DOI: 10.1534/genetics.109.113340

Natural Variation in Odorant Recognition Among Odorant-Binding Proteins in *Drosophila melanogaster*

Ping Wang,*,†,[1] Richard F. Lyman,*,† Trudy F. C. Mackay*,† and Robert R. H. Anholt*,†,‡,[2]

**Department of Genetics, †W. M. Keck Center for Behavioral Biology and ‡Department of Biology, North Carolina State University, Raleigh, North Carolina 27695*

Manuscript received December 18, 2009
Accepted for publication December 19, 2009

ABSTRACT

Chemical recognition is essential for survival and reproduction. Adaptive evolution has resulted in diverse chemoreceptor families, in which polymorphisms contribute to individual variation in chemosensation. To gain insights into the genetic determinants of individual variation in odorant recognition, we measured olfactory responses to two structurally similar odorants in a population of wild-derived inbred lines of *Drosophila melanogaster*. Odorant-binding proteins (OBPs) are the first components of the insect olfactory system to encounter odorants. Previously four single-nucleotide polymorphisms (SNPs) in the *Obp99* group were associated with variation in olfactory responses to benzaldehyde. Here, we identify six different SNPs that are associated with variation in responses to a structurally similar odorant, acetophenone, in the same *Obp* genes. Five SNPs are in coding regions of *Obp99b* and *Obp99d* and one SNP is in the 3′-untranslated region of *Obp99a* (A610G). We found that the 610G allele is associated with higher response scores to acetophenone than the 610A allele, but with lower expression of *Obp99a*, suggesting that binding of acetophenone to Opb99a might limit rather than facilitate access to odorant receptors. Our results show that overlapping sets of OBPs contribute to odorant recognition for structurally similar odorants, but that different SNPs are associated with odorant-specific individual variation. Thus, dual olfactory recognition where OBPs regulate odorant access to receptors may enhance olfactory discrimination.

ADAPTIVE evolution in diverse chemical environments has resulted in large multigene chemoreceptor families, including odorant-binding protein (*Obp*) genes, odorant receptor (*Or*) genes, and gustatory receptor (*Gr*) genes (Hekmat-Scafe *et al.* 2002; Robertson *et al.* 2003; Nozawa and Nei 2007; Nei *et al.* 2008; Su *et al.* 2009). Polymorphisms in these chemoreceptor genes contribute to individual variation in chemosensory behavior (Keller *et al.* 2007; Wang *et al.* 2007). At the same time, combinatorial recognition of odorants may contribute functional redundancy, which allows individual variation without compromising overall olfactory ability. This may be the reason why segregating null alleles of chemoreceptor genes can be maintained within a population (Takahashi and Takano-Shimizu 2005; Wang *et al.* 2007). *Drosophila melanogaster* presents a favorable model for investigating the genetic basis of individual variation in olfactory discrimination, because the genome can be manipulated readily. Furthermore, flies can be inbred, which enables repeated behavioral measurements on identical genotypes under controlled environmental conditions. In addition, both the olfactory and the gustatory systems of Drosophila have been well characterized (Su *et al.* 2009; Yarmolinsky *et al.* 2009). Convergent projections of olfactory neurons expressing distinct odorant receptors have been mapped to specific glomeruli in the antennal lobe (Gao *et al.* 2000; Vosshall *et al.* 2000), and detailed electrophysiological studies on transgenic flies have identified molecular response profiles of a large fraction of the odorant receptor repertoire (de Bruyne *et al.* 2001). Surprisingly, however, behavioral responses

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under the following accession numbers: *D. melanogaster Obp99a*, EU088877–EU088894, EU088896–EU0888902, EU0888904–EU088916, EU088918–EU088953, EU088955–EU088987, EU088989–EU089033, EU089035–EU089058, EU089061–EU089069, and FJ486990–FJ487101; *D. melanogaster Obp99b*, EU089070–EU089087, EU089089–EU089095, EU089097–EU089109, EU089111–EU089146, EU089148–EU089180, EU089182–EU089226, EU089228–EU089251, EU089254–EU089262, and FJ487102–FJ487213; *D. melanogaster Obp99c*, EU089263–EU089280, EU089282–EU089288, EU089290–EU089302, EU089304–EU089393, EU089395–EU089419, EU089421–EU089444, EU089447–EU089455, and FJ487214–FJ487314; *D. melanogaster Obp99d*, EU089456–EU089473, EU089475–EU089481, EU089483–EU089495, EU089497–EU089532, EU089534–EU089566, EU089568–EU089612, EU089614–EU089637, EU089640–EU089648, and FJ487315–FJ487426; and *D. simulans Obp99a*, *Obp99b*, *Obp99c*, and *Obp99d*, EU089658–EU089661, respectively.

[1]*Present address:* Department of Neurobiology, Duke University Medical Center, Durham, NC 27710.

