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Evidence of olfactory antagonistic imposition as a facilitator of evolutionary shifts in pheromone blend usage in *Ostrinia* spp. (Lepidoptera: Crambidae)

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Abstract

Olfactory receptor neuron (ORN) response was measured to assess why some males (''rare males'') of the Asian corn borer (ACB), *Ostrinia furnacalis*, have a broad behavioral response to fly upwind to both the ACB and the European corn borer (ECB), *Ostrinia nubilalis*, pheromone blends. We performed single-sensillum electrophysiological recordings on ACB males that had been behaviorally assessed for upwind flight response to the ACB blend [60:40 (*Z*)-12-tetradecenyl acetate (Z12-14:OAc) to (*E*)-12-tetradecenyl acetate (E12-14:OAc)], as well as to ECB (Z-strain) and ECB (E-strain) blends [3:97 and 99:1 (*Z*)-11-tetradecenyl acetate (Z11-14:OAc) to (*E*)-11-tetradecenyl acetate (E11-14:OAc)]. Sensilla from all types of males had large- and small-spike-sized ORNs responding strongly to Z12- or E12-14:OAc, but weakly to Z11- and E11-14:OAc. In the majority of males (''normal males'') that flew upwind only to the ACB blend, Z11-14:OAc elicited responses in an intermediate spike-sized ORN associated with behavioral antagonism that is mainly tuned to (*Z*)-9-tetradecenyl acetate (Z9-14:OAc). In the rare-type ACB males that flew to both the ACB and ECB pheromone blends, Z11-14:OAc did not stimulate this ORN. Increased responsiveness to ancestral pheromone components by ORNs associated with behavioral antagonism could be instrumental in reproductive character displacement, or in reinforcement and reproductive isolation during speciation by helping to increase assortative mating between males and females in derived populations that use novel sex pheromone blends.

Keywords: Olfactory receptor neurons; *Ostrinia nubilalis*; *Ostrinia furnacalis*; Asymmetric tracking; Pheromone shift; Behavioral antagonism

1. Introduction

Pheromone specificity can be a powerful means of reproductive isolation, being associated with species specificity in mating and/or aggregation behavior across a wide array of taxa (Roelofs and Comeau, 1969; Lanier and Wood, 1975; Costa et al., 1997; Kotani et al., 2001; Lemaster and Mason, 2003). Historically, it had often been assumed that pheromone systems involve strong stabilizing selection on emitters and receivers (Cardé and Baker, 1984). Theoretically, such stabilizing pressure would only allow small, incremental changes in pheromone systems (Paterson, 1980). However, investigations have revealed that there are large within- and between-species shifts in pheromone-related traits that tend to involve a simple genetic basis (Klun and Maini, 1979; Hansson et al., 1987; Roelofs et al., 1987; Löfstedt, 1990; LaForest et al., 1997; Roelofs et al., 2002; Domingue et al., 2006).

It was later proposed that many of these patterns might be explained better in the context of differential selection on the pheromone-emitting and receiving sexes with respect to their parental investment (Phelan, 1992, 1997). This model, called asymmetric tracking (Phelan, 1992, 1997),

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predicts that the non-limiting sex (usually males) experiences stronger selection and would more strongly track changes that occur in the limiting sex (usually females), regardless of which sex is the emitter. For female pheromone emission systems, this asymmetry allows high between-individual variation in the emitted pheromone blend quality and quantity (Löfstedt, 1990, 1993). At the same time, male responses tend to possess little heritable variation (individual variation), and all males stereotypically broadly bracket the emission ratios emitted by all the females in the population. Shifts in pheromone blends, therefore, are predicted to be initiated by males in the population that have broadened response profiles to include mutant or rare female pheromone blends as well as the normal female blends, rather than by immediately changing specificity to a new blend. The plausibility of this process has been confirmed for the cabbage looper moth, *Trichoplusia ni* (Liu and Haynes, 1994). When confronted with a mutant phenotype consisting of highly skewed, disparate ratios of female-emitted pheromone components, normal males in the population, over the course of 40 generations, succeeded in broadening their behavioral response profiles to include both the wild-type and mutant-type female blends (Liu and Haynes, 1994).

It is inherently difficult to find situations in which one can observe and characterize the genetic and olfactory changes that might be involved in dramatic shifts in pheromone blends implied by the diverse communication systems in taxa such as Lepidoptera (Baker, 2002). Additionally, most of the study systems described above that support the asymmetric tracking model involve intraspecific pheromone races where divergence has already occurred. In such cases, it will always remain uncertain whether genetic properties of the population are the cause or result of speciation processes. For example, where major genetic loci have been implicated in controlling pheromone blends between pheromone races, there is also the presence of a second level of interacting genetic variation (Zhu et al., 1996; Domingue et al., 2006). In this context, it is possible for initial divergence to be more incremental, with major genes arising later.

