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Altered Olfactory Receptor Neuron Responsiveness Is Correlated with a Shift in Behavioral Response in an Evolved Colony of the Cabbage Looper Moth, *Trichoplusia ni*

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Abstract There is little understanding of how sex pheromone blends might change during speciation events. For the cabbage looper, *Trichoplusia ni*, there is a mutant laboratory strain that has exhibited characteristics of a shift to a new pheromone blend. Mutant females produce a blend that is significantly different from wild-type females in having a much higher proportion of a minor pheromone component and lower quantity of the major component. Males in this colony have changed over the years to become more broadly tuned and fly upwind equally well to both the wild-type and mutant female pheromone blends. They also exhibit reduced overall sensitivity to pheromone, flying upwind to either blend at a lower success rate than is typical when wild-type males respond to the wild-type blend. Using single-cell recordings, we examined the olfactory receptor neurons (ORNs) of males from evolved and wild-type colonies for evidence of changes in response characteristics that might explain the above-described behavioral evolution. We found that in evolved-colony males the ORNs tuned to the major sex pheromone component exhibited a somewhat lower responsiveness to that compound than the ORNs of wild-type males. In addition, the minor pheromone component, emitted at excessively high rates by mutant females, elicited a drastically reduced ORN responsiveness in evolved-colony males compared to wild-type males. This alteration in ORN responsiveness may be responsible for allowing evolved males to tolerate the excessive amounts of the minor pheromone component in the mutant female blend, which would normally antagonize the upwind flight of unevolved males. Thus, peripheral olfactory alterations have occurred in *T. ni* males that are correlated with the evolution of the more broadly tuned, but less sensitive, behavioral response profile.

Keywords Lepidoptera · Electrophysiology · Pheromone · Evolution · Behavior · Olfaction · Antenna

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Introduction

The nature of pheromonal changes related to speciation has been speculated upon frequently (Phelan 1992; Baker 2002, 2008; Symonds and Elgar 2004; Symonds and Wertheim 2005). It has been particularly difficult to pinpoint what initial mutations to olfactory systems might occur that provide the raw material upon which selection could operate. According to the “asymmetric tracking” hypothesis (Phelan 1992), the nonlimiting sex (usually males) that experiences stronger selection will more strongly track changes that occur in the limiting sex (usually females). For systems in which females emit sex pheromones, this asymmetry manifests itself in high between-individual variation in emitted pheromone blend composition, while

males have high within-individual variation in behavioral response profiles to these blends that bracket the pheromone composition of most females in the population (Löfstedt 1990). Therefore, shifts in female pheromone blends might initially require that males in the population broaden their response profiles to include mutant female pheromone blends as well as the wild-type female blends.

Pheromone olfactory pathways logically can be inferred as playing a key role in allowing broad pheromone blend responsiveness of males in this first stage of the asymmetric tracking process. Indeed, olfactory receptor neuron (ORN) tuning profiles have been implicated in the broader responsiveness of "rare" males in *Ostrinia nubilalis* and *Ostrinia furnacalis* that fly upwind equally well to the quite different pheromone blends emitted by females of these two species (Roelofs et al. 2002; Linn et al. 2003, 2007; Domingue et al. 2007a, b).

A system that is particularly well suited for exploring the olfactory changes possibly involved in broader behavioral response related to speciation is the sex pheromone behavior of the cabbage looper moth, *Trichoplusia ni*. This species uses (*Z*)-7-dodecenyl acetate (Z7-12:OAc) as its major pheromone component along with five minor pheromone components (Bjostad et al. 1984; Linn et al. 1984). In 1990, a mutant female phenotype, caused by a single gene mutation, was reported to produce a 1:2 ratio of the minor pheromone component (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) to Z7-12:OAc compared to the normal wild-type proportion of 1:100 (Haynes and Hunt 1990). An "evolved" colony then was developed in which all females displayed this inherited mutant trait, which is controlled by a major autosomal gene (Haynes and Hunt 1990).

Initially, males from this mutant female colony were like wild-type males, in that they were more likely to fly upwind to and reach the source of the wild-type female pheromone blend than they were to the mutant female blend. Then, between generations 10 and 25, males from the evolved colony began to fly to the mutant female blend more frequently while retaining the same upwind flight success rate in response to the wild-type female blend. The success rate of upwind flight and source contact by males from the evolved colony in response to either the mutant or the wild-type blend became equal at generation 37, e.g., after approximately 3 years (Liu and Haynes 1994; Haynes 1997).

In an early attempt to see whether the ORNs of evolved-colony and wild-type males differed in their abundance or response profiles, recordings were performed on male sensilla from generations 14–17 (Todd et al. 1992). Most sensilla (type I) were found to house a larger-spike-size ORN responding primarily to Z7-12:OAc, with sometimes a lower-frequency response to (*Z*)-7-tetradecenyl acetate (Z7-14:OAc) and a smaller-spike-size ORN responding to (*Z*)-7-dodecen-1-ol (Z7-12:OH). There were also sensilla (type II) in which one ORN responded strongly to Z9-14:OAc. There was a rare sensillum type (type III) housing one ORN primarily tuned to another minor pheromone component, Z7-14:OAc. The degree to which behavioral evolution of the males had begun at this point is not clear, and physiologically tested males also were not behaviorally characterized. There were no obvious differences in the relative proportions of sensillum types in the two colonies. However, the firing frequencies on ORNs responsive to Z9-14:OAc appeared to be lower in evolved-colony vs. wild-type males. If the tendency to respond behaviorally to pheromone blends with greater proportions of Z9-14:OAc had begun at this point, a lower responsiveness to Z9-14:OAc could have facilitated such a response, assuming that the relative firing ratios of type I and type II ORNs influence behavior. In the ensuing 2 years, the evolved-colony males clearly began to exhibit the broad behavioral tuning consistent with the asymmetric tracking model (Phelan 1992). A behavioral tendency to fly successfully upwind to mutant- and wild-type female pheromone blends had become fixed in the population (Liu and Haynes 1994; Haynes 1997).

We checked the ORNs of these evolved-colony males vs. wild-type males in 1996 (Todd et al., previously unpublished) and again more thoroughly in 2007 in order to refine our knowledge about what, if any, changes in the peripheral receptor tuning profiles may have occurred that could explain the behavioral evolution. Recently, it was reported (Hemmann et al. 2008) that the more broadly behaviorally receptive males from the evolved colony also are less sensitive in response to either pheromone blend than wild-type males are to the wild-type blend. Thus, higher doses of either blend are required before evolved-colony males fly upwind. Here, we also examined whether changes in the responsiveness of the ORNs might have occurred that are consistent with this reduced behavioral sensitivity.

