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The roles of nicotine and natural enemies in determining larval feeding site distributions of *Manduca sexta* L. and *Manduca quinquemaculata* (Haworth) on tobacco

Karen M. Kester[1], Steven C. Peterson*[2], Frank Hanson[2], D. Michael Jackson[3] and R. F. Severson[†4]

[1]Department of Biology, Virginia Commonwealth University, 1000 W. Cary St., Richmond, VA 23284-2012, USA
[2]Department of Biological Sciences, University of Maryland, Baltimore, Maryland 21228, USA
[3]USDA-ARS, U.S. Vegetable Laboratory, 2875 Savannah Hwy, Charleston, SC 29414, USA
[4]USDA-ARS, Tobacco Safety Research Unit, Athens, Georgia 30604, USA

Summary. Field observations indicated that hornworms select feeding sites non-randomly on tobacco. We tested the hypotheses that differences in feeding site locations of larvae of *Manduca sexta* L. and *Manduca quinquemaculata* (Haworth) (Lepidoptera: Sphingidae) on tobacco could be explained by differential nicotine concentrations within plants and leaves, species-specific responses to nicotine, or pressure exerted by natural enemies. Results showed that third-instar larvae of *M. sexta* fed more proximally and centrally on the leaf, whereas *M. quinquemaculata* fed more distally. Within-plant selection of leaves did not differ; both species selected leaves in the middle region of the plant. Nicotine concentrations in a high nicotine genotype, NC95, varied within each leaf, increasing 2–3 fold from the basal to apical portion of the leaf, and within each plant, increasing 7–10 fold from the first fully expanded leaf to the twelfth (lowest) leaf. In laboratory bioassays, both *Manduca* species responded to nicotine as a feeding deterrent. Electrophysiological studies demonstrated that gustatory organs of both species responded to nicotine at concentrations found in tobacco leaves and that *M. quinquemaculata* generally showed a less vigorous response to nicotine than *M. sexta*. Field mortality of *M. sexta* due to parasitism by *Cotesia congregata* (Say) and to parasitism and predation combined differed among feeding sites; predation alone did not. Results suggest that although nicotine concentration and species specific responses to nicotine play a role in determining feeding site locations, pressure exerted by natural enemies, especially parasitism by *C. congregata*, is more important.

Key words. Feeding behavior – tritrophic interactions – chemoreception – electrophysiology – nicotine – tobacco – Lepidoptera: Sphingidae – *Manduca* – *Manduca sexta* – *Cotesia congregata*

Correspondence to: Karen M. Kester, e-mail: kmkester@saturn.vcu.edu
* Present address: Ecology and Environment, Inc., 368 Pleasantview Dr., Lancaster, NY 14086, USA
† Deceased

Introduction

Distribution of secondary plant chemicals varies among species, within species and even within individual plants. Highest concentrations of plant allelochemicals may be allocated to those tissues of greatest value to plant fitness and at greatest risk of attack by herbivores (Rhoades 1979; McKey 1979). However, herbivores possess physiological and/or behavioral adaptations that enable them to utilize chemically defended plants. For example, tobacco-feeding coleopterans and orthopterans can metabolize nicotine to cotinine and other alkaloids (Self *et al*. 1964a), and the aphid, *Myzus persicae* (Sulz.) avoids nicotine by selectively feeding in the phloem (Guthrie *et al*. 1962). *Manduca sexta* L. (Lepidoptera: Sphingidae), which commonly feeds on tobacco and other solanaceous plants, rapidly excretes most of the nicotine it ingests (Self *et al*. 1964b). Ingestion of dietary nicotine (0.75% wet weight) also induces midgut cytochrome P-450 activities in *M. sexta* and may represent an additional adaptation to tobacco feeding in this species (Snyder *et al*. 1993) and other lepidopterans (Rose *et al*. 1991). Additional physiological mechanisms, *e.g*., metabolic detoxification and/or carrier-mediated transport, have been hypothesized to detoxify or block entrance of nicotine in the central nervous system of *M. sexta* (Morris 1983, 1984). As discussed below, nicotine intake may be regulated behaviorally.

During the course of related work, one of us (KK) observed that early instars of *M. sexta* (''tobacco hornworm'') and *Manduca quinquemaculata* (Haworth) (''tomato hornworm'') feed preferentially on certain areas within tobacco leaves and that these feeding patterns appear to differ between the two species. One possible explanation for this observation is that the two species differ in their ability to detect or tolerate a chemical that is not equally distributed within the leaves. Since nicotine is found in large quantities in tobacco and is a poison that is differentially tolerated by various insects (Self *et al*. 1964a; Yang & Guthrie 1969), we considered it to be a likely cue. Nicotine

concentrations vary among wild *Nicotiana* species (Saitoh *et al*. 1985), cultivated tobacco varieties (Sisson & Saunders 1982) and within individual plants and leaves (Darkis *et al*. 1936; Jeffrey 1958). Also, *M. sexta* and *M. quinquemaculata* are sympatric in many areas but *M. quinquemaculata* is far less common in the southeastern USA (Hodges 1971) where tobacco is typically cultivated, suggesting that the two species may differ in their degree of adaptation to nicotine.

The status of nicotine as a feeding stimulant or deterrent is not clear. Behavioral responses to nicotine have been tested in the laboratory: DeBoer & Hanson (1987) showed that *M. sexta* did not discriminate among 1 mM, 10 mM and water, and therefore concluded that nicotine played no role in feeding behavior. However, natural concentrations of nicotine can be much higher than those tested. For example, Sisson & Saunders (1982) report total alkaloid concentrations of up to 6.6% dry weight for whole plants (at least 1.32% wet weight), 90–98% of which is nicotine. If available in simple solution this would be equivalent to 70–80 mM. Therefore, we tested the effect of nicotine on feeding behavior of *M. sexta* and *M. quinquemaculata* using concentrations likely to be encountered under field conditions.

The sensory information controlling this behavior is likely to be mediated by the maxillary sensilla styloconica (see DeBoer & Hanson 1984). Schoonhoven (1969) reported no activity of the maxillary gustatory organs of *M. sexta* in response to 1 mM nicotine, which at that time was thought to be representative of concentrations in the plant. More recently, Glendinning (1996) demonstrated chemosensory detection of 4.6 mM nicotine by these organs.

In the present study, we test the response of the maxillary gustatory organs of *M. sexta* and *M. quinquemaculata* electrophysiologically using the higher concentrations of nicotine now known to occur in tobacco plants. In view of this new information, we also re-investigate the chemical and physiological bases of feeding behavior observed in the field by asking the question: Does nicotine concentration explain differences in feeding site distributions of the two *Manduca* species on tobacco? In attempting to answer this question, we determined whether: 1) nicotine concentrations vary consistently within and among tobacco leaves, 2) preferred feeding sites differ between the two species on tobacco plants in the field, 3) behavioral and physiological responses to nicotine differ between the two species, and 4) risks of parasitism and predation vary with feeding sites.

Materials and methods

Insects. Hornworm eggs originated from stock USDA colonies (Oxford, NC); additional *M. sexta* eggs from another USDA colony (Beltsville, MD) and *M. quinquemaculata* eggs collected directly from tobacco plants in Oxford, NC were also used, as described below. Stock colonies of both species were maintained on diets based on Yamamoto (1969). Larvae used in experimental work were reared on a similar laboratory diet (BioServ) from hatching.