Recently revealed aspects of two *Ostrinia* species, the European corn borer (ECB), *Ostrinia nubilalis*, and the Asian corn borer (ACB), *Ostrinia furnacalis*, suggest a system wherein shifts in sex pheromone communication have occurred that are directly relevant to the plausibility of the asymmetric tracking process. ECB has pheromone races utilizing either a 97:3 or a 1:99 ratio of (*Z*)- to (*E*)-11-tetradecenyl acetate (Z11/E11-14:OAc) (Kochansky et al., 1975). ACB produce blends of (*Z*)- to (*E*)-12-tetradecenyl acetate (Z12/E12-14:OAc) ranging from 1:1 (Klun et al., 1980) to 2:1 (Ando et al., 1980). A mechanism has been uncovered (Roelofs et al., 2002) demonstrating that pheromone production between these species is controlled by the expression or lack of expression of two desaturase genes (plus other enzymes) that cause a simple divergence in the biosynthetic pathway to result in either a Z12/E12 or a Z11/E11 system.

A key co-factor of the mechanism for facilitating a shift to the divergent blend has also been discovered in the observation that there are ''rare males'' (Roelofs et al., 2002) in both species that fly upwind and are attracted to the cross-specific ECB or ACB pheromone blend. In wind tunnel tests, 3–5% of Z-strain or E-strain ECB males flew upwind and located an ACB pheromone source in addition to exhibiting the same response to their own blend (Linn et al., 2003). Similarly, 70% of ACB males flew upwind and located an ACB blend source, whereas 3–4% of ACB males also flew upwind and located either ECB(Z) or ECB(E) pheromone sources (Linn et al., 2007a). Also, 1% of ACB males flew upwind in response to both ECB blends as well as to the ACB blend. This pattern is consistent with the asymmetric tracking model because it shows the potential of individual males to be able to broadly respond to a wide variety of female-emitted compounds. These observations, though, present the question of what aspects of the olfactory perception system might account for the response behavior of the broadly responding rare males in such populations.

The peripheral olfactory system in the ECB has been well described, consisting of three olfactory receptor neurons (ORNs). Using single-cell electrophysiology, it has been observed that two co-compartmentalized ORNs in every trichoid sensillum respond to the pheromone components (Z11/E11-14:OAc) (Hansson et al., 1987, 1994; Hallberg et al., 1994; Cossé et al., 1995). There is also a third ORN co-compartmentalized in each sensillum that responds to (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), and, at least in ECB (Z-strain), also responds to (*Z*)-11-hexadecenal (Z11-16:Ald) (Linn et al., 2007b). These latter compounds act as behavioral antagonists to ECB attraction (Klun and Robinson, 1971; Struble et al., 1987; Glover et al., 1989; Gemeno et al., 2006).

Asian corn borers have sensilla containing two co-compartmentalized ORNs responsive to the two pheromone components of this species (Takanashi et al., 2006). However, in this case, one ORN responds to both Z12-14:OAc and E12-14:OAc. The second, small-spike-amplitude ORN is most sensitive to E12-14:OAc, but some also exhibit a weak response to Z12-14:OAc. As with ECB, there is a third co-compartmentalized ORN that is highly responsive to Z9-14:OAc. It is presumably through the input of this ORN that Z9-14:OAc acts as a behavioral antagonist in ACB (Takanashi et al., 2006; Linn et al., 2007a). Takanashi et al. (2006) also found that the ECB pheromone components (Z11/E11-14:OAc), were both capable of stimulating the attraction-related ORN in ACB males that is stimulated by Z12-14:OAc and E12-14:OAc, and importantly these ECB components also stimulate the neuron associated with behavioral antagonism tuned to Z9-14:OAc. They noted that the antagonistic response could be useful for avoiding cross-specific mating with numerous sympatric *Ostrinia* species in

Asia that utilize Z11/E11-14:OAc blends (Ishikawa et al., 1999).

Here, we examine the ORN response to Z12-14:OAc, E12-14:OAc, Z11-14:OAc, and E11-14:OAc by rare ACB males that have been behaviorally tested for response to ECB (E-strain) blend, ECB (Z-strain) blend and the ACB pheromone blend. First, we were interested in whether the broadening of response preference occurring in rare males would be manifested as changes in the broadening of peripheral ORN tuning curves. Second, the behavioral changes might be manifested as other changes in ORN tuning, such as shifts in the responsiveness of the ORN involved in behavioral antagonism that could allow or prevent attraction to other blends despite the broad stimulation of the ORNs involved in attraction (Löfstedt et al., 1990).

2. Materials and methods

For physiological analysis, male moths were obtained from a subset of those described in a previous study (Linn et al., 2007a). The colony of the ACB originated from Jin Kyo Jung, National Institute of Crop Sciences, South Korea. Moths were maintained at 25 °C, and 16:8 L:D photoperiod as previously described for ECB (Roelofs et al., 1985). Pupae were separated by sex, with males placed on a layer of vermiculite in plastic and screen emergence cages. Cages were separated daily to isolate cohorts by age.

