Sexual isolation of male moths explained by a single pheromone response QTL containing four receptor genes

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Long distance sexual communication in moths has fascinated biologists because of the complex, precise female pheromone signals and the extreme sensitivity of males to specific pheromone molecules. Progress has been made in identifying some genes involved in female pheromone production and in male response. However, we have lacked information on the genetic changes involved in evolutionary diversification of these mate-finding mechanisms that is critical to understanding speciation in moths and other taxa. We used a combined quantitative trait locus (QTL) and candidate gene approach to determine the genetic architecture of sexual isolation in males of two congeneric moths, Heliothis subflexa and Heliothis virescens. We report behavioral and neurophysiological evidence that differential male responses to three female-produced chemicals (29-14:Ald, 29-16:Ald, Z11-16:OAc) that maintain sexual isolation of these species are all controlled by a single QTL containing at least four odorant receptor genes. It is not surprising that pheromone receptor differences could control H. subflexa and H. virescens responses to 29-16:Ald and 29-14:Ald, respectively. However, central rather than peripheral level control over the positive and negative responses of H. subflexa and H. virescens to Z11-16:OAc had been expected. Tight linkage of these receptor genes indicates that mutations altering male response to complex blends could be maintained in linkage disequilibrium and could affect the speciation process. Other candidate genes such as those coding for pheromone binding proteins did not map to this QTL, but there was some genetic evidence of a QTL for response to Z11-16:OH associated with a sensory neuron membrane protein gene.

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volutionary diversification of sexual communication traits remains paradoxical (1, 2) because signal production and signal reception are under independent genetic control, and a mutation causing an alteration in one component of the system is predicted to reduce efficiency of communication and to cause a loss of fitness. The resulting stabilizing selection is expected to promote evolutionary stasis, not diversification (3–5). Systems in which changes in signals and responses are governed by the same genetic alterations (i.e., pleiotropy) should be less evolutionarily constrained in many cases (6), and studies of mating communication have revealed a few systems that appear to have this property (7–9). However, no pleiotropy has been found between signal production and response in moths (e.g., 5, 10). Because female and male moths with divergent signals and responses appear to be selected against (11, 12), we have no simple explanation for the great diversity of moths (~180,000 species) and moth pheromones (5, 13, 14).

Beyond capturing the attention of evolutionary biologists, the diversity of long distance, pheromone-based sexual communication traits in moths has become a focus of some molecular biologists, biochemists, neurophysiologists, and communications specialists because of the surprisingly high specificity of signals and responses within a species and the clear differentiation in signaling systems among species (15, 16). In the past 20 years, a great deal has been learned about the biosynthetic pathways that result in precise ratios of specific compounds in pheromone blends (17), and major breakthroughs have recently been made in our understanding of the mechanisms that enable a male to detect incredibly low concentrations of pheromone molecules (18, 19). We now know that peripheral reception of pheromones involves a number of proteins in male antennae, including pheromone binding proteins (PBPs), general odorant binding proteins (GOBPs), chemosensory proteins (CSPs), two classes of odorant receptors (ORs), pheromone degrading enzymes (PDEs), and sensory neuron membrane proteins (SNMPs) (19–21). Genetic changes in the structure/expression of any or all of these proteins could have been involved in evolutionary diversification of moth sexual communication systems, but it is also possible that the crucial changes were in the male moth central nervous system (5, 22).

One pioneering study tested two races of the pyralid moth Ostrinia nubilalis (European corn borer) for a relationship between genetic traits for antennal neuron spike patterns and male pheromone response but found none (23). A related study on Ctenopseustis moths (brownheaded leafrollers) did find a correlation (24) but did not examine the genes that could be involved. More recently, correlations have been found between neuron targeting and pheromone responses (25, 26), but their genetic basis is unknown. As pointed out by Smadja and Butlin (5), a critical next step in understanding how mate communication systems diversify is the determination of what kinds of changes in which genes resulted in divergent signals and responses of closely related species and races.