[2]*Corresponding author:* Department of Biology, North Carolina State University, Campus Box 7617, Raleigh, NC 27695-7617.
E-mail: anholt@ncsu.edu

to odorants do not necessarily conform to predictions based on electrophysiological response profiles (Keller and Vosshall 2007).

Whereas Drosophila odorant receptors have been studied extensively, less is known about the function of odorant-binding proteins (OBPs) in mediating odor recognition and olfactory discrimination. OBPs are secreted by support cells in olfactory sensilla into the aqueous perilymph that surrounds olfactory dendrites and are thought to facilitate solubilization and transport of hydrophobic odorants, thereby either promoting or limiting access of odorants to odorant receptors (Steinbrecht 1998). For example, the pheromone-binding protein of the silk moth, *Bombyx mori*, binds and releases bombykol in a pH-dependent manner at the membrane interface (Wojtasek and Leal 1999; Sakurai *et al.* 2004). In *D. melanogaster*, an OBP, Lush, is essential for activation of the Or67d receptor by the pheromone *cis*-vaccenyl acetate in trichoid sensilla of the Drosophila third antennal segment (Ha and Smith 2006; Kurtovic *et al.* 2007). Binding of the pheromone causes a conformational transition in Lush, which enables this OBP to activate the Or67d receptor (Laughlin *et al.* 2008). Lush also interacts with short chain alcohols (Kim *et al.* 1998), but recognition of alcohols by Lush does not involve a conformation change and, thus, proceeds via a different mechanism (Stower and Logan 2008).

Polymorphisms in *Obp* genes can serve as a substrate for natural selection and contribute to speciation. A polymorphism in *Obp57e* is responsible for differences in host plant preference between *D. sechellia* and *D. melanogaster*. *D. melanogaster* flies lacking the *Obp57e* and *Obp57d* genes were no longer repelled by hexanoic and octanoic acid, toxins produced by *Morinda citrifolia*, the host plant for *D. sechellia*. Here, inactivation of an *Obp* gene has enabled *D. sechellia* to occupy a specialist evolutionary niche (Matsuo *et al.* 2007). Differences in expression levels between *Or*s and *Obp*s between *D. sechellia* and *D. simulans* have also been reported and postulated to contribute to the evolution of host plant preferences (Kopp *et al.* 2008).

Despite the demonstrated importance of OBPs in pheromone and host plant recognition, little is known about how naturally occurring allelic variation in *Obp* genes affects individual variation in olfactory behavior. Previously, we identified polymorphisms associated with natural variation in olfactory behavior in response to benzaldehyde in *Obp99a*, *Obp99c*, and *Obp99d* in a population of wild-derived inbred lines of *D. melanogaster* (Wang *et al.* 2007). These studies indicated that these OBPs are likely to recognize benzaldehyde in a combinatorial manner, similar to odorant recognition by mammalian odorant receptors (Malnic *et al.* 1999). This observation enables us to begin to explore OBP odorant response profiles using a population genetics approach that capitalizes on naturally occurring mutations that affect behavior. As a first step, we asked whether variation in responses to odorants that are chemically similar would be associated with the same or overlapping sets of OBPs and, if so, whether the same or different polymorphisms in these OBPs would contribute to individual variation for olfactory behavior in response to these odorants. We focused on genes of the *Obp99* group, previously associated with phenotypic variation in response to benzaldehyde. We obtained complete sequences of these genes from 297 inbred lines from the same wild-derived inbred population of *D. melanogaster* and measured variation in olfactory behavior in response to acetophenone, which is structurally similar to benzaldehyde. These odorants occur in fruits from host plants on which flies from the Raleigh population feed (*e.g.*, apples and peaches). We find that overlapping sets of OBPs contribute to recognition of these two odorants, but that different SNPs are associated with odorant-specific individual variation.

MATERIALS AND METHODS

Drosophila stocks: We used flies from the same population previously used to identify polymorphisms in *Obp*s associated with variation in response to benzaldehyde (Wang *et al.* 2007). These flies were derived from isofemale lines collected from a natural Raleigh, North Carolina population in 2002 and inbred by 20 generations of full-sib mating. Flies were reared on cornmeal–molasses–agar medium under standard culture conditions of 25°, 70% humidity, and a 12-hr light/dark cycle.

***Obp* sequences:** DNA sequences for the *Obp99* genes were obtained previously for 185 of the lines (Wang *et al.* 2007) and for a *D. simulans* line originally collected in Florida City, Florida by Jerry Coyne to serve as outgroup for McDonald–Kreitman tests (McDonald and Kreitman 1991). To obtain sequences for additional lines, genomic DNA was extracted, purified, sequenced, and analyzed as described previously (Wang *et al.* 2007).