Field studies. Tobacco plants were started from seed in a greenhouse and then transplanted to the experimental research farm of the University of Maryland, Upper Marlboro (Prince George's County), Maryland in 1986 and 1987, as described in Kester & Barbosa (1994). Plants used in field experiments were chosen for their healthy and uniform appearance; experimental treatments within each patch were randomly assigned. Experiments were conducted 10 weeks after transplanting.

Newly-emerged third-instar larvae of the hornworm species (Oxford colonies) were released onto the undersurface of the third fully-expanded leaf from the top of each tobacco plant (one larva per plant) and then recaptured two days later. This leaf is usually the largest and tallest upright leaf on a tobacco plant and commonly used for oviposition. At recapture, we noted the leaf and within-leaf region on which the hornworm and feeding damage were found. The first year, both hornworm species were tested on high nicotine 'NC95' and its low nicotine derivative, 'LAFC53' a near-isogenic line derived from 'NC95' (Chaplin & Burk 1984); only 'NC95' was used the second year. Feeding site distributions of the two hornworm species on the two tobacco varieties were compared at two levels: 1) Within and among plant selections were compared with an $R \times C$ test of independence using a G-test (Sokal & Rohlf 1981) and 2) within-leaf feeding site selections (the leaf vein interval receiving the most feeding damage) of the two hornworm species were compared for the two tobacco varieties with a generalized Fisher's Exact test (Mehta & Patel 1983).

Chemical analysis. Five plants of each of the two tobacco varieties were sampled at 10 weeks and again at 14 weeks after transplanting by removing a (1 cm diameter) disk of leaf tissue with a cork borer from the 1st fully expanded, 6th and 12th leaves (counting from top of the plant). Samples were taken from each of three regions within each leaf: lateral leaf vein interval 1–3 (''basal''), lateral vein interval 3–7 (''central''), and lateral leaf vein 7 to tip (''apical''); the 6th and 12th leaves were sampled at both interior and edge positions, as shown Fig. 1.

Sample disks were immediately frozen with dry ice, then crushed and stored at -20°C until chemical analyses were performed. Additional samples were taken at corresponding positions from the other half of each sampled leaf for determination of dry weight. Nicotine was quantified for each sample using capillary gas chromatography (Severson *et al*. 1981).

Laboratory behavioral studies. Responses of hornworm larvae were assayed using the disk test (Jermy *et al*. 1968), as modified by DeBoer & Hanson (1984). Briefly, six disks cut from glass fiber filter paper (Whatman GF/A) were placed around the perimeter of a circular dish (10 cm diam.). The floor of this dish was layered with paraffin wax into which were inserted pins holding the glass fiber disks 1 cm above the substrate. Three disks spotted with nicotine and three control disks spotted with solvent only were placed alternately around the perimeter of the disk. After the solvent evaporated, the disks were re-wetted with water. A recently molted, unfed fifth-instar larva was placed in the center of each test chamber and allowed to feed until 50% of the area of disks in the most eaten category was consumed; at this time the area consumed of each disk was estimated visually. Each larva was tested only once, then discarded. Tests were conducted at ca. 27°C. Results are presented as an ''inhibition index'', in which larger positive values represent greater inhibition of feeding on treated disks:

$$\text{Inhibition Index} = [(\text{control} - \text{treated})/(\text{control} + \text{treated})] \times 100$$

The nicotine solutions tested were 0.1% and 1% by volume. To make these tests comparable to the electrophysiological tests, the nicotine was made up in 100 mM NaCl plus 10% ethanol. The ethanol did not affect behavior, since it evaporated when the disks were dried prior to re-wetting.

Electrophysiology. Sensory responses of hornworm larvae were obtained using standard electrophysiological techniques for tip recording from the styloconica of isolated head preparations (Frazier & Hanson 1986; Peterson *et al*. 1993). The recording/stimulating pipettes contained the nicotine solution made up in a final concentration of 100 mM NaCl for electrical conductivity and 10% ethanol for improved electrical contact with the sensilla. Control solutions were

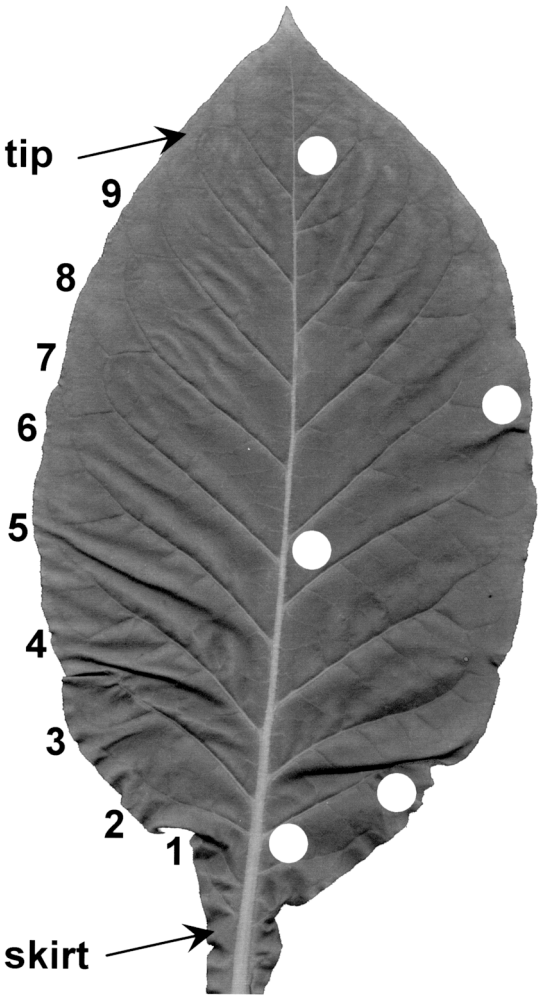

Fig. 1 Tobacco leaf illustrating leaf vein markings used in field experiments. Circles indicate typical locations of 1-cm tissue samples used in nicotine analyses

100 mM NaCl and 10% ethanol. Trials lasted 3 sec with intertrial intervals of 3 min. Each experiment consisted of a control trial, two nicotine trials and another control trial for each concentration (0.01, 0.1, 1.0, 3.0%). Responses were recorded using a high-impedence preamplifier with a baseline-restoring circuit, and a filter amplifier having a bandpass of 100–2000 Hz. Data were stored on an FM tape recorder and digitized for computer analysis and display (Frazier & Hanson 1986). High quality, repeatable recordings were obtained from five of the 10 *M. sexta* larvae tested and two of the six *M. quinquemaculata* tested.

Predation and parasitism study. Procedures used in this 1990 experiment were the same as those described above (''Field studies'') except that only *M. sexta* (Beltsville colony) was used and predation and parasitism status of each larva was noted. Predation was determined directly by hemolymph stains or partial remains of the larva on the leaf, or indirectly by the presence of feeding damage and failure to recover a hornworm. To determine parasitism status, all recovered hornworms were returned to the laboratory and reared until parasitoids egressed; the few that died were dissected. Main and interactive effects of tobacco genotype and feeding location on mean percent predation, mean percent parasitism and mean percent mortality (combined predation and parasitism) were compared among feeding sites with ANOVA (Proc GLM, Version 6, SAS Institute 1990). Means were compared using the PDIFF function and a Bonferroni's method for adjusting the level of significance ($P = 0.05$/number of comparisons).

