

BEHAVIORAL ADAPTATIONS INCREASE THE VALUE OF ENEMY-FREE SPACE FOR *HELIOTHIS SUBFLEXA*, A SPECIALIST HERBIVORE

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Abstract.—We investigated the importance of specialized behaviors in the use of enemy-free space by comparing the host-use behavior of two closely related moths, *Heliothis subflexa* Guenee and *H. virescens* Fabricius. *Heliothis subflexa* is a specialist on plants in the genus *Physalis*, whereas *H. virescens* is an extreme generalist, feeding on plants in at least 14 families. *Heliothis subflexa* uses the inflated calyx surrounding *Physalis* fruits as enemy-free space, and field rates of parasitism for *H. subflexa* on *Physalis* are much lower than for *H. virescens* on tobacco and cotton, common hosts found in the same habitat as *Physalis*. If *Physalis*' architecture were solely responsible for *H. subflexa*'s low rates of parasitism on *Physalis*, we predicted that *H. virescens* larvae experimentally induced to feed on *Physalis* would experience parasitism rates similar to those of *H. subflexa*. We found, however, that specialized host-use and host-acceptance behaviors are integral to the use of enemy-free space on *Physalis* and strongly augment the effects of the structural refuge. In laboratory assays, we found considerable differences between the larval behavior of the specialist, *H. subflexa*, and the generalist, *H. virescens*, and these contributed to *H. subflexa*'s superior use of enemy-free space on *Physalis*. We tested the importance of these behavioral differences in the field by comparing parasitism of *H. virescens* on *Physalis*, *H. virescens* on tobacco, and *H. subflexa* on *Physalis* by *Cardiochiles nigriceps* Vierick, a specialist braconid parasitoid. For *H. virescens*, a threefold decrease in parasitism occurred when feeding on *Physalis* (mean parasitism \pm SEM = $13 \pm 4\%$) rather than tobacco ($43 \pm 4\%$), a difference we attribute to the structural refuge provided by *Physalis*. However, parasitism of *H. virescens* on *Physalis* was more than ten times as great as that of *H. subflexa* on *Physalis* ($1 \pm 4\%$), supporting the hypothesis that specialized behaviors have a substantial impact on use of *Physalis* as enemy-free space. Behavioral adaptations may be central to the use of enemy-free space by phytophagous insects and may act as an important selective force in the evolution of dietary specialization.

Key words.—Behavioral adaptations, enemy-free space, *Heliothis subflexa*, *Heliothis virescens*, herbivore host range evolution, natural enemies, parasitoids, specialist herbivore.

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The question of why insect herbivores eat what they do has long intrigued biologists. With the huge variety of edible plants available in the world, why should a herbivore limit itself to one or a few plant species? Yet, the vast majority of phytophagous insects are dietary specialists (Farrell and Mitter 1993; Thompson 1994), feeding on plants from a single genus or a single family (Menken and Roessingh 1998). Less than 10% of all phytophagous insects are generalists, feeding on plants from three or more families (Bernays and Graham 1988). This predominance of specialist feeders suggests that insects with narrow host ranges may enjoy some selective advantage over generalist feeders.

The ability to use enemy-free space, defined by Berdegue et al. (1996, p. 203) as “a discrete moment in evolutionary time where the fitness of the prey, under selective pressure from the natural enemy, is increased by a change in habit,” has been invoked as an ecological force likely to result in the evolution of narrow host ranges. In most cases, insects obtain enemy-free space by means of substrate-specific adaptations: cryptic coloration in lepidopteran larvae (Heinrich 1979) is one well-known example. Because adaptations to one host plant may be inappropriate on another host plant, specialization can play a central role in the use of enemy-free space: cryptic coloration, for example, tends to vary inversely with an insect's host range (Jaenike 1990).

While the connection between dietary specialization and the use of enemy-free space has gained increasing attention (Denno et al. 1990; Mopper et al. 1995; Ohsaki and Sato 1994), the significance of host plant-specific insect behavior

in the use of enemy-free space has remained unclear. Behavior has been proposed as the “mechanism by which specialization is exercised” (Futuyma and Moreno 1988, p. 209), and several workers have suggested that behavioral, rather than morphological or physiological, barriers are responsible for constraints on the host range of herbivorous insects (Jaenike 1985; Futuyma and Philippi 1987; Karowe 1990). However, few studies have examined how host range may be constrained by the need for host plant-specific behaviors required for escape from natural enemies.

In this study, we investigate the role of host plant-specific behaviors in the use of enemy-free space. Separating the effects of behavior from the effects of physiology is challenging. For herbivorous insects, host-plant range encompasses both physiological mechanisms, such as the ability to ingest and incorporate plant material, and behavioral mechanisms, such as the willingness to feed on plant material. In spite of this difficulty, the potential for behavior and physiology to evolve separately has been demonstrated: Hawthorne (1999), for instance, found that several generations of selection on toxic host plants resulted in increased larval survivorship, but no change in oviposition preference. Wasserman and Futuyma (1981) found an opposite trend: Oviposition preference changed, while physiological tolerance did not. In testing the hypothesis that adaptations for the use of enemy-free space might confer a selective advantage to specialists as compared to generalists on the same host plant, we have tried to maintain a clear distinction between the effects of behavior and physiology.

To explore the importance of host-use behavior in allowing specialist herbivores to obtain enemy-free space from their host plants, we studied host-use traits in two closely related insects, *Heliothis virescens* Fabricius and *H. subflexa* Guenee. *Heliothis subflexa* is monophagous, feeding only on plants in the genus *Physalis* (Laster 1972). *Physalis* plants produce fruits enclosed within a thin-walled, inflated calyx (referred to herein as a "lantern"). When feeding on *Physalis* fruits within this lantern, *H. subflexa* larvae are protected from some natural enemies (Sisterson and Gould 1999). *Heliothis virescens*, by contrast, is an extreme generalist, feeding on 37 species of plants in at least 14 families (Sheck and Gould 1993), including tobacco, cotton, soybean, and other crops of agricultural importance. In spite of *H. virescens*'s extreme generalization, it has not been reported on *Physalis* plants in the field, although laboratory tests have demonstrated the ability of second instar (and older) *H. virescens* larvae to feed on *Physalis* (Sheck and Gould 1993; S. J. Oppenheim, pers. obs.) and the willingness of adult females to oviposit on *Physalis* (Mitchell et al. 1990; Tingle et al. 1990; Sheck and Gould 1995). The basis for this exclusion of *Physalis* plants from the host range of *H. virescens* is not known, but the scarcity of *Physalis* in comparison to other of *H. virescens*' hosts and *H. virescens*' relatively poor growth on *Physalis* may be contributing factors.