Here we focus on understanding the genetic architecture and genes involved in the sexual communication differences between two closely related, nonsister species of heliothine moths with a divergence time of approximately 2 million years based on CO-I divergence (27). Heliothis virescens (hereafter referred to as Hv) is a generalist, feeding on plants in over 14 families, whereas Heliothis subflexa (Hs) specializes on plants within the genus Physalis (28, 29). These two species are not attracted to each other in field locations where they cooccur because of differential response to pheromone blends (28), but they can be mated and backcrossed in


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those that did not inherit this chromosome. Of 90 males that presented with this blend in a wind tunnel, 36 more likely to respond positively to the blend with 9-14:Ald than those that did not inherit this chromosome. Of 90 males that presented with this blend in a wind tunnel, 36 flew at least halfway toward the pheromone source, suggesting that these males had inherited an Hv chromosome with gene(s) for response to 9-14:Ald. In a smaller experiment, Hv-BC males were tested for flight toward a blend with Z9-16:Ald, Z9-16:Ald, and Z11-16:OH, in a 1:0.05:0.1 ratio, which is attractive to F1 males but not to Hv males (31, 42). Fifteen of 28 males flew at least halfway to the pheromone source, suggesting that they had inherited Hv gene(s) for response to the Z9-16:Ald.

**Genetic Maps and QTL Analysis.** To determine which Hv chromosomes each Hv-BC male inherited and which Hv chromosomes each Hv-BC inherited, we used previously developed Amplified Fragment Length Polymorphism (AFLP) methods (29) to build genetic maps of the segregating BC families (BC to Hv, 230 AFLP markers, range 3–18 per chromosome; BC to Hs, 211 AFLP markers, range 3–19 per chromosome) (see SI Text, Table S1 for specific primers used).

Once the genotyping was completed, we used G-tests with Yates corrections (43) to check for associations between male response and the presence/absence of specific chromosomes from the nonrecurrent parent (44). In the Hs-BC, only one of the Hv chromosomes [chromosome 27 (Hv-C27)] showed a statistically significant association with male flight toward the blend. Of the males with one copy of Hv-C27, 34 of 40 had flown at least halfway toward the Z9-14:Ald-containing blend, but only 2 of 50 males without Hv-C27 had flown at least halfway toward the blend ($\chi^2 = 9.98, P = 0.0016;\text{Bonferroni}\), correction = 0.048). No other Hv or Hs chromosomes were associated with male response ($P = 0.05$ without Bonferroni correction). To better visualize the differences between responses of males with and without C27 from the nonrecurrent parental species Fig. 1 presents the degree of response by males of each genotype. Clearly, C27 contained one or more critical DNA sequences that differentiate male responses of the two species with respect to the preference of either Z9-14:Ald or Z9-16:Ald.

**Maps of Candidate Genes.** We mapped genes coding for 11 ORs, 2 PBPs, 1 SNMP, 2 CSPs, and 1 GOBP to individual autosomes or to the sex chromosome (Materials and Methods). We included some ORs that are expressed in both males and females because recent evidence indicates that females perceive certain components present in their own pheromonal mixtures (45, 46). We first mapped these genes in an Hv-BC family, testing for nonindependent segregation of the Hs allele of each candidate gene and the Hs form of one of the autosomes. Genes were mapped to the sex chromosome based on presence of the Hs allele of the candidate gene in BC males but not in females (females are the heterogametic sex). For confirmation, we then mapped the genes in an Hs-BC family. Table 1 presents the chromosomal locations of all 17 candidate genes. Five chromosomes had only a single OR gene, although chromosome 19 and chromosome 27 each had two and four OR genes, respectively. Genes for all of the PBPs, CSPs, and GOBP1 were found on the same chromosome (C19). H13, which is the OR for Z11-16:Ald (36), the most abundant pheromone component of Hv and Hs, was the only receptor that mapped to the sex chromosome. Genes coding for H14, H15, and H16, which have been associated with response to other critical Hv pheromone compounds (35, 47), all mapped to C27, which was the only chromosome that cosegregated with male response to the Z9-14:Ald and Z9-16:Ald in our QTL analysis above. HR6 was the only other candidate gene that mapped to C27. This OR could also be involved in pheromone response but its lack of tissue-specific expression makes it a less likely candidate (35, 47). These results suggest that changes in coding and/or regulatory regions of one or more of these OR genes are
males with Hs-C27 was 4.40 on our scale from 0 to 7 (Materials and Methods), whereas for those without Hs-C27 it was only 1.97.

