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Olfactory neuron responsiveness and pheromone blend preference in hybrids between *Ostrinia furnacalis* and *Ostrinia nubilalis* (Lepidoptera: Crambidae)

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ABSTRACT

The olfactory receptor neuron (ORN) and behavioral responses of hybrids between the Asian corn borer (ACB), *Ostrinia furnacalis*, and the E-strain European corn borer (ECB(E)), *Ostrinia nubilalis* were examined and compared to the parental populations. In hybrids and both parents, the large-spike-size ORN was capable of responding to all four pheromone components of ACB and ECB, despite differences in which compounds elicited the greatest spike frequency in each population. There was a small-spiking ORN more narrowly tuned to the minor pheromone components in both ACB and ECB(E). In hybrids the homologous small-spiking ORN was tuned primarily to the ECB(E) minor pheromone component, with some responsiveness to the ACB minor component. Both species and all the hybrids had an intermediate spike-size ORN tuned primarily to their common behavioral antagonist. Dominance of responsiveness to the ECB(E) versus the ACB minor pheromone component on the small-spiking ORN may explain the greater tendency of hybrids to fly upwind to the ECB(E) pheromone blend than the ACB blend. This finding points toward a distinct evolutionary role for this ORN in allowing a pheromone shift.

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1. Introduction

The degree of plasticity in the peripheral detection of olfactory stimuli has important implications for understanding how adaptive shifts in such systems occur. For a wide array of insect taxa, relatively broad responsiveness is exhibited by many general odorant olfactory receptor neurons (ORNs), whereas other types of ORNs are more narrowly tuned (Mustaparta, 1975; Priesner, 1979a,b; Stensmyr et al., 2001; Olsson et al., 2006; Qiu et al., 2006; Schlief and Wilson, 2007). It has also been discovered in *Drosophila* that more than one type of odorant receptor (OR) can be expressed on the same ORN (Goldman et al., 2005), increasing the potential breadth of ORN tuning. Pheromone sensitive ORNs are generally assumed to be more narrowly tuned than are ORNs tuned to general odorants. However, responsiveness to multiple compounds per ORN has become apparent in some moth sex pheromone systems when more fully investigated for this property. For example, in the genus *Yponomeuta* several species have ORNs that respond to multiple compounds including acetates showing considerable variation in chain length and structure (van der Pers, 1982; Löfstedt et al., 1990).

To understand the evolution of sex pheromone systems, in addition to characterizing the breadth of ORN tuning it is also important to describe how such systems can change. One approach to this problem has been to examine the behavior and ORN response profiles of closely related species and their hybrids. Crosses between different moth species have tended not only to show dominance of the olfactory and behavioral characteristics of one species in the hybrids (Gadenne et al., 1997), but also sometimes suggest more complicated epistatic effects (Hansson et al., 1989). The variable penetrance of the parental olfactory characteristics was clear in crosses between *Heliothis subflexa* × *Heliothis virescens* (Baker et al., 2006). These F_1 crosses exhibited patterns of ORN responsiveness that ranged from intermediate, to being more like either parental species. The pattern of responsiveness also suggested the possibility that different ORs from either parent are expressed in the hybrids.

Hybridization studies are valuable, because they provide evidence of evolutionary changes to the olfactory system that have already occurred. However, from such studies it is inherently difficult to interpret the role of the relevant characters for allowing signal divergence and causing reproductive isolation. Another approach to discern how pheromone systems change is to examine properties of rare individuals that exhibit unusual behavioral responses to novel pheromone blends. This approach has been applied to the 3–5% of "rare" Asian corn borers (ACB), *Ostrinia*

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furnacalis, and European corn borers (ECB), *Ostrinia nubilalis*, that will fly to the pheromones of the opposing species, as well as to their own pheromone (Roelofs et al., 2002; Linn et al., 2003, 2007).

Both species have two behavioral attraction-related ORNs that respond to their own pheromone components (*Z*)-12-tetradecenyl acetate (Z12-14:OAc) and (*E*)-12-tetradecenyl acetate (E12-14:OAc) for ACB or (*Z*)-11-tetradecenyl acetate (Z11-14:OAc) and (*E*)-11-tetradecenyl acetate (E11-14:OAc) for ECB. The pheromone binding proteins have identical amino acid sequences in ACB and ECB (Willett and Harrison, 1999), and thus are unlikely to affect cross-species ORN responsiveness. Both species also have a third behavioral antagonism-related ORN that responds strongly to (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), a compound that significantly reduces attraction when co-emitted at very small proportions in a blend (Hansson et al., 1987; Glover et al., 1989; Takanashi et al., 2006). Sensilla from more distal regions of ECB antenna often house only one or two ORNs, but the ORNs in these sensilla are always similar in response characteristics to one of the three ORN types found residing together in more basally located sensilla (Hallberg et al., 1994). A similar phenomenon is found in ACB, in which sensillar classes were found that were differentiable by whether or not all the commonly observed ORN responses were present (Takanashi et al., 2006).

There are two ECB strains. The E-strain (ECB(E)) utilizes a blend of 99% E11-14:OAc and 1% Z11-14:OAc in its blend, whereas the Z-strain (ECB(Z)) uses a reverse 97%:3% blend of Z11- and E11-14:OAc (Kochansky et al., 1975; Glover et al., 1987). Most other species in the genus use blends of Z11- and E11-14:OAc as components of their pheromone blends (Ishikawa et al., 1999). Because ACB is the only species in the genus to use Z12- and E12-14:OAc as pheromone components, it is likely that a shift occurred recently in its lineage from a pheromone blend consisting of Z11- and E11-14:OAc to one using Z12- and E12-14:OAc. The ACB pheromone blend consists of two isomers at relatively similar concentrations, although the composition is variable (Klun et al., 1980; Ando et al., 1980), as is the range of pheromone blends that will elicit behavioral attraction of ACB males in the field (Boo and Park, 1998) and wind-tunnel (Linn et al., 2007).

The ORNs of ACB and ECB regularly respond to the pheromone components of the opposing species, but specific changes in patterns of ORN responsiveness are linked to cross-species behavioral attraction of the 'rare males' (Domingue et al., 2007a,b). In normal ECB E-strain males, ACB components elicited responses from both of the neurons involved in attraction. On the ORN associated with the major pheromone component, E11-14:OAc, there were responses to Z12- and E12-14:OAc in all males, including the rare males. On the minor pheromone-component ORN tuned to Z11-14:OAc of the ECB(E) males, relatively lower spike frequency responses also occurred in response to the Z12-14:OAc. However, in the rare ECB(E) males that also flew to the ACB blend, the strength of the ORN response to Z12-14:OAc was reduced (Domingue et al., 2007b). This pattern is consistent with a hypothesis that the relative firing rates of the two relevant neurons serves as a critical factor for promoting upwind flight. In rare ECB(E) males this firing ratio is similar whether it occurs in response to the ECB E-strain blend, in which E11-14:OAc is the major component, or to the ACB blend, in which Z12-14:OAc is the major component.