The phylogenetic relationship between *H. subflexa* and *H. virescens* has been studied in some detail (Mitter et al. 1993; Poole et al. 1993; Fang et al. 1997). Morphologically, the two species are so similar that *H. subflexa* was only conclusively identified as a separate species in 1941 (McElvare 1941). Both *H. subflexa* and *H. virescens* are members of the *virescens* species group within the Heliothinae, a cosmopolitan subfamily of the Noctuidae. Polyphagy is ancestral in the *virescens* group, a conclusion supported by morphological (Matthews 1991), allozyme (Mitter et al. 1993), and nuclear gene (elongation factor-1a, Cho et al. 1995; dopa decarboxylase, Fang et al. 1997) character sets. Thus, *H. subflexa* and *H. virescens* apparently evolved from a shared, *H. virescens*-like, generalist ancestor (Poole et al. 1993).

Rates of parasitism differ widely between *H. subflexa* and *H. virescens*. Throughout the southeastern United States, the predominant parasitoid of both species is *Cardiochiles nigriceps* Vierick (Hymenoptera: Braconidae) (Lewis et al. 1967). This specialist endoparasitoid is only known to develop in *Heliothis* larvae (Lewis and Vinson 1971). All successfully parasitized larvae die prior to pupation. For *H. virescens* on tobacco, Johnson (1997) found parasitism rates of 25%, whereas Neunzig (1969) found 72% parasitism. On cotton, parasitism rates as high as 96% have been recorded (Lewis et al. 1972). For *H. subflexa* larvae, field collections have consistently shown much lower rates of parasitism: Roach (1975) found no parasitism, whereas Lewis et al. (1967) reported rates from 2% to 9%.

As a specialist parasitoid, *C. nigriceps* has evolved complex and highly successful strategies for finding and attacking host larvae. Recent work by DeMoraes et al. (1998) demonstrated that *C. nigriceps* females are preferentially attracted to plants damaged by *H. virescens* when offered a choice between cotton, corn, or tomato plants damaged by either *H. virescens* or *Helicoverpa zea*, a closely related nonhost. Re-

cent field observations (S. J. Oppenheim and F. Gould, unpubl. ms.) have confirmed that this preferential attraction by *C. nigriceps* extends to *H. subflexa*-damaged plants. In most plant species investigated to date, the release of herbivore-induced volatiles peaks approximately 48 h after herbivore feeding begins, but signaling from the damaged site may begin within minutes (Turlings et al. 1995). Given *C. nigriceps*'s strong attraction to these herbivore-induced volatiles, a larva's risk of being discovered and attacked by natural enemies increases dramatically following damage to the host plant. Larvae should therefore seek refuge either before feeding or as quickly as possible after starting to feed.

Sisterson and Gould (1999) demonstrated the importance of a structural refuge in protecting *H. subflexa* on *Physalis* from parasitoids. By cutting off the bottom of the lantern to allow natural enemies free access to larvae feeding on *Physalis* fruits, they eliminated the structural refuge. As a result, parasitism rates increased from a range of 6% to 15% on intact plants to a range of 48% to 60% on cut plants.

We wondered if this structural refuge was the principal explanation for enemy-free space on *Physalis*. If so, we predicted that both specialist and generalist species feeding within the lanterns of *Physalis* would experience a significant decrease in parasitism. Alternatively, specialized behaviors might be required for the best use of enemy-free space on *Physalis*, in which case we expected to find that specialists would be more protected than generalists on *Physalis*. To explore these issues, we posed two questions: (1) Are the benefits of enemy-free space on *Physalis* available to *H. virescens*? (2) Do specialized interactions between *H. subflexa* and *Physalis* increase the value of enemy-free space for *H. subflexa*?

While present-day circumstances may not mirror conditions when *H. subflexa* evolved from an *H. virescens*-like ancestor, an understanding of how current conditions might affect the host range of these two closely related species would add much to our understanding of the role of enemy-free space in the evolution of specialization.

MATERIALS AND METHODS

To address the questions above, we conducted three experiments: (1) Comparison of field rates of parasitism to determine the value of *Physalis* as enemy-free space for *H. subflexa* versus *H. virescens* and the value of *Physalis* as enemy-free space for *H. virescens* on tobacco versus *Physalis* (*H. subflexa* larvae fed on tobacco do not survive, so this treatment could not be included); (2) comparison of the larval behavior of *H. subflexa* and *H. virescens* on *Physalis* to determine whether *H. subflexa* have specialized behaviors for the use *Physalis* as enemy-free space; and (3) comparison of feeding and weight gain by *H. subflexa* and *H. virescens* on *Physalis* to clarify the impact of physiological differences on larval parasitism rates and behavior.

Field Rates of Parasitism

Insect strains and rearing

Heliothis subflexa larvae were obtained from North Carolina State University (NCSU) laboratory colonies estab-

lished using larvae originally collected in Orangeburg County, South Carolina, in 1997 (*H. subflexa*_{SC}). *Heliothis virescens* larvae were from an NCSU laboratory colony that had been in the lab for eight years (*H. virescens*_{LAB}). Larvae were individually reared on a cornmeal and soymeal-based diet, as modified from Burton (1970). Prior to testing, all larvae were held in a rearing room under 14:10 L:D. All insects were tested as newly molted third instars.

Plants

Although many species of *Physalis* are acceptable to *H. subflexa*, larvae are commonly found on *P. angulata* (referred to here as *Physalis*; Laster 1972; Yopez et al. 1990; Sisterson and Gould 1999), and we used this species for all experiments. *Physalis* plants were grown from seeds collected in Orangeburg County, South Carolina, in 1997. Seeds were planted in flats and maintained under greenhouse conditions until they were 5-cm high. Seedlings were then transplanted into 8-L pots, which were large enough to allow plants to attain sizes typical of field-grown plants. Only reproductive stage *Physalis* plants with more than 15 fruits were used. Seeds of a *H. virescens*-susceptible tobacco cultivar (variety MacNair 373) were planted in flats, and then transferred to 8-L pots. Only budding tobacco plants were used. Both tobacco and *Physalis* were grown in a large, screened outdoor cage in Tift County, Georgia. All plants were transported to the field site as needed for tests, and plants were discarded after being used in a single test.

Experiment site

Experiments were conducted in a 2-ha cotton field located in Irwin County, Georgia. During the time that tests were conducted, this field was being used as a research site by scientists from the Coastal Plains USDA Agricultural Research Service station in Tifton, Georgia, to evaluate the efficacy of trap crops in attracting natural enemies of lepidopteran crop pests.