Methods and Materials

Electrophysiological sampling of wild-type and evolved-colony males has occurred on separate occasions at three historical points, in generations 14–17 (1990, see Todd et al. 1992), in generations 86–90 (1996), and finally in about the 220th generation (2007). At the different time points, experiments were performed by different analysts by using slightly different methodologies. The methodology used in 1990 has been described previously (Todd et al. 1992). It was not until the recent 2007 experiments that we developed and performed a highly replicated and well-controlled experimental protocol to compare the relevant characteristics of the ORNs responsive to Z9-14:OAc and

Z7-12:OAc in wild-type and evolved-colony males. Thus, we primarily describe the methodology for this most recent experiment, noting how the previous experiments differed.

Insects The wild-type and evolved colonies were originally derived from a colony collected in Riverside, CA, USA (Haynes and Hunt 1990). Both colonies were used in the 1990 physiology experiments. In 1996 and 2007, the same evolved colony was used, but the wild-type colony of *T. ni* derived from a colony maintained at the US Department of Agriculture-Agricultural Research Service Insect Attractants, Behavior, and Basic Biology Research Laboratory in Gainesville, FL, USA (Hemmann et al. 2008). Colonies were reared on a pinto-bean-based diet (Shorey and Hale 1965), at ambient room temperature, humidity, and light conditions. Male pupae of each colony were shipped from Kentucky to California (1990), Iowa (1996), or Pennsylvania (2007) for physiological analysis.

For the 2007 experiment, the source of the evolved-colony and wild-type males was withheld from the physiological analyst until the experiment was completed. Pupae and emerging adults were incubated at 25°C, in plastic containers with a Petri dish full of cotton saturated with sugar water at a 16:8 h L:D photoperiod. Different containers were used to separate males emerging within each day, such that we could ensure that all males sampled were between 2 and 7 days old when analyzed.

Single-Cell Electrophysiology ORN responses were recorded from trichoid sensilla by using the cut sensillum technique (Kaissling 1974; van der Pers and den Otter 1978). Individual antennae were excised from the head and placed in a saline-filled recording electrode including a silver wire. When possible, recordings were made from both antennae of an insect. Each antenna was positioned with a micromanipulator such that a single trichoid sensillum rested on the tip of a vertically positioned tungsten knife. A second horizontally oriented glass knife, controlled with another micromanipulator, was used to cut the sensillum tip. The cut sensillum then was contacted and surrounded by a saline-filled glass micropipette containing a silver recording electrode.

A stream of purified humidified air blew continuously over the antenna (10 ml/s) through a 25-cm-long glass tube (8-mm inner diameter (ID)), the end of which was placed 2 cm from the antenna. A 50-ms air pulse at a 40-ml/s flow rate was injected through the odor cartridge and into the airstream by using a stimulus flow-controller device (SFC-2, Syntech). Linear flow through the air-stream was ~0.3 m/s. The AC signal from the recording electrode passed through a built-in amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) and recorded with a computer. An external loudspeaker coupled with computer software (Syntech Autospike v.32; Syntech, Hilversum, The Netherlands) allowed visual and auditory monitoring of neural activity as it was recorded. In the 1996 and 2007 experiments, we observed little spontaneous background activity, typically less than one spike per second (Fig. 1). To measure spike frequency, Syntech software was used to count the number of spikes occurring within 300 ms of the first appearance of a spike. If there were less than two spikes within a second of a puff, the response was considered negative.

ORN Classes in 1990 and 1996 A random search protocol for sampling the sensilla was performed at the first two time points in 1990 and 1996. In 2007, a different analyst knew more about the spatial distribution of sensillum types, which

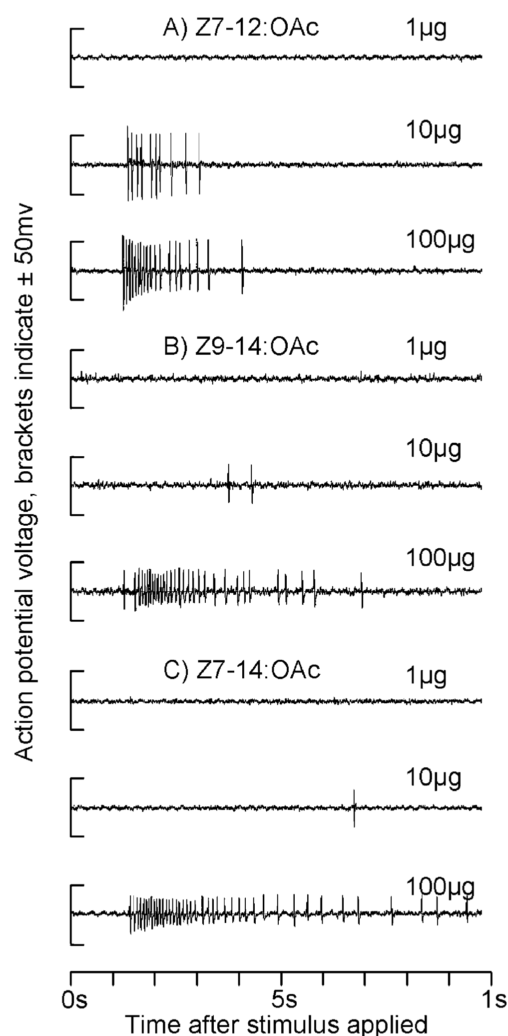

Fig. 1 Dose–response series (0.1-μg dose excluded) from ORNs housed within the three sensillum types commonly observed in *Trichoplusia ni*. The compounds inducing responses in these ORNs include Z7-12:OAc (*A*), Z9-14:OAc (*B*), and Z7-14:OAc (*C*), respectively. This series were obtained from different sensilla of the same male

precluded the same random sampling scheme (see Grant et al. 1998). The 1996 experiment followed the previously described protocol for the 1990 study (Todd et al. 1992). Compounds screened included Z7-12:OAc, Z7-12:OH, Z7-14:OAc, Z9-14:OAc, 12:OAc, 11–12:OAc, and Z5-12:OAc. Sensillum types were assigned based on responses to Z7-12:OAc and Z7-12:OH (type I), Z9-14:OAc (type II), and Z7-14:OAc (type III). The other minor pheromone components usually only weakly stimulate ORNs sensitive to these other compounds. In all colonies, there also have been <5% sensilla with ORNs broadly tuned to many compounds, which are not considered in our analyses. Thus, our current approach reduces the potential number of sensillum types compared to what was delineated in the 1990 study (Todd et al. 1992).

Comparisons of the relative proportion of sensilla belonging to each of the three sensillum types in the evolved and wild-type colonies in 1990 and 1996 were made by using logistic regression including *colony* (evolved vs. wild-type) and *year* (1990 vs. 1996) as factors that might explain the frequency of sensillum types observed. PROC LOGISTIC in SAS (version 9.3) was use to perform this analysis.