Males were tested in the sustained-flight tunnel during their second to third night as adults, under standard conditions for *Ostrinia* (Glover et al., 1989; Linn et al., 1997): 20–21 °C, 60–65% RH, 0.50 m/s air flow, and 11-lx of red light at the tunnel floor, during the third to sixth hour of scotophase. Adults were transferred individually to the flight tunnel in screen release cages prior to the start of the eighth hour of scotophase. There was a 1-h period of acclimation, in photophase, at 25 °C. Lights were turned off and the temperature dropped to 20–21 °C.

Adult moths were tested individually, and a positive response was counted if the male exhibited upwind flight in the odor plume and made contact with the rubber septum source. It was also noted when the moths flew partially toward a pheromone source consisting of the ECB Z-strain or E-strain blends without making contact. For flight tunnel and electrophysiological assays, we used various combinations of Z11-14:OAc, E11-14:OAc, Z12-14:OAc, E12-14:OAc, and Z9-14:OAc (Pherobank, the Netherlands). For flight-tunnel lures, mixtures were prepared in HPLC-grade hexane and applied to red rubber septa (Thomas Scientific, Swedesboro, NJ; Glover et al., 1989; Linn et al., 1997).

Following the behavioral analysis, ACB males were shipped via overnight courier from New York to Pennsylvania and analyzed between 3 and 7 days after emergence. ORN response was recorded from individual antennal sensilla of each behaviorally phenotyped male using the cut sensillum technique (Kaissling, 1974; van der Pers and den Otter, 1978). Antennae were excised from the head and placed in a saline-filled Ag recording electrode. The antenna was positioned using a micromanipulator such that a single trichoid sensillum rested on the tip of a vertically positioned tungsten knife. A second horizontally oriented glass knife, controllable with another micromanipulator, was used to cut the sensillum tip. The cut sensillum was then surrounded by a saline-filled glass micropipette containing an Ag recording electrode.

The AC signal from the recording electrode passed through a built-in amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) into a computer. Computer software (Syntech Autospike v.32; Syntech, Hilversum, the Netherlands) and an external loudspeaker allowed visual and auditory monitoring of neural activity.

A stream of purified, humidified air blew continuously over the antenna (10 ml/s) through a 25-cm-long glass tube (8 mm ID), the end of which was placed 2 cm from the antenna. A 50-ms air pulse at 40 ml/s flow rate was injected through the odor cartridge, and into the airstream using a stimulus flow-controller device (SFC-2, Syntech). Linear flow through the airstream was ~0.3 m/s. At least 30 s were allowed to elapse between stimulations. Syntech software was used for analyzing data by counting the number of spikes within 300 ms from the initiation of neuronal activity. Generally, there was little spontaneous background activity and initiation of response was easily discerned.

We created pheromone cartridges from serial dilutions (1, 3, 10, and 30 μg/μl) of Z11-14:OAc, E11-14:OAc, Z12-14:OAc, and E12-14:OAc in 1 ml of HPLC-grade hexane. We confirmed a >98% purity of the compounds, as well as equivalencies of the concentrations of each compound via gas chromatography. For each concentration, 10 μl was pipetted onto a $0.5 \times 2.0\,cm^2$ filter paper strip held in a 15-cm-long Pasteur pipette odor cartridge. The filter paper loadings thus were 10, 30, 100, and 300 μg for the four compounds. We similarly created a 100 μg cartridge for the behavioral antagonist Z9-14:OAc, using 10 μl from a 10 μg/μl solution.

Cross-stimulations were also performed to characterize the ORN-specific responses to the ACB pheromone components, E12-14:OAc and Z12-14:OAc, and the antagonist Z9-14:OAc in this population. For this purpose, we primarily used moths that were not behaviorally characterized. Air from two pheromone cartridges with loadings of 100 μg was pulsed as described above, with an interval of 300 ms between the pulse from the first cartridge followed by that from the second cartridge. Cartridge pairs used included E12-14:OAc and E12-14:OAc, Z12-14:OAc and E12-14:OAc, Z9-14:OAc and E12-14:OAc, E12-14:OAc and Z12-14:OAc, Z12-14:OAc and Z12-14:OAc, Z9-14:OAc and Z12-14:OAc, E12-14:OAc and Z9-14:OAc, Z12-14:OAc and Z9-14:OAc, Z9-14:OAc and Z9-14:OAc. At least eight replicates of all paired stimulations were performed. To assess the intensity of response to

stimulation by the first compound, spikes were counted within 300 ms of the initiation of neuronal activity. For the second compound, initiation of response was noted if a different spike size appeared, or if there was a lapse of more than 50 ms between spikes. When action potentials of a similar size were observed in a regular pattern for more than 300 ms after the initial response, the second response was counted between 300 and 600 ms. No more than three paired stimulations were performed per sensillum, and no more than six sensilla were used per antenna.

All dose–response curves we obtained were from individuals that had been behaviorally characterized. On each antenna, we began with the ECB pheromone components, Z11-14:OAc and E11-14:OAc at the 10 μg dose, alternating which compound was tested first. Higher concentrations of the two compounds were puffed in the same order (30, 100, and 300 μg/μl). If the contact could be maintained on the same sensillum, the procedure was repeated using sequentially increasing concentrations of Z12-14:OAc and E12-14:OAc. When the ORNs did not respond to Z12-14:OAc and E12-14:OAc components at 100 μg, the data were discarded and these compounds were tested on a new sensillum.