Experimental methods

To allow larvae to establish on plants, they were set on plants in a screened outdoor cage prior to field exposure. Third instars were used because both species feed well on *Physalis* at this stage. Because the release of herbivore-induced volatiles by a damaged plant peaks approximately 48 h after damage, we set larvae on plants 24 h before field exposure. Five larvae were set onto each *Physalis* plant, and two larvae were set onto each tobacco plant, ensuring larval access to protected feeding sites within the bud. The number of larvae placed on each treatment reflects typical field infestation levels (pers. obs.). Infested plants were randomly arranged in the experimental field, and were interspersed with flowering field-planted tobacco. Experiments were replicated over six consecutive days in July 1999, during a period of high field densities of *C. nigriceps*. Daily replication included 10 plants of each treatment (*H. virescens* on tobacco; *H. virescens* on *Physalis*; and *H. subflexa* on *Physalis*). For experiments conducted on days 1–3, plants were left in the field for 24 h. Because a low percentage of larvae were recovered

from this 24-h exposure period, we subsequently collected larvae after 12 h of field exposure (days 4–6).

Following field exposure, we collected larvae and reared them on an artificial diet to monitor parasitoid emergence. Larvae that died prior to either pupation or parasitoid emergence were dissected as described in Lewis and Brazzel (1966) to check for the presence of parasitoid eggs and larvae. After collecting larvae, we assessed percent of larvae initially set out that were recovered from plants, percent of recovered larvae dying prior to pupation from causes other than parasitism, and percent of recovered larvae parasitized (i.e., a parasitoid either emerged or was detected upon dissection of a dead larva).

Statistical analysis

Data were analyzed using the SAS procedure for analysis of fixed and random effects (PROC MIXED; SAS Institute 1999). All data were converted to percentages prior to analysis, then arcsine-square-root transformed to achieve variance homogeneity. Individual ANOVAs were performed for percent recovery, percent mortality, and percent parasitism. We modeled each of these dependent variables as a function of the fixed effects of plant-herbivore combination, the duration of field exposure, and the interaction between plant-herbivore combination and duration of field exposure. Date, nested within duration of field exposure, was treated as a random effect. Parameter estimates were obtained for the transformed data, then backtransformed to get the population means and other summary statistics reported.

Larval Behavior on *Physalis*

Insect strains and rearing

Heliothis subflexa larvae were obtained from NCSU laboratory colonies. One strain was originally collected in Orangeburg County, South Carolina, in 1997 (*H. subflexa*_{SC}); the second was collected in Wayne County, North Carolina, in 1998 (*H. subflexa*_{NC}). Larvae were individually reared on a cornmeal and soymeal-based diet (as modified from Burton 1970) with one exception: plant-acclimated larvae were maintained on fruits of *Physalis* for at least 48 h prior to testing. *Heliothis virescens* larvae were also from NCSU laboratory colonies. One strain had been in the lab for more than eight years (*H. virescens*_{LAB}), whereas the second was collected in 1999 from Johnson County, North Carolina (*H. virescens*_{NC}). *Heliothis virescens* larvae were fed in the same manner as *H. subflexa*. Prior to testing, all larvae were held in a rearing room under 14:10 L:D at 22°C.

Plants and experiment site

Plants and experiment site were as described for the parasitism experiment.

Experimental methods

The behavior of all larval instars of *H. subflexa* and *H. virescens* on *Physalis* was tested as follows: Larvae were manually put on *Physalis* plants and observed continuously for up to 4 h. The time each larva took to complete each

TABLE 1. Sample of ANOVA results for effect of herbivore species, strain, instar, and diet on fruit colonization behaviors. Results are shown for the variable total, the total time from setting larva on plant to completion of entry into lantern.

Source	df	F	P
<i>Heliothis virescens</i>			
Strain	1, 16.0	0.02	0.8966
Strain × instar	2, 12.1	0.16	0.8538
Strain × diet	2, 15.6	0.00	0.9507
<i>Heliothis subflexa</i>			
Strain	1, 21.3	5.23	0.0325
Strain × instar	2, 12.5	0.00	0.9969
Strain × diet	1, 58.7	1.17	0.2838
All larvae			
Instar	4, 33.3	0.87	0.4948
Species	1, 98.6	34.67	0.0001
Species × instar	4, 97.0	0.58	0.6802
Diet	1, 122.0	0.44	0.5079
Species × diet	1, 135.0	0.00	0.9576

stage of fruit colonization was recorded. On each experimental day we conducted paired observations, using *H. subflexa* and *H. virescens* larvae at identical stages of development. Tests were conducted from April through September 1999.

To ensure that differences observed between the two species were neither strain specific nor artifacts of laboratory rearing, both newly collected (*H. subflexa*_{NC}, collected in September 1998, and *H. virescens*_{NC}, collected in June 1999) and older (*H. subflexa*_{SC}, maintained for two years in the laboratory, and *H. virescens*_{LAB}, maintained for eight years in the laboratory) strains were tested. To eliminate the possibility that plant material might act as a deterrent to larvae reared on artificial diet, several tests were conducted with plant-acclimated larvae (maintained on *Physalis* fruit for 48 h prior to observation).

For all tests, neonates or newly molted second, third, fourth, or fifth instar larvae were removed from feeding cups containing either artificial diet or *Physalis* fruit. Larvae were placed on plant stems 7.5–15 cm inches away from the nearest fruit and observed continuously for 4 h or until they colonized a lantern. No more than five larvae were observed simultaneously.

Heliothis subflexa larvae colonize *Physalis* lanterns by carrying out a predictable set of behaviors. We divided colonization behavior into the following discrete phases, and used the indicated measure for each phase:

(1) fruit selection: (a) number prefruits: the number of lanterns visited prior to colonization; (b) to fruit: elapsed

time (in minutes) from when larva is set onto plant to its arrival at the lantern it will colonize; (2) prebore: amount of time (in minutes) spent on the lantern before boring an entry hole through the lantern; (3) bore: elapsed time (in minutes) from commencement to completion of boring entry hole; (4) entry: elapsed time (in minutes) from beginning entry to full enclosure of larva within lantern; and (5) total time: elapsed time (in minutes) from when larva was put on the plant to completion of entry.

Some larvae made no attempt to colonize a lantern during the 4-h observation period. We recorded these as failures and report their fates and behavior.