Results

Feeding site distributions. Field studies showed that within-leaf feeding sites differed between the two *Manduca* species; *M. sexta* fed more proximally than *M. quinquemaculata* on both high and low nicotine genotypes (Fig. 2, $P \leq 0.002$, $N = 118$). This difference was somewhat more pronounced on the high than on the low: Larvae of *M. sexta* tended to feed more proximally on the high than on the low nicotine genotype ($P = 0.077$, $N = 59$), and *M. quinquemaculata*, more distally ($P = 0.0761$, $N = 59$) (Fig. 2B vs. 2A).

In contrast, the two *Manduca* species did not differ in their within-plant locations for either year. In Year 1, larvae moved downward from their release site on Leaf 3 on both of the tobacco genotypes (Fig. 3A and B), whereas, in Year 2 they remained at the same level (Fig. 3C). Climatological data show that Year 1 was much cooler (high/low recorded temperatures were 28.3/12.2°C) than Year 2 (33.6/21.7°C), and since lowest temperatures occur in the upper portion of plants (DM Jackson, unpubl.), these year to year differences in leaf position selections may reflect larval responses to temperature rather than or in addition to phytochemical cues.

Chemical analyses. Nicotine concentrations varied consistently within leaves of 10-week-old plants of both tobacco genotypes. Nicotine gradients increased from basal to apical, and usually from medial to edge (Table 1). Another nicotine gradient was seen among leaves of the high nicotine genotype ('NC95') along the vertical axis, with highest concentrations in the bottom leaf and lowest concentrations in the top leaf; nicotine concentrations did not follow this gradient in the low nicotine genotype ('LAFC53').

Laboratory behavioral studies. In choice tests, both *Manduca* species were deterred by 1% (60 mM) nicotine solutions ($P < 0.01$) and perhaps by 0.1% (6 mM; $P < 0.5$) (Fig. 4). These bioassays were highly successful with *M. sexta* in that 96% of the larvae from both colonies fed on the glass fiber disks in the choice test. In contrast, only 62% of the laboratory colony and 0% of field collected *M. quinquemaculata* fed on the glass fiber disks.

Electrophysiology. The maxillary sensilla styloconica, the chemosensory organs most associated with control of feeding, responded to nicotine in a quasi dose-dependent manner over the range of nicotine tested: Response threshold occurred at ca. 0.1% nicotine, the maximum response at 1% nicotine and inhibition at 3% nicotine (Figs. 5 and 6). The lateral styloconica were much more responsive to nicotine than the medial styloconica (Fig. 5A vs. 5B, and Fig. 6A vs. 6B). Responses of *M. sexta* (Fig. 5) contained more spike activity than those of *M. quinquemaculata* (Fig. 6). Just as in the behavioral assays, more of the *M. sexta* preparations were functional (9/10) compared to *M. quinquemaculata* (2/6 laboratory strain, 0/8 field collected animals). Intense spike activity of short duration (''bursting'') was occasionally seen in both species

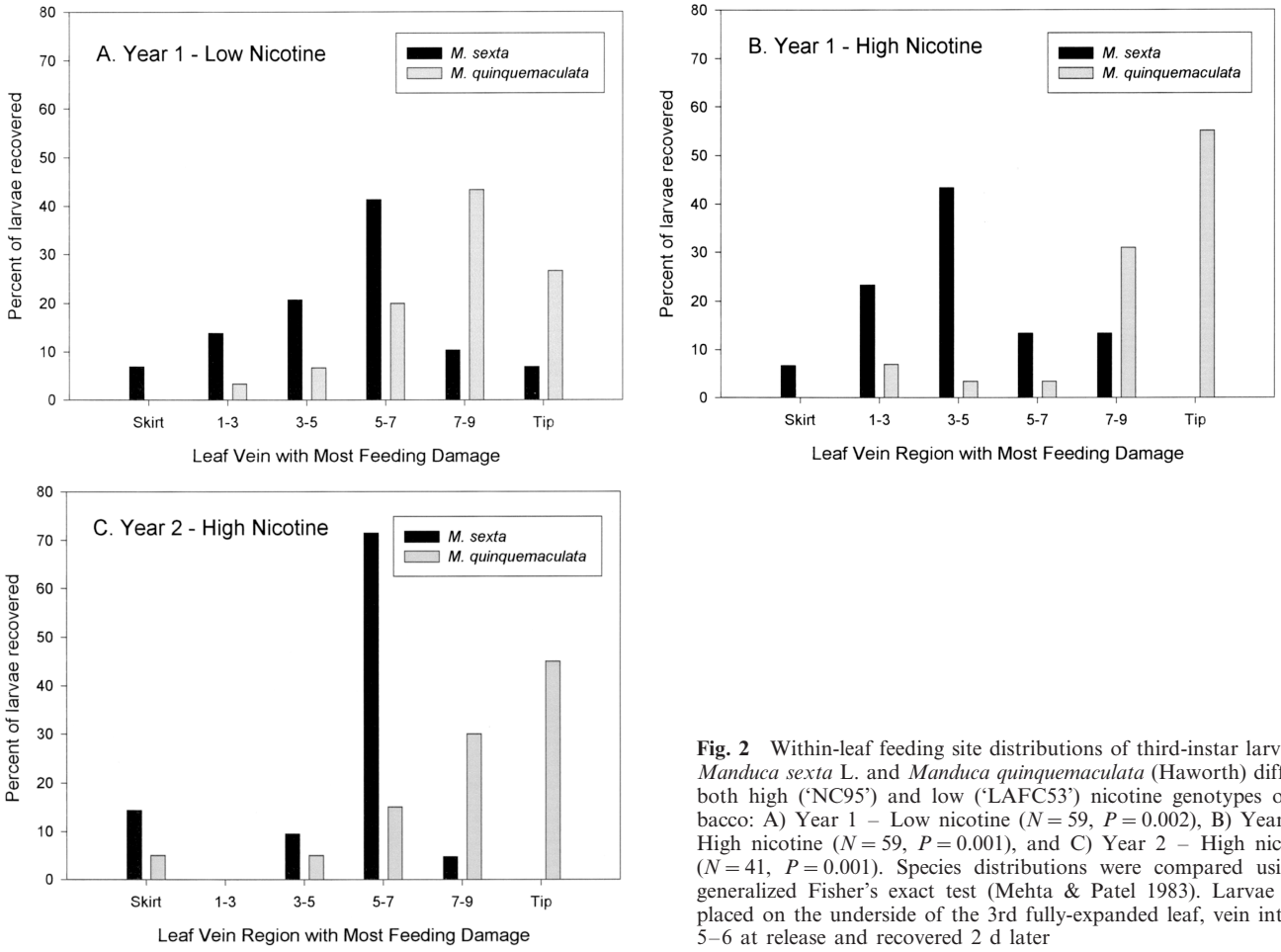

Fig. 2 Within-leaf feeding site distributions of third-instar larvae of *Manduca sexta* L. and *Manduca quinquemaculata* (Haworth) differ in both high ('NC95') and low ('LAFC53') nicotine genotypes of tobacco: A) Year 1 – Low nicotine ($N = 59$, $P = 0.002$), B) Year 1 – High nicotine ($N = 59$, $P = 0.001$), and C) Year 2 – High nicotine ($N = 41$, $P = 0.001$). Species distributions were compared using a generalized Fisher's exact test (Mehta & Patel 1983). Larvae were placed on the underside of the 3rd fully-expanded leaf, vein interval 5–6 at release and recovered 2 d later

in response to some of the higher concentrations of nicotine stimuli (not illustrated).

