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Altered olfactory receptor neuron responsiveness in rare *Ostrinia nubilalis* males attracted to the *O. furnacalis* pheromone blend

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Abstract

Three percent of E-strain *Ostrinia nubilalis* males fly upwind in response to the *Ostrinia furnacalis* pheromone blend [a 40:60 ratio of (*E*)-12-tetradecenyl acetate to (*Z*)-12-tetradecenyl acetate (E12-14:OAc to Z12-14:OAc)], in addition to their own pheromone blend [a 99:1 ratio of (*E*)-11-tetradecenyl acetate to (*Z*)-11-tetradecenyl acetate) (E11-14:OAc to Z11-14:OAc)]. We assessed the olfactory receptor neuron (ORN) responses of these behaviorally "rare" males versus those of normal males. For the three ORNs housed within each sensillum, we tested responsiveness to Z12-14:OAc, E12-14:OAc, Z11-14:OAc, E11-14:OAc, and the behavioral antagonist (*Z*)-9-tetradecenyl acetate (Z9-14:OAc). Z11-14:OAc, E11-14:OAc, and Z9-14:OAc stimulated ORNs exhibiting distinct small, large, and medium spike sizes, respectively. For rare and normal males, both Z12-14:OAc and E12-14:OAc usually elicited responses from the largest-spiking ORN. In many ORNs of normal males, Z12-14:OAc or E12-14:OAc stimulated the smaller-spiking ORN that is responsive to Z11-14:OAc. In rare males, detectable ORN responses from the smaller-spiking ORN in response to Z12- and E12-14:OAc were virtually non-existent. These differences in ORN tuning in rare males will tend to create an ORN firing ratio between the large- and small-spiking ORNs in response to the *O. furnacalis* blend that is similar to that elicited by the *O. nubilalis* blend.

Keywords: Olfactory receptor neurons; European corn borer; Asian corn borer; Asymmetric tracking; Pheromone shift; Antagonism; Flight tunnel

1. Introduction

Within the genus *Ostrinia* (Lepidoptera: Crambidae), the Asian corn borer, *Ostrinia furnacalis*, stands out as having a markedly distinct pheromone system. *O. furnacalis* females produce blends of (*E*)-12- and (*Z*)-12-tetradecenyl acetate (E12/Z12-14:OAc) at ratios ranging from 1:1 (Klun et al., 1980) to 40:60 (Ando et al., 1980). Highly variable ratios of (E12/Z11-14:OAc) have been shown to be attractive to *Ostrinia furnacalis* males in field trapping (Boo and Park, 1998) and in wind-tunnel experiments (Linn et al., 2007a). The other members of the genus all use blends comprised of (*E*)-11- and (*Z*)-11-tetradecenyl acetate (E11/Z11-14:OAc) as their sex pheromone (Ishikawa et al., 1999), with the exception of *Ostrinia latipennis*, which uses a corresponding alcohol, (*E*)-11-tetradecen-1-ol (E11-14:OH) (Takanashi et al., 2000). Attraction of male *Ostrinia* spp. using Z11- and E11-14:OAc is generally much more tightly tuned to narrow ratios than is the attraction of *O. furnacalis* males to ratios of their E12- and Z12-14:OAc components, as exemplified by the European corn borer, *Ostrinia nubilalis*. This species consists of two pheromone strains, E-strain and Z-strain *O. nubilalis*, which produce and respond maximally to 99:1 and 3:97 ratios of E11/Z11-14:OAc, respectively (Kochansky et al., 1975; Glover et al., 1987). Two other species, *Ostrinia zealis* and *Ostrinia zaguliaevi*, also use a third component in their pheromone blends, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc). Importantly, Z9-14:OAc has been shown to act as a behavioral antagonist, reducing attraction

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of males of other species of the genus, such as *O. furnacalis* (Takanashi et al., 2006; Linn et al., 2007a, b) and *O. nubilalis* (Glover et al., 1989).

Comparison of the *O. furnacalis* pheromone production and response system with that of other *Ostrinia* spp. provides an illuminating framework for understanding how relatively large shifts in pheromone blend composition potentially can occur between closely related moth species (Roelofs et al., 2002; Baker, 2002). Such insight may help to explain the evolution of such a large diversity of pheromone systems used by moths in general. Many previous studies support the close evolutionary relationship between *O. furnacalis* and *O. nubilalis*, including evidence from morphological characters (Mutuura and Munroe, 1970) and allozyme frequencies (Wang et al., 1995). Also the amino acid sequences in the pheromone-binding proteins (Willett and Harrison, 1999) and Δ11-desaturases (Roelofs et al., 2002) are identical in *O. furnacalis* and *O. nubilalis*.