Statistical analysis

We used a factorial design to analyze the effects of larval species, strain, developmental stage, and feeding substrate (i.e., diet- or plant-acclimated) on colonization behavior. Replication of these factors was uneven, an artifact of our experimental protocol: In the plant-acclimated treatment, only insects aged second instar or older could be used because 48 h of fruit feeding were required prior to testing; for all treatments, tests involving fifth instars had few replicates because these older larvae frequently crawled to the soil to pupate, making no attempt to colonize a lantern.

Prior to analysis, data were transformed to homogenize variances. The number of prefruits was square-root transformed; all other data were log transformed. Means and summary statistics reported here were backtransformed after analysis.

The effects of larval strain and species could not be analyzed simultaneously (a consequence of uneven replication; C. Brownie, pers. comm.). We therefore carried out preliminary analyses of the comparative magnitude of intraspecific (i.e., strain) and interspecific (i.e., species) effects to determine if strain effects could be ignored. Within each species, the effects of strain, strain × instar interaction, and strain × diet interaction were assessed (Table 1). We found that intraspecific differences were quite small compared to differences between the two species (Table 2). Although two variables (number prefruits for *H. virescens* and time to fruit for *H. subflexa*) did exhibit significant intraspecific strain differences, the effects of strain were not consistent (e.g., recently collected strains performed no better than strains maintained in the laboratory for several years, and plant-acclimated larvae performed no better than larvae from diet). We therefore adopted a reduced model that did not include strain effects. Because our model included both fixed and random effects, we used the SAS procedure mixed. Denominator de-

TABLE 2. Strain and species means for larval fruit colonization behaviors. Means for prefruits represent number of fruits visited prior to colonizing a fruit; all other means are time (in minutes) required to complete each behavior.

Species _{STRAIN}	Prefruits	To fruit	Prebore	Bore	Entry	Complete
<i>H. virescens</i> _{LAB}	1.62 ± 0.12	35.03 ± 4.35	4.62 ± 1.02	3.90 ± 0.42	16.04 ± 3.09	59.68 ± 5.18
<i>H. virescens</i> _{NC}	1.70 ± 0.22	43.90 ± 10.23	3.10 ± 0.81	4.20 ± 0.57	14.10 ± 3.08	65.20 ± 11.45
<i>H. virescens</i> _{ALL}	1.64 ± 0.11	37.05 ± 4.08	4.25 ± 0.81	3.85 ± 0.34	15.23 ± 2.49	58.68 ± 4.76
<i>H. subflexa</i> _{SC}	1.25 ± 0.07	9.63 ± 1.98	1.96 ± 0.55	2.82 ± 0.30	2.20 ± 0.25	16.61 ± 2.24
<i>H. subflexa</i> _{NC}	1.17 ± 0.08	25.35 ± 5.96	2.57 ± 0.47	3.65 ± 1.09	1.78 ± 0.31	33.35 ± 6.16
<i>H. subflexa</i> _{ALL}	1.23 ± 0.05	14.20 ± 2.35	2.14 ± 0.41	3.06 ± 0.38	2.08 ± 0.20	21.48 ± 2.52

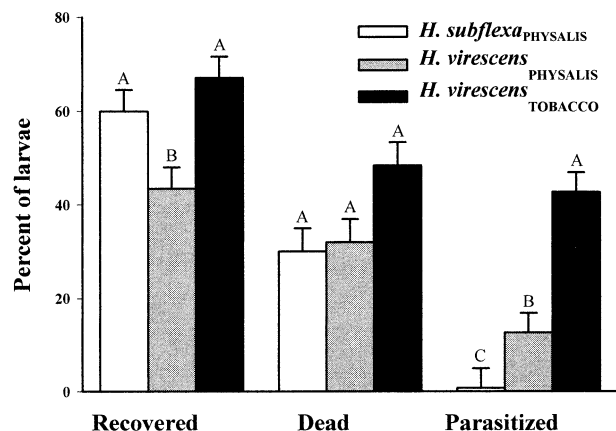


FIG. 1. Rates of recovery, mortality and parasitism for *Heliothis virescens* and *H. subflexa* larvae. Recovery rates show percent of larvae set on plants that were recovered after exposure in the field; mortality rates show mortality from sources other than parasitism; and parasitism rates show percent larval parasitism by the specialist endoparasitoid *Cardiochiles nigriceps*. Mortality and parasitism were calculated as the percent of recovered larvae suffering each fate.

degrees of freedom were generated using the Satterthwaite equation.

Separate ANOVA models were used for final analysis of number prefruits, time to fruit, prebore, bore, entry, and total. Each model included species, and other explanatory variables were included only when significant in the model described above (see Table 4).

Of 265 larvae tested, 77 never attempted to colonize a fruit. A chi-square test of the association between herbivore species and probability of failure to colonize was conducted.

Larval Feeding on *Physalis*

Insects and plants

As in previous experiments, *H. subflexa*_{SC} and *H. virescens*_{LAB} were used. *Physalis angulata* fruits were obtained from greenhouse-grown plants.

Experimental methods

Larvae were fed on artificial diet until the third instar, then transferred to plastic petri dishes containing one *Physalis* fruit. *Physalis* fruits were removed from their lanterns to ensure that larval ability to penetrate lanterns did not affect feeding performance. Fruit weight and larval weight were recorded at the beginning of each assay and recorded again after 24 h (we chose this duration to mirror exposure duration in field tests of parasitism). Sixty larvae of each species were tested on three different dates in March–April 2001.

Statistical analysis

Weight change values for fruits and larvae were converted to proportions (start weight minus end weight divided by start weight for fruits; end weight minus start weight divided by start weight for larvae) prior to analysis. We used the SAS procedure GLM to perform a two-way ANOVA, treating species and date as main effects. We modeled the proportional

TABLE 3. ANOVA results for mortality, recovery, and parasitism rates of *Heliothis virescens* on tobacco, *H. virescens* on *Physalis angulata*, and *H. subflexa* on *P. angulata*. Results shown are for Type III tests of fixed effects.

Dependent variable	df	F	P
Proportion of recovered larvae dead from sources other than parasitism			
Herbivore-plant combination	5, 20	2.09	0.1097
Duration of exposure in the field	1, 4	0.07	0.8000
Combination × exposure	2, 20	1.83	0.5260
Proportion of larvae recovered after field exposure			
Herbivore-plant combination	5, 20	5.88	0.0017
Duration of exposure in the field	1, 4	0.75	0.4344
Combination × exposure	5, 20	1.29	0.3087
Proportion of recovered larvae parasitized by <i>Cardiochiles nigriceps</i>			
Herbivore-plant combination	5, 24	11.25	<0.0001
Duration of exposure in the field	1, 4	3.68	0.0669
Combination × exposure	5, 24	0.60	0.6979

change in larval weight as an effect of larval species, fruit start weight, larval species × fruit start weight interaction, date, and species × date interaction. Proportional change in fruit weight was modeled as an effect of larval species, larval start weight, larval species × larval start weight interaction, date, and species × date interaction. Because the calculation of larval (or fruit) proportionate change in weight included start weight, these factors were not independent; thus, analysis of proportional change as a function of start weight was not undertaken. We regressed larval weight change (larval start weight minus end weight) on the amount of fruit material consumed (fruit start weight minus end weight) to test whether assimilation efficiency varied between species.