A second effect of nicotine that we observed was an increase in spike activity in response to control stimuli (0.1M NaCl) following nicotine stimuli, suggesting that nicotine hypersensitized one or more taste neurons in the styloconicum (Figs. 5A and 6A: compare NaCl control before and after exposure to nicotine). This effect was seen in the lateral (Figs. 5A and 6A) but not in the medial styloconica (Figs. 5B and 6B).

Parasitism and predation. Percent parasitism by *C. congregata* differed significantly ($P = 0.0001$, $N = 73$) among within-leaf feeding locations; hornworms feeding on the skirt area had the lowest percent parasitism (Fig. 7A; tobacco genotype alone had no effect and there was no significant ($P \geq 0.05$) interaction among within-leaf feeding locations (Table 2A). In contrast, nonspecific predation was not significantly ($P > 0.05$, $N = 73$) affected by any of these factors (Table 2B, Fig. 7B). However, combined mortality due to parasitism by *C. congregata* and non-specific predation differed significantly with respect to both leaf position ($P = 0.0001$) and tobacco genotype ($P = 0.04$) (Table 2C). Hornworms feeding on the skirt and middle area of the leaf (veins 4–7) suffered lowest combined mortality (Fig. 7C). Overall, hornworms on the high nicotine variety were significantly ($P = 0.04$) less likely to be eaten or parasitized ($34 \pm 6\%$, $N = 48$) than those on the low nicotine variety ($51 \pm 6\%$, $N = 48$) (Table 2, Fig. 7C).

Discussion

The foregoing clearly demonstrates that hornworm larvae select specific parts of the tobacco leaf on which to feed and that these feeding site selections differ between the two *Manduca* species: larvae of *M. quinquemaculata* tend to feed towards the apical tip, where nicotine concentrations are highest, and larvae of *M. sexta* tend to feed more towards the basal region, where nicotine concentrations are lowest ($P = 0.02$; Fig. 2). These results are consistent with the hypothesis that nicotine concentration is responsible for differences in selection of within-leaf feeding sites of these two species. If so, it follows that these differences should be accentuated on the high nicotine genotype ('NC95') due to its wider range of within-leaf nicotine concentrations compared to the low-nicotine genotype ('LAFC53'), but this pre-

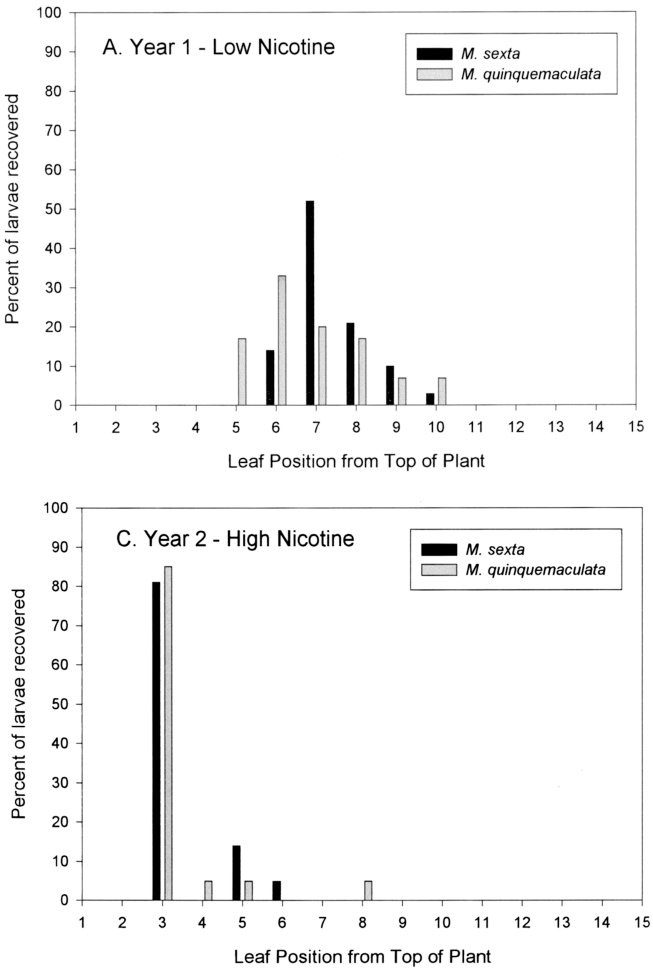

Fig. 3 Within-plant feeding site distributions of third-instar larvae of *Manduca sexta* L. do not differ from those of *Manduca quinquemaculata* (Haworth) on either low ('LAFC53') or high ('NC95') nicotine genotypes of tobacco in the field: A) Year 1 – Low nicotine ($N = 59$, 0.05), B) Year 1 – High nicotine ($N = 59$, 0.05), and C) Year 2 – High nicotine ($N = 41$, 0.05). Species distributions were compared with an R × C test for independence using a G-test (Sokal & Rohlf 1981). Larvae were released onto the underside of the 3rd fully expanded leaf from the top and recovered 2 d later

diction is not well supported by our field data ($P = 0.07$, Fig. 2). Indeed, given the large disparity in mean nicotine concentrations between the two tobacco varieties, *M. sexta* would not be expected to feed at all on the high nicotine genotype yet it feeds extensively and shows clear within-leaf selectivity on the high nicotine variety, just as it does on the low nicotine variety (Fig. 2). This suggests that nicotine plays no role in selection of feeding sites; alternatively, this suggests that *M. sexta* can acclimate to higher average nicotine levels and still discern relative concentrations of nicotine among leaf regions. Since the two tobacco genotypes have similar ratios of basal/apical concentrations (Table 1), a likely explanation is that the larvae are selecting feeding sites on the basis of these ratios irrespective of the background level. This is a typical solution of a simple signal-to-noise problem common to many sensory-based behaviors.

In addition to within-leaf selectivity, hornworm larvae also exhibit clear preferences for specific regions of the tobacco plant. In our field experiments, both species fed almost exclusively on the middle region of plants of both high and low nicotine varieties. Results of our chemical analyses show that in the high nicotine genotype ('NC95'), nicotine concentrations varied along a vertical gradient with lowest concentrations in the new fully expanded leaves near the top of the plant and highest concentrations in the lower, older leaves (Table 1). This pattern was reported previously by Jeffrey (1958) and is opposite of that predicted by plant defense theory for optimal distribution of secondary compounds (McKey 1979; Rhoades 1979). However, this top-bottom gradient was not found in the low nicotine genotype ('LAFC53'), suggesting that some factor other than nicotine concentration – perhaps localized temperature – must account for the vertical distribution of hornworms on tobacco plants.