The converse situation was also examined, where ACB rare males also fly to ECB(E) or ECB(Z) blends (Domingue et al., 2007a). The ORNs of normal ACB males responded to ECB attraction-related components, but the behaviorally antagonism-related ORN normally associated with Z9-14:OAc also responded to the ECB component Z11-14:OAc (Takanashi et al., 2006; Domingue et al., 2007a). It has been proposed that such responsiveness to ECB components on the behaviorally antagonism-related ORN prevents upwind flight to ECB blends (Takanashi et al., 2006). This property of ACB males may serve as a premating isolating mechanism that precludes mating attempts between ACB males and females of ECB or other *Ostrinia* that emit pheromone blends containing Z11- and E11-14:OAc. Further supporting this hypothesis, we found that the behavioral antagonism-related ORN of rare ACB males did not respond to Z11- or E11-14:OAc (Domingue et al., 2007a). Thus, normal ACB males experience the imposition of antagonistic ORN input from ECB pheromone components, which is not exhibited by ACB rare males.

The results of these studies suggested distinct changes to the peripheral olfactory system that may have been at work at the beginning (relative firing rates) and end (antagonist imposition) of a shift from a pheromone system using Z11- and E11-14:OAc to result in the ACB system using Z12- and E12-14:OAc. Perhaps, as currently observed in the rare ECB(E) males, there was some change in an ORN associated with the minor pheromone component (Domingue et al., 2007b). Subsequently, reproductive isolation of the new population would have been achieved by the imposition of antagonism to the pheromone blend of the ancestral population (Domingue et al., 2007a). In the current study, we performed hybridization studies between the ACB and ECB(E) to determine whether the above scenario might be consistent with the genetic differences between the species that are observable in the hybrids, or if other changes in the olfactory system can be inferred to have been required for such a shift.

2. Materials and methods

2.1. Insects

ECB(E) male moths were obtained from a colony of the bivoltine E-strain of ECB that has been maintained in the laboratory of W.L. Roelofs as previously described (Roelofs et al., 1985). ACB male moths were obtained from a subset of those described in a previous study (Linn et al., 2007). 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). Reciprocal hybrid crosses were obtained using similar rearing procedures. For the crosses between ACB females and ECB(E) males (ACB♀ × ECB(E)♂), males were behaviorally assayed in Geneva, NY before being shipped overnight to University Park, PA for physiological analysis. For the cross between ECB(E) females and ACB males (ECB(E)♀ × ACB♂), males that were tested for electrophysiological responses were a separate group from those tested in the flight tunnel. In preparation for behavioral analyses, male pupae for both crosses were placed on a layer of vermiculite in screened emergence cages. Shipping occurred either after the behavioral assay as adults in individual plastic cups for ACB♀ × ECB(E)♂, or as pupae in cotton-lined Petri dishes for ECB(E)♀ × ACB♂.

2.2. Single-cell electrophysiology

ORN responses were recorded from individual antennal sensilla using the cut sensillum technique (Kaissling, 1974; van der Pers and den Otter, 1978). Antenna 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, controlled 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.

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 air stream using a stimulus flow-controller device (SFC-2, Syntech). Linear flow through the air stream was ~0.3 m/s.

We created pheromone cartridges from dilutions of isomerically pure Z11-, E11-, Z12-, E12-, and Z9-14:OAc (Pherobank, The Netherlands) in HPLC-grade hexane. Using gas chromatography, we confirmed that each of the preparations of compounds had no identifiable traces of the other behaviorally relevant compounds, and were of relatively similar concentrations. For each concentration (0.1, 1, 3, or 10 μg/μl), 10 μl was pipetted onto a 0.5 cm × 2.0 cm filter paper strip held in a 15-cm long Pasteur pipette odor cartridge. The filter paper loadings thus were 1, 10, 30, or 100 μg. For some experiments, a single compound of a particular concentration was pulsed on the antennae. In other cases, there were paired staggered stimulations at an interval of 300 ms using two 100 μg cartridges. After a single or a paired stimulation, there was at least a 30 s delay before further testing.

The AC signal from the recording electrode passed through a built-in amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) into 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. Although there may be different sensillum types for ECB(E) (Hallberg et al., 1994) and ACB (Takanashi et al., 2006), nearly all the sensilla we sampled at the basal areas of the antennae housed three co-compartmentalized ORNs, as described in the introduction. For data presentation we consider each population of males as having a single-sensillum type.

2.3. Response patterns across ORNs

Paired staggered stimulations were performed to clarify which compounds stimulated the same ORNs and accordingly to the levels of differential adaptation caused by such compounds. In the current study, this objective was only of interest for the hybrids because the distinct response characteristics of the three co-compartmentalized ORNs from ACB and ECB(E) males have been previously described (Hansson et al., 1987; Takanashi et al., 2006; Domingue et al., 2007a). Because there were five compounds of interest for hybrids, it was impractical to perform paired stimulations of each possible pair in either order. Thus, we performed stimulations of all five compounds before the ECB components, E11- and Z11-14:OAc, and before the antagonist Z9-14:OAc. To confirm that Z12- and E12-14:OAc were stimulating the same ORN, we also performed paired stimulations with all combinations of these compounds that assessed potential cross- and self-adaptation. F_1 hybrids from reciprocal crosses were used in the paired stimulations. Combining data from both F_1 types, there were 9–13 replicates of each specific combination of compounds. No more than three such paired stimulations were performed per sensillum, and no compound was used more than twice on the same sensillum.

Generally, we have observed little spontaneous background activity for ACB, ECB, or hybrid ORNs (Fig. 1). To measure spike