Dose–Response Relationships Dose–response relationships were investigated in 1996 and more thoroughly in 2007. In both cases, we made pheromone cartridges from dilutions of Z7-12:OAc, Z9-14:OAc, and Z7-14:OAc in high-performance liquid chromatography (HPLC)-grade hexane. For each concentration (0.01, 0.1, 1, or 10 µg/µl), 10 µl was pipetted onto a 0.5 × 2.0-cm^2 filter paper strip held in a 15-cm-long Pasteur pipette odor cartridge. The filter paper loadings thus were 0.1, 1, 10, or 100 µg, a range that includes the 10-µg dose used successfully previously with the cut-tip method (Todd et al. 1992). The same cartridges were used for all recordings over an approximately 1-month period within a given year.

After a single stimulation with one such cartridge, there was at least a 30-s delay before further testing. For a typical sensillum, we began by administering the three compounds at the lowest concentration in random order. We then continued at each successively higher concentration by using the same order of compounds. When it became clear which sensillum type we had encountered, we usually completed the dose–response curve for the relevant compound only. For verification of the sensillum type, the other two compounds were puffed at the highest concentration at the end of the experiment. This protocol was also important to prevent biases in the pretest exposure of these compounds to other sensilla because we usually sampled multiple sensilla per antenna.

A total of 25 type I sensilla and 12 type II sensilla were sampled from wild-type males in 1996. During this same period, 12 type I sensilla and four type II sensilla were sampled from evolved-colony males. In the 2007 experiment, sensilla sampled included 149 type I, 36 type II, and 62 type III from wild-type males. We also sampled 88 type I, 33 type II, and 11 type III sensilla from evolved-colony males in 2007.

Additional precautions were employed for the 2007 experiment. The larger sample in 2007 allowed a more thorough exploration of statistical differences between the ORN characteristics of ORNs tuned to Z7-12:OAc vs. Z9-14:OAc in the populations, which might be relevant to their behavioral differences. By using the pattern of the distribution of sensillum types described by Grant et al. (1998), sampling was focused on the medial portion of the antenna where sensilla housing ORNs responsive to Z7-12:OAc and Z9-14:OAc should be found with high frequency. At the beginning of the experiment, sensilla were sampled randomly from this region. Sensilla were encountered with ORNs responsive to Z9-14:OAc much less frequently than those for Z7-12:OAc, and, thus, antennae were sampled as many times as possible in order to ensure that Z9-14:OAc-responsive ORNs were encountered at least once per moth. As the experiment proceeded, the analyst developed the ability to visually distinguish the sensillum types. To maintain an unbiased approach, pairs of ORNs responsive to Z7-12:OAc or Z9-14:OAc were sampled per antennae in random order, before sampling sensilla likely to have ORNs responsive to Z7-14:OAc. Thus, throughout the experiment, we ensured that the order of sampling of sensilla with ORNs responsive to Z7-12:OAc vs. Z9-14:OAc remained random. Also, sampling from the two colonies, coded only by number, was regularly alternated as much as possible, with approximately equal proportions of the moths sampled from the two colonies over each of the 3 weeks of the experiment.

To analyze the data, spike frequency dose–response curves were calculated for mutant vs. wild-type males in the 1996 and 2007 populations. Further hypotheses were tested concerning the response characteristics of Z7-12:OAc and Z9-14:OAc in the 2007 experiment. First, the maximum spike frequencies obtained within each dose series were compared for the ORNs responsive to Z7-12:OAc and Z9-14:OAc in wild-type vs. evolved-colony males. Usually, the greatest response was elicited at 100 µg, but sometimes there was a slight decline at 100 µg after a stronger response at 10 µg. Data were log-transformed to ensure that the distributions were normal and of homogeneous variance. Analysis of variance (ANOVA) was performed, using *ORN type*, *colony*, and *ORN type×colony* as factors. The analysis was performed such that per individual rather than per sensillum differences were considered. Thus, the latter two factors were nested within the individual sampled from (*Moth*).

The other hypothesis of interest was whether there were any differences between the colonies with respect to the threshold for responses to Z9-14:OAc and Z7-12:OAc. For this purpose, the minimum concentration eliciting responses to Z9-14:OAc or Z7-12:OAc was noted for all the relevant ORNs. Possible shifts in the threshold response doses were assessed by logistic regression, assuming a cumulative logit model, using the SAS procedure GENMOD. Contrasts were evaluated by comparing the distributions of thresholds of wild-type to evolved-colony males for ORNs in type I or type II sensilla. The interaction effect between *colony* and *ORN type* also was evaluated. It also must be considered that the unbalanced sampling of varying numbers of sensilla across males might bias the results. Thus, rather than using all sensilla without considering such subsampling, we weighed each observation proportionally to the number of sensilla sampled for that moth.

Cartridge Emissions and ORN Spike Frequencies in Type I and Type II Sensilla Compounds emitted from odor cartridges were collected as they were issued from the pipette tip in 25-cm-long glass collection tubes (3-mm ID). The tip of an odor cartridge containing the filter paper strip dosed with one of the pheromone components was inserted into a collection tube, and the connection was sealed with Teflon tape. The collection tube was placed in a container (20 cm long × 3-cm ID) filled with dry ice. The odor cartridge was connected to the flow controller that generated 20-ms puffs of air with the flow set at 15 ml/s through the cartridge. Collection tubes were washed with 30 µl of HPLC-grade hexane containing (*Z*)-10-pentadecenyl acetate (50 pg/µl) as an internal standard. Collected amounts were analyzed by using gas chromatography–mass spectrometry (GC–MS) in selective ion mode. Collected amounts were calculated as mean picogram per puff and corrected for differences in relative abundance of the selected ions relative to the internal standard. Trap breakthrough was checked and confirmed negative for all odor cartridges at the highest dose level by analyzing collected material in a second, in-series-connected, glass collection tube.

Collection protocol was as follows: Z7-12:OAc was collected by using 512 pulses for each dose. Collection of Z9-14:OAc was performed with 4,096 pulses of each dose. Collections were analyzed by using a 30-m DB-1 capillary column. All collection tubes were stored at −20°C prior to analyses by GC–MS.

For the 1996 and 2007 ORN data, ORN firing frequencies were plotted in response to emitted amounts of Z7-12:OAc and Z9-14:OAc from the cartridges. We interpolated the Z7-12:OAc to Z9-14:OAc ORN relative firing ratios in response to emitted amounts of Z7-12:OAc and Z9-14:OAc that had been measured from mutant and wild-type females (Haynes and Hunt 1990) in order to relate the peripheral olfactory input experienced by evolved-colony and wild-type males to their upwind flight behavior when exposed to the evolved-colony and wild-type pheromone blends (Liu and Haynes 1994; Hemmann et al. 2008).