For nicotine to play a role in feeding site selection, the animal must detect it. Results of our laboratory behavioral assays show that nicotine deters feeding at concentrations of 1%, well within levels typically present in cultivated tobacco (Sisson & Saunders 1982). Our electrophysiological experiments demonstrate that chemosensory neurons in the lateral styloconica of the maxillae are clearly activated by nicotine, although the response does not appear to be a "normal" chemosensory response. For example, it is not at all similar to the response to caffeine, which is also a deterrent and elicits

Table 1 Results of chemical assays of nicotine concentration among and within leaves of low nicotine ('LAFC53') and high nicotine ('NC95') genotypes of tobacco. Means shown are μg per 1 cm (diameter) sample ± SE of five 10-week old plants as quantified by GC using the methods of Severson *et al.* 1981. Key: Basal = veins 1–3, Central = veins 4–8, Apical = veins 9 – tip; * = edge sample not taken because of small leaf. See Fig. 1 for sampling scheme

Leaf position	Within-leaf	Nicotine concentration (μg) per sample	
(from top)	region	LAFC53	NC95
Leaf 1*	Basal medial	11.4 ± 0.6	40.1 ± 8.2
	Central medial	13.2 ± 0.5	55.5 ± 13.2
	Apical medial	21.2 ± 2.3	96.7 ± 21.4
Leaf 6	Basal medial	7.8 ± 0.3	48.5 ± 10.6
	Basal edge	10.4 ± 1.2	59.0 ± 14.9
	Central medial	10.7 ± 1.31	60.2 ± 10.6
	Central edge	13.9 ± 1.3	70.6 ± 8.5
	Apical medial	15.0 ± 1.4	91.8 ± 13.0
	Apical edge	17.2 ± 2.1	102.3 ± 13.8
Leaf 12	Basal medial	7.5 ± 0.6	58.8 ± 8.5
	Basal edge	8.5 ± 0.7	67.0 ± 7.3
	Central medial	12.9 ± 2.1	70.6 ± 6.6
	Central edge	11.2 ± 1.8	99.7 ± 13.0
	Apical medial	19.4 ± 1.5	147.5 ± 15.8
	Apical edge	21.5 ± 1.6	161.6 ± 16.6

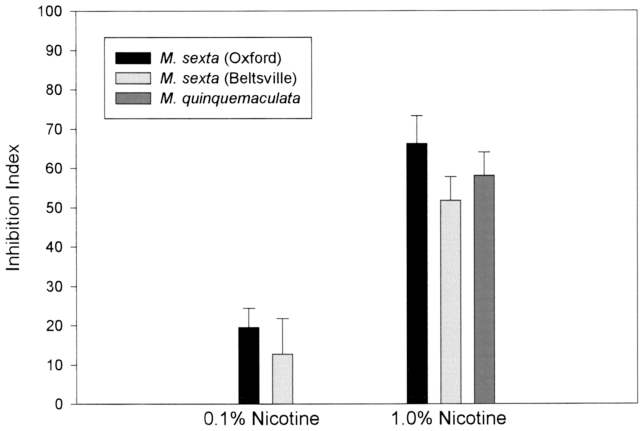

Fig. 4 Feeding deterrence by nicotine in laboratory bioassays with fifth-instar larvae of *Manduca sexta* L. and *Manduca quinquemaculata* (Haworth). Bars represent means ± SE. For 1.0% nicotine experiments: *M. sexta*, Oxford strain ($N = 18$), *M. sexta*, Beltsville strain ($N = 27$), and *M. quinquemaculata*, Oxford strain ($N = 18$); for 0.1% nicotine, $N = 13$, 7 and 0, respectively

a classic "deterrent neuron" response of action potentials from a single chemosensitive neuron characterized by a spike frequency that rises slowly to a peak followed by a nearly steady state or gentle decline (Frazier 1986; Schoonhoven 1987; Glendinning 1996). Instead, as shown in Figs. 5 and 6, nicotine activates more than one receptor neuron with a phasic pattern of spikes. Nicotine also appears to have a hypersensitizing effect on these neurons such that they also become responsive to other stimuli as well, *e.g.*, a dilute salt solution (Figs. 5A and 6A). Note, however, that this nicotine effect is not general for all sensory neurons nor for all deterrent neurons, since the medial styloconicum, which also contains a deterrent cell (Peterson *et al.* 1993), does not respond to nicotine nor show any hypersensitization following a strong nicotine stimulus (Figs. 5B and 6B). The simplest neural mechanism that can explain the observed phagodeterrence by nicotine is that one or more chemosensory neurons sensitive to or hypersensitized by nicotine are deterrent neurons which, when activated, stimulate the caterpillar's central nervous system to decrease feeding. Alternatively, Glendinning (1996) suggests that the nicotine-sensitive cell is a "stimulant neuron" that increases feeding in the short term (minutes), but that post-ingestive feedback decreases feeding over the longer term (hours) as monitored in our experiments.

Our finding that both hornworm species detect nicotine via their gustatory organs and respond to it behaviorally provides at least a minimal foundation for the hypothesis that nicotine concentration underlies the within-leaf feeding site distributions we observed in the field. Furthermore, electrophysiological recordings showed that *M. quinquemaculata* generally has a less active sensory response to nicotine than *M. sexta* (Fig. 6 vs. Fig. 5), suggesting that *M. quinquemaculata* has a higher chemosensory threshold for nicotine. This is consistent with our field observations that *M. quinquemaculata* tends to feed more apically on tobacco leaves, where nicotine concentrations are 2–3 fold higher, than on areas of the leaf where *M. sexta* feeds (Table 1, Fig. 2), and with the observation by Cheng (1977) that early instars of *M. quinquemaculata* feed more distally on tobacco. The basis of this feeding pattern may be a preference for or tolerance to higher nicotine concentrations by *M. quinquemaculata*, or alternatively, may be due to a higher activity level ("restlessness") or undirected random movement that is arrested when a larva reaches a leaf extremity. Additionally, there may also be a behavioral barrier that tends to retain the animal on a single leaf, thus forcing the animal to select a feeding site on the basis of relative concentrations rather than preferences for an absolute concentration. Within-plant distributions of larvae may be determined by other leaf constituents or properties such as temperature (see "Results") or physical constraints, *e.g.*, more mature larvae may rest on leaf midveins that can support their weight (McFadden 1968).

While physiological and behavioral mechanisms may affect feeding site selections proximately, selective pressures operating at the population level are more likely ultimate causes. Just as predators may influence the evolution of herbivore host plant ranges (Bernays 1989), natural enemies may also influence the choice of feeding sites within the host plant. For example, *C. congregata* is the primary parasitoid of *M. sexta* in southeastern USA, and parasitism levels are often > 90% (Kester, unpubl.); therefore, this parasitoid species exerts considerable selective pressure on hornworm populations.

Perhaps the most cogent reason to consider nicotine as a factor in determining feeding site selections is that its ingestion by host larvae deleteriously affects the survival and development of parasitoids, including *C.*