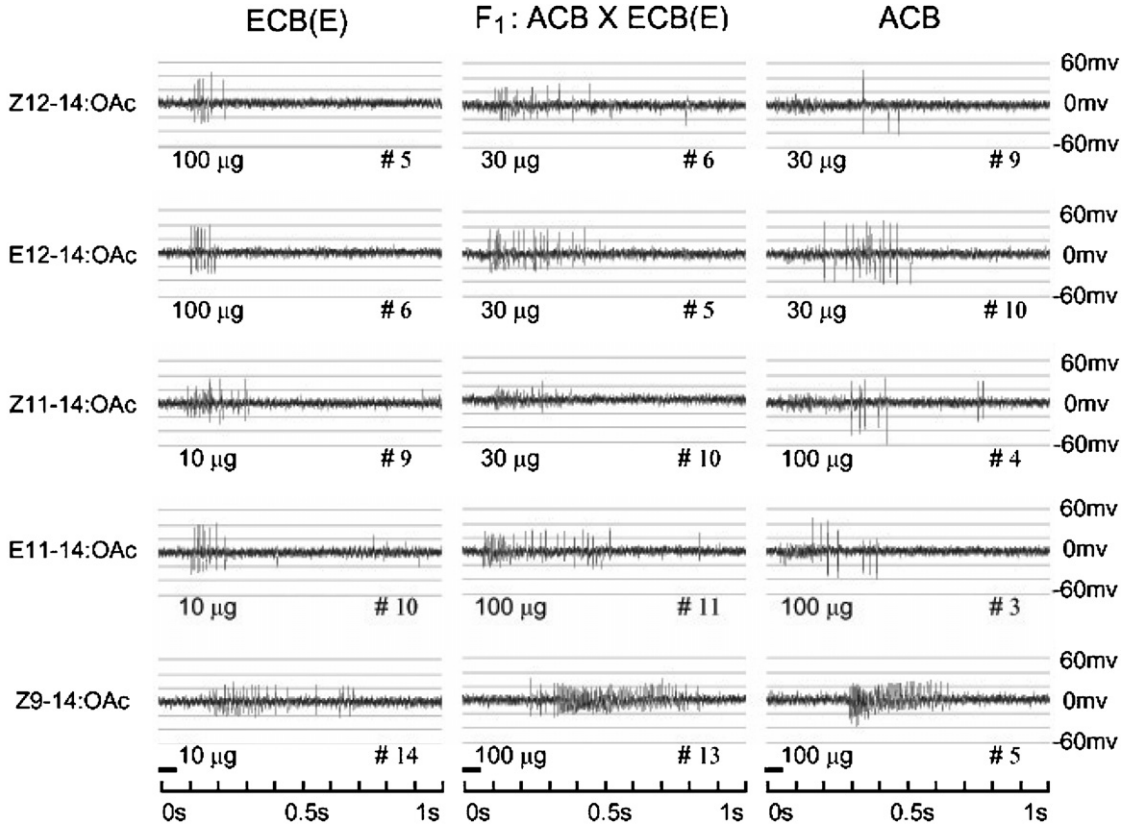

Fig. 1. ORN response traces from single sensilla of a typical ECB(E), F_1, and ACB males. The F_1 is an offspring of an ECB female and an ACB male. For each compound listed to the left, the cartridge loading and the order of that particular application in the original sequence are shown below each trace. A large-spiking ORN can be observed in response to the ACB and ECB pheromone components in most instances (top four rows). In the hybrid, Z11-14:OAc only elicits response on a small-spiking ORN. ACB and ECB(E) had large- and small-spiking ORN responses to Z11-14:OAc, as did ACB and the hybrid to E12:14:OAc. For all three examples Z9-14:OAc elicits an intermediate spiking ORN (bottom row). Stimulus duration was always 0.05 s at the beginning of each trace, as indicated by a black bar above the time scale.

frequency, Syntech software was used to count the number of spikes occurring within 300 ms of the first appearance of a spike. When two compounds were used in paired stimulations as described above, we measured spike frequencies similarly. We counted spikes within 300 ms of the first appearance of neuronal activity and then again for the second burst of activity, which was usually approximately 300 ms later. Occasionally, the spike trains associated with these paired stimulations overlapped. These cases usually involved different ORNs such that the spikes arising from stimulation by the first compound could be separated from those associated with the second compound by spike amplitude and/or the tempo of the spike train. ANOVA with the Tukey adjustment for multiple paired comparisons was performed to compare spike frequency elicited by the second compound according to which initial compound was used. Data were pooled for F_1 males derived from either cross direction because the response characteristics during exposure to all the compounds were similar between these groups.

We were also interested in quantifying the spike amplitude differences within and between the species and hybrids. The paired stimulation data is better suited than separate applications of the pheromones for such an endeavor, because spike amplitude tends to change, usually decreasing, over the course of a connection to a sensillum. Often such changes affect the different ORNs independently of one another. Thus, observing the firing of multiple ORNs shortly after making contact with the sensillum allows a more accurate assessment of their relative spike amplitudes.

2.4. Determining relative spike amplitudes of ORNs

The F_1 paired stimulation data, described above in the context of assessing differential adaptation, was also analyzed for comparative spike amplitude. For ACB, paired-stimulation experiments had also been previously performed using identical laboratory conditions for E12-, Z12-, and Z9-14:OAc (Domingue et al., 2007a). These cross-stimulation traces were re-analyzed for spike amplitude. For the current study, additional paired stimulations were performed on ECB(E) males, using E11-, Z11-, and Z9-14:OAc. Because ECB(E) moths were readily available, it was only necessary to perform a single-paired stimulation per sensillum. Up to six sensilla per antenna were tested, never repeating the possible combinations of the pairs of the three compounds.

For calculating relative spike amplitude we exported the data from the traces of interest from Autospike as ASCII data files. These files were analyzed in Labview using the peak-finder function. The locations of peaks were visually checked against the Autospike trace to confirm whether all relevant peaks were being considered, and to categorize the spikes as belonging to one of the three ORN classes. Amplitudes of each peak were recorded in spreadsheet form.

The paired stimulation traces were separated by whether they offered a comparison between the large-spike versus small-spike-size attraction-related pathway ORNs, the large-spike-size attraction-pathway versus behavioral antagonistic-pathway ORNs, or between two different stimulations of the large-spike-size agonistic-pathway. Data were then normalized with respect to the mean spike amplitude for the large-spiking attraction-related pathway ORN. When two paired stimulations of the large-spiking ORN occurred, we normalized data with respect to the first of the two stimulations, considering the variation in the spike amplitudes of the second stimulation in our comparisons. The relative spike amplitudes of the small-spike-size agonistic-pathway ORN, the antagonistic-pathway ORN, and the second of the large-spike-size agonistic-pathway ORN were compared using ANOVA for ACB, ECB(E), and the hybrid populations. Because the experiment had a nested design with multiple spikes being measured per sampling event, our ANOVA included the factors: Population, Spike-Size Category, Population × Spike-Size Category and the Sampling Event (nested in Population × Spike-Size Category). Pairwise comparisons between all of the Population × Spike-Size Category combinations were performed using the error associated with Sampling Event and Tukey's test to correct for multiple comparisons.

For many of the above comparisons, different compounds were often grouped under the categories of large-spiking ORNs, especially for the hybrids. Also both Z11-14:OAc and E12-14:OAc stimulated the small-spiking ORN in the hybrids. To ensure that the spikes elicited by these various compounds were indeed of the same amplitude classes as we had presumed, we performed additional ANOVA. For the three different size amplitude comparisons within each particular species, we tested the effect of the compound combination used.

2.5. Dose–response relationships

For obtaining dose–response curves for F_1 hybrids we used a protocol in which the ACB pheromone components, E12-14:OAc and Z12-14:OAc, were first applied. We began with the two compounds in either order at 10 μg, followed by applications of the 30 and 100 μg doses. ACB components were used first because we already had a strong indication that the behavioral responses were more strongly oriented toward the ECB(E) blend than the ACB blend. Our prior experiences with ACB and both ECB strains had shown that responses to the non-behaviorally active pheromone blend components were weak, and detectable only if presented before the behaviorally active compounds. When possible, we continued stimulating the same sensillum with the ECB components using a similar alternating pattern with respect to E11-14:OAC and Z11-14:OAc at increasing doses. At the end we applied Z9-14:OAc at 100 μg. However, as is typical for both parental species (Domingue et al., 2007a,b) connections were often lost before the protocol could be completed on a single sensillum.

Because of the limited availability of moths, it was not possible to sample until we could complete the protocol for many of the sensilla. Thus when contacts were lost after completing all doses for the ACB components, we contacted a new sensillum and began by using the ECB components and Z9-14:OAc. If possible, we would begin the protocol again with the ACB components on another sensillum. By using this approach, the data are most strongly robust for comparing the relative responsiveness to Z12-14:OAc versus E12-14:OAc and Z11-14:OAc versus E11-14:OAc. The protocol was performed similarly for hybrids of both directions. However, the ACB♀ × ECB(E)♂ hybrids were behaviorally characterized before being analyzed (see below). Both types of F_1 were analyzed at similar ages (2–7 days old), but those that had been behaviorally phenotyped were shipped as adults rather than as pupae.