The mechanism by which Z12/E12-14:OAc versus Z11/E11-14:OAc blends are produced has been demonstrated to be controlled primarily through biosynthetic pathways involving either action of a Δ14-desaturase followed by chain-shortening or by chain-shortening first and then action of a Δ11-desaturase (Roelofs et al., 2002), plus additional reductase activities that are specific to certain substrates. Additionally, cross-specific behavioral attraction occurs with surprising regularity. In both *O. nubilalis* strains, some males (3–5%; called "rare" males; Roelofs et al., 2002) are attracted to the *O. furnacalis* blend as well as to their own blend (Linn et al., 2003). Similarly, in addition to being attracted to the *O. furnacalis* blend, 3–4% of *O. furnacalis* males fly upwind to either of the *O. nubilalis* blends, and 1% of *O. furnacalis* males respond to both *O. nubilalis* blends (Linn et al., 2007a).

Investigations of olfactory receptor neurons (ORNs) of *O. furnacalis* males revealed an olfactory-pathway-related mechanism by which the rare males are able to be attracted to *O. nubilalis* pheromone blends (Domingue et al., 2007). Normal *O. furnacalis* male ORNs exhibited responses to Z11-14:OAc on a large-spiking ORN that normally responds to both of its pheromone components, Z12- and E12-14:OAc. However, Z11-14:OAc also elicited action potentials from an intermediate-sized ORN that is highly responsive to the behavioral antagonist, Z9-14:OAc. Rare males differ from normal males only in the lack of responsiveness to Z11-14:OAc by this antagonistic-pathway ORN. It was also proposed that rare males, with this type of ORN, represented a relict of an evolutionary transition when a Z11/E11-based *Ostrinia* spp. ancestor included males with broadened behavioral attraction to also be able to track mutant females emitting Z12/E12 blends. Thus, the normal *O. furnacalis* males were hypothesized to represent a second stage in the evolution of the *O. furnacalis* pheromone system, in which "olfactory antagonistic imposition" had occurred to create assortative mating across the Z12/E12 population and finalize reproductive isolation between *O. furnacalis* and its ancestral *Ostrinia* species.

The rare *O. furnacalis* males thus suggested an olfactory mechanism by which behavioral responsiveness might be broadened or reduced quite simply, even when it involves compounds as different as the Δ11- and Δ12-14-OAc's. This interpretation also provided neuroethological support for a key element of the asymmetric tracking model for moth pheromone evolution (Phelan, 1992, 1997). This model predicts that the non-limiting male sex experiences stronger selection and would more strongly track changes that occur in the limiting female sex. This asymmetry would allow high between-individual variation in female-emitted pheromone blend quality and quantity (Löfstedt, 1990, 1993). At the same time, male responses should broadly bracket typical female emission blends. Males that have further broadened their behavioral response profiles would be necessary to facilitate shifts in pheromone blend composition.

In the current study we were interested in determining any possible differences in ORN response profiles between normal and rare E-strain *O. nubilalis* males that might explain the ability of rare males also to fly upwind in response to the *O. furnacalis* pheromone blend. In E-strain, *O. nubilalis* males there are often three co-compartmentalized ORNs in the trichoid sensilla. Two of these ORNs respond to either of the two pheromone components (Z11/E11-14:OAc) and the third responds to the behavioral antagonist, Z9-14:OAc (Hansson et al., 1987; Cossé et al., 1995). Some sensilla that are usually shorter and more distally located have only one or two ORNs that are also responsive to Z11-14:OAc, E11-14:OAc, or Z9-14:OAc (Hallberg et al., 1994).We used the first sensillum type to compare the responsiveness of each class of ORN to the *O. furnacalis* components in normal E-strain *O. nubilalis* males versus rare males that flew upwind in response to both the *O. furnacalis* and E-strain *O. nubilalis* pheromone blends.

2. Materials and methods

E-strain *O. nubilalis* male moths were obtained from a colony of the "bivoltine E" strain of *O. nubilalis* that has been maintained in the laboratory of W.L. Roelofs as previously described (Roelofs et al., 1985). Pupae were separated by sex, with males placed on a layer of vermiculite in plastic and screen emergence cages. Pupae and emerging males were maintained at 25 °C on a 16:8 L:D photoperiod, 40–50% RH.

Males were tested in the sustained-flight tunnel during their second to third night as adults, under standard conditions for *O. nubilalis* (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 3–6 h of scotophase. Adults were transferred individually to the flight tunnel in screen release cages 1 h prior to the start of scotophase. Thus, there was a 1-h period of acclimation during photophase at 25 °C and typical work-room

fluorescent white-light illumination levels. When scotophase began, illumination was reduced to 11 lx of red incandescent light, and the temperature was reduced to 20–21 °C.