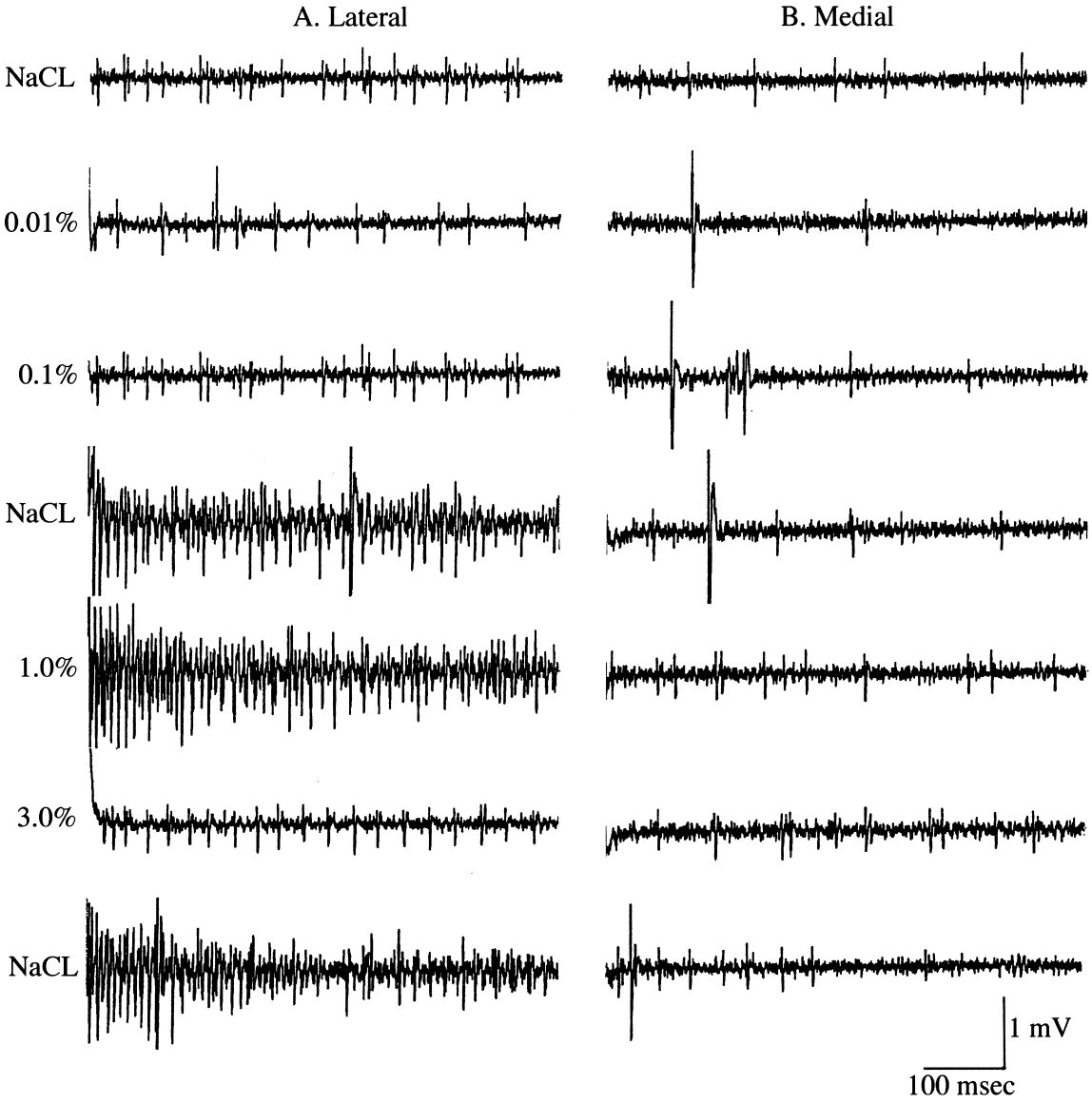

Fig. 5 Electrophysiological responses to nicotine by maxillary chemosensory organs of A) lateral and B) medial styloconicum of *Manduca sexta* L. Representative traces shown are for the first 0.5 sec of representative responses of four nicotine concentrations interspersed with controls (0.1 M NaCl) in the same order as stimuli were presented in the experiment. Traces shown were recorded from right maxillum of one larva (Oxford colony)

congregata (Thurston & Fox, 1972; Barbosa *et al.* 1986; Kester & Barbosa 1991). Thorpe & Barbosa (1986) report that over a 2-week exposure period, higher levels of parasitism by *C. congregata* occurred on hornworms feeding on the low than on the high nicotine genotypes also used in our study.

Results of our field experiment support the hypothesis that feeding site distributions are due, at least in part, to selective pressures exerted by natural enemies, particularly, parasitism by *C. congregata*. Because hornworms feeding on locations with higher nicotine concentrations suffered higher levels of parasitism than those feeding on the skirt, nicotine does not appear to confer direct protection against parasitism by *C. congregata* at this scale. Larvae of *M. sexta* feeding on the leaf skirt, where nicotine concentrations are lowest, had significantly ($P = 0.03$, $N = 73$) lower levels of parasitism than those feeding within any other area (Table 2A, Fig. 7A). Possibly, hornworms feeding on the skirt may be more difficult for parasitoids to locate or to attack due to physical obstruction or leaf morphology. Alternatively, feeding on low-nicotine regions of tobacco leaves may constitute a counter-adaptation for evasion of parasitism by tobacco-adapted *C. congregata*. For example, females of *C. congregata* from this same "tobacco population" show a positive dosage-dependent searching response to nicotine that differs from that of females collected from a "tomato population" and these differential responses can be linked to parasitism levels of *M. sexta* on tobacco and tomato in the field (Kester & Barbosa 1991, 1994).