RESULTS

Field Rates of Parasitism

Rates of parasitism and recovery varied with the host plant–herbivore combination (Fig. 1). Parasitism rates were greatest for *H. virescens* on tobacco (mean ± SEM = 43 ± 4.2%) and lowest for *H. subflexa* on *Physalis* (1 ± 4.2%). Parasitism of *H. virescens* on *Physalis* was intermediate between these extremes (13 ± 4.2%). In *t*-tests of least-squared means, parasitism rates for each of the host plant–herbivore combinations differed significantly at the 0.05 level. Neither date nested within exposure duration nor the interaction between host plant–herbivore combination and exposure duration affected parasitism (Table 3).

Recovery rates were greatest for *H. virescens* on tobacco (67 ± 4.6%), intermediate for *H. subflexa* on *Physalis* (60 ± 4.6%), and lowest for *H. virescens* on *Physalis* (43 ± 4.6%; Fig. 1). In *t*-tests of least-squared means, recovery rates for *H. virescens* on *Physalis* were significantly lower than for the other two host plant–herbivore combinations. Mortality of recovered larvae from sources other than parasitism did not differ significantly between host plant–herbivore combinations ($F = 2.09$; $df = 5, 20$; $P = 0.1097$).

Larval Behavior on *Physalis*

Fruit colonization behavior varied significantly with larval species. After being set onto a *Physalis* plants, *H. subflexa*

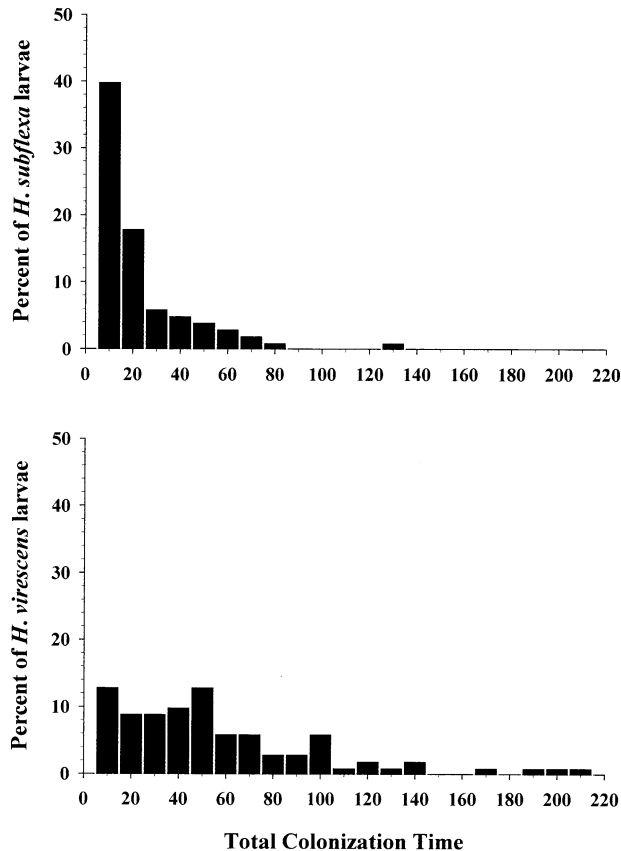


FIG. 2. Frequency distribution of total fruit colonization times (in minutes) for *Heliothis subflexa* (22 ± 22 min) and *H. virescens* (mean \pm SD = 59 ± 45 min) on *Physalis angulata*.

larvae completed colonization of *Physalis* lanterns about three times more quickly than *H. virescens* (*H. subflexa* total time \pm SEM = 21.5 ± 2.5 min; *H. virescens* = 58.7 ± 4.8 min). Furthermore, variability between larvae in total colonization time was much lower for *H. subflexa* (SD = 22.3 min) than for *H. virescens* (SD = 44.5 min; Fig. 2). *Heliothis subflexa* visited fewer fruits before choosing one to colonize, arrived at that fruit more quickly, spent less time on the outer lantern before starting to bore, and entered through the hole they bored in less time than *H. virescens* (Fig. 3). Larval species affected all behaviors except bore time, whereas instar affected time to fruit, prebore duration, entry time, and total time. Instar effects did not differ between species—overall, younger larvae were slower than older larvae. Although other variables affected some behaviors, they affected both species in the same way (Table 4).

Larvae that failed to colonize a lantern did so for a variety of reasons. Some were attacked by predatory wasps or ants while still on the plant, some silked off the plant and did not return, some climbed to the soil and dug pupation tunnels, some wandered around on the plant without attempting colonization, and the rest sat immobile for the entire observation period (Fig. 4). A chi-square test of association between herbivore species and the probability of failing to colonize a lantern was significant at the 0.05 level ($\chi^2 = 4.69$, df = 1; $\chi^2_{\text{CRIT}} = 3.84$).

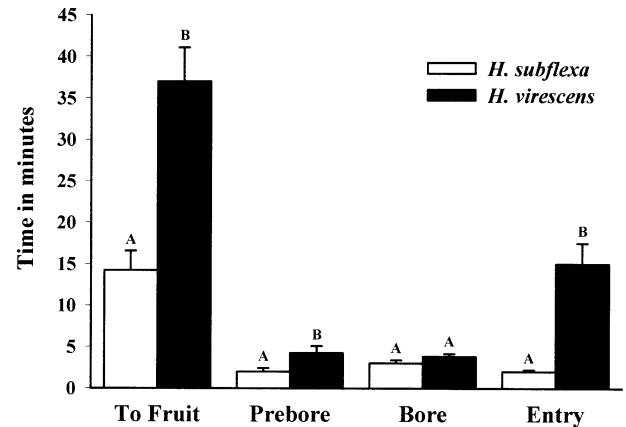


FIG. 3. Time required for *Heliothis virescens* and *H. subflexa* larvae to carry out each colonization behavior on *Physalis angulata*.