Results

Compared to males in the wild-type colony, the ORNs of evolved-colony males exhibited lower mean spike frequencies in response to Z9-14:OAc and Z7-12:OAc at all concentrations in both 1996 and 2007 (Figs. 2 (A, B) and 3 (A, B)). However, the reduction in spike frequency was much greater for the evolved-colony male ORN response to Z9-14:OAc vs. that to Z7-12:OAc. Lower responsiveness of the Z9-14:OAc ORN in evolved-colony vs. wild-type males had been observed in the 1990 populations, although dose–response curves were not collected and sample sizes were smaller than in 2007 (Todd et al. 1992). In contrast, the 2007 spike frequencies in wild-type vs. evolved-colony males responsive to Z7-14:OAc appeared similar at all odorant doses (Fig. 3 (C)).

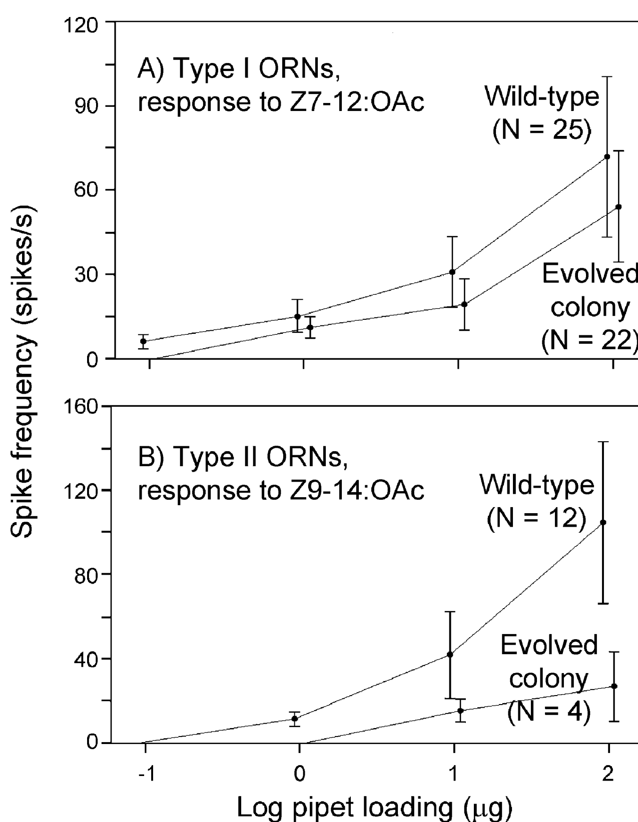

Fig. 2 Dose–response relationships for ORNs of type I (*A*) and type II (*B*) sensilla to their respective ligands Z7-12:OAc and Z9-14:OAc (mean spike frequency+SE). Means reflect pooled data from all individuals in which ORNs were sampled in 1996, between generations 86 and 90

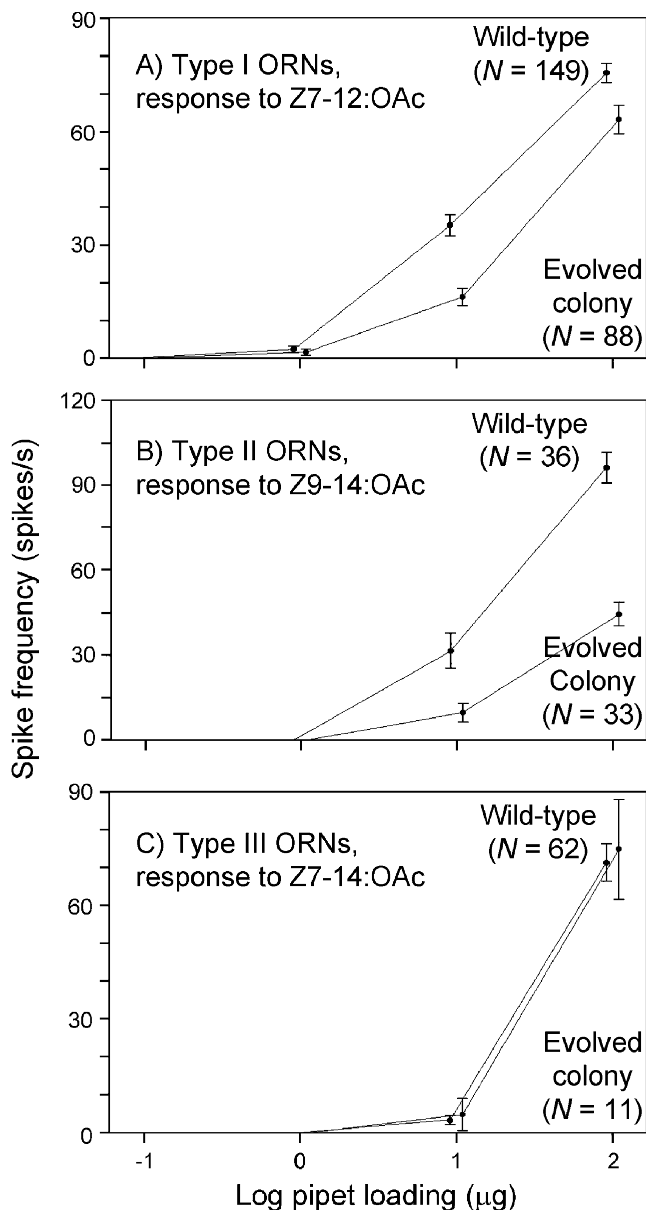

Fig. 3 Dose–response relationships for ORNs of type I (*A*), type II (*B*), and type III (*C*) sensilla to their respective ligands Z7-12:OAc, Z9-14:OAc, and Z7-14:OAc (mean spike frequency+SE). Means reflect pooled data from all individuals in which ORNs were sampled in 2007 (about 220 generations after the colony was initiated)

There were relatively fewer type II vs. type I sensilla located in the random search for evolved-colony vs. wild-type males as indicated by the corresponding significant regression coefficient (Tables 1 and 2). There was no similarly statistically significant relationship between the frequencies of type III vs. type I sensilla with respect to the *colony* effect (Table 2). However, it is also relevant to note that in 2007 we became able to locate any of these three sensillum types based on their consistent location and morphology. After achieving this capability, we could not detect any differences in the frequencies of these sensillum types. In the regression model presented in Table 2, only the *colony* effect is shown. Models also were considered where *year* and *colony*×*year* interactions were considered. In a full model that used all three factors, the model did not converge when the iterative method for obtaining maximum likelihood estimates of the parameters was performed. Models were also considered where either *year* or *colony-by-year* interactions were removed, which in both cases did not exhibit such a lack of convergence. Regardless of the model selected, *colony* was the only effect that was ever significant. Thus, we present the simplest model containing only this effect.