Additionally, there was also often an absence of responsiveness to a given compound at 300 μg after strong responses at 100 μg. In such cases, non-responses at the higher concentration were discarded from the data set. For this reason, sample sizes were not consistent for all compounds and among all concentrations of a given compound with respect to the behavioral types. At the end of many sequences within a sensillum Z9-14:OAc was puffed at 100 μg, to ensure that the ORN associated with behavioral antagonism was active. For all the sensilla we investigated, an intermediate sized ORN response was always found if Z9-14:OAc was used.

Finally, one behaviorally unusual male of particular interest was subjected to slightly different procedures, but the data were retained. This ACB male was tested and flew upwind toward the ECB(Z) pheromone blend, and also did respond when subsequently tested with the ACB blend. For electrophysiology, pipette loadings of 1, 10, and 100 μg were used to test four sensilla in a procedure otherwise similar to that described above. Only data from the 10 and 100 μg are reported, with no responses having occurred at 1 μg. We used this alternative methodology for many moths early in the study when we were still developing the optimal experimental protocols, but retain only this moth because of its unusual upwind flight toward the ECB(Z) blend.

3. Results

Cross-stimulation experiments showed that there were three responsive ORNs per sensillum trichodeum, a large-amplitude-spiking ORN responding to both Z12-14:OAc and E12-14:OAc, an intermediate-amplitude-spiking ORN responding to Z9-14:OAc, and a small-spiking ORN responding to only E12-14:OAc. The relative spike sizes of these three ORNs can be seen in rapid cross-stimulation involving E12-14:OAc and Z9-14:OAc (Fig. 1A), as well as in separate responses to Z11-14:OAc, E11-14:OAc, E12-14:OAc and Z12-14:OAc (Fig. 1B). The response frequency of the small-spiking neuron in response to E12-14:OAc was only affected by prior exposure to that compound (Fig. 2). Similarly, Z9-14:OAc caused self-adaptation of the intermediate spike-sized ORN, which did not appear to be influenced by other compounds. There was very little post-stimulus response of the large-spiking ORN by E12-14:OAc or Z12-14:OAc if it was previously exposed to either compound (Fig. 2).

Different ACB males were grouped into three behavioral categories based on their flight-tunnel responses to the ACB and ECB pheromone blends (Fig. 3). The first category, designated ACB responders, included all individuals that did not show any upwind flight responses to the ECB(Z) or the ECB(E) pheromone blends; these males flew upwind to and located the ACB pheromone source just as normal ACB males do. Twenty-one sensilla from 12 individuals were analyzed electrophysiologically from this group (Fig. 3A). The second phenotype, designated non-responders, were individuals that did not fly upwind to any of the three pheromone sources. Fourteen sensilla from 12 of these individuals were examined electrophysiologically (Fig. 3B). A final group of ACB males, designated rare males, were able to fly upwind and locate their ACB blend source, but they also flew upwind in response to ECB blends (Fig. 3C). We were able to analyze electrophysiologically eight sensilla from three individuals in this group.

Males within the rare-male group exhibited individual variation with respect to flight (Fig. 4A–C). The first such male (A) flew partially to the ECB (Z-strain) source, and was not tested behaviorally to the ECB (E-strain) blend. This male, however, exhibited a complete upwind flight to the ACB source. The second rare male (B) flew upwind to the ECB (E-strain) and ACB sources, but did not fly upwind to the ECB (Z-strain) source. A third male (C) flew upwind part way in response to the ECB (E-strain) source and completely upwind in response to the ACB source; it did not fly upwind at all in response to the ECB (Z-strain) source. A final male (D) was not grouped with any of the others. This individual exhibited a partial flight in the plume of both the ECB Z-strain and E-strain blends, but did not fly upwind at all in response to its own ACB blend (Fig. 4D).

The ECB pheromone component Z11-14:OAc often stimulated both the large ORNs (agonistic pathway) and medium-spiking ORNs (antagonistic pathway) of ACB pheromone blend behavioral responders (Figs. 1B, 3A). Both of these responses also occurred regularly for the pheromone non-responders (Fig. 3B). On the other hand, while showing large-spiking ORN responses to Z11-14-OAc, rare-male ACB/ECB behavioral responders exhibited negligible stimulation of the intermediate-sized spiking ORN by Z11-14:OAc (Fig. 3C). When we consider each of