Larval Feeding on *Physalis*

Herbivore species did not affect whether or not larvae fed on *Physalis*, but had a strong impact on change in larval weight ($P = 0.0204$). Larval weight gain was much more variable for *H. subflexa* larvae (SD = 56.5%) than for *H. virescens* (SD = 16.3%; Fig. 5). Thirteen percent of the *H. virescens* larvae tested never fed on a fruit versus 20% of the *H. subflexa* (Fig. 6). A chi-square test of the association be-

TABLE 4. ANOVA models and results for larval colonization of *Physalis angulata* fruits by *Heliothis subflexa* and *H. virescens*. Values shown are from Type III sums of squares.

Dependent variable	df	F	P
Number of prefruits			
Species	1	11.46	0.0010
Diet	1	5.50	0.0210
Date \times diet (species \times instar)	51	0.97	0.5463
$R^2 = 0.3870$ CV = 149.86			
Time to fruit			
Species	1	13.45	0.0004
Instar	4	2.94	0.236
Date (instar)	34	1.64	0.0279
$R^2 = 0.4560$ CV = 43.19			
Prebore interval			
Species	1	4.76	0.0012
Instar	4	21.66	<0.0001
$R^2 = 0.2031$ CV = 107.45			
Bore duration			
Species	1	0.62	0.4334
Date (instar)	38	1.79	0.0112
Date \times diet (species \times instar)	14	1.94	0.0305
$R^2 = 0.5110$ CV = 53.89			
Entry duration			
Species	1	127.42	<0.0001
Instar	4	4.16	0.0032
$R^2 = 0.5530$ CV = 43.32			
Total			
Species	1	24.31	<0.0001
Instar	4	2.13	0.0815
Date (instar)	34	1.76	0.0144
$R^2 = 0.5721$ CV = 20.87			

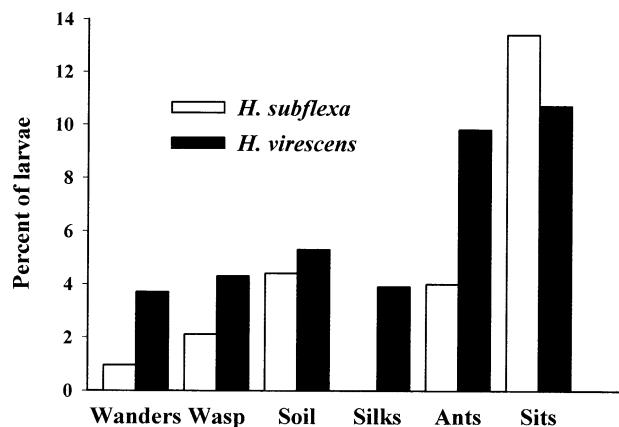


FIG. 4. Fates of *Heliothis virescens* and *H. subflexa* larvae that failed to colonize a fruit during 4 h of observation. Wanders, active on plant but never colonizes a fruit; wasp, eaten by vespid wasp while on plant; soil, climbs down plant to soil, does not return; silks, silks off plant, does not return; ants, eaten by ants while on plant; sits, inactive for entire period.

tween larval species and the likelihood of feeding on *Physalis* was not significant ($\chi^2 = 1.19$, $df = 1$; $P = 0.2744$). Larval weight gain, however, did vary with species. *Heliothis subflexa* larvae gained $53 \pm 4\%$ (mean \pm SEM) of their start weight, whereas *H. virescens* gained only $14 \pm 4\%$ of their start weight. Fruit start weight ($P = 0.0352$), date ($P < 0.0001$), and the species \times date interaction ($P < 0.0001$) each affected larval weight gain. Regression of larval weight gain on amount of fruit consumed resulted in an intercept of 0.0009 and slope of 0.30 for *H. subflexa* and an intercept of 0.002 and slope of 0.09 for *H. virescens*. *Heliothis subflexa*, in other words, gained three times more weight per unit of fruit consumed than *H. virescens*.

There was no main effect of species on the amount of fruit each larva consumed. Although mean consumption over the three test dates differed between *H. subflexa* and *H. virescens* (mean consumption \pm SEM for *H. subflexa* = $20 \pm 2\%$; *H. virescens* = $11 \pm 2\%$; $P = 0.0004$ for a t -test of H_0 : no effect of species), the full ANOVA model showed no main effect of species. In the full model, the effects of date ($P = 0.0005$) and the date \times species interaction ($P = 0.0002$) far outweighed the effect of species. Date and date \times species interaction effects were attributable to variability in *H. subflexa*'s performance over test dates (Table 5).

DISCUSSION

Investigations of the mechanisms underlying the adaptation of specialist herbivores to their host plants have traditionally emphasized physiological differences between specialists and generalists in their ability to feed on these plants. Here, we report findings on the role of behavior in allowing specialist herbivores to make use of enemy-free space. *Heliothis subflexa* larvae use the inflated lantern of *Physalis* as a refuge from natural enemies (Sisterson and Gould 1999). If specialized behaviors increase the value of this refuge, such behaviors might make specialists better able to avoid natural enemies than generalists feeding on the same host plant. Be-

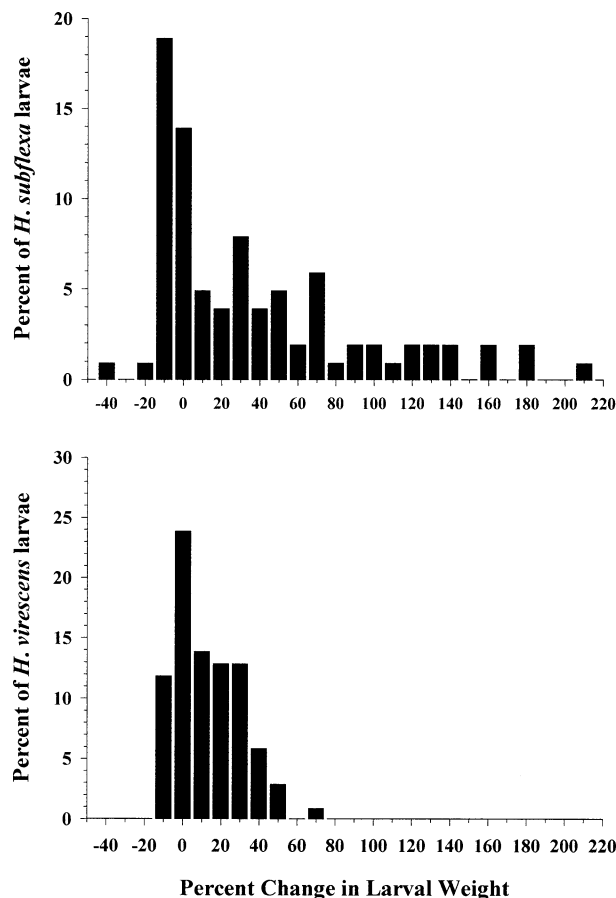


FIG. 5. Frequency distribution of change in larval weight for third instar *Heliothis virescens* (mean \pm SD = $14 \pm 16\%$) and *H. subflexa* ($53 \pm 57\%$) fed on *Physalis angulata* fruits for 24 h.