Olfactory behavior assay: Olfactory behavior was quantified with the "dipstick" method as described previously (Anholt *et al.* 1996). All assays were conducted between 2:00 and 4:00 pm in a behavioral chamber at 25° and 70% humidity under white light. Olfactory behavior of 4- to 10-day-old flies from 297 wild-derived inbred lines was measured in single-sex groups of five flies per replicate and 10 replicates per sex. To determine an optimal acetophenone concentration for assessing variation among the 297 wild-derived inbred lines with maximal resolution, pilot experiments were performed separately on 5 of the lines over a range of acetophenone concentrations. Subsequently, measurements for each line were obtained at 3.5% (v/v) acetophenone in a randomized design over several days to average environmental effects.

Quantitative genetic analysis of olfactory behavior: We used ANOVA to partition sources of variation in olfactory behavior in response to acetophenone according to the model $Y = \mu + L + S + L \times S + E$, where Y is the observed value, μ is the overall mean, L is the random effect of line, S is the fixed effect of sex, $L \times S$ is the random effect of the line-by-sex interaction, and E is environmental error. The total genotypic variance among lines and the broad sense heritability were estimated as described previously (Wang *et al.* 2007). The genetic correlation between males and females was calculated

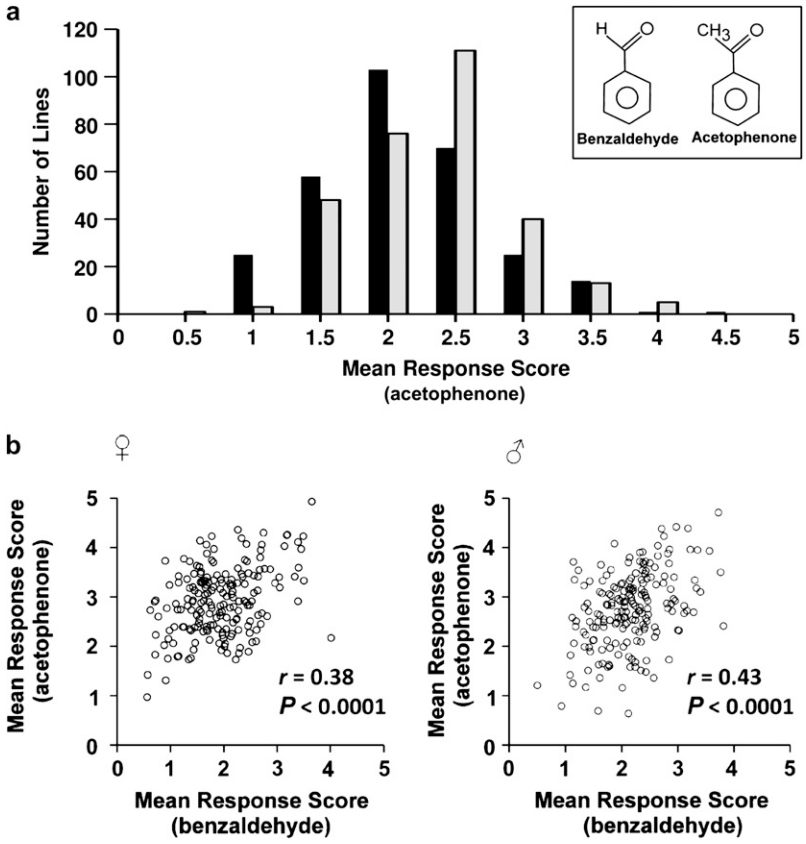

FIGURE 1.—Olfactory responses of 297 wild-derived inbred lines. (a) Distribution of mean olfactory response scores for male (solid bars) and female (shaded bars) flies to 3.5% (v/v) acetophenone. The inset in a shows the structural similarity between benzaldehyde and acetophenone. (b) Correlations between olfactory response scores to acetophenone and benzaldehyde for females (left graph) and males (right graph).

as $r_{MF} = cov_{MF}/\sigma_M\sigma_F$, where cov_{MF} is the covariance of line means for the two sexes and σ_M and σ_F are the square roots of the genotypic variances for each sex (FALCONER and MACKAY 1996). Analyses of variance and tests of significance were calculated using the Proc GLM procedure, and variance components were estimated using the Proc VARCOMP procedure in SAS (SAS Institute, Cary, NC).

Genotype–phenotype associations: Associations between polymorphisms and line means were analyzed by two-way factorial ANOVA according to the model $Y = \mu + S + M + M \times S + E$, in which Y is the observed value, μ is the overall mean, marker (M) and sex (S) are fixed effects, and E indicates error variance. Similarly, for the analysis of haplotypes, we used two-way ANOVA with the model $Y = \mu + S + H + H \times S + E$, in which haplotype ($H$) and sex ($S$) are fixed factors and E indicates error. We used least-squares means to assess the effect of haplotypes on olfactory behavior in response to acetophenone and *post hoc* Tukey tests to determine the significant mean phenotypic differences among haplotypes.