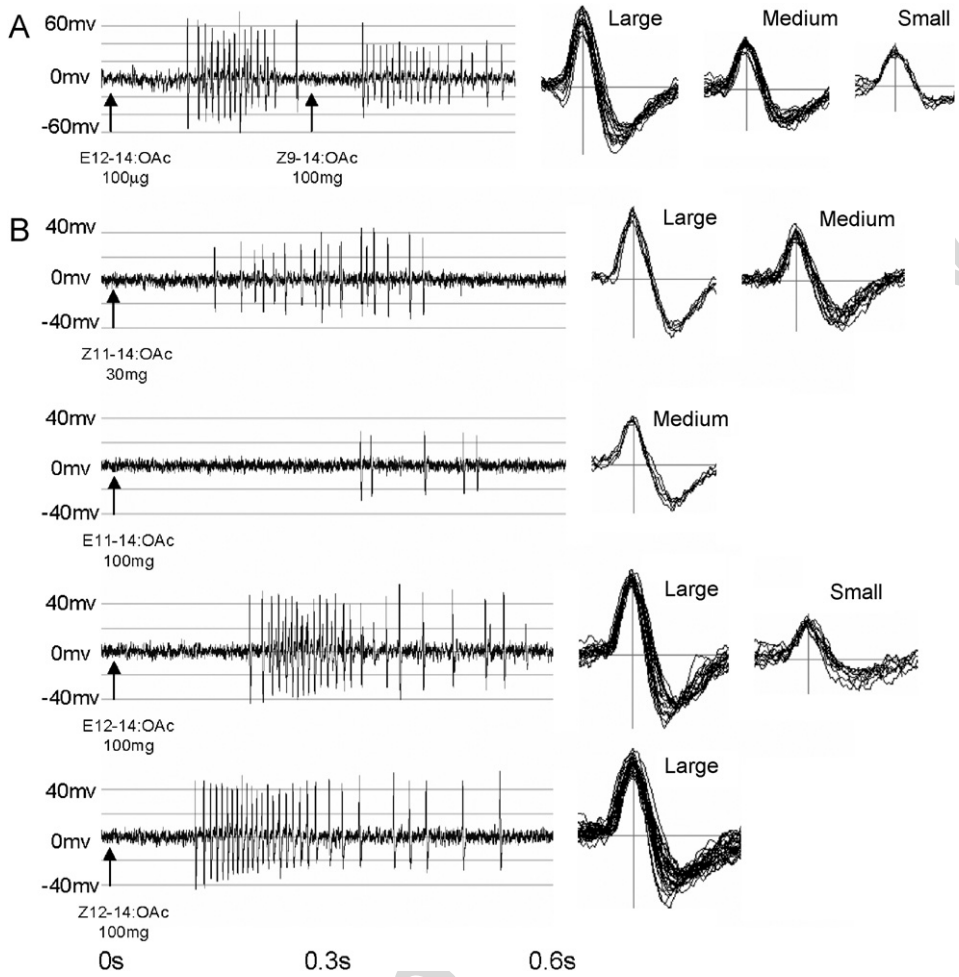

Fig. 1. ORN response traces for the ACB. In one individual, (A) all three ORNs are stimulated during cross-stimulation by E12-14:OAc (large and small ORNs) and Z9-14:OAc (medium ORN) at an interval of 0.3 s. In another individual, ORNs are observed responding to (B) Z11-14:OAc (large and medium ORNs), E11-14:OAc (medium ORN) E12-14:OAc (large and small ORNs) and Z12-14:OAc (large ORN).

the rare-male ACB/ECB responders separately, Z11-14:OAc always stimulated the large-spiking ORN (Fig. 4A–D). In one case, the small-spiking ORN (agonistic pathway) was also stimulated (Fig. 4B). The intermediate-spike-sized ORN associated with behavioral antagonism was stimulated at a very low level in just one of four sensilla from the first rare male, producing just two spikes at a 100 µg loading. The average stimulation of the ORN associated with behavioral antagonism by Z11-14:OAc at this concentration was thus very low for both this individual (Fig. 4A) and the pooled data for rare males (Fig. 3C).

The other ECB pheromone component, E11-14:OAc, was less active in stimulating ORN responses from the ACB males. This compound stimulated the medium-spiking ORN in the example shown in Fig. 1B, but stimulation of this ORN was actually extremely rare. It was more common for E11-14:OAc to stimulate the large-spiking ORN, but at low spike frequencies. At 100 µg loadings, such a large-spiking ORN response to E11-14:OAc was observed at about 2 spikes/0.3 s for the ACB behavioral responders and non-responders as well (Fig. 3A, B). In the one rare male that did not fly upwind in response to its own ACB blend but did exhibit partial flights in response to both of the ECB blends, the medium-spiking ORN responded with an unusually high firing frequency to Z12-14:OAc (Fig. 4D). This response occurred in both sensilla tested for this moth, at levels of 5 and 17 spikes/0.3 s at 100 µg loadings of Z12-14:OAc.

The known behavioral antagonist, Z9-14:OAc, stimulated the intermediate spiking ORN at 100 µg equally strongly in all three of the male behavioral groupings (Fig. 5). The response of this ORN to Z9-14:OAc was roughly an order of magnitude larger than the highest firing frequency obtained in response to Z11-14:OAc in any of the groups of the ACB-behavioral responders (Fig. 3A).

