, such as the predators of their primary hosts or primary hyperparasitoids. In this scenario, parasitoids depend on the host possessing behavioral defenses that enable them to repel attackers or else in their hosts occupying microhabitats where the risk of attack is low. Several studies have reported that the ecology of parasitized hosts and healthy cohorts differ, presumably due to the regulation of host behavior as a

result of parasitism (Stamp 1981; Fritz 1982; Brodeur and McNeil 1989, 1992; Eberhard 2000; Matsumoto 2009).

Extrinsic threats to the parasitoids also occur during the pupal stage, when this stage is also potentially vulnerable to attack from predators and secondary hyperparasitoids (Brodeur and Vet 1994). Because the pupa of the parasitoid is largely defenseless, parasitoids have evolved several strategies to reduce the risk of being attacked. These include cocoon formation in cryptic sites (Stamp 1981, Tagawa and Fukushima 1993), the construction of cocoons that are colored in such a way as to mimic bird droppings (Shaw et al. 2009), and the weaving of dense silk threads to make the cocoons more impervious to penetration from the ovipositors of hyperparasitoids or the jaws of predators (Tagawa and Sato 2009). These strategies, however, are limited in that a defensive mechanism that is more effective against hyperparasitoids may be less effective against predators and vice versa. Consequently, optimal defense strategies in parasitoid pupae may be association-specific and based on frequency-dependent selection balancing risks from different kinds of attackers.

The caterpillars of many lepidopteran species harbor multiple parasitoid species, each of which may dispose of resources differently. For example, larvae of *Mythimna separata* Walker (Lepidoptera: Noctuidae)

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are attacked by several species of braconid parasitoids. The mature larvae of two of its gregarious parasitoids, *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae) and *Cotesia ruficrus* Haliday (Hymenoptera: Braconidae) emerge from the intact host, which dies within several hours of parasitoid egression (Harvey et al. 2008b). However, a closely related solitary parasitoid, *Microplitis* sp. (Hymenoptera: Braconidae) emerges from intact caterpillars that remain alive for up to a week or more after parasitoid egression. The parasitoid cocoon is attached to the surface of a leaf, and the caudal appendages of the host caterpillar remain physically attached to the parasitoid cocoon. The parasitized caterpillar exhibits extremely aggressive behavior when it is disturbed, including frequent bouts of head thrashing, and biting (Harvey et al. 2011). Still another solitary braconid wasp, *Meteorus pulchricornis* Wesmäl (Hymenoptera: Braconidae), also emerges from an intact host which drops from the food plant and dies within a few hours. The parasitoid larva produces a cocoon at the end of a strong, single silk thread that enables it to hang from a leaf. Here, we investigate the significance of the pupation behavior of the two solitary endoparasitoids of *M. separata* against a hyperparasitoid.

The success of different strategies in reducing the risk of predation, hyperparasitism, or both has received increasing attention in recent years. Several studies have shown that retaining the use of the surviving host as a "bodyguard" is an effective strategy against hyperparasitoids (Harvey et al. 2008a,b) and predators (Kester and Jackson 1996, Grosman et al. 2008). However, the results of other studies have been less conclusive (Tanaka and Ohsaki 2006). A recent study also reported that *M. pulchricornis* cocoons that were suspended from threads suffered lower predation from ants than cocoons that were attached to the leaf surface (Shirai and Maeto 2007).

Using a single host species, *M. separata*, this study compares the success of the two pupation strategies described above in the parasitoids *Microplitis* sp. and *Me. pulchricornis* against attack from the secondary hyperparasitoid *Gelis agilis* F. (Hymenoptera: Ichneumonidae). We discuss how selection pressure generated by natural enemies such as predators and hyperparasitoids influences optimal pupal defense strategies in primary parasitoids.

Materials and Methods

Insects. All insects were maintained in a climate room at $25 \pm 2^\circ\text{C}$ and a photoperiod of 16:8 (L:D) h. Individuals of *M. separata*, *Me. pulchricornis*, and *Microplitis* sp. were originally collected from field sites located in Aichi and Kagoshima prefectures, Japan. The biology of *M. separata* is described in Kawaguchi and Tanaka (1999). Female moths lay clusters of eggs onto withered leaves of grass. Newly hatched larvae were placed in groups of up to several hundred in petri dishes (2 by 9 cm) containing blocks of artificial diet (INSECTA-LF Nihon Nohsan, Kanagawa, Japan) as described in Suzuki and Tanaka (2007). Larvae of *M.*

separata complete six instars before pupation. When the larvae reached the final instar, they were transferred to large plastic boxes (30 by 22.5 by 6 cm) that were covered by a lid to prevent escape. Pupae were collected from the rearing boxes, and after emergence the moths were placed into rearing cages (36 by 24 by 36 cm) with 2–3% honey solution absorbed into cotton wool provided as a source of adult nutrition. Mated female moths were allowed to oviposit directly onto folded parafilm paper sheets that were placed inside the cages.