Adult males were tested individually, and a positive response was scored if a male exhibited upwind flight in the odor plume and made contact with the rubber septum source. It was also noted whether or not the male flew upwind at least 10 cm from the release point toward pheromone sources consisting of either the Z-strain or E-strain *O. nubilalis* blends, or the *O. furnacalis* blend, 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).

Behaviorally phenotyped rare and normal males were shipped via overnight courier from New York to Pennsylvania and their ORN responses were recorded when the males were between 3 and 7 days post-emergence. A small portion of other E-strain *O. nubilalis* males that were not behaviorally tested were transported to Pennsylvania as adults before ORN recordings and analyses were performed. Other behaviorally non-phenotyped males were shipped from New York to Pennsylvania as pupae, and stored in similar conditions to those described for Geneva, before testing ORN responsiveness when males were at from 3 to 7 days post-emergence. While the latter males did not undergo behavioral phenotyping, on average only 3% of them will have been likely to be rare males in our ORN response profile analyses. Thus, a sample of behaviorally untested males should not be significantly different from males that do not fly to the *O. furnacalis* blend. Thus, we will also hereafter refer to these untested males taken randomly from the colony as "normal males".

ORN responses were recorded from individual antennal sensilla using the cut sensillum technique (Kaissling, 1974; van der Pers and den Otter, 1978). The base of an excised antenna was 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 by the movements of another micromanipulator, was used to cut the sensillum tip. The cut sensillum was then contacted with 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; linear velocity ~0.3 m/s) through a 25-cm-long glass tube (8 mm ID), the outlet 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 using a stimulus flow-controller device (SFC-2, Syntech). A period of at least 30 s was 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 in any of the preparations, and initiation of the response was easily discerned by the sudden appearance of spike activity after stimulation.

We created pheromone cartridges from solutions of Z11-14:OAc, E11-14:OAc, Z12-14:OAc, E12-14:OAc, and Z9-14:OAc in 10 μg/μl concentrations in 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 compound, 10 μl of solution 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 were thus all 100 μg for these five compounds.

Results of previous research were considered in deciding to use only the 100 μg loading for stimulation. It had been previously shown that 100 μg provides the most effective loading for obtaining repeatable, definitive ORN response differences to the pheromone compounds in Z-strain *O. nubilalis* (Domingue et al., 2006) and *O. furnacalis* (Domingue et al., 2007). In preliminary attempts to obtain ORN responses from E-strain *O. nubilalis*, we experienced difficulty in maintaining connections of sufficient duration to test dosage series of each compound. During these attempts, 100 μg also appeared to be the definitive dosage for obtaining strong, comparative responses among ORNs.

On each antenna, we began with one of the *O. furnacalis* pheromone components, Z12-14:OAc or E12-14:OAc at 100 μg, alternating which compound was tested first. The procedure was continued using cartridges containing Z11-14:OAc, E11-14:OAc, and Z9-14:OAc at 100 μg. Both antennae per moth were examined in this manner. At most, we were able to analyze two sensilla per antenna. We used longer sensilla from near the base of the antenna that were likely to be the type containing all three ORNs for the major pheromone components and the antagonist (Hallberg et al., 1994). Furthermore, our analysis only considered sensilla exhibiting responses to puffs of Z12-14:OAc or E12-14:OAc, which readily occurred in fresh preparations. When the Δ12-14:OAcs did not elicit responses, responses to the Δ11-14:OAcs were obviously weaker, indicating a poor connection. Usually, we were able to obtain ORN responses to all five compounds of interest. We could not rule out that some sensilla were of the type that did not have an ORN for the antagonist, because we sometimes lost connections before the antagonist was tested. Because of the scarcity of these males, all such data were retained for analysis. In none of these truncated sequences were the spike-size identities of the responses to Z12-14:OAc or E12-14:OAc in doubt since

they were clearly emanating from only the largest-spiking ORN.

2.1. Statistical analyses

We were primarily interested in the differences with respect to ORN responses to Z12-14:OAc and E12-14:OAc that might help explain rare male behavior, and thus focused our analyses on these compounds. First, we considered the proportion of sensilla containing ORNs responding or not responding to each treatment. Significance was evaluated using χ^2-tests comparing the number of responding versus non-responding ORNs in sensilla of rare males versus the numbers expected from the proportion of ORNs responding in sensilla from normal males.