After the fifth BC, males that were heterozygous (one copy of Hs-C27 and one of Hv-C27) were crossed to Hv females to allow recombination within C27. No recombination among the four OR genes was found among the 62 offspring analyzed, but we were able to use this male BC to map the general location (within approximately 6 cm) of the OR genes within C27 by use of 14 recombining AFLP markers and the HR14 codominant marker (Fig. S1). In a final attempt to separate the OR genes through recombination, we analyzed a total of 574 BC offspring (six male Hv–BC families) for Hv and Hs alleles of HR14, HR15, and HR16, but we did not find a single recombinant. This suggests that the HR genes are a tightly linked cluster of duplicated genes.

Further Backcrossing to Isolate a Smaller QTL Region for Response to Z9-16:Ald. Backcrossing of males that were positive for the Hs-HR14 allele, as determined by PCR, was continued for 15 more generations for allow for recombination and eventual loss of segments of Hs-C27 that were not tightly linked to the OR gene cluster. One half of the male offspring from the 15th BC generation of backcrossing were expected to be identical to Hv for all autosomes, sex chromosomes, and mitochondria, with the exception of being heterozygous for Hs genes that are physically linked to Hs-HR14.

The other half of the offspring were expected to be genetically identical to Hv. Male offspring (n = 80) were flown in a wind tunnel to blends containing Z9-16:Ald. If a male did not respond to this blend (by more than simply taking flight), it was flown to a blend with Z9-14:Ald. Of 58 males with one copy of the Hs-HR14 allele, 45 responded to the blend with Z9-16:Ald with a mean degree of response equal to 3.89. The 13 males with Hs-HR14 that did not respond to this blend also did not respond to the blend with Z9-14:Ald. Of 22 males without the Hs-HR14 allele, none responded to the blend containing Z9-16:Ald and 19 responded to the blend with Z9-14:Ald blend with a mean degree of response of 5.58. AFLP analysis of nine markers on C27 revealed that four of the males with the Hs-HR14 allele only had one or two of the AFLP markers from Hs. All of these males responded to the Z9-16:Ald blend. Furthermore, no relationship was found between the number of Hs-C27 AFLP markers (range 1–9) and the degree of response (0–7 scale) to the Z9-16:Ald-containing blend (Spearman rank order correlation = -0.18; P = 0.203). This indicates that only the four HR genes and genes tightly linked to them could be controlling male response to the Z9-16:Ald.

Response to the Acetate, Z11-16:OAc. The Z11-16:OAc component of the Hs blend enhances Hs male response but decreases that of Hv (28, 49). Grosse-Wilde et al. (35) found, evidence that Z11-16:OAc is a ligand for HR14 based on expression of HR14 in receptor neurons neighboring those expressing HR16 (also see ref. 47) as well as binding affinity in a heterologous system. These results suggest that the Hv and Hs alleles of HR14 on C27 could impact male response to Z11-16:OAc. Therefore, we flew BC males to pheromone blends with and without this odorant. Previous work had established that F1 males were partially deterred by Z11-16:OAc (42), so we considered its deterrent effect to be codominant. In one test, single pair crosses were conducted between males and females that were each genetically identical to Hv except for being heterozygous for the full Hs-C27 chromosome (as determined by the codominant HR14 genetic marker). The offspring of this cross were expected to segregate into three genotypes: homozygotes for Hs-C27, heterozygotes, and homozygotes for Hs-C27. Male offspring from these crosses (n = 62) were first flown to a blend of Z11-16:Ald/Z9-16:Ald/Z11-16:OH/Z11-16:OAc in a 1:0.5:0.1:0.1 ratio. They were then flown to a similar blend that lacked the acetate, which was expected to be more attractive to males that were heterozygous for Hs-C27. Males that did not respond to either of these blends were flown to the Hv blend of Z11-16:Ald.