The biology of *Microplitis* sp. is described in Tanaka et al. (1984). The species was originally classified as *Mi. mediator*, but recent taxonomic studies indicated that this identification is incorrect and the species is not known (M. Shaw and K. Maeto, personal communication). In the laboratory the parasitoid was reared exclusively on second stage (L2) larvae of *M. separata*. Groups of ≈ 50 host larvae were placed into plastic cylinders (14 by 13 cm) containing diet and 10–20 mated female wasps that were >5 d old. Wasps were allowed to parasitize caterpillars over the course of several hours; larvae were then placed in rearing boxes (as described above) until parasitoid egression and pupation. Parasitoid pupae (cocoons) were maintained in age-specific cohorts in groups of 10–20 in glass tubes (15 by 4 cm) that were closed at both ends with foam plugs. Honey was smeared on the walls of the tubes and water absorbed into cotton wool as food for adult wasps.

The biology of *Me. pulchricornis* is described in Wu et al. (2008) and Harvey et al. (2010). The species reproduces sexually over much of the western Palearctic, whereas in central and eastern Asia populations reproduce asexually (Lui and Li 2006). Here, an asexual strain was used. The parasitoid was reared exclusively on L3–L4 larvae of *M. separata*. Groups of ≈ 50 larvae were placed into plastic cylinders (14 by 13 cm) containing diet and 10–20 female wasps that were >6 d old. Wasps were allowed to parasitize caterpillars for several hours; larvae were then placed in rearing boxes (as described above) until parasitoid egression and pupation. Parasitoid pupae were maintained in age-specific cohorts in glass beakers containing 20% sugar solution absorbed into paper towels. The solution was refreshed once weekly.

G. agilis was originally obtained from cocoons of *Cotesia glomerata* L. (Hymenoptera: Braconidae) recovered from leaves of cabbage, *Brassica oleracea* L., growing in a garden plot adjacent to the Institute of Ecology, Heteren, The Netherlands. *G. agilis* is an asexually reproducing parasitoid that is widespread across most of Eurasia. Like many ectoparasitic idiobionts, adult females of *G. agilis* perforate the host cocoon with their ovipositor and inject permanently paralyzing venom into the prepupa or pupa. After envenomation, the wasp lays a single egg on the moribund host. After the parasitoid egg hatches, the larva ruptures the host cuticle with its mandibles and imbibes hemolymph, and as it grows it begins attacking other tissues indiscriminately and eventually consumes the entire host, pupating within the cocoon of

C. glomerata. In culture, *G. agilis* were maintained exclusively on 1–2-d-old pupae of *Microplitis* sp. Parasitoids were kept in petri dishes (2 by 9 cm) at room temperature until use.

Choice Experiments in Petri Dishes: *Microplitis* sp. and *M. separata*. Adult *Microplitis* sp. parasitoids were taken from the holding cages and were individually placed in glass vials (5 by 3 cm). Larvae of *M. separata* were removed from the culture as early L2 and were individually presented to *Microplitis* sp. females at the end of a brush. Parasitism was visually confirmed by insertion and removal of the ovipositor into the host. Between 400 and 500 larvae were parasitized over the course of 4 h. Groups of ≈ 100 parasitized caterpillars were placed into large plastic cages (30 by 22.5 by 6 cm) containing artificial diet. A single sheet of blotting paper was placed over the feeding caterpillars to provide a pupation substrate for the parasitoids. When the parasitoid larvae are mature, they emerge through the side of the host caterpillar and construct a brown, papery cocoon that is attached by cocoon silk to the blotting paper. The parasitoid larva chooses a pupation site directly below the body of the caterpillar which habitually rests its caudal appendages on the cocoon (Fig. 1A). All body segments of the caterpillar posterior to the parasitoid emergence hole are paralyzed, whereas those anterior to the hole are not and remain active when disturbed.