We had previously obtained dose–response curves for ACB (Domingue et al., 2007a), but not for ECB(E) males. A similar procedure to that used for the hybrids was used for obtaining dose–response curves for ECB(E) male response. Again ACB components were presented first, followed by the ECB components, and finally Z9-14:OAc. It was even more difficult to maintain the connection long enough to test all doses of the five compounds on one sensillum for this *Ostrinia* species. Thus, we followed the same procedure as above for the hybrids, in that we continued to a second sensillum if responses to the ACB components were successful, but we still needed to obtain responses to the ECB components and Z9-14:OAc. We did not sample more than one sensillum per antenna for the ECB(E) species because we never

obtained responses to the ACB components after the ECB components had been puffed on the antenna. We also modified the dose series to include 1, 10, and 100 μg cartridge loadings for E11- and Z11-14:OAc, which better provides a range of low, intermediate and strong responses. The effect of order of stimulation was also considered, by comparing dose–response curves in which E12-, and E11-14:OAc were puffed before Z12-, and Z11-14:OAc, versus the reciprocal pattern. This effect was tested for spike frequency of responses at 100 μg using ANOVA.

2.6. Behavioral assay

The males from both reciprocal F_1 crosses 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 to the room housing the flight tunnel and placed individually in screen release cages 1-h prior to the start of the 8-h 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. Males were tested sequentially to the three pheromone blends: 97:3 Z:E11-14:OAc (Z race ECB), 1:99 E:Z11-14:OAc (E race ECB), and 2:1 Z:E12-14:OAc (ACB). Mixtures were prepared in HPLC-grade hexane and 30 μg of the appropriate blend were applied to red rubber septa (Thomas Scientific, Swedesboro, NJ; Glover et al., 1989; Linn et al., 1997).

2.7. ORN responses of behavioral variants

The F_1 males arising from ACB♀ × ECB(E)♂ crosses were analyzed after being behaviorally characterized. Statistical comparisons of the ORN response data were made using the groupings of ACB♀ × ECB(E)♂ F_1 males as responders to (1) ECB(E) blend alone, (2) the ECB(E) and ACB blends, or (3) none of the pheromone blends presented. ANOVA using the Tukey's correction was used to compare the spike frequency of the most regularly observed responses, including the large-spike-size agonistic ORN to E12-, Z12-, and E11-14:OAc at 100 μg loadings and the small-spike-size behavioral attraction-related ORN to Z11-14:OAc at a 30 μg loading. Other infrequent, weak responses occurred to E12-14:OAc on the small-spike-size attraction-related ORN and to Z11-14:OAc on the large-spike-size agonistic ORN. Such statistical distributions were not suitable for ANOVA. In these cases χ^2 tests were performed to assess the differences in the frequency with which each sensillum would show such response to these compounds at any concentration.

3. Results

The ORN response profiles of the hybrids exhibited characteristics of both parental types (Fig. 1). A large-spike-size ORN responded strongly to Z12-, E12-, and E11-14:OAc as in the parental populations. A small-spike-size ORN was stimulated by E12-14:OAc as it was in the ACB males, and also by Z11-14:OAc as it was in ECB E-strain males. The hybrids and both parental species had one type of ORN that was highly responsive to Z9-14:OAc. The reciprocal F1 crosses responded to the same compounds on the same ORNs.

When staggered presentations of two different compounds were presented to the hybrids at a 0.3-s interval, similar patterns were observed. Data were combined for the reciprocal crosses, because no statistical differences in spike frequency in any treatment could be found in this experiment. Z12-, E12-, and E11-14:OAc (and more weakly Z11-14:OAc) stimulated the large-spike-size ORN (Fig. 2). When E11-14:OAc was presented after any of these compounds, the spike frequency was lower than if presented after Z11-14:OAc, which primarily stimulated a small-spike-size ORN (Fig. 2A). Z11- and E12-14:OAc both stimulated a small-spike-size ORN that was about two-thirds the size of the large-spike-size ORN (Table 1). However, the response elicited by E12-14:OAc was quite weak and only Z11-14:OAc caused a reduction in spike frequency to a second application of Z11-14:OAc (Fig. 2B). Z9-14:OAc elicited responses of an intermediate spike-size ORN that was approximately three-quarters the relative amplitude of the large-spiking ORN (Table 1). The presentation of Z9-14:OAc caused a reduction in

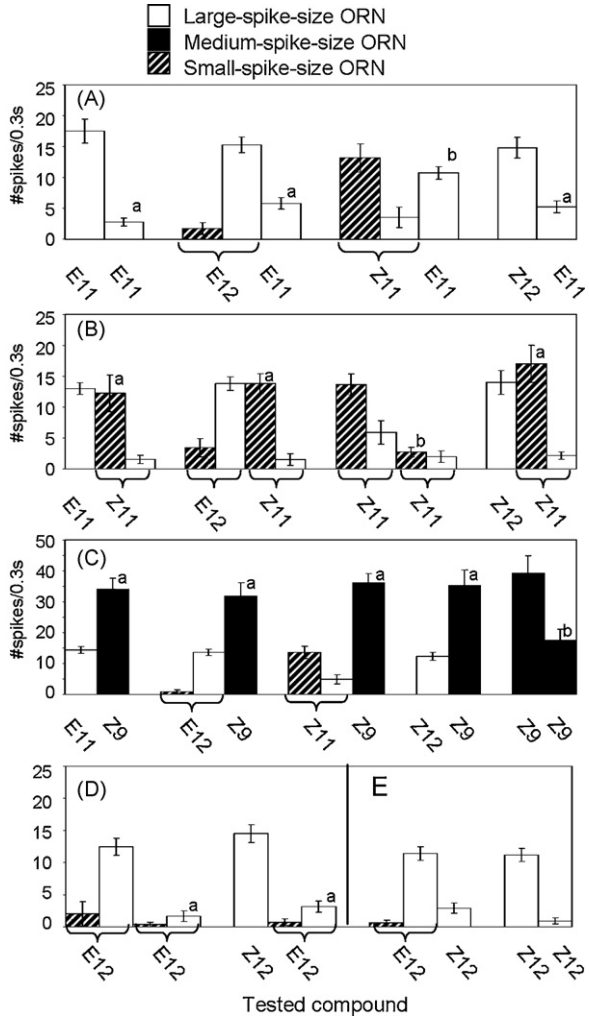

Fig. 2. Spike frequency of ORN responses for the combined reciprocal F_1 crosses after cross-stimulation with paired combinations of compounds at a 300 ms interval. (A) E11-, E12-, Z11-, and Z12-14:OAC before E11-14:OAc with $N = 10$, 11, 11, and 11, respectively. (B) E11-, E12-, Z11-, and Z12-14:OAC before Z11-14:OAc with $N = 13$, 14, 11, and 12, respectively. (C) E11-, E12-, Z11-, Z12-, and Z9-14:OAc before Z9-14:OAc with $N = 12$, 10, 11, 10, and 10, respectively. (D) E12- and Z12-14:OAc before E12-14:OAc with $N = 11$ and 14, respectively. (E) E12- and Z12-14:OAc before E12-14:OAc with $N = 10$ and 12, respectively. Shading pattern indicates ORN spike size; white is the largest ORN, black the intermediate ORN, and striped the smallest ORN. Lowercase letters within parts A, B, C, and D indicate pairwise comparisons of the spike frequencies of the ORNs primarily associated with the second compound (Tukey's correction, $\alpha = 0.05$). Responses to Z12-14:OAc in part (E) violated normality assumptions required for such tests.