Quantitative reverse transcriptase PCR: Flies (8–10 days old) from five low-responding and five high-responding lines with alternative alleles in *Obp99a* associated with variation in response to acetophenone were frozen on dry ice. Total RNA was isolated independently from three extracts of 25 males and females for each line using Trizol reagent. cDNA was generated from 125 ng RNA of each sample, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Expression levels of *Obp99a* were quantified using the SYBR Green method (Applied Biosystems) on an ABI 7900 instrument. Three technical replicates were measured for each extract of each line. The primers used for *Obp99a* were 5′-CGATCGCTGGAGGAATACAT-3′ and 5′-TTTTTCCCCACT GAATCGAG-3′.

RESULTS

Phenotypic variation in olfactory behavior in an inbred wild-derived *D. melanogaster* population: Previously, we identified polymorphisms in *Obp99a*, *-c*, and *-d* that were associated with variation in olfactory responsiveness to benzaldehyde in a population of inbred wild-derived lines of *D. melanogaster*. To assess to what extent the same polymorphisms would be associated

TABLE 1

Variance components and quantitative genetic parameters from ANOVA of olfactory behavior in response to acetophenone

	Source	d.f.	MS	*F*-value	Pr > *F*	Variance components σ^2
Sexes pooled	Sex	1	71.46	71.46	<0.0001	
	Line	296	6.22	8.89	<0.0001	0.26
	Sex × line	296	1.00	1.43	<0.0001	0.03
	Error		0.70			0.70
Female	Line	296	3.32	4.93	<0.0001	0.26
	Error	2673	0.67			0.67
Male	Line	296	3.9	5.38	<0.0001	0.32
	Error	2673	0.73			0.73
Cov_{MF}						0.26
H^2						0.29
r_{MF}						0.90