Small sections of blotting paper containing cocoons and caterpillars were cut from the paper with a pair of fine scissors. From half of the cocoons the caterpillars were gently removed using a pair of soft tweezers. Two sets of cocoons with or without caterpillars were individually placed into petri dishes (2 by 9 cm) at opposite ends of the dish (thus there were four cocoons per dish). Individual *G. agilis* wasps that were 5–10 d old and that had previous experience with host cocoons (for host-feeding purposes) were then placed into each of the dishes and their behavior was visually monitored over the next 60 min. When a cocoon was mounted by a hyperparasitoid and stung, this was recorded. At this point, the observation was ended and the cocoons were removed from the arena and placed in plastic vials that were sealed with lids. The fate of these cocoons was determined. This experiment was replicated 101 times with new wasps. The numbers of *G. agilis* wasps emerging from these cocoons were statistically analyzed using a chi-square test, with H_0 being the number of successful parasitisms is equal in host cocoons with and cocoons without an attending caterpillar.

The number of times a hyperparasitoid female physically contacted a cocoon in arenas before rejecting it (in cocoons with caterpillars) or accepting it (in cocoons without caterpillars) also was determined. Data for wasp contacts on the three cocoons (out of 84) in which a caterpillar was present but which was successfully hyperparasitized was dropped, as were observations in which the first cocoons contacted (that was caterpillar-unattended); thus, the data are based exclusively on unattended cocoons in which an attended cocoon was first parasitized. Data comparing

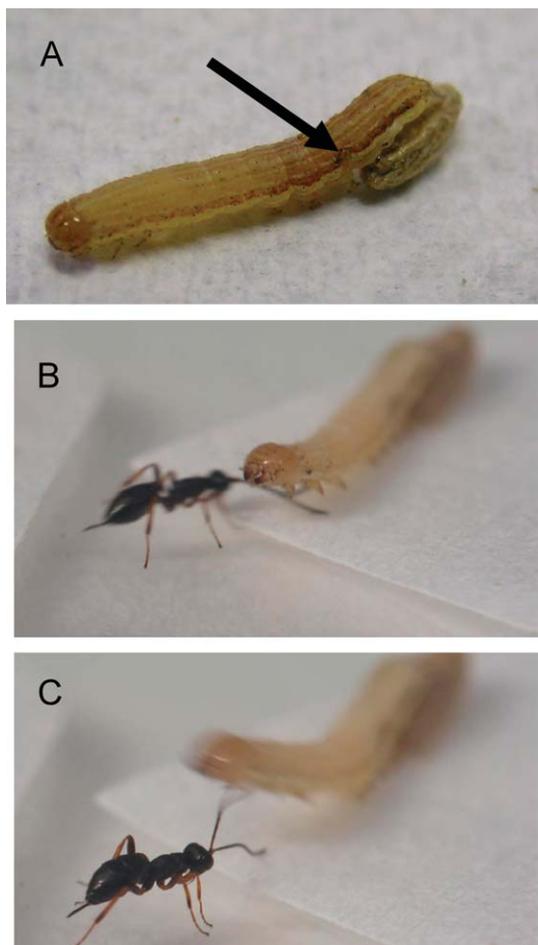


Fig. 1. Photographs showing aggressive behavior of a *M. separata* caterpillar that deters attack by *G. agilis* on a cocoon of *Microplitis* sp. (A) Parasitized caterpillar of *M. separata* with caudal appendages attached to a cocoon of *Microplitis* sp. Note egression hole of parasitoid larva in ninth segment (dark arrow) of *M. separata* caterpillar. All appendages behind this hole are effectively paralyzed. (B) Parasitized *M. separata* larva attempting to bite *G. agilis*. (C) *M. separata* larva vigorously thrashing its head capsule at *G. agilis*. (On-line figure in color.)

the number of cocoon contacts before parasitism (for caterpillar-unattended cocoons only) or being rebuffed (for caterpillar-attended cocoons) were compared using an unpaired *t*-test.