4. Discussion

Our results show that there were distinguishable differences in the stimulation of the antagonistic-pathway-related ORN between the behavioral classes of ACB males, providing an explanation for the rare ACB phenotype and insights into the evolution of a new

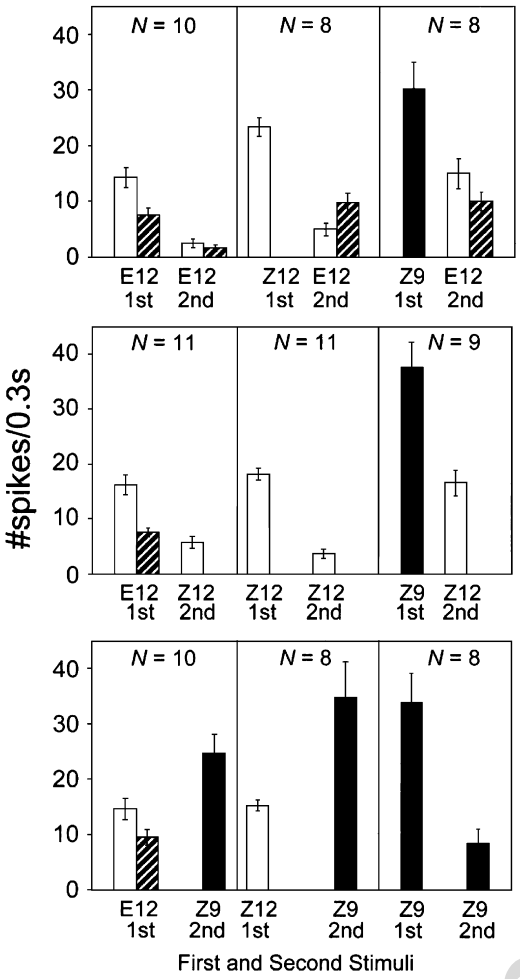

Fig. 2. Cross-stimulation of ORNs by all possible paired combinations of Z12-14:OAc, E12-14:OAc, and Z9-14:OAc in ACB from 100 μg cartridges at a 0.3 s interval (mean ± SE). Shading pattern indicates ORN spike size; white is the largest ORN, black the intermediate ORN, and striped the smallest ORN.

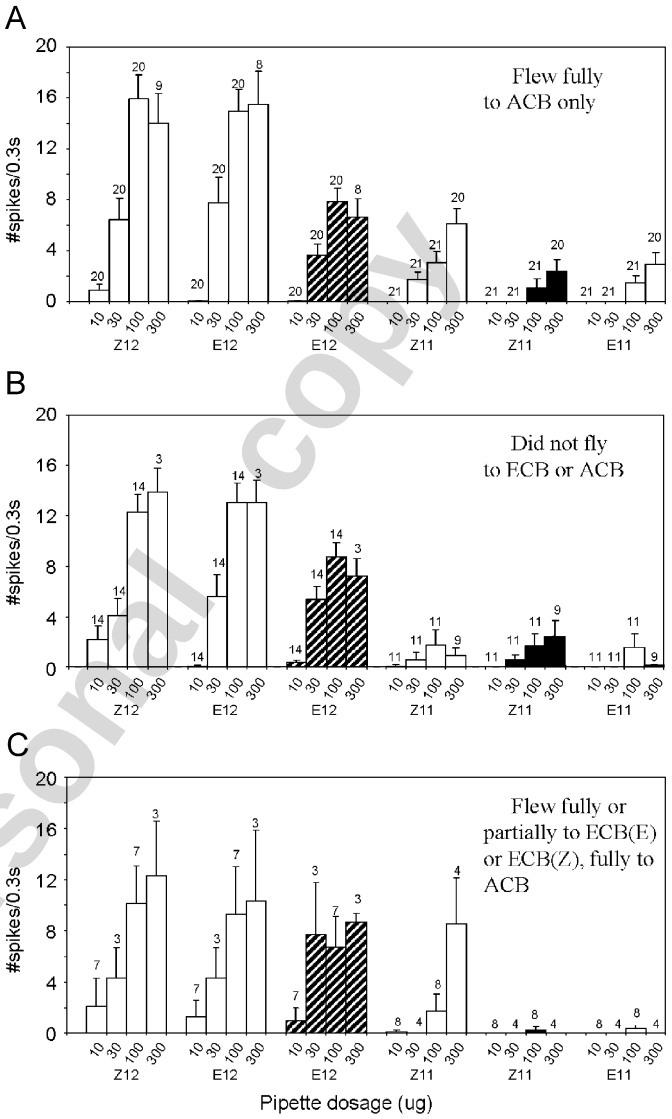

Fig. 3. ORN response (mean + SE) to increasing pipette loadings of Z12-14:OAc, E12-14:OAc, Z11-14: OAc, and E11-14:OAc for behavioral variants of ACB. ORN-specific responses are presented for compounds if the average is greater than 1 spike/0.3 s for at least one loading level in one of the behavioral categories. Sample size for each loading of a compound given above each data point. Shading pattern indicates ORN spike size; white is the largest ORN, black the intermediate ORN, and striped the smallest ORN.

pheromone system. For ACB-only responders, the ECB component Z11-14:OAc elicited a weak, but consistent response from the ORN associated with antagonism (Fig. 3A). In contrast, for the rare-male responders, Z11-14:OAc did not stimulate this antagonistic pathway ORN (Fig. 3C). Otherwise Z11-14:OAc appeared to have a similar affect on stimulation of the large-spiking ORN across all three of our behavioral classes. Of additional interest is the one ACB male that exhibited partial flights in response to both of the ECB blends, but did not fly upwind in response to its own ACB blend (Fig. 4D). The ORN associated with behavioral antagonism for this moth responded with high spike frequency to puffs of its own pheromone component, Z12-14:OAc, thus explaining the lack of upwind flight to its own pheromone blend.