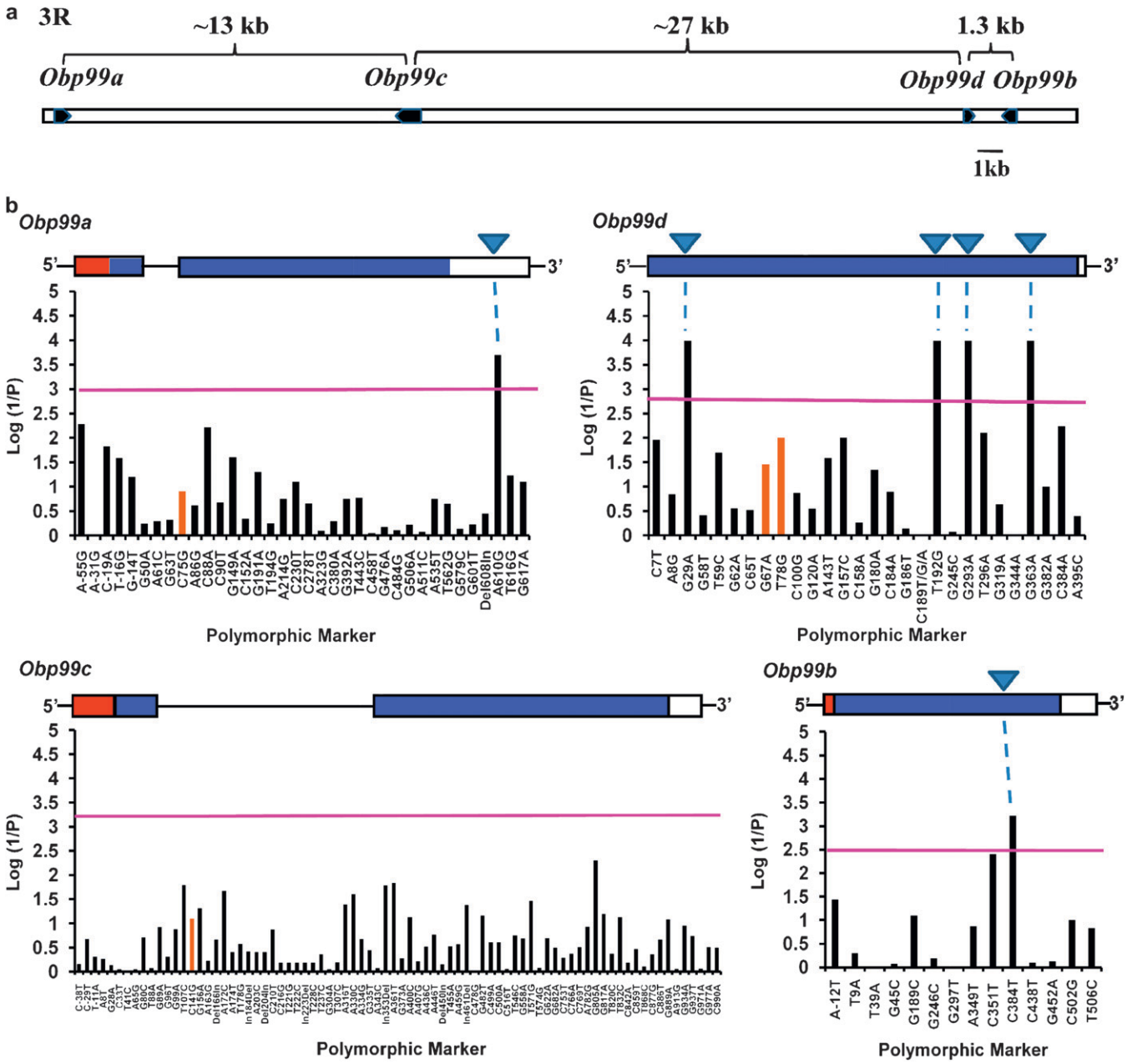

FIGURE 2.—Associations of polymorphisms in the *Obp99* gene group with variation in behavioral responses to acetophenone. (a) Relative locations of the four *Obp99* genes on the right arm of the third chromosome (3R) with arrows showing the direction of transcription. The distances between the genes are indicated. (b) Schematic representations of the *Obp99a*, *Obp99d*, *Obp99c*, and *Obp99b* genes. Blue boxes, exons; red boxes, 5′-untranslated regions; white boxes, 3′-untranslated regions; and intervening black lines, introns. The purple horizontal line in each graph indicates the significance threshold for association determined by Bonferroni correction for multiple testing. Blue arrowheads indicate the locations of SNPs with significant associations with variation in olfactory responses to acetophenone. For comparison, orange bars indicate polymorphic markers in the same genes previously associated with olfactory response to benzaldehyde in a subset of the same lines (WANG *et al.* 2007).

with variation in olfactory responsiveness to a structurally closely related odorant, we measured in 185 of the same inbred lines plus an additional 112 lines from the same population behavioral responses to acetophenone. This odorant differs from benzaldehyde in a methyl group that transforms the aldehyde group on the benzene ring into an acetyl moiety (Figure 1a, inset). Behavioral responses to acetophenone showed a similar broad range of phenotypic variation within this population as previously observed for benzaldehyde (Figure 1a) (WANG *et al.* 2007). Response scores for the two odorants showed significant correlations between behavioral responses to benzaldehyde and acetophenone both for males and for females (Figure 1b). Analysis of variance of responses to acetophenone shows significant variation for line, sex, and the sex-by-line interaction

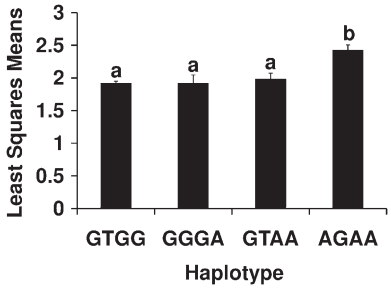

FIGURE 3.—Haplotype analysis for polymorphic markers associated with variation in response to acetophenone in the *Obp99d* gene. The bar graphs show significant differences in olfactory behavior to acetophenone of the haplotypes GTGG, GGGA, and GTAA from AGAA. The haplotypes analyzed are composed of the four SNPs associated with variation in response to acetophenone in *Obp99d*. Additional haplotypes were not observed or present at low frequency. Data were analyzed by ANOVA and haplotypes that differ significantly in olfactory behavior in response to acetophenone from one another were identified by *post hoc* Tukey's test and are indicated with different letters above the bars.

term (Table 1). The environmental error variance component σ_E^2 is high (0.70), similar to that measured previously for benzaldehyde (0.62) (WANG *et al.* 2007), which reflects the sensitivity of olfactory behavior to environmental variance, as expected for this trait. There is, however, a substantial genetic contribution to the observed phenotypic variation with broad sense heritability $H^2 = 0.29$ (Table 1), which presents a favorable scenario for association analyses. The significant sex-by-line interaction term indicates sexual dimorphism in the response to acetophenone, similar to that observed previously for benzaldehyde. However, the cross-sex genetic correlation is high ($r_{MF} = 0.90$; Table 1).

TABLE 2

McDonald–Kreitman tests

	Synonymous substitutions		Nonsynonymous substitutions		
Genes	Between species	Within species	Between species	Within species	*P*-value
Obp99a[a]	8	10	2	5	0.6592
Obp99b[a]	6	10	0	8	0.0663
Obp99c[b]	2	20	2	13	1.0000
Obp99d[a]	15	9	8	24	**0.0066**

Significant deviation from neutrality is indicated in boldface type.
[a] Values obtained from 297 lines.
[b] Values obtained from 286 lines.