Parasitism Preference Experiments on Corn, *Zea mays* L., Plants: *Me. pulchricornis* and *M. separata*. Adult *Me. pulchricornis* parasitoids were taken from the holding cages and were individually placed in plastic vials (5 by 3 cm). Larvae of *M. separata* were removed from the culture as early L3 and were individually presented to *Me. pulchricornis* females at the end of a brush. Parasitism was visually confirmed by insertion and removal of the ovipositor into the host. Approximately 200 L3 larvae of *M. separata* were individually parasitized by females of *Me. pulchricornis*

over 2 h (as described above). Groups of ≈ 100 parasitized caterpillars were placed into large plastic cages (30 by 22.5 by 6 cm) containing artificial diet. Approximately 24 h before the wasps began emerging from their caterpillar hosts, they were removed from the diet and placed in groups of 20–30 on 3-wk-old corn plants, one of the main food plants of *M. separata* in nature. Each corn plant was cultivated separately in plastic cups (250 ml) containing water absorbed into cotton wool. Natural daylight was supplemented by metal-halide lamps ($225 \mu\text{mol photons/m}^2/\text{s}$) during the 16-h photoperiod. The plants were watered daily. Parasitoids were allowed to emerge from their hosts and to pupate by dangling from threads on the food plants. Approximately half of the cocoons were allowed to remain suspended from the plants, whereas threads of the remaining cocoons were clipped and they were gently attached to the leaf surfaces with water-soluble glue (Bond, Konishi Co., Osaka, Japan). The reason was to clarify whether cocoons that dangled from leaves with threads reduced the risk of hyperparasitism. One day after the cocoon treatment, two adult female *G. agilis* wasps were released at the bottom stem of an individual corn plant ($n = 7$). The wasps were allowed to freely forage on the plants for 24 h and were then removed. Cocoons were then collected and placed in marked vials detailing plant number and whether they were attached to or dangled from the leaf. The fate of these cocoons was monitored as in the previous experiments. Because we were interested in whether cocoon suspension is effective against hyperparasitism, we determined the fraction of cocoons that produced *Me. Pulchricornis* wasps for suspended and leaf-attached cocoons separately in each of the seven trials. A paired *t*-test was used to compare the fractions within a trial with H_0 ; the difference between the ratios is equal to zero.

Results

When unattended *Microplitis* sp. cocoons and cocoons attended by larvae of *M. separata* were simultaneously offered to *G. agilis*, significantly more unattended than attended cocoons were successfully parasitized by the hyperparasitoid ($\chi^2_1 = 72.40, P < 0.001$). In fact, very few cocoons with attending caterpillars were successfully parasitized at all (Fig. 2A). Out of 84 cocoons mounted and stung by *G. agilis*, 72 produced adult hyperparasitoids, and the remainder died. By contrast, all of the cocoons that were not stung by *G. agilis* successfully produced adult *Microplitis* sp. wasps.

The mean number of contacts of *Microplitis* sp. cocoons by *G. agilis* differed significantly depending on whether the cocoon was attended by a caterpillar or not ($t_{48} = 4.70, P < 0.001$). Several *G. agilis* females made >10 attempts to parasitize cocoons with attending caterpillars before parasitizing unattended cocoons (Fig. 2B).

In contrast, in within-plant preference tests the proportion of *Me. pulchricornis* cocoons that produced adult primary parasitoids did not differ significantly with treatment (mean difference of *Me. pulchricornis*