Next, we made comparisons involving rare and normal males with respect to the spike frequencies elicited over all the sensilla tested. For responses to the *O. furnacalis* components, the included responses often consisted of no spike activity. Due to the resulting non-normality of the data, we used the MULTEST procedure in SAS 9.1 to perform a bootstrapping technique involving 10,000 re-sampling events. For responses to the *O. furnacalis* compounds on the smaller-spiking ORN, activity occurred in the normal males but not the rare males, making it impossible to statistically compare spike frequency in the two treatments. Here, we employed a *Z*-test, using bootstrap estimates of the mean and standard error, to examine whether the average response was greater than zero in the normal males. A Bonferroni adjustment was applied to correct for the multiple comparisons employed.

Finally, we calculated the ratio of activity on the large- versus small-spiking attraction-related ORNs at 100 μg for the normal and rare E-strain *O. nubilalis* males. This ratio should be roughly similar to the relative ratio of ORN firing experienced by males flying upwind in response to a 1:1 blend of E12/Z12:14:OAc, which is normally highly attractive to *O. furnacalis* males (Linn et al., 2007a). We did not challenge ORNs with these compounds in a blend because we were primarily interested in discerning the specific compound-specific pathway stimulated by each compound. Furthermore, we have found that both E12- and Z12-14:OAc can often stimulate both of the attraction-related ORNs. Thus, our calculations of spike frequency ratios will reflect uncertainty about how the two compounds could possibly interact when presented as blend. For this reason, we calculated the ratio of firing three ways. First, we assumed that in a blend, the responses of ORNs to both compounds would be additive on a given ORN. Next, we assumed that on either ORN, only the maximum level of stimulation by either compound would be achieved. Finally, we assumed that responses will be additive on the smaller-spiking ORN, but will be determined by the maximum stimulation level on the larger-spiking ORN, which tends to be much more highly responsive. We, respectively, abbreviated these calculations as "additive", "non-additive", and "partially additive".

3. Results

We analyzed 22 sensilla from 17 antennae of 15 normal males. We also obtained responses of 13 sensilla from 11 antennae of 11 rare males. As previously reported, we observed strong responses to E11-14:OAc, Z11-14:OAc, and Z9-14:OAc on ORNs having three distinct spike sizes (Fig. 1). E11-14:OAc always evoked activity from the ORN with the largest spike size. As also observed by Cossé et al. (1995), the response to the known antagonist Z9-14:OAc occurred on an ORN that was usually of intermediate spike size, with response to Z11-14:OAc occurring on the smallest ORN. However, in accordance with previous results the spike amplitude was sometimes slightly greater for Z11-14:OAc versus Z9-14:OAc (Hansson et al., 1987; Cossé et al., 1995). Responses of the ORN excited by Z9-14:OAc occurred at a similarly high spike frequency whenever tested on an active connection in either normal (35.6 ± 3.6 spikes/0.3 s) or rare (28.0 ± 4.3 spikes/0.3 s) males. Because responses to any of the *O. furnacalis* and *O. nubilalis* components could never be elicited from this antagonistic-pathway-related ORN, we do not consider its importance any further with respect to behavioral differences that we saw between rare and normal males.

Responses of ORNs to Z12 and E12-14:OAc occurred primarily on the largest-spiking neuron, which is the ORN that responds to E11-14:OAc (Figs. 1 and 2). An additional response to Z12-14:OAc (Fig. 1) or E12-14:OAc often occurred on the smaller-spiking ORN that strongly responds to Z11-14:OAc. These less frequent responses were limited almost entirely to normal males (Fig. 2B). In one single case for a rare male sensillum, one spike attributable to this ORN was observed occurring within an intense spike train from the largest-spiking ORN. The proportion of sensilla housing ORNs responding to Z12-14:OAc in rare males was significantly different than

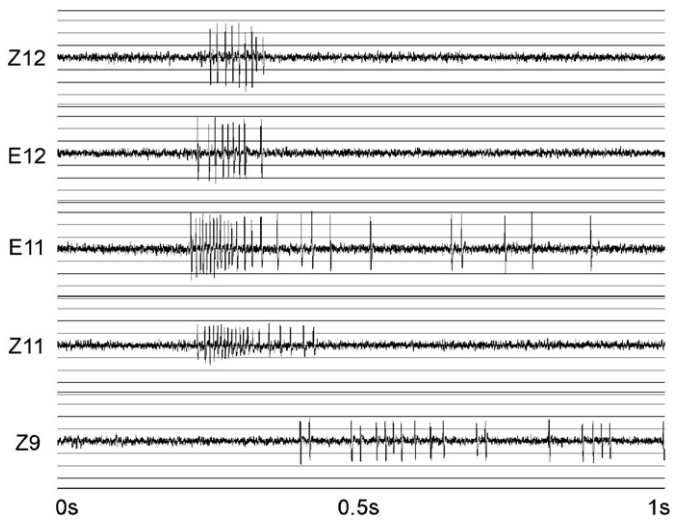

Fig. 1. Sample of recordings from the sensilla of a normal male including response to Z12-14:OAc, E12-14:OAc, E11-14:OAc, Z11-14:OAc, and Z19-14:OAc at a 100 μg cartridge loading.