The pattern observed for differences in ORN response characteristics between wild-type and evolved-colony males in the dose–response curves is further reinforced by statistical analysis of the maximum spike frequencies for the Z9-14:OAc- and Z7-12:OAc-responsive neurons in wild-type and evolved-colony males from 2007. The average maximum spike frequency in response to Z9-14:OAc was greater than that for Z7-12:OAc in wild-type males (Fig. 4). This measurement was lower for both compounds in evolved-colony males. However, the reduced maximum spike frequency in evolved-colony males was much greater for responses to Z9-14:OAc than for those to Z7-12:OAc, thus resulting in lower response levels to Z9-14:OAc. The interaction effect indicative of this observation is statistically significant (Table 3).

There were significant differences in the distribution of observed threshold of responses for the evolved-colony and wild-type populations of ORNs for both type I and type II sensilla (Table 4). In both cases, more ORNs have a higher threshold of response to Z7-12:OAc or Z9-14:OAc in evolved-colony male vs. wild-type male sensilla. There was

Table 1 Proportion of sensilla of each ORN type

Sensillum type	Wild-type colony		Evolved colony	
	1990	1996	1990	1996
I (Z7-12:OAc)	77/112 (69%)	58/80 (72%)	83/96 (87%)	51/55 (95%)
II (Z9-14:OAc)	26/112 (23%)	12/80 (15%)	10/96 (10%)	4/55 (7%)
III (Z7-14:OAc)	3/112 (3%)	6/80 (7%)	3/96 (3%)	0/55 (0%)

The colonies were sampled in 1990 (gens. 14–17) AND 1996 (gens. 86–90)

Table 2 Results of logistic regression involving the proportion of sensilla of each ORN type, as described in Table 1

Test[a]	*Wald* X^2	*df*	*P*
Colony	10.7	2	0.005
Colony effect on type I vs. type II frequency	8.72	1	0.003
Colony effect on type I vs. type III frequency	2.59	1	0.107

[a] Test statistics and hypothesis tests are for maximum-likelihood-derived estimates of regression coefficients

no significant interaction effect between population and sensillum type in this analysis.

The amounts emitted per puff from the odor cartridges for a given filter paper loading conformed to previous measurements involving pheromone components that differed in chain length by two carbons (Cossé et al. 1998). The amount per puff of Z9-14:OAc emitted was approximately ten times lower than that of Z7-12:OAc at 1-, 10-, and 100-µg loading, respectively (Fig. 5).

Emitted amounts from cartridges were used not only for calculating ORN response thresholds (Table 4) but also for comparing the Z7-12:OAc to Z9-14:OAc ORN spike frequency ratios of evolved-colony compared to wild-type males in response to the mutant- and wild-type-female-emitted blends (Fig. 6). Amounts of Z7-12:OAc and Z9-14:OAc emitted by wild-type and mutant females (Hunt and Haynes 1990) fell between the emitted cartridge amounts for the 1996 and 2007 ORN data, as shown in Fig. 6 for our 2007 recordings only.

In both 1996 and 2007, wild-type males that, as a group, exhibited good upwind flight in response to their wild-type female pheromone blend (Liu and Haynes 1994; Hemmann et al. 2008) had a slightly greater than 1:1 Z7-12:OAc to Z9-14:OAc ORN firing ratio in response to this blend (Table 5). Similarly, evolved-colony males that had evolved the ability to fly upwind successfully in response to either the mutant or wild-type female blends exhibited ratios of firing of Z7-12:OAc to Z9-14:OAc ORNs that were approximately 1:1 or 3:1, respectively (Table 5). In contrast, in both years, wild-type males challenged to fly upwind in response to the mutant female blend exhibited poor upwind flight (Liu and Haynes 1994; Hemmann et al. 2008). It is of interest that the ORN firing ratio of this group of males in response to the mutant blend was the only one in which the Z7-12:OAc-tuned ORN's contribution to the ratio was only one half that of the Z9-14:OAc-tuned ORN (a 1:2 firing ratio; Table 5).

Discussion

Three aspects of ORNs responsive to Z9-14:OAc and Z7-12:OAc on male *T. ni* antennae were examined that could be relevant to the historical broadening of the behavioral response of evolved-colony males. This response now includes upwind flight to both the unusual pheromone blend produced by mutant females as well as the blend emitted by wild-type females (Haynes and Hunt 1990; Liu and Haynes 1994). These aspects include: (1) the relative abundance of each sensillum type; (2) the response threshold of each ORN within each type of sensillum; and (3) the spike frequencies exhibited by these ORNs in response to a given stimulus. Of these factors, all showed some statistically significant differences between evolved-colony and wild-type populations.

The most obvious difference we observed was the severe reduction in firing frequency of evolved-colony male exhibited by ORNs tuned to Z9-14:OAc (Figs. 2, 3, and 4, Table 3). Evolved-colony males also exhibited significantly reduced sensitivity and firing frequency of the ORN tuned to Z7-12:OAc compared to this type of ORN in wild-type males. However, there was a much greater reduction in responsiveness of the Z9-14:OAc-tuned ORN, underscored by the statistically significant interaction comparing alterations to ORN responsiveness in type I (Z7-12:OAc) vs. type II (Z9-14:OAc) sensilla in evolved-colony vs. wild-type males in 2007 (Fig. 4, Table 3). While the studies of evolved-colony and wild-type males in 1990 (Todd et al. 1992) and 1996 were not replicated and controlled well enough to demonstrate a similar statistically significant

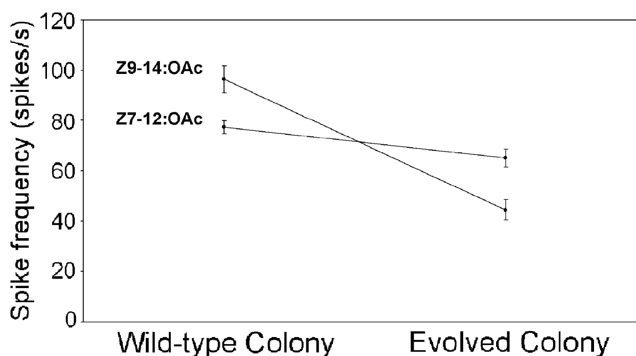

Fig. 4 Average of the maximum ORN response within each dosage series (mean spike frequency+SE) to Z7-12:OAc and Z9-14:OAc in the wild-type and mutant colonies. Means are weighted using per individual variation

Table 3 Analysis of variance testing the effect of the ORN sampled from and colony source as factors in the maximum spike amplitude within each dose–response series

Source	*df*	Sum of squares	Mean square	*F*	*P*
ORN type[a]	1	21.65	21.65	0.29	0.589
Error	248	18,360	74.05		
Colony[b]	1	4,608	4,608	32.51	<0.001
ORN type×colony[b]	1	904.6	904.6	6.38	0.014
Moth (colony)	53	7,511	141.7		

[a] The per sensillum error is used for hypothesis testing

[b] The latter two factors were nested within the individual sampled from (*Moth*), the error which is used for hypothesis testing for these two factors

effect, the trend also can be observed in the 1996 data (Fig. 2) and in 1990 (Todd et al. 1992). In the 1990 study, the ORN spike frequency in type I sensilla screened for response to Z7-12:OAc was lower in evolved-colony than in wild-type males by about 25% (134.6/s vs. 98.8/s). At the same time, a screening of type II ORN response to Z9-14:OAc showed a spike frequency in mutant male ORNs that was about 50% lower in evolved-colony than in wild-type males (142.3/s vs. 72.7/s).