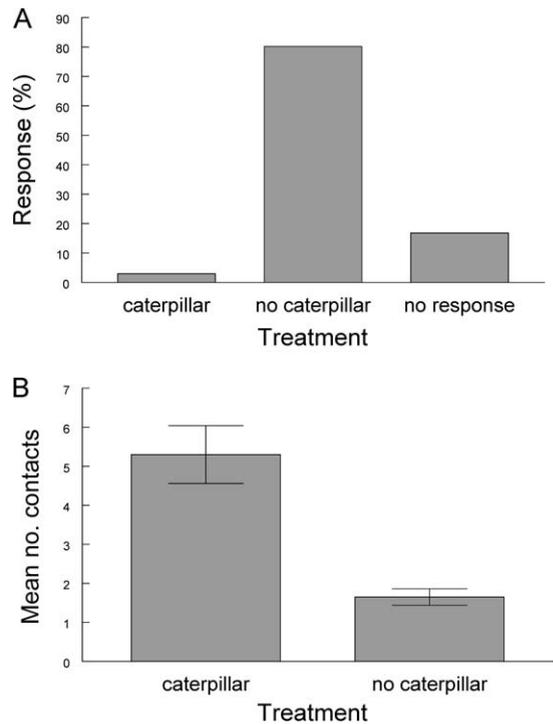


Fig. 2. (A) Percentage of *Microplitis* sp. cocoons mounted and parasitized by *G. agilis* females that were either attended or unattended by parasitized *M. separata* in a choice situation in petri dishes ($n = 101$). Single *G. agilis* females were released in petri dishes with either two caterpillar-attended or two nonattended *Microplitis* sp. cocoons at the opposite sides of the dishes. Mounting and stinging of host cocoons was visually monitored over a 60 min. observation period. (B) Mean counts of *Microplitis* sp. cocoons that were physically contacted by *G. agilis* females that were either attended or unattended by parasitized *M. separata* in a choice situation in petri dishes ($n = 46$). Line bars represent SEM.

eclosion fractions [suspended – attached] = 0.076, SD =, $t_6 = 1.46, P = 0.19$). Regardless of whether the cocoons were attached to the leaf surface or hanging with threads from the leaf, similar proportions produced primary parasitoids (Fig. 3).

Discussion

The results of this study reveal that there were differences in the effectiveness of the two pupation defense strategies in the primary parasitoids, *Microplitis* sp. and *Me. pulchricornis* against attack from the secondary hyperparasitoid *G. agilis*. In choice tests, susceptibility of the primary parasitoid cocoons to the hyperparasitoid was strongly correlated with retention or removal of the caterpillar after parasitoid egression and pupation (also see Harvey et al. 2011). *G. agilis* significantly preferred to mount and oviposit in cocoons of *Microplitis* that were not attended by parasitized *M. separata* larvae. In contrast, when hosts were not limiting, there was little difference in the rates of hyperparasitism of *Me. pulchricornis* cocoons

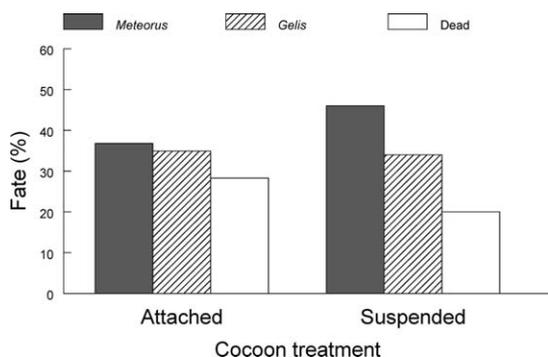


Fig. 3. Fate of *Me. pulchricornis* cocoons on corn plants that were either attached to the leaf surface or hanging by a thread when exposed to *G. agilis* females for 24 h in a choice situation. The fate of the hyperparasitoid-exposed cocoons was determined as adult *Meteorus* emerged, adult *Gelis* emerged, or dead cocoon in which neither species emerged. Seven trials were performed (see Materials and Methods).

by *G. agilis*, irrespective of whether they were attached to plant leaves or hanging from them.

Several recent studies have found reduced rates of predation, hyperparasitism, or both when parasitoid cocoons were attended by parasitized caterpillars (Kester and Jackson 1996; Grosman et al. 2008; Harvey et al. 2008a,b). In all of these studies, however, the primary parasitoids were gregarious, and the effectiveness of the host caterpillar in protecting the brood was far from absolute (Tanaka and Ohsaki 2006, 2009; Harvey et al. 2008a,b). Moreover, Harvey et al. (2008b) found that the *Pieris brassicae* L. (Lepidoptera: Pieridae) caterpillars themselves did not protect cocoons of the parasitoid *C. glomerata* against attack from a hyperparasitoid *Lysibia nana* Gravenhorst (Hymenoptera: Ichneumonidae). Instead, the caterpillars habitually spun a thick silk layer over *C. glomerata* cocoons that did interfere with the hyperparasitoid's ability to gain access to the cocoons. In the case of *Microplitis* sp., mature parasitoid larvae always egress through the host's ninth abdominal tergite (Fig. 1A) and construct a cocoon on which the caudal appendages of the host caterpillar rests. The four posterior segments of the host are effectively paralyzed, whereas the anterior nine segments are not. The parasitized caterpillar can remain highly aggressive for several days when disturbed, biting, regurgitating fluids from the gut, and vigorously swinging the head capsule, even under limited stimuli. This behavior was very effective in repelling attacks from *G. agilis* (Fig. 1B and C).