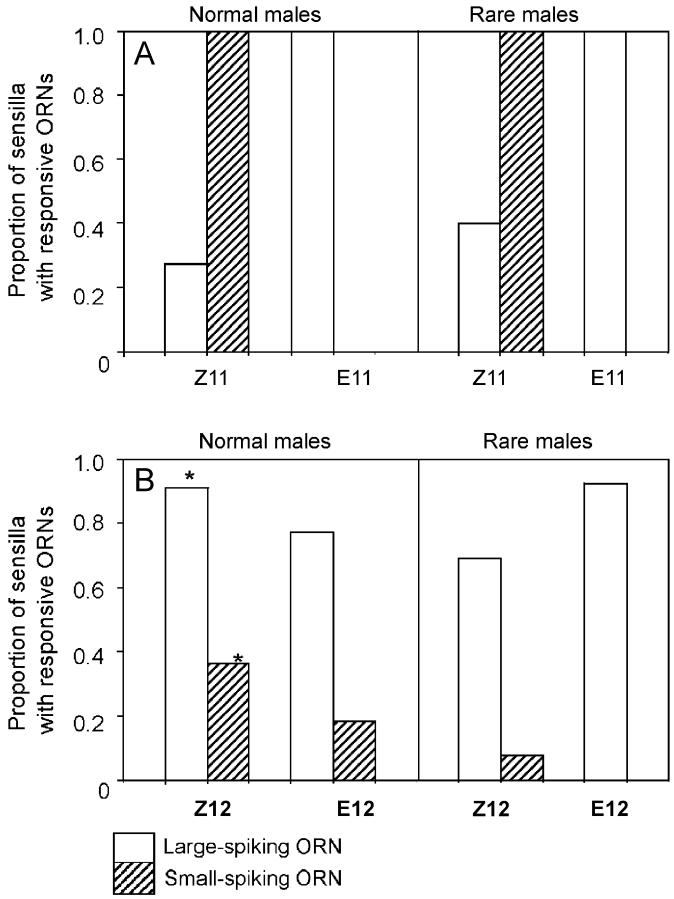

Fig. 2. Proportion of sensilla responding to (A) the *O. nubilalis* components Z11-14:OAc and E11-14:OAc for normal ($n = 22$) and rare males ($n = 10$) and (B) the *O. furnacalis* components Z12-14:OAc and E12-14:OAc for normal ($n = 22$) and rare males ($n = 13$). Clear and shaded bars indicate large- and small-spiking agonistic-pathway neurons, respectively. *Significant difference between rare and normal males (χ^2-test, $\alpha = 0.05$).