Table 1
Relative spike amplitude of the different co-compartmentalized ORNs of ECB E-strain, ACB, and hybrid males

Amplitude comparison	ECB E-strain	F_1 hybrids	ACB
Agonist(L): Agonist(L)	0.933 ± 0.098ab (N = 6)	0.993 ± 0.024a (N = 75)	1.024 ± 0.036a (N = 29)
Agonist(S): Agonist(L)	0.637 ± 0.026d (N = 31)	0.656 ± 0.016d (N = 74)	0.598 ± 0.026d (N = 41)
Antagonist: Agonist(L)	0.848 ± 0.019b (N = 31)	0.744 ± 0.013c (N = 38)	0.670 ± 0.016d (N = 30)

The measurements were made by paired stimulations of combinations of E11-, Z11-, Z12-, E12-, and Z9-14:OAc. All compounds were used for hybrids, but the heterospecific pheromone components were not used for either parental species. The large agonistic ORN was stimulated by E11- or Z11-14:OAc in ECB, Z12- or E12-14:OAc in ACB, and any of these compounds in the hybrids. The small agonistic ORN was stimulated by Z11-14:OAc in ECB, E12-14:OAc in ACB, and either compound in the hybrid. Only Z9-14:OAc caused responses to the antagonistic ORN. Different letters indicate significantly different amplitude ratios (α = 0.05, Tukey's test).

spike frequency to a second presentation of Z9-14:OAc, whereas no other compounds did (Fig. 2C). When combinations of E12- and Z12-14:OAc were included in the paired stimulations, there was always a strong response by the large-spike-size ORN and a reduction in spike frequency to the second compound (Fig. 2D).

The spike amplitude relationships in the hybrids were generally similar to those of the parental species. The type of ORN exhibiting the smallest spike size, which was associated with the minor pheromone components, gave relative response amplitudes that were not significantly different between populations (Table 1). The ORN type stimulated by the antagonist was, on average, intermediate in relative spike amplitude for ACB, ECB E-strain, and the hybrid males. The relative amplitude of this intermediate-sized antagonistic ORN was significantly larger than that of the small-spike-size agonistic ORN in ECB E-strain and hybrid males, but not ACB males. Relative spike amplitude was never significantly affected (α = 0.05) by the particular compounds used within the species and ORN combinations shown in Table 1 (details of analyses not shown).

The mean spike frequency ± standard error for responses to 100 μg loadings of Z9-14:OAc on the antagonistic ORN were of a similar large magnitude for each genetic group (ACB: 38.3 ± 1.77; ACB × ECB(E) F_1: 39.4 ± 2.80; ECB(E) × ACB F_1: 31.2 ± 2.40; ECB(E): 31.3 ± 1.97). Thus, we did not include results for this compound in our presentation of the dose–response curve since the data provide little comparative value for the behaviorally and genetically distinct populations.

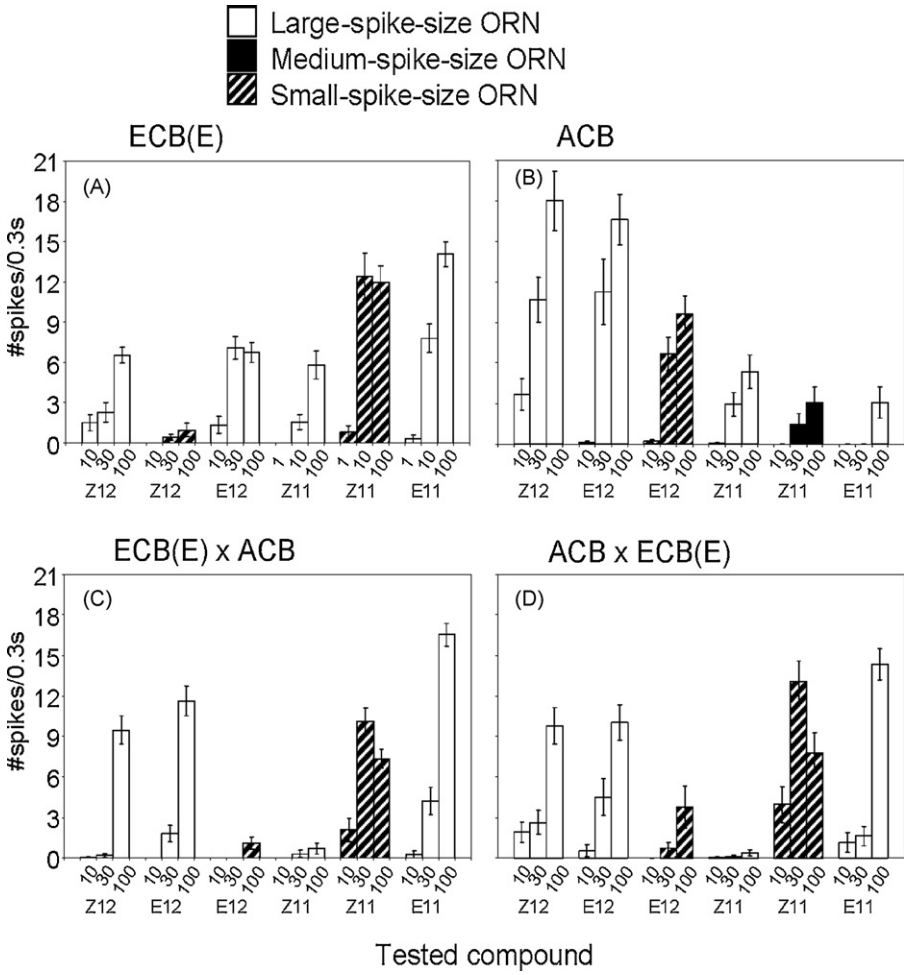

Fig. 3. ORN response (mean ± S.E.) to increasing pipette loadings of Z12-14:OAc, E12-14:OAc, Z11-14:OAc, and E11-14:OAc for (A) ECB E-strain males (N = 21 for all compounds), (B) ACB males (N = 21 for all compounds), (C) ACB♀ × ECB(E)♂ derived F_1 males (N = 21 for E12-/Z12-14:OAc, N = 23 for E11-/Z11-14:OAc), and (D) ECB(E)♀ × ACB♂ derived F_1 males (N = 22 for E12-/Z12-14:OAc, N = 21 for E11-/Z11-14:OAc). Shading pattern indicates ORN spike size; white is the large agonistic ORN, striped the small agonistic ORN, and black the antagonistic ORN.