It appears that there has been the evolution of a highly significant dampening of ORN responsiveness to Z9-14:OAc in males from the mutant colony. Thus, a behavioral response to a greater proportion of Z9-14:OAc in the female blend of mutant females is correlated with a diminished physiological response to that same component by the males. This alteration seems to have helped place the ratio of Z7-12:OAc to Z9-14:OAc peripheral inputs within a range acceptable to integrative centers in the brain. Therefore, a simple selective attenuation of a peripheral response to one component may explain the behavioral response.

However, a reduction in sensitivity of the Z9-14:OAc ORN pathway cannot also explain how evolved-colony males have simultaneously retained their ability to fly successfully upwind in response to the wild-type pheromone blend. The Z7-12:OAc to Z9-14:OAc spike frequency ratios in wild-type ORNs exposed to the wild-type female blend range from 1.13:1 to 1.41:1, whereas the ratios of evolved-colony male ORNs flying upwind to wild-type or mutant blends were somewhat lower or higher than this range, respectively. In evolved-colony males exposed to the wild-type blend, the ratio of spiking activity would be more heavily skewed (3:1) toward the Z7-12:OAc ORN than in wild-type males exposed to this same blend. Thus, ORN spike frequency ratios, which are somewhat higher (3:1 for evolved-colony male ORNs to the wild-type blend) or lower (0.83:1 or 1.05:1 for evolved-colony male ORNs to the mutant blend) than the ratio range shown above for the wild-type male ORNs to the wild-type blend, are at least adequate for upwind flight. The poorest upwind flight and source contact was exhibited by wild-type males in response to the mutant blend, and this ratio was low (1:2) and dominated by the activity of the Z9-14:OAc ORN, apparently making it out of the normally acceptable range to promote flight.

It is difficult to reconstruct precisely the sequence of olfactory changes that occurred at the inception of the evolved colony along with the evolution of behavioral responsiveness to both the mutant and wild-type pheromone blends. Many critical tests that would have been

Table 4 Threshold of responses in type I and type II sensilla from 2007

	Weighted threshold frequencies[a]					
	Z7-12:OAc (type I)			Z9-14:OAc (type II)		
Pipette load (µg)	Emission (ng)	Wild	Evolved	Emission (ng)	Wild	Evolved
1	0.0039	3.37	1.39	0.0003	0	0
10	0.54	11.4	17.9	0.016	11.2	7.50
100	8.4	8.20	27.7	0.86	5.83	15.5
Within sensillum[b]		X^2 = 4.64	P < 0.031		X^2 = 4.39	P = 0.036
Sensillum type by colony interaction		X^2 = 0.10	P = 0.753			

[a] Frequencies are weighted proportionally to the number of sensilla sampled per moth

[b] Results from logistic regression involving type I and type II sensillum are included (see text for details)

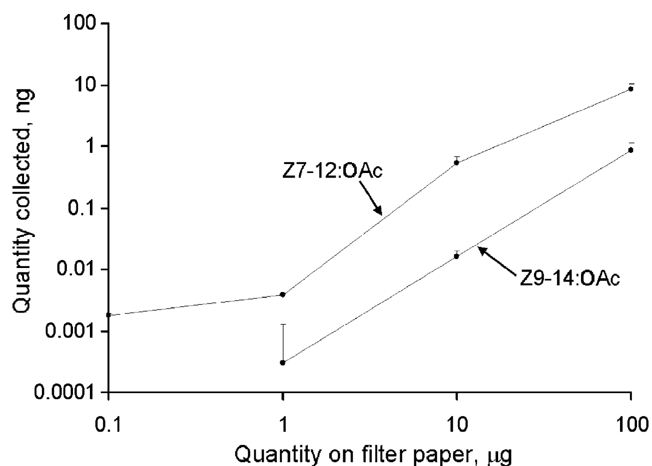

Fig. 5 Relationship between the dosage applied to filter papers in the odor cartridges and the amounts emitted from the cartridges per single puff (mean+SE). Sample sizes vary for each concentration ($N = 2$ for Z7-12:OAc at 1 µg, $N = 3$ for Z7-12:OAc at 0.1 µg, and Z9-14:OAc at 1 µg and 10 µg, $N = 4$ for Z7-12:OAc at 10 µg and 100 µg, and Z9-14:OAc at 100 µg)

useful in the earliest generations did not become apparent until later on. Despite such inherent difficulties, a reasonable historical scenario is beginning to emerge.

At the initiation of the evolved colony line, male ORNs from the first few generations were not sampled to provide a baseline reading of ORN firing frequency ratios. However, because males were randomly selected from the wild-type population to mate with mutant females to initiate the mutant colony, it is reasonable to assume that the ORN firing ratios in response to different pheromone blends did not differ initially between the two colonies, and this is consistent with behavioral observation at the time. A new pattern of behavioral responsiveness to the mutant female blend by males began to evolve between 10 and 25 generations after the colony was founded (Liu and Haynes 1994). Males from this colony have exhibited a shift from an approximately 1:2 Z7-12:OAc to Z9-14:OAc ORN firing ratio in response to the mutant pheromone blend to a 1:1 ratio in response to this mutant blend. Concomitantly, a ratio of nearly 3:1 of Z7-12:OAc to Z9-14:OAc ORN firing in response to the wild-type blend was established as a result of this shift in ORN responsiveness. This ratio likewise is coincident with the retention of adequate levels of upwind flight by evolved-colony males to the wild-type female blend. Although we can provide only strong statistically confirmation of this physiological alteration from more recently employed experiments, it is likely to have occurred by 1990 (generations 14–17), when limited sampling pointed to this trend (Todd et al. 1992).