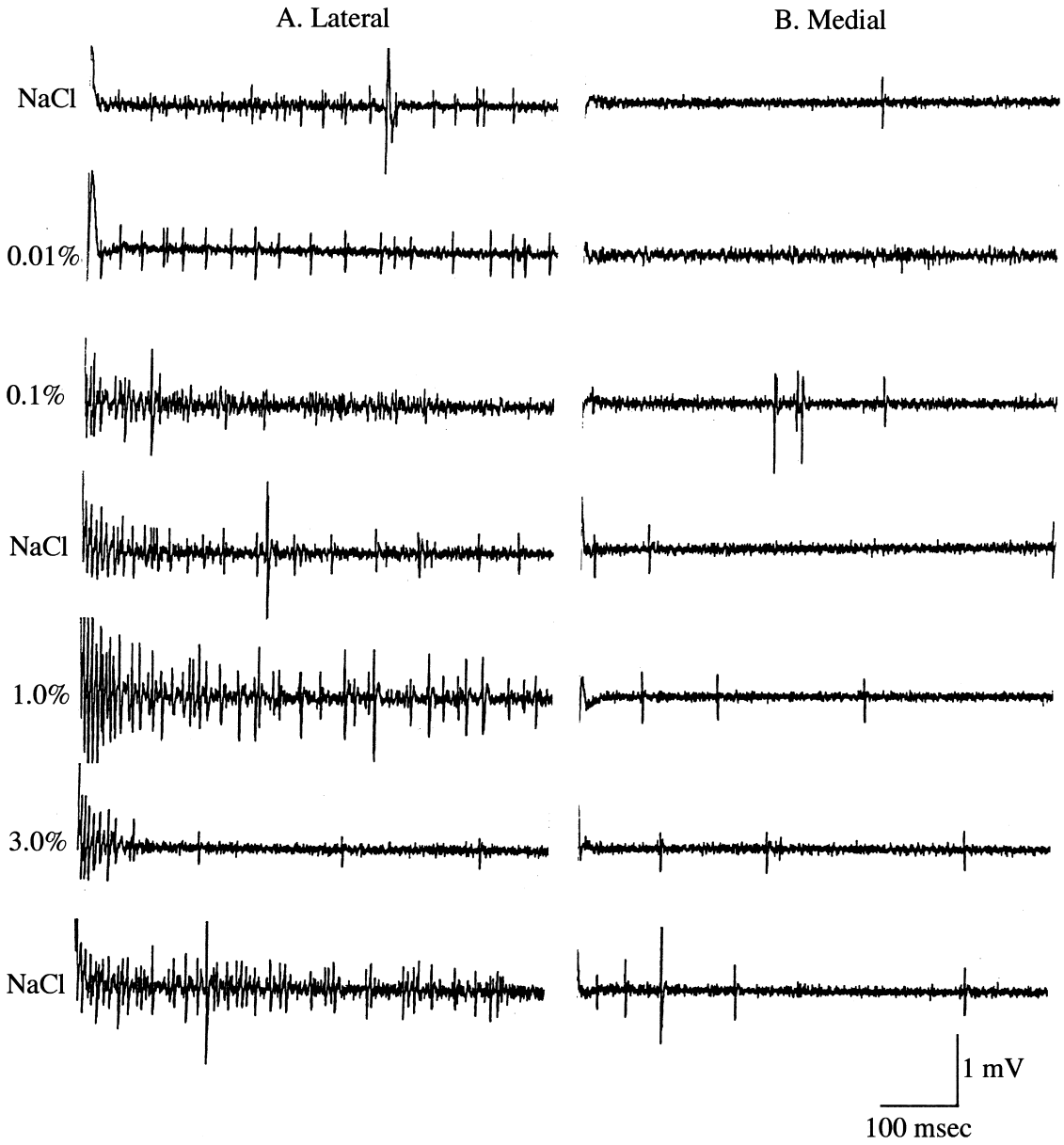

Fig. 6 Electrophysiological responses to nicotine by maxillary chemosensory organs of A) lateral and B) medial styloconicum of *Manduca quinquemaculata* (Haworth). Representative traces are shown for the first 0.5 sec of representative responses of four nicotine concentrations interspersed with controls (0.1 M NaCl) in the same order as stimuli were presented in the experiment. Recordings shown were obtained from the right maxillum of one larva (Oxford colony)

Predation risk alone did not differ significantly among within-leaf feeding site locations ($P > 0.05$, $N = 73$; Table 2B, Fig. 7B) but when combined with parasitism, overall mortality varied significantly with respect to feeding location ($P = 0.04$; Table 2C, Fig. 7C). Hornworms feeding on the low nicotine genotype suffered greater mortality ($51 \pm 6\%$) due to predation and parasitism combined than those feeding on the high nicotine genotype ($34 \pm 6\%$). Overall, this pattern corroborates Thorpe & Barbosa's (1986) findings that parasitism by *C. congregata* was higher on the low nicotine than on the high nicotine varieties also used in our study.

In conclusion, our laboratory experiments demonstrate that larvae of *M. sexta* and *M. quinquemaculata* can detect and behaviorally respond to naturally occurring concentrations of nicotine. Further, our work demonstrates that in the field, nicotine concentrations vary within leaves and plants, and that feeding site distributions of these hornworm species differ within but not among tobacco leaves. Nicotine concentration does not explicitly explain feeding site distributions of *M. sexta* and *M. quinquemaculata* in the absolute sense, in that each species does not feed preferentially on leaf tissue containing a particular concentration of nicotine; however, they may choose feeding sites on the basis of relative concentrations within a single leaf. Although this may be the proximate explanation for this behav-

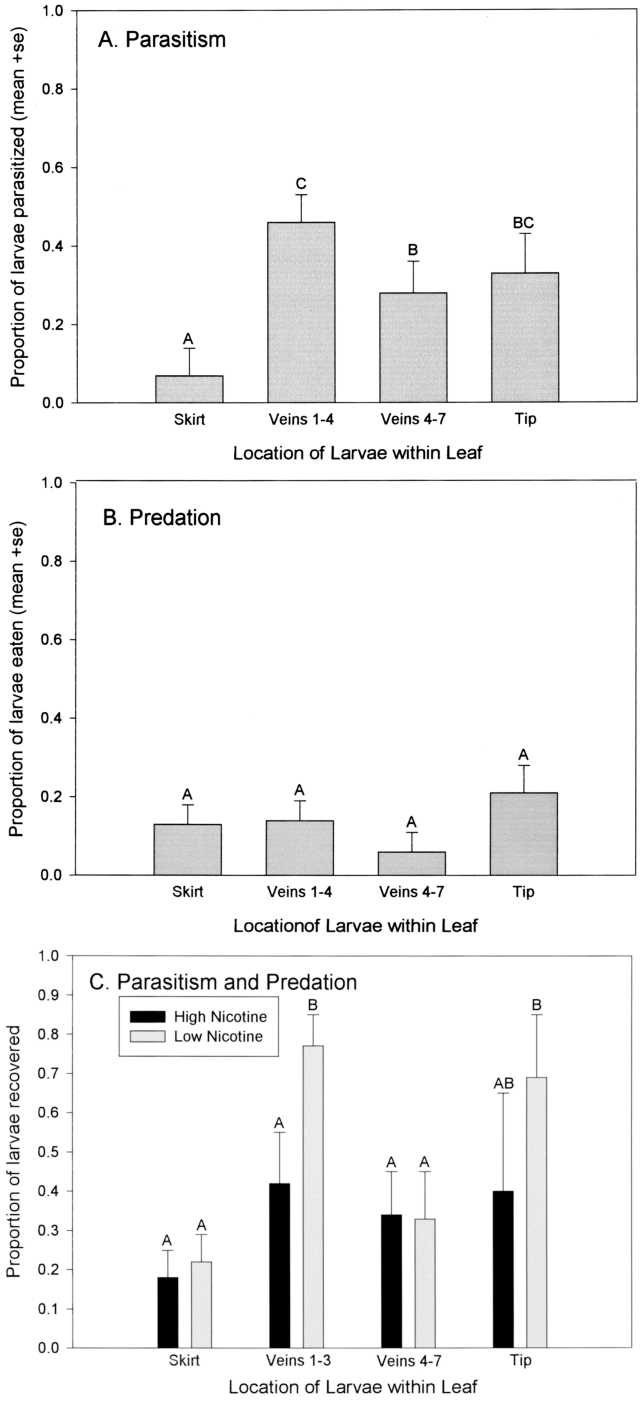

Fig. 7 Field mortality of *Manduca sexta* L. on high ('NC95') and low ('LAFC53') nicotine tobacco with respect to within-leaf feeding sites: A) Mortality due to parasitism differs significantly ($P = 0.0001$, $N = 73$), B) Mortality due to predation does not differ ($P > 0.05$), and C) Combined mortality due to parasitism and predation differs significantly ($P = 0.0001$, $N = 73$). Bars represent means of 10–18 replications for each treatment group (240 larvae released, 43–54 larvae per treatment group recovered); means with different letters are statistically different ($P \leq 0.03$)

ior, the ultimate explanation is probably cumulative selection pressure exerted by natural enemies, particularly the parasitoid, *C. congregata*.