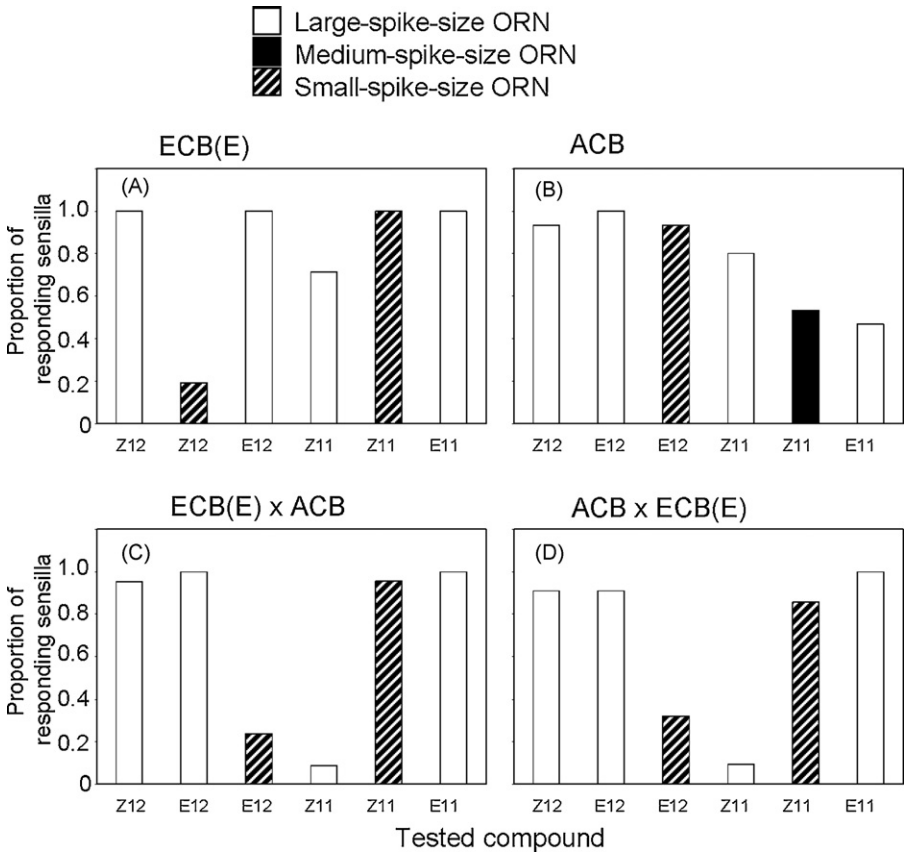

Fig. 4. Proportion of sensilla exhibiting ORN responses (mean ± S.E.) to Z12-14:OAc, E12-14:OAc, Z11-14:OAc, and E11-14:OAc for (A) ECB(E) males, (B) ACB males, (C) ACB♀ × ECB(E)♂ derived F_1 males, and (D) ECB(E)♀ × ACB♂ derived F_1 males. Sample sizes are as listed in legend to Fig. 3. Shading pattern indicates ORN spike size; white is the large agonistic ORN, striped the small agonistic ORN, and black the antagonistic ORN.

The dose–response curves for ECB(E) males (Fig. 3(A)) exhibit a similar pattern to those observed at only the 100 μg loading in a previous study (Domingue et al., 2007b). The large-spiking ORN was not only most strongly tuned to E11-14:OAc but also was responsive to Z12-, E12-, and Z11-14:OAc (Fig. 3(A)). All four responses occurred on a large proportion of sensilla (Fig. 4(A)), but the spike frequency at 10 μg to E11-14:OAc was about the same as that to 100 μg for the other compounds (Fig. 3(A)). The small-spiking ORN responded strongly to Z11-14:OAc in ECB(E), but it otherwise only showed a low and more variable spike frequency in response to Z12-14:OAc (Figs. 3(A) and 4(A)).

Likewise, ACB sensilla possessed a large-spike-size ORN that responded to all four pheromone components (Figs. 3(A) and 4(A)). However, this ORN responded with greater spike frequency and regularity to E12- and Z12-14:OAc than to E11- and Z11-14:OAc. We observed a smaller-spike-size ORN that was responsive only to the minor pheromone component E12-14:OAc. Furthermore, the intermediate spike-size ORN, normally highly responsive to Z9-14:OAc, often responded to Z11-14:OAc with a low-spike frequency.

The dose–response curves for both types of hybrids (Figs. 3(C), 3(D), 4(C), 4(D)) were much more similar to those of the ECB(E) parents (Figs. 3(A) and 4(A)) than they were to the ACB parents (Figs. 3(B) and 4(B)). The large-spiking ORNs of hybrids were most strongly tuned to E11-14:OAc (Fig. 3(C) and (D)), but the responses were of lower frequency at 10 μg than they had been in the ECB(E) parents. Although responses to Z12-14:OAc and E12-14:OAc by the hybrids on this ORN were weaker than to E11-14:OAc, the tuning difference was not as great as in the ECB(E) parents. Also, the responses of the hybrid large-spike-size ORN to Z11-14:OAc occurred rarely and with low-mean spike frequency, despite their being somewhat greater in both parental types and for hybrids in the paired stimulation experiment (Fig. 2). The small-spiking ORNs of hybrids responded strongly to Z11-14:OAc as they did in the ECB parents. There were rare, low-frequency responses of this ORN to E12-14:OAc (Figs. 3(C), 3(D), 4(C), 4(D)), unlike the more consistent high-frequency responses to E12-14:OAc on the small-spike-size attraction-related ORN in the ACB parents (Figs. 3(B) and 4(B)). Generally, the ACB♀ × ECB(E)♂ derived F_1 males, which had been transported as adults, exhibited responses of lower spike frequencies than those for the ECB(E)♀ × ACB♂ derived F_1 males, which were transported as pupae (Fig. 3(C) and (D)). There was no difference in the proportion of successful responses observed between the groups (Fig. 4(C) and (D)).

When order of stimulation is considered in ANOVA for ORN responses at 100 μg in each population, only in ECB(E) for the large-spiking ORN was there a significant interaction effect between the compound presented and the order of presentation ($p = 0.0143$, full details of all ANOVA not shown). For ECB(E), individual comparisons of spike frequency were made considering each ORN response to any compound and whether the Z or E isomers were presented first. The only significance at the $\alpha = 0.05$ level ($p = 0.012$) involved the large-spike-size ORN with respect to Z11-14:OAc. When E11-14:OAc was presented first, the spike frequency in response to Z11-14:OAc at 100 μg was lower (mean = 4.00, S.E. = 1.37) than when Z11-14:OAc was presented first (mean = 8.22, S.E. = 1.32).