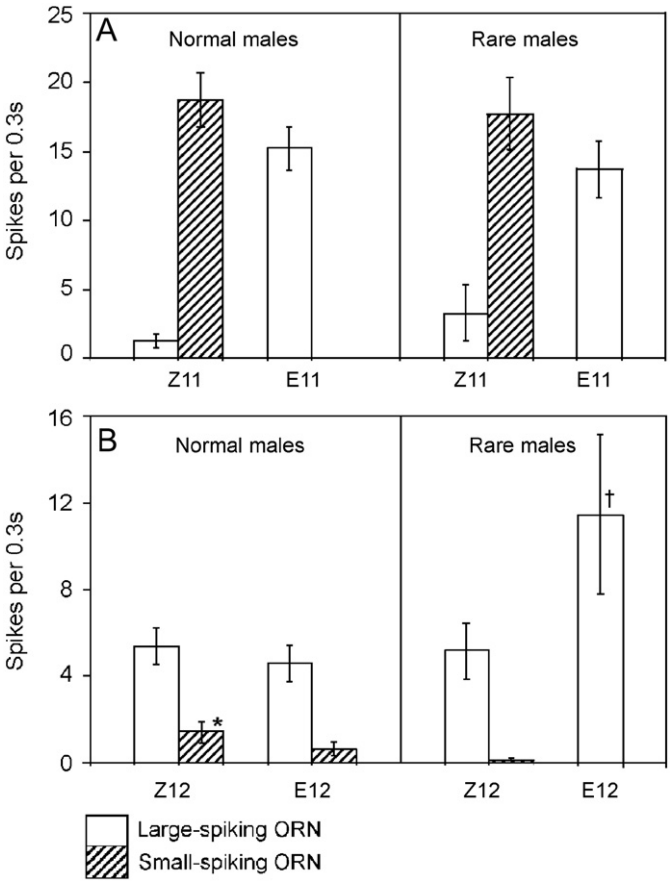

Fig. 3. Average spike frequency in response to (A) the *O. nubilalis* components Z11-14:OAc and E11-14:OAc for normal ($n = 22$) and rare males ($n = 10$) and (B) the *O. furnacalis* components Z12-14:OAc and E12-14:OAc for normal ($n = 22$) and rare males ($n = 13$). Clear and shaded bars indicate large- and small-spiking neurons, respectively. *Average spike frequency is greater than zero, as tested for only the Z12- and E12-14:OAc responses on the small agonistic-pathway ORN in normal E-strain *O. nubilalis* males (*Z*-test, $p = 0.012$). †Spike frequency is significantly greater for rare compared to normal males (*t*-test, $p = 0.044$).

that observed for normal males with respect to responses by both the large-spiking (χ^2, $p = 0.007$) and the small-spiking (χ^2, $p = 0.032$) ORNs (Fig. 2B). No such differences were found with respect to the frequency of ORNs responding to E12-14:OAc.

Large- and small-spiking ORN responses to E11-14:OAc and Z11-14:OAc were of similar frequency in both normal and rare males (Fig. 3A). These response frequencies were much greater than what we observed in response to the *O. furnacalis* components (Fig. 3B). However, Z11-14:OAc also stimulated the largest-spiking ORN in 27% of the sensilla of normal males and 40% of the sensilla of rare males (Fig. 2A). The average spike frequency in response to Z11-14:OAc on the large ORN was similarly weak in normal and rare males (Fig. 3A).

Spike frequency in response to Z12-14:OAc from the large-spiking ORNs was similar in rare and normal males (Fig. 3B) despite the lower percentage of sensilla containing ORNs that were responsive to Z12-14:OAc (Fig. 2B). Also the mean spike frequency of ORNs in response to Z12-14:OAc in normal males from the small-spiking ORN was significantly greater than zero ($p = 0.012$). As described above there was essentially no responsiveness to Z12-14:OAc by the small-spiking ORN in rare males. The response occasionally seen in normal males from the small-spiking ORN to E12-14:OAc did not have a mean frequency greater than zero ($p = 0.102$). The large-spiking ORN of rare males was significantly more responsive to E12-14:OAc than that of normal males (Fig. 3) ($p < 0.044$).

The range of estimates for relative spike frequency on the large and small ORNs were between 2.62:1 and 4.87:1 for normal males (Table 1). These estimates were much larger for rare males, being well over 100:1.

4. Discussion

The first important finding of our study is that E-strain *O. nubilalis* rare and normal males are able to detect heterospecific pheromone components on the same ORNs that respond to their own pheromone components. This

Table 1
Ratio of firing of the large and small agonistic-pathway ORNs at a 100 μg pipette loading of E12- and Z12-14: OAc.

Blend interaction model[a]	E-strain *O. nubilalis* (normal males)	E-strain *O. nubilalis* (rare males)
Additive	4.87	216
Non-additive	3.81	149
Partially additive	2.62	149

[a]Models are defined by whether stimulation by both components would be additive in a blend, occur at the level of the greatest response to either compound (non-additive), or is additive at the small, but not the large agonistic-pathway ORN (partially additive).

property was similarly found in *O. furnacalis* males when presented with *O. nubilalis* components (Domingue et al., 2007). Thus, in both species there is no evidence for the presence of unique ORNs in rare-responding versus normal-responding males that explain the two phenotypes. E-strain *O. nubilalis* males are clearly able to respond to the *O. furnacalis* pheromone components, E12- and Z12-14: OAc, on the same large- and small-spiking ORNs that are responsive to their own pheromone components, E11- and Z11-14:OAc. In particular, the responses of the large-spiking ORN to both E12- and Z12-14:OAc were remarkably strong and consistent, and similar to those that were evoked on the large-spiking ORN of *O. furnacalis* (Takanashi et al., 2006; Domingue et al., 2007). A previous study had shown responses by Z-strain *O. nubilalis* male ORNs to E12- and Z12-14:OAc, but in a much lower percentage of sensilla and with lower overall spike frequencies relative to what we found in this study of E-strain *O. nubilalis* male ORNs (Domingue et al., 2006). In *O. furnacalis*, responses to E11- or Z11-14:OAc occur on all three co-compartmentalized ORNs (Takanashi et al., 2006; Domingue et al., 2007).

A second important finding of our study is that differences in ORN responsiveness that are correlated with rare versus normal male behavior occur on the attraction-pathway ORNs. This result contrasts with the finding in *O. furnacalis* that the differences in ORN responsiveness that are correlated with rare versus normal male behavior occur on the antagonist-related pathway ORNs. The *O. furnacalis* components never stimulated the antagonistic-pathway ORN in E-strain *O. nubilalis* males, even though all the sensilla tested were either demonstrated to have the antagonistic ORN or likely to have this ORN based on sensillum morphology and location.