Table 2 Results of analyses for testing the interactive effects of tobacco variety (Low Nicotine 'LAFC53' or High Nicotine 'NC95') and feeding site location (skirt, veins 1–3, veins 4–7, or vein 9 to tip) on parasitism, predation and combined predation and parasitism of *Manduca sexta* L. (PROC GLM, SAS Institute Inc., version 6, 1990)

Source	df	F Value	*P* Value
A. Parasitism by *Cotesia congregata* (Say)			
Tobacco genotype	1	1.21	0.2700
Feeding location	3	4.83	0.0001
Genotype × location	3	0.20	0.8900
Residual	65		
B. Non-specific predation			
Tobacco genotype	1	2.09	0.1500
Feeding location	3	1.05	0.3700
Genotype × location	3	1.09	0.3600
Residual	65		
C. Combined mortality due to parasitism by *C. congregata* and non-specific predation			
Tobacco genotype	1	4.07	0.0400
Feeding location	3	5.50	0.0001
Genotype × location	3	1.23	0.3000
Residual	65		

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References

Barbosa P, Saunders JA, Kemper J, Trumbule R, Olechno J, Martinat P (1986) Plant allelochemicals and insect parasitoids: Effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae). J Chem Ecol 12:1319–1328

Bernays EA (1989) Host range in phytophagous insects: the potential role of generalist predators. Evol Ecol 3:299–311

Chaplin JF, Burk LG (1984) Agronomic, chemical and smoke characteristics of flue-cured tobacco lines with different levels of tobacco alkaloids. Agron J 76:133–136

Cheng HH (1977) Flue-cured tobacco losses caused by the tomato hornworm, *Manduca quinquemaculata* (Lepidoptera: Sphingidae), at various infestation levels in Ontario. Canad Entomol 109:1091–1095

Darkis FR, Dixon LF, Wolf FA, Gross PM (1936) Correlation between composition and stalk position of tobaccos produced under varying weather conditions. Ind Eng Chem 28:1214–1223

DeBoer G, Hanson FE (1984) Foodplant selection and induction of feeding preference among host and non-host plants in larvae of the tobacco hornworm, *Manduca sexta*. Entomol Exp Appl 35:177–193

DeBoer G, Hanson FE (1987) Feeding responses to solanaceous allelochemicals by larvae of the tobacco hornworm, *Manduca sexta*. Entomol Exp Appl 45:123–131

Frazier JL (1986) The perception of plant allelochemicals that inhibit feeding. Pp 1–42 *in* Brattsten LB, Ahmad S (eds) Molecular Aspects of Insect-Plant Associations. New York: Plenum

Frazier JL, Hanson FE (1986) Electrophysiological recording and analysis of insect chemosensory responses. Pp 285–330 *in* Miller JR, Miller TA (eds) Insect-Plant Interactions. New York: Springer-Verlag

Fraenkel GS (1959) The *raison d'être* of secondary plant substances. Science 129:1466–1470

Glendinning JL (1996) Is chemosensory input essential for the rapid rejection of toxic foods? J Exper Biol 199:1523–1524

Guthrie FE, Campbell WV, Baron RL (1962) Feeding sites of the green peach aphid with respect to its adaptation to tobacco. Ann Ecol Soc Amer 55:42–46

Hodges RW (1971) The Moths of America North of Mexico, Fasc. 21 Sphingoidae. London: EW Classey Limited

Jeffrey RN (1958) Buildup and conversion of alkaloids within the tobacco leaf. Cigar Manuf Assn Research Session, Washington, DC

Jermy T, Hanson FE, Dethier VG (1968) Induction of specific food preference in lepidopterous larvae. Entomol Exp Appl 11:211–230

Kester KM, Barbosa P (1991) Behavioral and ecological constraints imposed by plants on insect parasitoids: Implications for biological control. Biol Control Theor Appl 1:94–106

Kester KM, Barbosa P (1994) Behavioral responses to host foodplants of two populations of the insect parasitoid, *Cotesia congregata* (Say). Oecologia 99:151–157

McFadden MW (1968) Observations on feeding and movement of tobacco hornworm larvae. J Econ Entomol 61:352–356

McKey D (1979) The distribution of secondary compounds within plants. Pp 56–133 *in* Rosenthal GA, Janzen D (eds) Herbivores: Their Interaction with Secondary Plant Chemicals. New York: Academic Press

Mehta CR, Patel NR (1983) A network algorithm for the exact treatment of Fisher's exact test in R × C contingency tables. J Amer Stat Assn 78:427–434

Morris C (1983) Uptake and metabolism of nicotine by the CNS of a nicotine-resistant insect, the tobacco hornworm (*Manduca sexta*). J Insect Physiol 29:807–817

Morris C (1984) Electrophysiological effects of cholinergic agents on the CNS of a nicotine-resistant insect, the tobacco hornworm (*Manduca sexta*). J Exper Zool 229:361–374

Peterson SC, Hanson FE, Warthen JD (1993) Deterrence coding by a larval *Manduca* chemosensory neurone mediating rejection of a non-host plant, *Canna generalis* L. Physiol Entomol 18:285–295

Rhoades DF (1979) Evolution of plant chemical defense against herbivores. Pp 3–54 *in* Rosenthal GA, Janzen DH (eds) Herbivores: Their Interaction with Secondary Plant Metabolites. New York: Academic Press

Rose RL, Gould F, Levi PE, Hodgson E (1991) Differences in cytochrome P-450 activities in tobacco budworm larvae as influenced by resistance to host plant allelochemicals and induction. Comp Biochem Physiol 99B:535–540

Saitoh F, Noma M, Kawashima N (1985) The alkaloid contents of sixty *Nicotiana* species. Phytochemistry 24:477–480

SAS Institute (1990) SAS/STAT User's Guide, Version 6, Cary: SAS Institute.

Self LS, Guthrie FE, Hodgson E (1964a) Metabolism of nicotine by tobacco-feeding insects. Nature 204:300–301

Self LS, Guthrie FE, Hodgson E (1964b) Adaptation of tobacco hornworms to the ingestion of nicotine. J Insect Physiol 10:907–914

Severson RF, McDuffie KL, Arrendale RF (1981) Rapid method for the analysis of tobacco nicotine alkaloids. J Chrom 211:111–121

Schoonhoven LM (1969) Gustation and foodplant selection in some lepidopteran larvae. Entomol Exp Appl 12:555–564

Schoonhoven LM (1987) What makes a caterpillar eat? The sensory code underlying feeding behavior. Pp 69–97 *in* Chapman RA, Bernays EA, Stoffolano JG (eds) Perspectives in Chemoreception and Behavior. New York: Springer-Verlag

Schoonhoven LM, Jermy T, van Loon JJA (1998) Insect-Plant Biology: Chapter 6. London: Chapman & Hall

Sisson VA, Saunders JA (1982) Alkaloid composition of the USDA tobacco (*Nicotiana tabacum* L.) introduction collection. Tob Sci 26:117–120

Snyder MJ, Hsu EL, Feyereisen R (1993) Induction of cytochrome P-450 activities by nicotine in the tobacco hornworm, *Manduca sexta*. J Chem Ecol 19:2903–2915

Sokal RR, Rohlf FJ (1981) Biometry. 2nd edn. New York: W.H. Freeman Pp 744–745

Stadler E, Hanson FE (1978) Food discrimination and induction of preference for artificial diets in the tobacco hornworm, *Manduca sexta*. Physiol Entomol 3:121–133

Thorpe K, Barbosa P (1986) Effects of consumption of high and low nicotine tobacco by *Manduca sexta* (Lepidoptera: Sphingidae) on the survival of the gregarious parasitoid, *Cotesia congregata* (Hymenoptera: Braconidae). J Chem Ecol 12:1329–1337

Thurston R, Fox PM (1972) Inhibition of emergence of *Apanteles congregatus* from its host, the tobacco hornworm. Ann Entomol Soc Amer 65:547–550

Yamamoto RT (1969) Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. J Econ Entomol 62:1427–1431

Yang RSH, Guthrie FE (1969) Physiological responses of insects to nicotine. Ann Entomol Soc Amer 62:141–146

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