Among the ACB♀ × ECB(E)♂ derived F_1 males behaviorally analyzed, about half flew upwind to the source only in response to the ECB(E) pheromone blend, one-quarter only to the ECB(E) and

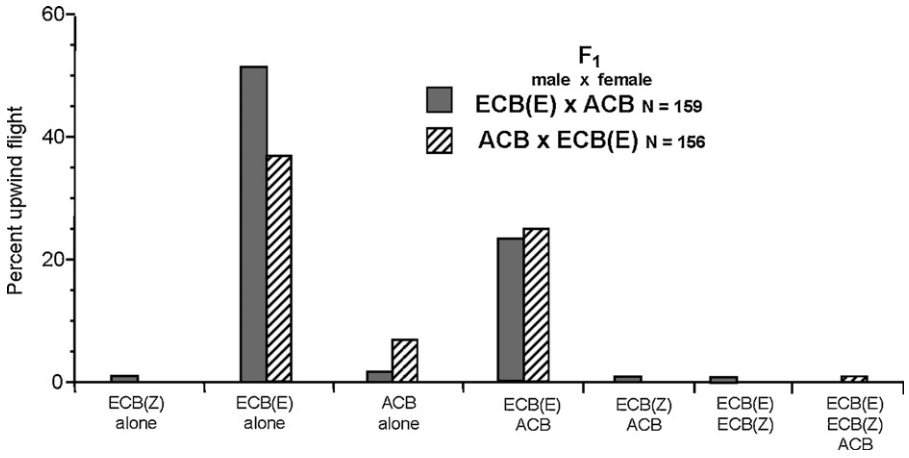

Fig. 5. Phenotypic distribution of F_1 hybrids with respect to whether they flew upwind to the ECB(E), ECB(Z), or ACB pheromone blends alone or to more than one blend. Percentages given out of the total number of males tested in either of the bidirectional crosses.

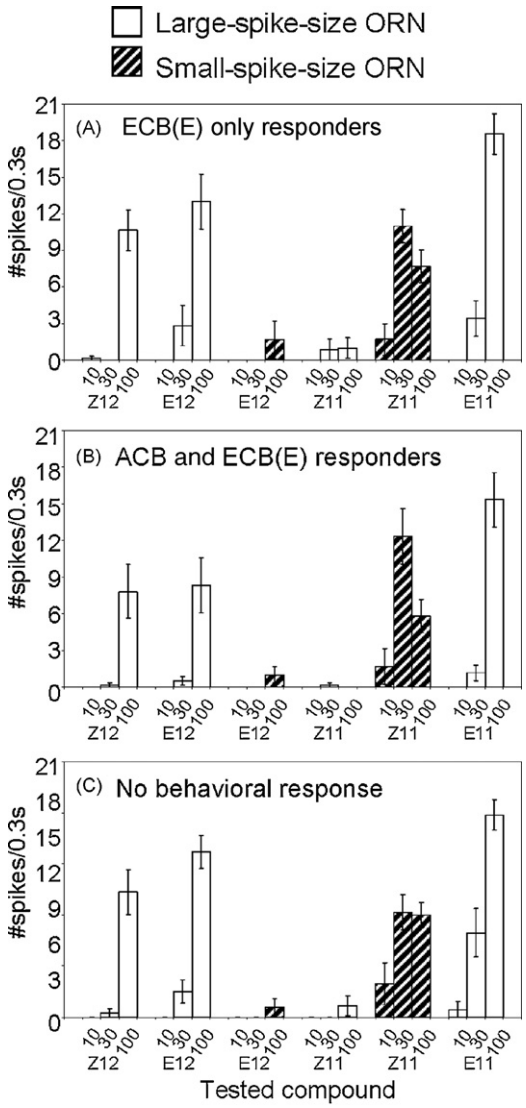

Fig. 6. ORN response (mean ± S.E.) to increasing pipette loadings of Z12-14:OAc, E12-14:OAc, Z11-14:OAc, and E11-14:OAc for behaviorally characterized ACB♀ × ECB(E)♂ F1 offspring; (A) males responding only to the ECB E-strain blend (N = 6 for E12- and Z12-14:OAc, N = 7 for E11- and Z11-14:OAc), (B) males responding to the ACB and ECB E-strain blends (N = 6 for all compounds), and (C) males not responding to ACB or ECB blends (N = 9 for E12- and Z12-14:OAc, N = 10 for E11- and Z11-14:OAc). Shading pattern indicates ORN spike size; white is the large agonistic ORN and striped the small agonistic ORN.

ACB pheromone blends, and another one-quarter did not fly to any pheromone blends tested (Fig. 5). Very small percentages exhibited other phenotypes such as flying to the ACB blend alone, or flying to the ECB(Z) blend either alone or in combination with the ACB or ECB(E) blends. In the reciprocal crosses (ECB(E)♀ × ACB♂), males also fit into the above categories of responding to the ECB(E) blend alone (37%), the ECB(E) as well as the ACB blend (25%), or not responding at all (18%) (Fig. 5). The distribution of behavioral responses differed significantly, using a χ^2 contingency test, which did not include the very rare phenotypes that occurred less at frequencies than 1% ($\chi^2 = 9.58$, $p = 0.02$, 7 d.f.).

The dose–response curves of ORNs among the behavioral groups of ACB♀ × ECB(E)♂ derived F_1 males were nearly identical (Fig. 6). There were no significant differences in spike frequency among the groups with respect to stimulation by 100 μg E11-14:OAc, E12:14:OAc, or Z12-14:OAc (each compound tested separately, $\alpha = 0.05$). There were also no significant differences in spike frequency of the small-spiking ORN to Z11-14:OAc at 30 μg. Responses to Z11-14:OAc at the large-spike-size ORN or E12-14:OAc at the small-spike-size ORN were similarly low, occurring in only one or two of the individuals tested per behavioral phenotype. The probability of the occurrence of such rare responses did not differ significantly among the behavioral categories for either compound (χ^2 tests, Bonferroni correction at $\alpha = 0.05$).

4. Discussion

There are several apparent homologies in the peripheral olfactory systems of ACB and ECB(E) that are also observed in the hybrids. Despite differences in the relative amplitudes of the antagonistic versus the large-spike-size ORNs, spike size relationships remain generally similar across all three populations (Table 1). We observed that there is a large-spike-size ORN that tends to be most broadly tuned, an intermediate spike-sized ORN that is primarily responsive to the behavioral antagonist Z9-14:OAc, and a small-spike-sized ORN that is associated with the minor pheromone components, Z11-14:OAc in ECB(E), E12-14:OAc in ACB, and both compounds in the hybrids (Fig. 7). This result contrasts with crosses between ECB(E) and ECB(Z), in which spike amplitudes are practically indistinguishable among all F_1 hybrids, and among many of the F_2 and backcross progeny (Hansson et al., 1987; Roelofs et al., 1985, 1987; Cossé et al., 1995).

It was previously observed that the large-spike-sized ORN is broadly tuned to ACB and ECB components in ACB (Takanashi et al.,

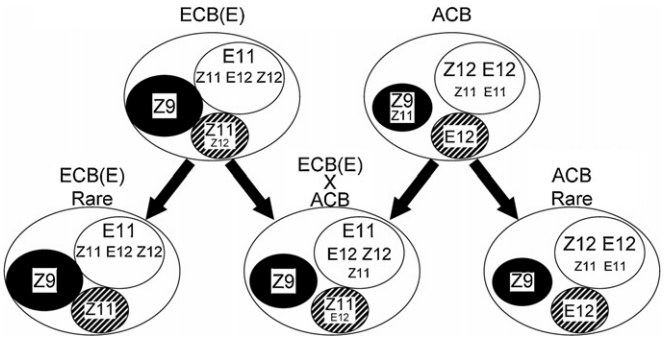

Fig. 7. Summary of ORN responsiveness of ECB(E), ACB, rare ACB (Domingue et al., 2007a) and ECB(E) (Domingue et al., 2007b) that fly to the pheromone of both species, and the F_1 hybrids. The circles within the males schematically represent the ORNs within the trichoid hairs of each species. The size of each circle is approximately proportional to the spike amplitude of each ORN. Abbreviations for the tetradecenyl acetate compounds are included within the ORN they are associated with. Compounds eliciting higher spike frequency responses are listed in larger font and above those eliciting lower frequency responses. The reciprocal F_1 crosses are not distinguished, despite some minor differences.