**Table 1. Candidate genes mapped to Heliothis chromosomes**

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<td>27</td>
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<td>CSP2</td>
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<td>HR11</td>
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<td>GOBP1</td>
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<td>HR12</td>
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<td>PB1</td>
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Z9-14:Ald in a 1.0:0.05 ratio. We specifically chose this order of exposure because males generally respond more strongly to their first exposure and preliminary tests indicated that this order resulted in more response to blends with the acetate. Nine males did not respond at all, and of the 16 responsive males that were homozygous for Hv-C27 chromosome, 15 only responded to the Hv blend. Of the males that were heterozygous for Hs-C27 (n = 25), 80% responded less to the blend with the acetate than to the blend lacking the acetate. In contrast, only 25% of males homozygous for the Hs-C27 (n = 12) responded less to the acetate-containing blend ($\chi^2$ with Yates correction = 7.80; $P = 0.0052$—no need for Bonferroni correction).

To confirm this finding, we tested males from a typical BC of a hybrid female to an Hv male where there were only two C27 genotypes, those that were homozygous for the Hv-C27 chromosome and those that were heterozygous (one copy of C27 from Hs and one copy from Hv). We tested these males to a blend of Z11-16:Ald/Z9-14:Ald/Z11-16:OH/Z11-16:OAc in a 1:0.1:0.1:0.1 ratio and then to this blend without the acetate. We expected the blend with acetate to be more deterrent to homoyzogous for Hv-C27. Of males homozygous for Hv-C27 (n = 34), 44.1% responded less to the blend with the acetate. In contrast only 6.9% of heterozygous males (n = 29) responded less to the blend with the acetate ($\chi^2$ with Yates correction = 11.37, $P = 0.0007$). For both heterozygotes and homozygotes, more males responded positively to the blend with acetate than we had expected. The higher relative responsiveness to the acetate blend could be due to the fact that their first exposure was to this blend.

**Response to the Alcohol, Z11-16:OH.** Results from Grosse-Wilde et al. (35) demonstrate that Z11-16:OH is a ligand for HR16, the gene for which is found on C27. Therefore, we also tested the potential involvement of C27 in the differential response of Hv and Hs to Z11-16:OH. Hs males require this compound to be attracted, although Hv males respond to blends without it (32). Because F1 hybrid males are more responsive to blends without Z11-16:OH than Hs males, we treated response to this compound as a codominant trait and tested response of male offspring from a first female BC to Hs. About 50% of these males were expected to be heterozygotic for C27, and the other 50% were expected to be homozygous for Hs-C27. Males were first flown to a blend of Z11-16:Ald/Z9-16:Ald in a 1.0:0.5 ratio, after which they were flown to a similar blend with the addition of Z11-16:OH at a 0.1 ratio. We tested whether males that were heterozygous for Hs-C27 were more likely to respond to the blend without the alcohol than males homozygous for the Hs form. Of 55 heterozygotes, 67% responded to the blend without the alcohol, and of 48 homozygotes, 77% responded ($\chi^2 = 1.228, P = 0.2678$). This indicates that Hs homozygote and heterozygote males do not differ. The data from this experiment were also analyzed based on degree of response. Of 54 heterozygotes that could be scored, 72% had a higher score when the alcohol was present, although 57% of 47 homozygous males had a higher score with the alcohol present ($\chi^2 = 2.394, P = 0.1218$). This indicated clearly that C27 did not affect the requirement for Z11-16:OH by males.

To follow up on these results, we used AFLPs to map all 30 autosomes from this BC to Hs. We tested each autosome for effects on the ratio of males that responded more to the blend that included the alcohol. Only chromosome 24, which includes an SNMP gene, showed a significant $P$ value ($\chi^2 = 6.88, P = 0.0087$) but when adjusted using a Bonferroni correction the $P$ value became 0.261. Therefore, no single autosome appears to strongly affect male response to Z11-16:OH. We could not test for a relationship between the species origin of two male Z sex chromosomes and response to the alcohol because all males in this BC are homozygous for the Z chromosome of Hs.