Thus, the normal versus rare E-strain *O. nubilalis* responses must be considered in the context of their behavioral preferences for a 99:1 E11/Z11-14:OAc blend ratio (Glover et al., 1987; Linn et al., 1997). Rare E-strain *O. nubilalis* males have increased responsiveness to E12-14: OAc on the large-spiking, attraction-related pathway ORN, while concomitantly there is reduced responsiveness to Z12-14:OAc on the small-spiking, attraction-related pathway ORN. The firing ratio between the two attraction-related ORNs occurring in response to mixed blends of *O. furnacalis* compounds would be thus highly skewed towards the large-spiking ORN in rare males when compared to normal males (Fig. 3; Table 1). This altered large-to-small-spiking ORN firing ratio could explain the relative propensity of rare E-strain *O. nubilalis* males to be attracted to this blend. Under ordinary circumstances, high relative stimulation of the large- versus small-spiking agonist-pathway ORNs is expected to occur in response to the normal 99:1 E11:Z11-14:OAc blend, because of the extremely low percentage of Z11-14:OAc. Thus, the CNS of rare males would be expected to integrate the ratios from an *O. furnacalis* blend as being more similar to that received from the E-strain *O. nubilalis* blend. It matters little whether we always recorded from the A versus B type sensilla that, respectively, do or do not have the antagonistic-pathway ORN co-compartmentalized with the agonistic-pathway ORNs (Hallberg et al., 1994). The alteration does not involve the antagonistic pathway, and could potentially involve any agonistic ORNs in any of the sensilla types.

In contrast, for *O. furnacalis* rare males, broad behavioral responsiveness appears to mediate upwind flight to the *O. nubilalis* blends if there is reduced responsiveness to the *O. nubilalis* components on the ORN related to the heterospecific antagonism-related pathway (Domingue et al., 2007). Unlike E-strain *O. nubilalis* males, *O. furnacalis* males' upwind flight behavior does not appear to be strongly constrained by CNS interpretations of blend ratios on their attraction-pathway ORNs in the field (Boo and Park, 1998) or wind-tunnel (Linn et al., 2007a). In this context, it was not surprising that rare versus normal *O. furnacalis* males did not show differences in the activities of their attraction-pathway-related ORNs.

Understanding the process by which co-receptivity to both the *O. furnacalis* and *O. nubilalis* pheromone components has occurred on these attraction-related ORNs is critical to understanding how behaviorally correlated changes in ORN responsiveness have occurred to affect the evolution of *O. furnacalis* and *O. nubilalis* pheromone systems. Historically, it has been assumed that there is one olfactory receptor (OR) per ORN. More recently, the potential of insects to express multiple olfactory receptors (ORs) on single ORN dendrites has been demonstrated in the general-odorant-responsive ORNs in *Drosophila melanogaster* and *Drosophila. pseudoobscura* (cf., Goldman et al., 2005). Conclusive evidence of such co-expression of multiple ORs per ORN does not yet exist for any moth pheromone systems. However, one type of ORN on the antenna of *Heliothis subflexa* × *Heliothis virescens* hybrids exhibits patterns of co-responsiveness to two different components that cannot be explained by a mere broadening of responsiveness by a single OR gene (Baker et al., 2006).

For E-strain *O. nubilalis*, it remains reasonable that the traditional singly expressed OR model explains the responses to both *O. furnacalis* and *O. nubilalis*

components on the attraction-pathway ORNs. In this case, the ability of the attraction-related ORs on E-strain *O. nubilalis* male ORNs to accept both the Δ12 and Δ11 components would be due to the chemical structural similarities of the pheromone components of the two species, plus a favorable receptor interaction. In the context of the single OR expression model, it is of particular interest to consider the response profile of the large-spiking ORN of E-strain *O. nubilalis*, which was frequently responsive to Z12-14:OAc, E12-14:OAc, and E11-14:OAc. In a previous study, *O. furnacalis* was found to have an ORN tuned to Z12-14:OAc, E12-14:OAc, and Z11-14:OAc (Domingue et al., 2007). Responding to three of these compounds but not to Z11-14:OAc for E-strain *O. nubilalis* and not to E11-14:OAc for *O. furnacalis* would seem to require two different single OR conformations in the two species.