Identification of polymorphisms associated with variation in olfactory responsiveness to acetophenone: To identify polymorphisms in *Obp99* genes that could be associated with olfactory responsiveness to acetophenone, we obtained and aligned complete DNA sequences for these four genes from 297 fly lines. We used ANOVA of line means to assess whether polymorphic markers were associated with variation in olfactory behavior in response to acetophenone. Association analyses revealed individual genotype–phenotype associations for olfactory responsiveness to acetophe-

TABLE 3

Variance components attributable to markers associated with olfactory behavior

	In response to acetophenone						
Parameter estimate	*Obp99a* A610G	*Obp99b* C384T	*Obp99d* G29A	*Obp99d* T192G	*Obp99d* G293A	*Obp99d* G363A	H_{All}[g]
q[a]	0.138	0.078	0.098	0.16	0.24	0.236	
$2a$[b]	0.265	0.316	0.486	0.31	0.26	0.23	
σ_{AM}^{2}[c]	0.004	0.004	0.010	0.006	0.006	0.005	
$\sigma_{AM}^{2}/\sigma_{A}^{2}$[d]	0.028	0.028	0.072	0.040	0.040	0.034	
σ_{M}^{2}[e]	0.033	0.046	0.114	0.046	0.033	0.025	0.038
$\sigma_{M}^{2}/(\sigma_{M}^{2} + \sigma_{E}^{2})$[f]	0.087	0.115	0.253	0.117	0.087	0.066	0.101

	In response to benzaldehyde			
Parameter estimate	*Obp99a* C75G	*Obp99c* C141G	*Obp99d* G67A	*Obp99d* T78G
q[a]	0.036	0.161	0.057	0.280
σ_{M}^{2}[e]	0.415	0.057	0.115	0.034
$\sigma_{M}^{2}/(\sigma_{M}^{2} + \sigma_{E}^{2})$[f]	0.443	0.09	0.176	0.060

[a] Frequency of the rare allele.
[b] Difference in mean olfactory behavior between homozygous genotypes for common and rare alleles.
[c] Additive genetic variance attributable to the marker.
[d] Fraction of total additive genetic variance attributable to the marker.
[e] Variance attributable to the marker.
[f] Fraction of total variance attributable to the marker.
[g] Haplotypes derived from all combinations of haplotypes of the *Obp99d* gene.