We caution that it is not clear if the physiological alteration is a direct cause of the behavioral evolution of the mutant colony population or is one in addition to other potentially important factors. These physiological assays of earlier generations had sample-size limitations and were not experimentally linked to behavioral observations, while neither trait was likely to be fixed in the population. Further experimental inquiries into the degree of genetic linkage between the mutant behavior, the ORN firing ratio alteration observed here, and perhaps yet unknown factors are still possible and could contribute to understanding how this novel behavior evolved. It also would be interesting to investigate the potential linkage of the female pheromone and male ORN and behavioral response traits. With these caveats, the current focus on peripheral ORN response shifts do not preclude the role of olfactory alterations such as changes in antennal lobe interneuron response profiles or the central nervous system olfactory integration of the incoming ORN activity.

Nevertheless, profound shifts in pheromone behavioral response profiles have been shown to have occurred in other species such as the E and Z strains of *O. nubilalis* due to alterations of the ORNs alone (Kárpáti et al. 2008). In that case, behavioral blend preferences are correlated with shifts in the primary afferents' tuning profiles, with

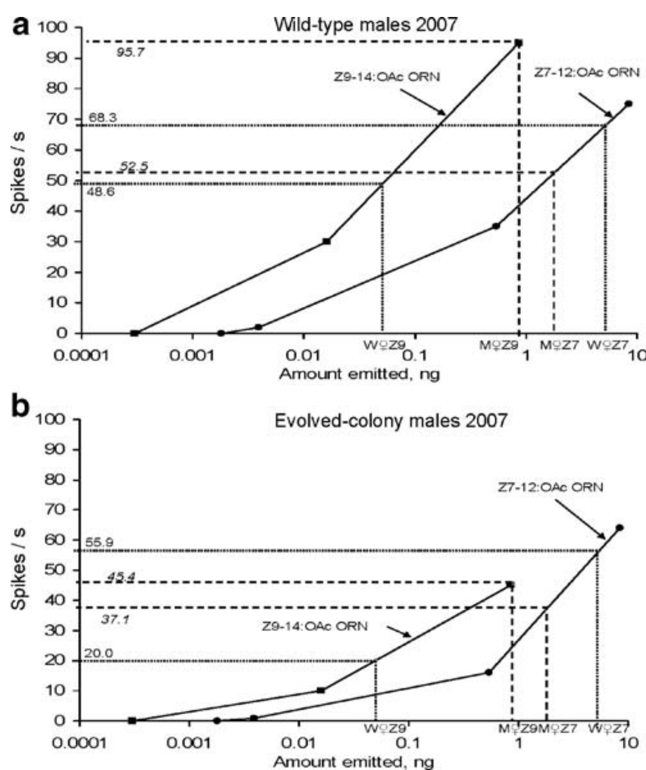

Fig. 6 Dose–response curves for the Z7-12:OAc- and Z9-14:OAc-tuned ORNs in **a** wild-type and **b** evolved-colony males using the amounts emitted from the odor cartridges. Interpolations were made to construct the firing frequencies of the ORNs in response to the amounts of Z7-12:OAc and Z9-14:OAc emitted by wild-type and mutant females in their blends (Haynes and Hunt 1990). The interpolated ratios of Z7-12:OAc/Z9-14:OAc ORNs are shown in Table 5. *Dashed line* shows mutant female emission rate. *Dotted line* shows wild-type emission rate

Table 5 Z7-12:OAc/Z9-14:OAc ORN spike frequency ratios and flight performance

2007[a]			
Wild-type males			
Wild-type female blend	69:49 spikes per second	(1.41:1)	Good flight
Mutant female blend	53:96 spikes per second	(0.55:1)	Poor flight
Evolved-colony males			
Wild-type female blend	56:20 spikes per second	(2.80:1)	Adequate flight
Mutant female blend	38:46 spikes per second	(0.83:1)	Adequate flight
1996			
Wild-type males			
Wild-type female blend	72:64 spikes per second	(1.13:1)	Good flight
Mutant female blend	54:109 spikes per second	(0.49:1)	Poor flight
Evolved-colony males			
Wild-type female blend	56:20 spikes per second	(2.80:1)	Adequate flight
Mutant female blend	39:37 spikes per second	(1.05:1)	Adequate flight

[a] See Fig. 6 for derivation of ratios

no changes in glomerular wiring (Kárpáti et al. 2008). Furthermore, the ORN alteration documented here for *T. ni* is similar in many respects with others observed that involve unusual behavioral variants of the corn borer species *O. nubilalis* and *O. furnacalis* that included broadened behavioral receptivity, characterized by upwind flight to conspecific and heterospecific pheromone blends. These rare behavioral phenotypes had correlated alterations to their ORNs, which were likely to impact relative ORN firing rates (Domingue et al. 2007a, b), as we observed here for *T. ni*. Together, the results of such studies support the generality of the "balanced olfactory antagonism" (Baker 2008) model for insect olfactory systems. In this model, there is said to be a set point for a behaviorally optimal balanced ratio of neuronal activity along mutually antagonistic odorant-specific olfactory pathways. The model predicts that a previously narrow range of pheromone component blend ratios can be expanded to include a wider range after alterations in olfactory activity occur, often at the ORN level. Differential changes in the sensitivities of differently tuned ORN classes may allow previously unacceptable pheromone blend ratios to evoke ratios of firing that fall within the acceptable balanced range (Baker 2008).

In both the *T. ni* and *Ostrinia* study systems, reductions rather than increases in ORN responsiveness appear to be the critical factors that promote flight to rare or mutant pheromone blends in laboratory populations. More specifically, these significant reductions in responsiveness tend to occur on ORNs associated with the minor pheromone components as described here for *T. ni* (also, *O. nubilalis* Domingue et al. 2007b) or with heterospecific behavioral antagonists (*O. furnacalis*, Domingue et al. 2007a). After such alterations have occurred, compounds that were previously antagonistic at low proportions in the pheromone blend can become acceptable and even promote flight at higher ratios.

The molecular basis for the observed reductions in ORN responsiveness in the rare *T. ni* males is speculative given the current level of knowledge of the functionality and expression of odorant receptors (ORs) and other factors influencing signal transduction in this species. Possible explanations might include, but are not limited to, changes to the conformations of ORs or reductions in the numbers of normal ORs expressed on the dendrites. Interestingly, if it were not for the severe decline in responsiveness of the Z9-14:OAc-tuned ORNs, the evolved males' slight reduction in responsiveness of the Z7-12:OAc-tuned ORNs alone may have actually created a less optimal ORN firing ratio for upwind flight to the mutant blend.