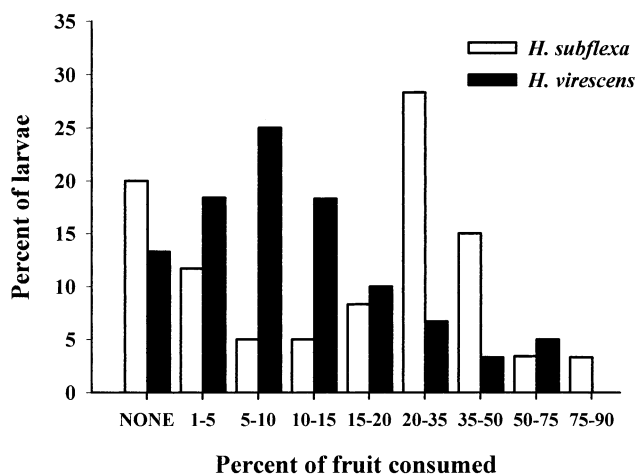


FIG. 6. Frequency distribution of consumption of *Physalis angulata* fruits by third instar *Heliothis virescens* and *H. subflexa* larvae. Proportion consumed shows fruit start weight minus fruit end weight divided by fruit start weight. Fruits that were not fed on at all lost less than 1% of their weight.

TABLE 5. Mean percent of *Physalis angulata* fruit consumed by third instar *Heliothis virescens* and *H. subflexa* and resulting percent change in larval weight. Data shown are for three separate days.

Variable	Replicate	N	Mean \pm SE
Larval fruit consumption			
<i>H. subflexa</i>	1	20	5.82 \pm 7.85%
	2	20	31.57 \pm 24.14%
	3	20	24.89 \pm 13.37%
<i>H. virescens</i>	1	20	9.59 \pm 11.35%
	2	20	13.23 \pm 20.09%
	3	20	9.711 \pm 5.66%
Larval weight gain			
<i>H. subflexa</i>	1	20	7.28 \pm 20.10%
	2	20	106.16 \pm 56.72%
	3	20	45.73 \pm 32.44%
<i>H. virescens</i>	1	20	9.58 \pm 21.07%
	2	20	17.42 \pm 11.75%
	3	20	14.76 \pm 14.53%

havior might thus act as an adaptive force in the evolution of specialization in *H. subflexa*.

We show here that considerable, highly consequential, differences do exist in the host-use behavior of *H. subflexa* and *H. virescens*. Although both species are capable of colonizing and feeding upon *Physalis* fruits, *H. subflexa* secures enemy-free space from the lantern of *Physalis* much more efficiently than *H. virescens*. The hypothesis that specialized behaviors are integral to the use of enemy-free space on *Physalis* is supported by our demonstration that rates of parasitism on *Physalis* are significantly higher for *H. virescens* than for *H. subflexa*.

The relative ability of *H. subflexa* and *H. virescens* to secure enemy-free space from the lantern of *Physalis* might reflect dietary constraints: If *H. virescens* larvae refuse to feed on *Physalis*, their failure to colonize a lantern could be a side effect of their rejection of *Physalis* as a feeding substrate. In our behavioral assays, 35% of *H. virescens* larvae failed to attempt colonization versus 23% of *H. subflexa*. In the laboratory, we carried out feeding assays to determine if these behavioral differences (and, by extension, differences in field parasitism rates) were a direct result of feeding preferences. Although there was no statistical difference in the likelihood of feeding by *H. virescens* versus *H. subflexa*, we did observe two unexpected trends. First, in spite of being a specialist on *Physalis*, *H. subflexa*'s feeding performance was extremely variable when compared to that of *H. virescens*. When data for each species were analyzed separately, the effects of date on larval fruit consumption were significant for *H. subflexa* ($P = 0.0002$) but not for *H. virescens* ($P = 0.4125$). Why *H. subflexa* should be so variable across trials is unclear. It is possible that *H. subflexa* larvae are more sensitive to plant quality than *H. virescens*, but additional experiments are needed to clarify the basis of this variability.

A second interesting (but statistically insignificant) finding was that *H. virescens* larvae were more likely than *H. subflexa* to feed on *Physalis* fruits (87% of *H. virescens* fed within 24 h versus 80% of *H. subflexa*), and their mean consumption of *Physalis* fruits was statistically indistinguishable from that of *H. subflexa*. This result was surprising because *H. virescens*

gain only 0.09 mg per milligram of fruit material consumed, versus a gain of 0.30 mg per milligram consumed by *H. subflexa*. Thus, although *Physalis* is a poor host for *H. virescens*, this is not generally reflected in *H. virescens*'s willingness to feed on *Physalis* fruits. *Heliothis virescens*'s failure to secure enemy-free space from *Physalis* appears to depend at least in part on behavioral rather than physiological differences between *H. subflexa* and *H. virescens*.

The comparative inefficiency of *H. virescens*'s fruit colonization behavior may stem from its lack of sensory specialization. Recent work on larval sensory and behavioral responses to secondary plant compounds (Bernays et al. 2000) demonstrated that *H. subflexa* is more sensitive than *H. virescens* to many toxic compounds: *H. subflexa* larvae typically accepted or rejected compounds after a single bite, whereas *H. virescens* fed for extended periods before rejecting a compound. This sensitivity may allow *H. subflexa* larvae to distinguish between host and nonhost plant material more quickly than *H. virescens*, and thus begin colonization more rapidly. Bernays (1998) has suggested that specialist feeders make more rapid decisions than generalists, resulting in more efficient host use. Bergelson and Lawton (1988) found that exposure while moving over a plant's surface is far more dangerous than exposure while stationary—adaptations reducing the amount of time a larva spends traveling on a plant before accepting it as a host should thus decrease a larva's vulnerability to attack by natural enemies.

For *H. virescens* and *H. subflexa*, differences in exposure time were particularly striking in how long each species took to arrive at a lantern: *H. virescens* larvae took almost three times as long as *H. subflexa* to arrive at the lantern that was eventually colonized, thus increasing their risk of detection by natural enemies. A similar difference was seen in the amount of time larvae spent on a lantern before boring an entry hole: *H. virescens* larvae took almost twice as long as *H. subflexa* to initiate boring after arriving at a lantern. In this study, precolonization movement was almost three times more protracted in *H. virescens* than in *H. subflexa*.