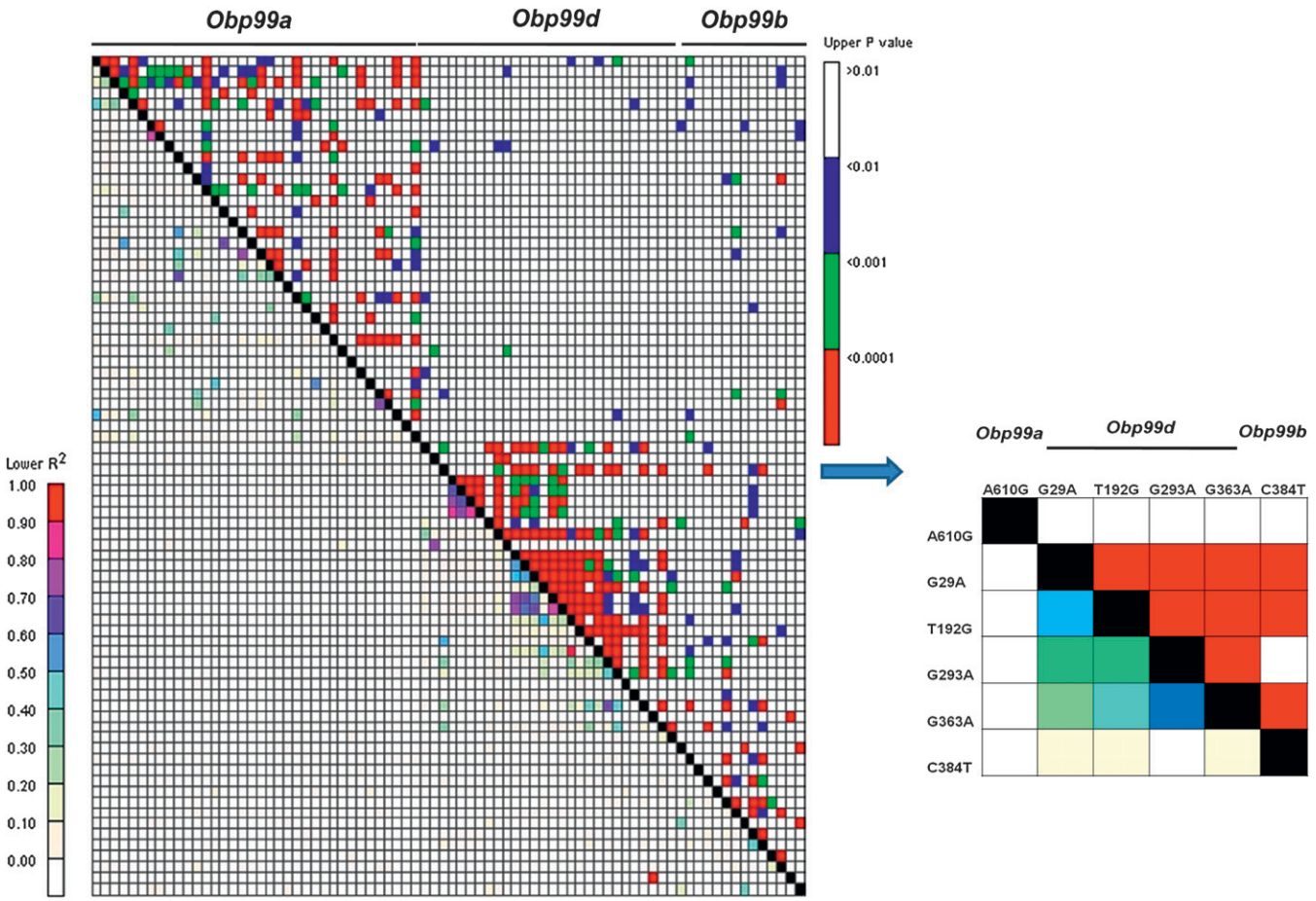

Figure 4.—Linkage disequilibrium analysis of SNPs associated with variation in olfactory response to acetophenone. Linkage disequilibrium analysis for all possible pairwise combinations of SNPs in *Obp99a*, *Obp99d*, and *Obp99b* was based on complete sequence data for these genes from 297 wild-derived inbred lines. Color-coded boxes above the diagonal indicate corresponding *P*-values for the marker combinations, whereas R^2 values are indicated below the diagonal. Note the overall large extent of historical recombination. SNPs T192G, G293A, and G363A in *Obp99d*, which are associated with variation in olfactory response to acetophenone, are in linkage disequilibrium with each other and with C384T of *Obp99b*, which is located adjacent on the chromosome (Figure 2). Linkage disequilibrium among these SNPs is indicated on the right.

none that exceeded the Bonferroni threshold for multiple testing in *Obp99a*, *Obp99b*, and *Obp99d* (Figure 2). Six SNPs are associated with variation in response to acetophenone, four of which are located in the exon of the *Obp99d* gene, including G29A (a cysteine–tyrosine substitution in the computationally predicted signal peptide), T192G (a synonymous substitution), G293A (an arginine–glutamine substitution), and G363A (a synonymous substitution). The other two SNPs were in the 3′-untranslated region of the *Obp99a* gene (A610G) and a synonymous substitution in the exon of the *Obp99b* gene (C384T). Haplotype analysis of *Obp99d* revealed that the mean olfactory response is greater for haplotype AGAA than for haplotypes GTGG, GGGA, and GTAA (Figure 3). Since we found a significant line-by-sex interaction when we analyzed phenotypic variation in olfactory behavior in this population (Table 1), we included a marker-by-sex interaction term in the association model. However, none of the significant SNPs had significant marker-by-sex interactions. Polymorphisms in the *Obp99d* gene are of particular interest as McDonald–Kreitman tests (McDonald and Kreitman 1991) reveal a strong signature of balancing selection for this gene (Wang *et al.* 2007) (Table 2).

We estimated the fraction of the total genotypic variance attributable to each marker associated with olfactory behavior in response to acetophenone (Table 3). We found that G29A in the *Obp99d* gene contributes 25% of the total variance, while the remaining SNPs each explain 6–11% of the total genotypic variance. Linkage disequilibrium analysis shows that G29A, which changes a cysteine into a tyrosine in the signal peptide, is in linkage disequilibrium with the other three SNPs in *Obp99d* and the SNP in the adjacent *Obp99b* gene that are also associated with olfactory behavior in response to acetophenone (Figure 4; $P < 0.0001$).

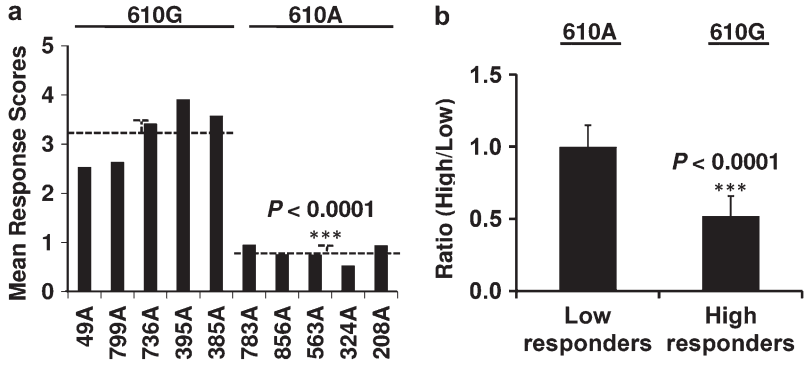

FIGURE 5.—Correspondence between behavioral response to acetophenone and differences in expression of *Obp99a* correlated with the A and G alleles of the A610G SNP. Five high-responding lines with the G allele and five low-responding lines with the A allele of the polymorphic marker A610G in the 3′-untranslated region of *Obp99a* were selected (a) and expression levels of *Obp99a* were assessed (b). The dashed lines in a indicate the mean response scores for the five high- and low-responding lines, respectively (***, $P < 0.0001$; two-tailed Student's *t*-test). The *y*-axis in b shows the mean expression level of the five high-responding lines standardized to the mean expression level of the five low-responding lines, set at 1.0. Mean expression levels across lines with high response scores to acetophenone corresponding to the 610G allele showed ~48% lower expression levels of the *Obp99a* transcript than lines with low response scores to acetophenone and the 610A allele (***, $P < 0.0001$; two-tailed Student's *t*-test).