As described above, evolved-colony *T. ni* males are equally likely to fly upwind to mutant or wild-type blends, but respond positively to the wild-type blend less often than wild-type males do (Hemmann et al. 2008). It has been previously determined that this particular shift in behavioral response patterns can cause assortative mating between members of the two strains under certain rearing conditions (Zhu et al. 1997). It is thus possible that the physiological alteration described here represents a neuroethological cause of reproductive isolation, which involves a tradeoff between the breadth and sensitivity of the pheromone response.

Formidable challenges remain for explaining how the final stages of a speciation event could occur after the initial stages of reproductive isolation evolve because of an olfactory alteration of the particular types uncovered in the laboratories. How could the novel broadly tuned pheromone response system characteristic of the intermediate behavioral state further evolve into another highly sensitive and narrowly tuned system typical of most closely related species or races found in nature (Kárpáti et al. 2008), where reproductive isolation from the parent population is strong? Perhaps certain saltational events, such as radical changes in OR conformation, the

substitution of different ORs on dendrites, or reorganization of aspects of the central nervous system will be more likely to be adaptive when most males of a population are already in a broadly tuned state similar to that currently in existence for the evolved *T. ni* colony. After such a saltational event, it may become possible for selective pressures again to allow increases in the specificity and sensitivity of the pheromone system in a manner that continues to facilitate reproductive isolation from the parent population.

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References

Baker, T. C. 2002. Mechanism for saltational shifts in pheromone communication systems. *Proc. National Acad. Sci. U. S. A.* 99:13368–13370.

Baker, T. C. 2008. Balanced olfactory antagonism as a concept for understanding evolutionary shifts in moth sex pheromone blends. *J. Chem. Ecol.* 34:971–981.

Bjostad, L. B., Linn, C. E., Du, J. -W., and Roelofs, W. L. 1984. Identification of new sex pheromone components in *Trichoplusia ni*, predicted from biosynthetic precursors. *J. Chem. Ecol.* 10:1309-1323.

Cossé, A. A., Todd, J. L., and Baker, T. C. 1998. Neurons discovered on male *Helicoverpa zea* antennae that correlate with pheromone-mediated attraction and interspecific antagonism. *J. Comp. Physiol. A* 182:585–594.

Domingue, M. J., Musto, C. J., Linn Jr., C. E., Roelofs, W. L., and Baker, T. C. 2007a. Evidence of olfactory antagonistic imposition as a facilitator of evolutionary shifts in pheromone blend usage in *Ostrinia spp.* (Lepidoptera: Crambidae). *J. Insect Physiol.* 53:488–496.

Domingue, M. J., Musto, C. J., Linn Jr., C. E., Roelofs, W. L., and Baker, T. C.. 2007b. Altered olfactory receptor neuron responsiveness in rare *Ostrinia nubilalis* males attracted to the *O. furnacalis* pheromone blend. *J. Insect Physiol.* 53: 1063–1071.

Grant, A. J., Riendeau, C. J., and O'connell, R. J. 1998. Spatial organization of olfactory receptor neurons on the antenna of the cabbage looper moth. *J. Comp. Physiol. A* 183:433–442.

Haynes, K. F. 1997. Genetics of pheromone communication in the cabbage looper moth, *Trichoplusia ni*, pp. 525–534, in R. T. Cardé and A. K. Minks (eds.). Insect Pheromone Research: New Directions. Chapman and Hall, New York.

Haynes, K. F. and Hunt, R. E. 1990. A mutation in pheromonal communication system of the cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:1249–1257.

Hemmann, D. J., Allison, J. D., and Haynes, K. F. 2008. Trade-off between sensitivity and specificity in the cabbage looper moth response to sex pheromone. *J. Chem. Ecol.* 34:1476–1486.

Hunt, R. E., and Haynes, K. F. 1990. Periodicity in the quantity and blend ratios of pheromone components in glands and volatile emissions of mutant and normal cabbage looper moths, *Trichoplusia ni*. *J. Insect Physiol.* 36:769–774.

Kaissling, K. -E. 1974. Sensory transduction in insect olfactory receptors, pp. 243–273, in L. Jaenicke (ed.). Biochemistry of Sensory Functions. Springer, Berlin.

Kárpáti, Z., Dekker, T., and Hansson, B. S. 2008. Reversed functional topology in the antennal lobe of the male European corn borer. *J. Exp. Biol*. 211:2841–2848.

Linn, C. E. Jr., Bjostad, L. B., Du, J. W., and Roelofs, W. L. 1984. Redundancy in a chemical signal: behavioral responses of male *Trichoplusia ni* to a 6-component sex pheromone blend. *J. Chem. Ecol.* 10:1635–1658.

Linn, C. E. Jr., O'connor, M., and Roelofs, W. L. 2003. Silent genes and rare males: a fresh look at pheromone response specificity in the European corn borer moth, *Ostrinia nubilalis*. *J. Insect Sci*. 3:15 www.insectscience.org/3.15/

Linn, C. E. Jr., Musto, C. J., and Roelofs, W. L. 2007. More rare males in *Ostrinia*: response of Asian corn borer moths to the sex pheromone of the European corn borer. *J. Chem. Ecol.* 33:199–212.

Liu, Y. -B. and Haynes, K. F. 1994. Evolution of behavioral response to sex pheromone in mutant laboratory colonies of *Trichoplusia ni*. *J. Chem. Ecol.* 19:503–515.

Löfstedt, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

Phelan, P. L. 1992. Evolution of sex pheromones and the role of asymmetric tracking, pp. 265–314, in B. D. Roitberg and M. B. Isman (eds.). Insect Chemical Ecology: An Evolutionary Approach. Chapman and Hall, New York.

Roelofs, W. L., Liu, W. T., Hao, G. X., Jiao, H. M., Rooney, A. P., and Linn, C. E. 2002. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl. Acad. Sci. U. S. A.* 99:13621–13626.

Shorey, H. H. and Hhale, R. L. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

Symonds, M. R. E. and Elgar, M. A. 2004. The mode of pheromone evolution: evidence from bark beetles. *Proc. R. Soc. Lond. Biol.* 271:839–846.

Symonds, M. R. E. and Wertheim, B. 2005. The mode of evolution of aggregation pheromones in *Drosophila* species. *J. Evol. Biol.* 18:1253–1263.

Todd, J. L., Haynes K. F., and Baker, T. C. 1992. Antennal neurons specific for redundant pheromone components in normal and mutant *Trichoplusia ni* males. *Physiol. Entomol.* 17:183–192.

Van Der Pers, J. N. C. and Den Otter, C. J. 1978. Single cell responses from olfactory receptors of small ermine moths to sex-attractants. *J. Insect Physiol.* 24:337–343.

Zhu, J., Chastain, B. C., Spohn, B. G., and Haynes, K. F. 1997. Assortative mating in two pheromone strains of the cabbage looper moth, *Trichoplusia ni*. *J. Insect Behav.* 10:805–808.