Not every behavioral difference between *H. virescens* and *H. subflexa* is equally consequential, however; larvae are particularly vulnerable after they have initiated feeding (Milinski 1990). Many plants release herbivore-induced volatiles within hours of being attacked (Turlings et al. 1995). Herbivore-induced volatiles are often used by predators and parasitoids as location cues and attractants (Turlings et al. 1990; McCall et al. 1993), because larvae themselves have typically evolved to be inconspicuous to natural enemies (Vet and Dicke 1992). Volatile cues can be extremely informative to natural enemies, making it possible for them to distinguish between host and nonhost larvae (DeMoraes et al. 1998).

For larvae feeding on *Physalis*, the emission of herbivore-induced volatiles is critical throughout larval development—first, as larvae enter the lantern that they have damaged; subsequently, as they abandon consumed fruits and travel over the plant to new ones. In addition, larvae may hatch on a plant that is already infested, thus releasing herbivore-induced volatiles. Rapid exploitation of enemy-free space is therefore particularly important once a plant is damaged, and the greatest differences in the fruit colonization behavior of *H. subflexa* and *H. virescens* occur after a larva has damaged

a plant. Damage to *Physalis* first occurs as larvae bore through the lantern surrounding a fruit. We think it likely that the strongest selection pressure for refuge use comes in the wake of boring. The behavior of *H. subflexa* larvae reflects this—*H. subflexa* larvae typically complete entry within 5 min, leaving very little time for natural enemies to exploit the location cues provided by herbivore-induced volatiles. *Heliothis virescens*, however, sometimes treat the lantern of *Physalis* like a cotton boll, boring in just far enough to begin feeding on the fruit. Thus, they begin to feed on fruits while their bodies are outside the lantern, and take more than six times as long as *H. subflexa* to fully enter the lantern. This mode of feeding is dangerous for larvae: Increasing emission of herbivore-induced volatiles attracts parasitoids to the damaged plant, while the enclosure of the larva's sensory apparatus within the lantern reduces its ability to detect and evade searching parasitoids. Because larval feeding maintains high levels of volatile emission, subsequent movement from fruit to fruit is also risky.

Investigation of the value of *Physalis* lanterns as enemy-free space for *H. virescens* illustrates the potential importance of sensory and behavioral differences between *H. subflexa* and *H. virescens*. As expected, rates of parasitism were high for *H. virescens* on tobacco and low for *H. subflexa* on *Physalis*. If structural refuge alone were responsible for *H. subflexa*'s low levels of parasitism, we would expect similarly low rates for *H. virescens* on *Physalis*. Instead, parasitism of *H. virescens* on *Physalis* was much higher than for *H. subflexa*, and overall levels of attack by other natural enemies were also higher for *H. virescens* on *Physalis* than for *H. subflexa*.

Additional experimentation in the field (S. J. Oppenheim and F. Gould, unpubl. ms.) has eliminated two alternative explanations for *H. subflexa*'s reduced rate of parasitism. First, we looked into the possibility that *C. nigriceps* may have an inherent preference for *H. virescens* larvae over *H. subflexa*. We tested the attractiveness of *H. virescens*, *H. subflexa*, and *H. zea* (a nonhost) on tobacco, *Physalis*, and cotton to naïve *C. nigriceps* and found that, although *C. nigriceps* consistently prefers host species to nonhosts, no distinction is made between the two host species. Second, we investigated whether *C. nigriceps* might be more attracted to volatiles released by *H. virescens*-damaged plants than to those released by *H. subflexa*-damaged plants. In the field tests mentioned above, we found no difference in *C. nigriceps*' attraction to *H. virescens*-and *H. subflexa*-infested plants.

In spite of their relative inefficiency in carrying out host-related behaviors on *Physalis*, *H. virescens* larvae on *Physalis* do experience a significant reduction in parasitism compared to *H. virescens* on tobacco. *Heliothis virescens* larvae on tobacco are more than three times as likely to be parasitized as *H. virescens* larvae on *Physalis*, and we consider it likely that such an escape from parasitism could exert positive selection on *H. virescens* for adaptation to *Physalis*. Intriguingly, we found that *H. subflexa* is less variable than *H. virescens* in colonization behavior, but much more variable than *H. virescens* in feeding performance—possible evidence that behavioral avoidance of natural enemies is under strong selection in the specialist. Currently, agricultural monocultures may provide *H. virescens* with abundant food, and this may offset *H. virescens*'s vulnerability to natural enemies. A

change in selection regime, however, such as an increase in natural enemy populations or resource limitations due to highly toxic transgenic crops, might render a host shift by *H. virescens* onto *Physalis* adaptive. Use of *Physalis* could become fixed even if alternative hosts were of greater physiological utility, if escape from natural enemies had a sufficiently beneficial impact on fitness (Templeton and Rothman 1981). In many systems, the provision of enemy-free space by a host plant compensates for other, less favorable, plant characteristics (Damman 1987; Ohsaki and Sato 1994; Feder 1995). Geitzner and Bernays (1996), for example, found that a predatory wasp, *Polistes arizonensis*, was less attracted to *H. virescens*-infested *Physalis pubescens* than to *H. virescens*-infested *Helianthus annuus* (sunflower), and we (unpubl. ms.) found that *H. virescens* on *Physalis* is significantly less attractive to parasitoids than *H. virescens* on tobacco. This plant effect, coupled with the structural enemy-free space provided by *Physalis*, might provide *H. virescens* larvae with rates of survival on *Physalis* comparable to those experienced on tobacco, cotton, and other presently used hosts. The compensatory value of enemy-free space on *Physalis* would be particularly high in habitats where natural enemies are a significant source of larval mortality.

The evolution of specialized host acceptance and host use behaviors may have served as an important step in the evolution of specialization on *Physalis*. By feeding on *Physalis* plants instead of tobacco, the generalist ancestor of *H. subflexa* could, like present-day *H. virescens*, experience an immediate reduction in vulnerability to parasitoids. The subsequent evolution of behavioral adaptations to *Physalis* would, as we have shown here, further increase the value of this enemy-free space. Although contemporary ecological conditions may not correspond to conditions at the time of speciation by *H. subflexa* from a *H. virescens*-like generalist ancestor, we suggest that behavioral use of enemy-free space is likely to have been a strong force in this system. Behavioral constraints on the use of enemy-free space may be more common than previously realized and may constitute an effect equal in importance to morphological and physiological aspects of specialization.

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