The characteristics of rare male ORN responses documented here are consistent with the "asymmetric tracking" model for the evolution of sex pheromone communication systems (Phelan, 1992, 1997). The rare ACB males are behaviorally broadly responsive and better able to track widely varying pheromone blends. In the Lepidoptera, these blends are known to vary more widely among individual females of a species than do the broad response profiles among individual males (Löfstedt, 1990, 1993; Phelan, 1992, 1997).

The ACB is considered to be a derived species (Ishikawa et al., 1999) and is the only *Ostrinia* species that uses Z12- and E12-14:OAc as its sex pheromone components. Our results suggest that the rare ACB males represent a relict of the type of male that existed during the first stage of asymmetric tracking, in which males can be attracted to the unusual females emitting Z12- and E12-14:OAc, while retaining their responsiveness to the ancestral pheromone

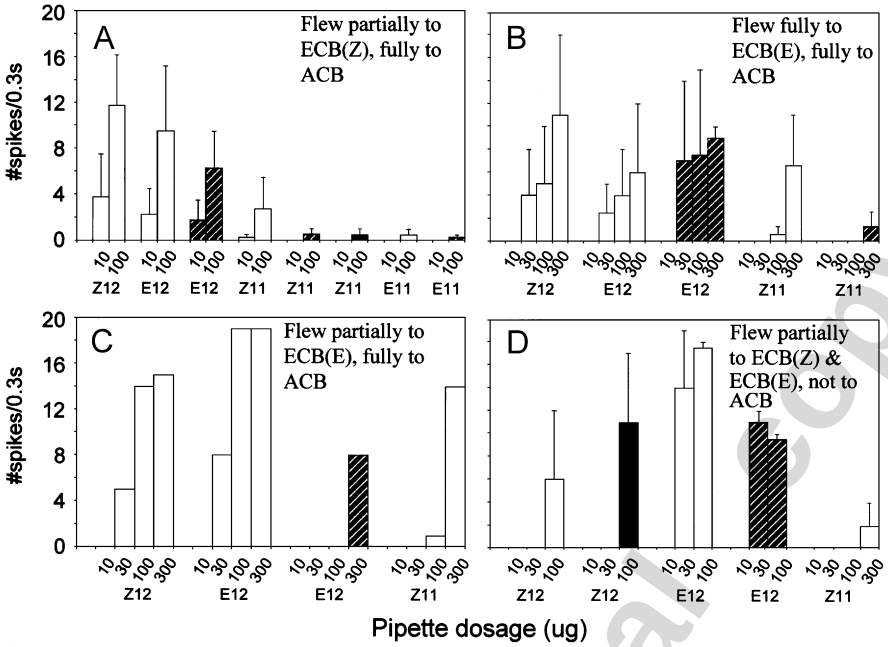

Fig. 4. ORN response (mean + SE) to increasing pipette loadings of Z12-14:OAc, E12-14:OAc, Z11-14: OAc, and E11-14:OAc for individual ACB with unusual behavioral responses to ECB(Z) or ECB(E) blends as labeled. Sample sizes were as follows for each moth: (A) $N = 4$ for all loadings of each compound, (B) $N = 2$ for each loading of Z12/E12-14:OAc and $N = 3$ for each loading of Z11-14:OAc), (C) $N = 1$ for all loadings of each compound, and (D) $N = 2$ for all loadings listed for each compound. Shading pattern indicates ORN spike size; white is the largest ORN, black the intermediate ORN, and striped the smallest ORN.

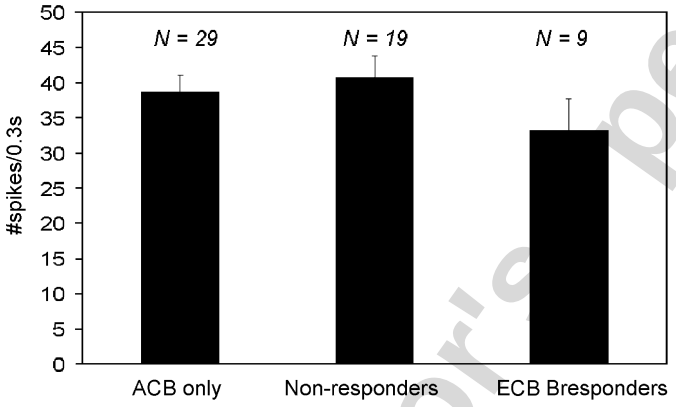

Fig. 5. Response (mean + SE) of the intermediate spiking neuron to 100 μg of Z9-14:OAc for behavioral variants of ACB. Number of sensilla investigated per group indicated.

blend comprised of Z11- and E11-14OAc. This stage of divergence of pheromone blends had been previously noted as producing "asymmetrical reproductive isolation" (Löfstedt et al., 1991) (as represented by the rare ACB males in our study) because males from the derived population could respond to both the derived and the ancestral females, whereas ancestral population males could only respond to ancestral females.