**Recordings from Single Antennal Neurons.** Previous single cell recordings from male antennal sensilla have shown differences in response of male Hv and Hs neurons to specific pheromone components (50). We hypothesized that if alleles of the HR genes in Hv and Hs were responsible for the behavioral differences among male genotypes, then we should find distinct electrophysiological responses in moths with Hs versus Hv receptor alleles. We compared the electrophysiological responses of the following types of males: (i) pure Hv, (ii) pure Hs, (iii) Hv except for being homozygous for Hs-C27. We recorded independently from B-type and C-type sensilla that are described in Baker et al. (50). As expected from Baker et al. (50), the neurons in B-type sensilla of pure Hv were activated by low concentrations of Z9-14:Ald but not Z9-16:Ald (Fig. 2 A and B). B-type sensilla of Hs males were somewhat more sensitive to Z9-16:Ald than to Z9-14:Ald. The males that were identical to Hv except for having Hs-C27 responded much like the pure Hs males. The neurons of C-type sensilla of Hv responded strongly to both Z11-16:OH and Z11-16:OAc (Fig. 2 C and D). Hs C-type sensilla also responded strongly to Z11-16:OH but only weakly to Z11-16:OAc. The response to Z11-16:OAc by Hv males with Hs-C27 was generally similar to the pure Hs males. Curiously, the Hs-C27 males had a stronger response to Z11-16:OH than the pure Hv and Hs males, which were quite similar to each other (Fig. 2D). These electrophysiological results reinforce the behavioral genetic studies in that the C27 genotype affected response to the two aldehydes and the acetate, but did not affect response to the alcohol.
Discussion

DNA sequence differences in a tightly linked portion of C27 cause major differences between Hs and Hv males in their responses to Z9-16:Ald and Z9-14:Ald, and are also responsible for at least a substantial portion of the difference between the two species in their responses to Z11-16:OAc. It is surprising to find that a change in receptors for Z11-16:OAc could be responsible for the switch between the positive behavioral response of Hs and the negative response of Hv to this compound. In *Drosophila* where cis-vaccenyl acetate is attractive to females but repulsive to males, it is a central nervous system difference that controls the switch between positive and negative response (22). Furthermore, previous work showed that acetate receptor amino acid sequence differences in the neurons of *Drosophila melanogaster* (19). If this assumption is incorrect, it could be that changes in the acetate receptor amino acid sequence could alter the targeting of the receptors. We have not yet determined if the differential glomerular targeting in Hv and Hs maps to C27.

From work with *Drosophila* and other insect genomes it is apparent that OR genes are often clustered within small chromosomal regions and appear to have arisen from gene duplication (52). Our finding of four OR genes in a segment of C27 that rarely if ever recombines is in line with this previous information. However, our recombination data cannot differentiate between linkage due to close tandem genes versus species-specific insertions (53). A phylogenetic analysis of Hv OR proteins indicates that HR6, HR14, HR15, and HR16 are more closely related to each other than any other known Hv OR proteins, arguing for gene duplication (34). HR11 and HR13 are also closely related to this group of four, but their genes map to chromosome 3 and the Z sex chromosome, respectively. HR13 is the receptor for the major component of the Hv and Hs pheromones and HR11 has recently been shown to have time and cell-specific expression patterns linked with HR13 (37). The receptor for bombykol, the major component of the silkmoth (*Bombyx mori*) pheromone, clusters phylogenetically with HR13 (54) and is also located on the sex chromosome (55). Functional and comparative genomic studies of Hv, Hs, and related moth species will help to determine the evolutionary processes that have shaped the location and sequence divergence of these pheromone-related OR genes.

Our study revealed that the genes for three other classes of proteins associated with peripheral reception of odors (CSP1, CSP2, GOBP1, PB1, and PB2) are all found on the same chromosome. We have yet to perform fine-scale mapping of chromosome 19 to determine their locations relative to each other. Although these genes could all be involved in differential perception and response to species-specific pheromones, our QTL analysis found no evidence of their involvement in differences between Hv and Hs male response. Although an SNMP gene on chromosome 24 may influence response to Z11-16:OH, the data are not strong because the multiple tests involved increase the chance of type I errors. An independent test of this association would be useful.

The tight genetic linkage of receptors that are critical to species isolation could have important evolutionary consequences both in terms of the build-up of linkage disequilibrium between novel alleles of multiple receptor genes early in the process of speciation (56) and for evolution of strong isolation after speciation occurs (57). A number of theoretical analyses suggest that the number of independently assorting sexual isolation genes can affect the likelihood of speciation (e.g., 6, 58, 59). Our current data cannot test whether the tight linkage of OR genes involved in pheromone reception is simply an outcome of genome evolution that has a side-effect on speciation, or if selection acted to build this linkage through species-specific inversions (60). Our data also bring up the question of whether selection or a general property of the genome led to HR13 and HR11 being on different chromosomes. Only more detailed comparative studies will shed light on these questions.