2006; Domingue et al., 2007a) and ECB(E) (Domingue et al., 2007b). However, responses to Z11-14:OAc on the large-spike-sized ORN were either not observed in earlier studies of ECB(E) (Hansson et al., 1987, 1994; Cossé et al., 1995) or were weak (Domingue et al., 2007b). A careful consideration of the methods employed in this and previous experiments explains why such strong responsiveness to this compound was not found in previous recordings of ECB(E). As noted above, this species is particularly prone to the effects of long-term neuronal adaptation. Once stronger responses have been obtained to the compound that an ORN is primarily tuned to, it tends not to respond to any other compounds. Thus, it is not surprising that responses to Z11-14:OAc on the large-spike-size ORN were not noted in prior studies of ECB(E) males (Hansson et al., 1987; Cossé et al., 1995) when there was not a concerted effort during the testing protocol to minimize the effects of prolonged neuronal adaptation caused by exposure to E11-14:OAc. Furthermore, the response to Z11-14:OAc at 100 μg was significantly greater whenever Z11-14:OAc was presented before E11-14:OAc, in comparison to the reverse order.

In previous studies, the very weak response observed to Z11-14:OAc in ECB(E), in combination with stronger responses to E12-, Z12, and E11-14:OAc seemed difficult to reconcile with the expression of broadly tuned, single ORs on this ORN (Domingue et al., 2007b), prompting consideration that separate, co-expressed ORs might explain this broader ORN responsiveness pattern (Domingue et al., 2007b). However, our new data showing a stronger response to Z11-14:OAc on the large-spike-size ORN, seems more consistent with the possibility that there is a single OR most responsive to E11-14:OAc, but also broadly tuned enough to respond to the other ECB and ACB pheromone components.

In ACB the large-spike-size ORN also is relatively broadly tuned, but the responses to Z11- and E11-14:OAc in ACB are relatively less repeatable and of lower spike frequency than are the responses to Z12- and E12-14:OAc in ECB(E). A reduction in responsiveness to these compounds on the large-spike-size ORN of ACB may have assisted in selection against accidental upwind flights to the females of such species. Responsiveness to Z11-14:OAc on the behavioral antagonism-related ORN may also have arisen from the same selective pressure (Takanashi et al., 2006; Domingue et al., 2007a).

The large-spike-size ORN is also broadly tuned in the F_1 hybrids. In the hybrids, E11-14:OAc stimulated this large-spike-size ORN most strongly, but the responses to Z12- and E12-14:OAc were also of high-spike frequency. In the dose–response experiments, responses to Z11-14:OAc were of relatively low frequency (Figs. 3(C), 3(D), 4(C), 4(D)), but they were of much higher frequency when presented first in paired stimulations of the ORNs (Fig. 2).

With respect to the small-spike-size ORN, ACB is tuned with high specificity to E12-14:OAc, whereas ECB(E) is tuned to Z11-14:OAc, with some low-spike frequency responsiveness to Z12-14:OAc. Responsiveness to Z11-14:OAc on this ORN appears to be strongly dominant in the hybrids. In hybrids, the mean spike frequency (Fig. 3(C) and (D)) and the repeatability of responses (Fig. 4(C) and (D)) to E12-14:OAc are lower than those to Z11-14:OAc, despite the fact that the ACB pheromone components were administered before the ECB components.

We cannot discern from the ORN tuning profiles any differences that would explain particular individual behavioral phenotypes (Fig. 6). It is also important to consider that ACB (Linn et al., 2007) will fly upwind in response to a much greater variety of geometric isomer blends than will ECB (Glover et al., 1987). Thus, the phenotypic variation in F_1 hybrid behavior may be caused by variations in whatever neurophysiological characteristics may have promoted these differences in ratio specificity, or by other unknown physiological factors that may reflect varying thresholds of response. Similarly it is difficult to explain the differences in the frequencies of phenotypic classes between the reciprocal crosses. Because males are the homogametic sex in Lepidoptera, the reciprocal F_1 crosses were identical with respect to chromosomal inheritance. Thus, the incongruities must have derived from either peculiarities of the parents sampled for creating the crosses, other maternal or paternal factors, or environmental interactions. However, it is interesting to note that the ECB(E)♀ × ACB♂ F_1 male offspring had stronger responses to E12-14:OAc on the minor pheromone component associated ORN, and were more likely to respond to the ACB blend alone, and less likely to respond to the ECB(E) blend alone or to the ECB(Z) blend (Fig. 5).

An obvious exception to the overall pattern of homology between the peripheral olfactory systems of ACB and ECB(E) occurs in the tuning of the small-spike-size behaviorally agonistic ORN, which changes abruptly from Z11-14:OAc in ECB(E) to E12-14:OAc in ACB. Also suggesting the important role of this ORN, differences in responsiveness of the small-spiking attraction-related ORN in rare ECB(E) males were correlated to unusual behavioral responses to the ACB pheromone blend (Domingue et al., 2007b). However, in the ECB(E) rare males, responsiveness to the ACB components had decreased rather than increased on the small-spiking attraction-related ORN, making it obvious that other changes in the olfactory system separate ECB(E) and ACB (Domingue et al., 2007b).

Nevertheless, both the current study and the rare male study (Domingue et al., 2007b) point toward the narrowly tuned small-spike-size ORN as having a distinctly important role in facilitating evolutionary change in communication channels. Because the large-spike-size ORN is so broadly tuned, slight changes in responsiveness may not have the large behavioral impact as when changes occur to the more narrowly tuned small-spike-size ORN. In this sense, alterations to the minor pheromone component-related ORN have "evolutionary traction", meaning that they are capable of causing significant shifts in upwind flight behavior characteristic of a potential speciation event. A distinct evolutionary role for elements of the olfactory system associated with minor pheromone components is also supported by studies of the antennal lobe functional morphology in the cabbage looper, *Trichoplusia ni*. There is more variation in the topographical coding patterns for minor pheromone components, which may be indicative of their more recently derived functionality (Anton and Hansson, 1999).

Our results also do not conflict with the hypothesis that responses to Z11-14:OAc on the behaviorally antagonistic ORN

assist in maintaining reproductive isolation by preventing mating between ACB males and ECB females (Takanashi et al., 2006; Domingue et al., 2007a). This trait appears to be recessive and does not affect F_1 hybrids. The absence of this effect in hybrids is clear both from the failure of these compounds to stimulate the antagonistic ORNs of the hybrids, and also from the ease by which F_1 males fly upwind in response to the ECB pheromone blend. Thus, like the ORN associated with the minor pheromone component, the ORN associated with behavioral antagonism also appears to exhibit evolutionary traction.

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