The second stage of asymmetric tracking involves the occurrence of assortative mating between females that emit the new blend and the derived males that respond to it. The impetus for assortative mating would be a fitness disadvantage that arises in hybrids resulting from matings between the ancestral population females and males from the derived population (Phelan, 1992, 1997). Ancestral females should then be selected to reject derived males for mating and such males should subsequently be selected to not be attracted to these females because of the (ultimately) fruitless mating encounters with these females. The lack of responsiveness in derived males to the ancestral blend (here represented by our normal ACB males) could be accomplished by the emergence of behavioral antagonism to the old blend. We suggest that the responsiveness of the antagonistic-pathway-related ORNs to Z/E11-14:OAc in these normal ACB males is evidence for this second step. We would call this "olfactory antagonistic imposition". After antagonistic imposition has occurred, full premating reproductive isolation would result (Löfstedt et al., 1991; Phelan, 1992, 1997).

Behavior-related antagonistic pathways in the Lepidoptera have been previously recognized as having arisen through an adaptive response by the males in a population to avoid mating mistakes with females from other populations (Löfstedt, 1990, 1993; Löfstedt et al. 1991). An increase in the number of compounds that can stimulate ORNs involved in behavioral antagonism will narrow the range of potential pheromone blends that can evoke upwind flight. The resulting increase in male pheromone blend response specificity would facilitate reproductive character displacement or reinforcement during speciation (Butlin, 1989; Löfstedt, 1990, 1993; Butlin and Trickett, 1997). This is what we suggest may have occurred in the ACB antagonistic-pathway-related

ORN that responds strongly to the known behavioral antagonist Z9-14:OAc, and now also includes responsiveness to Z11-14:OAc.

We would also propose that a reduction of activity of a previously antagonistic ORN might broaden males' abilities to respond to a wider spectrum of blends. We would describe this process as "olfactory antagonistic release". We do not know of any examples of antagonism-pathway-related ORNs that might have evolutionarily lost or reduced their responsiveness to compounds, but we think this possibility should be kept in mind in future neuroethological studies of olfaction and also in re-interpreting previous studies. We think it is possible that this framework of olfactory antagonistic imposition and release might provide insights into the mechanisms in other species groups that may have facilitated shifts in many of their sex pheromone communication systems.

The sensitivities of the ORNs of our ACB males were somewhat lower in response to all compounds than found by Takanashi et al. (2006), who used antennae from restrained, whole-body males. Also, Takanashi et al. (2006) found that nearly one-third of their sensilla did not exhibit a response from the small-spiking ORN to E12-14:OAc. We found such a response in all the sensilla from which we recorded. Sensilla from more distal regions of ECB antenna often have only one or two rather than three ORNs (Hallberg et al., 1994). If a similar pattern exists in ACB, a sampling bias might explain this difference.

For E12-14:OAc and Z12-14:OAc, we found no distinct differences in ORN responsiveness between the ACB that had flown upwind normally to the conspecific pheromone blend (Fig. 3A) and those not responding or flying upwind at all (Fig. 3B). It is interesting to note that in the pheromone non-responder group, the 300 μg loadings of Z11-14:OAc and E11-14:OAc elicited a lower spike frequency than did the 100 μg loadings. Furthermore, there were often no positive responses to puffs of Z12-14:OAc and E12-14:OAc at 300 μg after stimulation by the 100 μg cartridges. These observations suggest a susceptibility of these ORNs to adaptation that was not present in the normal ACB pheromone-only responders (Fig. 3A). However, other hormonal, neurosecretory, or photoperiod-related factors not controlled for in this study could be related to the behavioral inactivity observed in these otherwise healthy males.

Takanashi et al. (2006) also found stronger responses than we did of ACB ORNs to Z11-14:OAc and E11-14:OAc on the large- and intermediate-sized-spiking ORNs. Historical selection pressures on the populations tested may influence this discrepancy from our two groups' findings. Takanashi et al. (2006) used a Japanese population of ACB moths, where the congeneric species *O. orientalis* and *O. scapulalis* are both present and utilize pheromone blends consisting of Z11-14:OAc and E11-14:OAc (Ishikawa et al., 1999). Only the latter species is present in South Korea from where our colony originates. Thus, there could be strong selection for responses to Z11-14:OAc and E11-14:OAc on the antagonistic-related ORN in Japan to influence ACB males to avoid attraction-related mating mistakes with female *O. orientalis*. In addition, two other *Ostrinia* species that use Z9-14:OAc as a component in their pheromone blends are also present in Japan (Ishikawa et al., 1999).

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