Even though our study suggests that a few genes can strongly impact differential male response to important pheromone components, we do not know how many independent changes in base pairs, or indels within these genes affect male behavior. Sequence comparisons among moth species and heterologous expression of mutated receptor genes would help in assessing which differences in the C27 receptor gene region have significant impacts on male behavior.

Materials and Methods

**Mapping Candidate Genes.** The DNA processing and assignment of specific AFLP markers to specific chromosomes followed procedures described in Sheck et al. (29). The primers used and the number of markers per chromosome are provided in Table S2. We designed PCR primers for 11 ORs, 2 PBPs, 1 SNMP, 2 CSPs, and 1 GOBP based on Hv sequences in GenBank (Table S2). Only primers that reliably amplified both Hv and Hs genomic DNA and produced bands with species-specific migration patterns on agarose gels were used as codominant markers (see Table S2 and S3 for specific genes, restriction enzymes, primers, PCR conditions, and migration values). PCR products were sequenced to check for identity with the candidate gene sequence in GenBank. DNA of Hv-BC offspring and the Hz-BC offspring was amplified using each of the 17 primer pairs separately, and the products were run on agarose gels (number of BC offspring tested for each candidate gene: mean (SD) = 19.3 (1.5)). For each of the candidate genes, the correspondence was assessed between the presence of bands representing Hv and Hs alleles of the candidate gene in the BC and the presence/absence of bands from the Hv or Hs AFLP markers for the 30 autosomal linkage groups in the same insects (see ref. 61). In all cases, an obvious match between the codominant marker for a candidate gene and a single AFLP-identified chromosome was found (three mismatches in 328 comparisons). Fine scale mapping of C27 was done with JoinMap (62) using default parameter settings.

**Chemicals.** Test compounds for both flight tunnel assays and neurophysiology were obtained from Bedoukian Research, Inc. (<95% purity by gas chromatography), and stock solutions were diluted in steps from 100 ng to 1 mg in hexane and stored at −20 °C until use. Five compounds were used in the wind tunnel assays: Z11-16:Ald, Z9-14:Ald, Z9-16:Ald, Z11-16:OAc, and Z11-16:OH. The blends were created by admixing the required components directly onto a circular piece of filter paper (1-cm diameter). Stimulus cartridges for single sensillum recording had single compounds loaded on filter paper at dosages between 100 ng and 500 ug.

**Wind Tunnel Assays.** Wind tunnel assays followed procedures in Vickers (32) in which the filter paper with the pheromone blend being tested is placed 1 m from a platform upon which the test male is initially placed. As in Vickers (32, 42) males were typically 3–5 days old when tested. On the day of testing, males were placed in the wind tunnel for at least 1 h to acclimate to the conditions therein. Responses of individual male moths ranges from simply remaining on the take-off platform without taking flight to landing, casting back and forth, locking-on to the pheromone plume, upward flight in a zigzag pattern and eventual contact with the pheromone source. To quantify the degree to which an individual male responded to a pheromone blend we established a ranking of the successive behavioral responses as follows: 0 = no flight, 1 = took flight without orientation, 2 = casting flight above take-off platform, 3 = lock-on to the pheromone plume, 4 = upward flight less than halfway to source, 5 = upward flight at least halfway to the source, 6 = close approach to source (<5 cm), 7 = contact with source. The mean degree of response of a genotype was calculated by multiplying each rank number by the number of males with that response, taking the sum of all of these products and dividing by the total number of males.

**Electrophysiological Recordings from Single Sensilla.** The general methods used are described in Hillier et al. (63). Briefly, male moths were restrained on a depression slide and a reference electrode introduced to the contralateral eye. Long trichoid sensilla were selected randomly from the proximal ventral surface of the antenna and cut using a glass capillary piezoelectric cutter (64). Once cut, a saline-filled silver-chloride glass electrode was placed over the sensillum tip and activity of sensory neurons within recorded during odor presentation.
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