After egression from the host, *Me. pulchricornis* larvae first anchor a thread of silk to the leaf surface and then crawl across the leaf until they reach its edge, dropping from the leaf and hanging suspended by the thread by 1–2 cm (Fig. 4A). When encountering the threads, *G. agilis* wasps vigorously antennated and followed them to the leaf edge and then descended down the thread head-first, eventually climbing onto and parasitizing the pupa (Fig. 4B). Similar silk-descending behavior has been observed in both primary

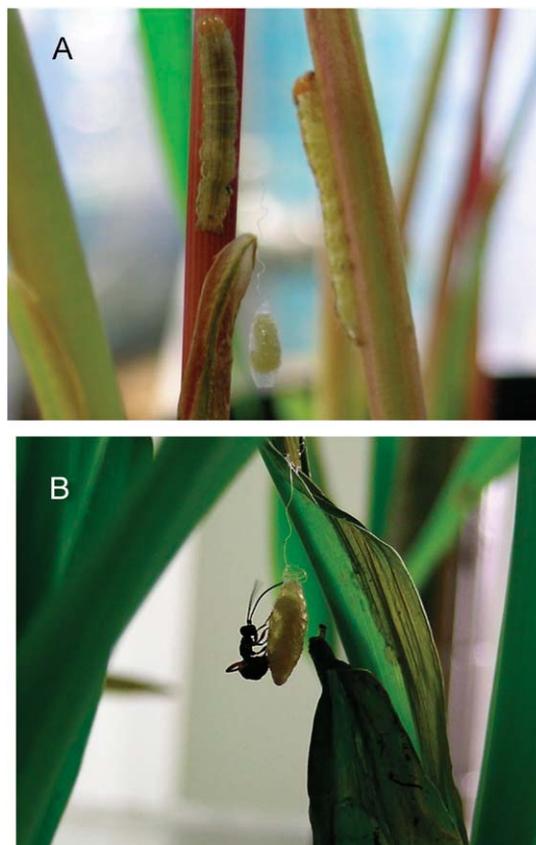


Fig. 4. Photographs showing the pupation strategy of *Me. pulchricornis* on the food plant of its host *M. separata*. (A) Parasitoid larva has emerged from the host caterpillar (see Fig. 1) and has attached itself to the leaf via a silk thread that enables it to hang suspended from the plant. (B) A *G. agilis* wasp is ovipositing into a newly constructed cocoon of *Me. pulchricornis* that is suspended by a thread from the plant. (Online figure in color.)

parasitoids and hyperparasitoids (Yeagan and Branham 1986, 1989; Godfray 1994).

The varying success of the two pupal defense strategies in *Microplitis* sp. and *Me. pulchricornis* against *G. agilis* suggests that these parasitoids may be under different selection pressures from predators and hyperparasitoids. A recent field study reported that attached cocoons of *Me. pulchricornis* were much more susceptible to attack from foraging ants than suspended cocoons (Shirai and Maeto 2007), although rates of hyperparasitism were not determined. Another potentially important factor favoring the retention of an attending caterpillar as a "bodyguard" is that many hyperparasitoids have limited host ranges and are therefore under much stronger selection to locate and exploit their parasitoid hosts than are predators, most of which are highly generalist feeders. Once hyperparasitoids detect the presence of a host, they may be exceedingly persistent and make repeated attempts to gain access to host cocoons. In the current study, some *G. agilis* females that were initially re-

buffered by *M. separata* caterpillars on individual cocoons made repeated attempts to mount the cocoons over the course of an hour or more.

Previous studies have shown that larvae of closely related parasitoids in the braconid subfamily Microgasterinae emerge from hosts which remain alive and active for variable periods after their use as a nutritional reservoir has been exhausted (Brodeur and Vet 1994). The adaptive significance of retaining a living host was discussed by Brodeur and Vet (1994). They suggested two possible functions: first, behavior of the parasitized host may be "usurped" by the parasitoid as a defense against its own natural enemies such as predators and hyperparasitoids; and second, the parasitized host presents a more inviting visual target for generalist predators than the parasitoid cocoon. As shown here, a growing body of evidence is providing support for the first hypothesis (Kester and Jackson 1996; Grosman et al. 2008; Harvey et al. 2008a,b; Janssen et al. 2010; this study) but the second has, as far as we know, not yet been tested. Future research should aim to test the second hypothesis, as well as elucidating the potential factors that might lead to trade-offs in the evolution of different pupal defense strategies in parasitoids.

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