When we compare E-strain *O. nubilalis* rare versus normal male responses on this large-spiking ORN, there was no difference in responsiveness to E11-14:OAc, while spike frequency in response to E12-14:OAc was elevated in rare males (Fig. 3). Similarly responses on the small-spiking ORN to Z11-14:OAc did not differ significantly between rare and normal males, but responses to Z12-14:OAc were significantly diminished in rare males (Fig. 3). These particular aspects of the results could potentially be explained by differences in the co-expression of multiple ORs on the dendrites of rare versus normal males' ORNs. On the other hand, it is also fully possible that such variation could arise from particular conformational differences between singly expressed ORs on the males' ORNs.

Responsiveness to multiple compounds per ORN in some systems appears to be directly related to ecologically important aspects of signaling such as behavioral antagonism in response to heterospecific pheromone components (Löfstedt et al., 1991; Cossé et al., 1998; Takanashi et al., 2006; Domingue et al., 2007; Linn et al., 2007b). However, in some cases ORNs respond to additional non-target compounds, many of which have never been shown to play a part in any moth sex pheromone systems. For instance, (*E*)-6-tetradecenyl acetate causes action potential activity in *Yponomeuta rorellus* ORNs that are tuned to its sex pheromone component tetradecyl acetate (Löfstedt et al., 1990). Likewise, 1-(*Z*)-12-heptadecadiene and (*Z*)-9-tetradecenyl formate stimulate the *Helicoverpa zea* ORN tuned to its major component (*Z*)-11-hexadecenal (Grant et al., 1989).

We would propose that whether ORs are singly or co-expressed is indicative of how likely it is that ORN responsiveness to multiple compounds has evolved adaptively versus non-adaptively. From the perspective of the co-expression model, OR specificity would be very high, with ORN responses to particular compounds unlikely to exist unless directly selected for. However, for singly expressed OR systems, cases of multiple responsiveness to compounds require more broadly tuned ORs, which could often accommodate compounds that are not involved in behavioral attraction or antagonism.

The finding of responses to the Δ12-14:OAc's in E-strain *O. nubilalis* on the attraction-related pathway ORNs of normal E-strain *O. nubilalis* raises the interesting evolutionary question of why such non-adaptive heterospecific responses occur. We believe this question cannot be answered until more is known about how olfactory receptors are expressed on the dendrites. If responses to the Δ12-14:OAcs on the attraction-related pathway ORNs of E-strain *O. nubilalis* are based upon co-expression of additional ORs, we must primarily consider adaptive evolutionary explanations for this trait. From a purely adaptationist perspective, responses to the Δ12-14:OAcs in E-strain *O. nubilalis* could only be presumed to be a relict of use of these compounds in the pheromone systems of ancestors of E-strain *O. nubilalis*. This hypothesis is difficult to reconcile with the likelihood that use of the Δ12-14:OAcs in *Ostrinia* recently evolved just once in the lineage leading to *O. furnacalis*, the only species employing such compounds as pheromone components (Ishikawa et al., 1999).

It is thus appropriate to caution that, given the single OR expression model, ORN responses of E-strain *O. nubilalis* males to *O. furnacalis* components may have no history of prior adaptive value. The ORs that had evolved for detection of the Δ11-14:OAcs may also be accommodating enough to allow ORN responses to structurally similar compounds like the Δ12-14:OAcs. Applying this paradigm, moth populations may express a latent potential for significant shifts in pheromone usage to novel compounds that have never been encountered before. The responsiveness to the Δ12-14:OAcs we observe in E-strain *O. nubilalis* would demonstrate the potential of an evolutionary shift toward the usage of the Δ12-14:OAc compounds. Based on this evidence alone, it should not be presumed that E-strain *O. nubilalis* and *O. furnacalis* are directly linked phylogenetically. The potential for shifts to the use of the Δ12-14:OAcs is not unique to the E-strain *O. nubilalis* population. There are also Z-strain *O. nubilalis* rare males capable of responding to *O. furnacalis* pheromone blends (Linn et al., 2003).

Ultimately the question of whether there are distinct co-expressed ORs for the Δ11- and Δ12-14:OAcs or singly expressed more broadly tuned ORs can be resolved if the genes for the pheromone-component-related ORs of *O. furnacalis* and *O. nubilalis* can be characterized and then their level of co-expression on ORNs analyzed using double-labeling in situ hybridization techniques. It will also be important to consider that either system or aspects of both systems could be applicable in different scenarios. Regardless of which OR expression model is best suited to the attraction-related ORNs of E-strain *O. nubilalis*, our data shows that ORNs are malleable enough to allow males to maintain their original target ORN response patterns while tracking other prospective communication channels. It is this malleability that is needed to explain the

historically noted paradox of extensive moth sex pheromone communication diversity despite strong stabilizing selection between pheromone emitters and receivers (Phelan, 1992, 1997; Löfstedt, 1990, 1993; Baker, 2002).

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