The six SNPs associated with variation in olfactory response to acetophenone are distinct from those previously found to be associated with variation in response to benzaldehyde (indicated by orange bars in Figure 2). Our results implicate *Obp99a* and *Obp99d* (and possibly *Obp99b*, although the association in this *Obp* gene could be due to linkage disequilibrium with the SNPs in *Obp99d*) in the recognition of acetophenone. *Obp99a*, *Obp99c*, and *Obp99d* are associated with recognition of benzaldehyde (WANG *et al.* 2007). These results corroborate our previous assessment that multiple OBPs may recognize the same odorant (WANG *et al.* 2007) and indicate that structurally similar odorants can interact with overlapping sets of OBPs, but that variation in their interactions is governed by distinct polymorphic sites.

Effects of SNPs in noncoding regions on *Obp* gene expression: Nonsynonymous SNPs in coding regions can affect interactions between OBPs and their ligands by modifying the structure of the protein. Synonymous SNPs in coding regions (assuming that they are not in linkage disequilibrium with nonsynonymous SNPs) might affect the structure and stability of mRNA (NACKLEY *et al.* 2006; WANG *et al.* 2007), whereas SNPs in regulatory regions could affect transcript levels. The effect of the synonymous SNP in *Obp99b* could be explained by linkage disequilibrium with nonsynonymous SNPs in *Obp99d* (Figure 4).To test whether the A610G SNP in the 3′-untranslated region of *Obp99a* contributes to phenotypic variation by influencing transcript levels, we selected five lines with high response scores to acetophenone carrying the 610G allele and five lines with low response scores to acetophenone carrying the 610A allele and used quantitative reverse transcriptase (qRT)–PCR to measure transcript levels in these lines (Figure 5). Surprisingly, we found that lines with high response scores and the *Obp99a* 610G allele had lower mean expression levels of *Obp99a* than lines with the 610A allele, which had lower response scores but a higher mean expression level ($P < 0.01$; sexes pooled). It should be noted that this SNP is not associated with variation in olfactory behavior in response to benzaldehyde (WANG *et al.* 2007).

DISCUSSION

Phenotypic variation in olfactory behavior: Previously, we characterized natural variation in behavioral responses to a standard odorant, benzaldehyde, in 193 inbred wild-derived lines of *D. melanogaster* and identified four polymorphisms in *Obp99a*, *Obp99c*, and *Obp99d* that were associated with phenotypic variation in olfactory behavior (WANG *et al.* 2007). Here, we measured responses to a chemically similar compound, acetophenone, in an expanded sample of 297 lines, including 185 of the 193 lines previously assessed for responsiveness to benzaldehyde. Both odorants are common components of fruits on which Drosophila feed. Benzaldehyde is a major component of bitter almond extract and can be extracted from apricots, cherries, and the seeds of peaches and apples, whereas acetophenone is found in apples, apricots, and bananas. Variation in responses to these odorants enables selective interactions of individuals within the population with different host plants and reflects the potential of a generalist feeder to adapt to a variety of food sources guided by chemosensory cues, which may be either repellent or attractant. Behavioral responses to these two structurally similar odorants were correlated (Figure 1b). This correlation likely reflects mostly the structural similarities between these compounds rather than generalized differences in olfactory ability among the lines, as no significant correlations were observed between responses to benzaldehyde or acetophenone and an unrelated odorant, hexanol (data not shown), and since we found associations between variation in responses to both odorants with SNPs in the same *Obp99* genes, *Obp99a* and *Obp99d*. Analysis of variance showed significant sexual dimorphism but a high genetic correlation between the sexes ($r_{MF} = 0.9$; Table 1), as

observed previously for responses to benzaldehyde (Anholt *et al.* 1996; Wang *et al.* 2007). It should be noted that additional OBPs not analyzed in this study may also contribute to recognition of benzaldehyde and acetophenone, as could OBPs that bind these odorants without contributing to phenotypic variation in the behavioral response in this population.

Polymorphisms associated with variation in olfactory behavior in response to acetophenone: We identified six polymorphic markers with significant associations with variation in olfactory behavior in response to acetophenone. These markers were distinct from those associated with response to benzaldehyde (Wang *et al.* 2007) and can be categorized in two groups: a single SNP in the downstream regulatory region of *Obp99a* (A610G) and five SNPs in linkage disequilibrium with it, including four SNPs in *Obp99d* (G29A, T192G, G293A, and G363A) and one in the adjacent gene *Obp99b* (C384T). Previous population genetic analyses of *Obp99d* in this population showed a strong signature of balancing selection (Wang *et al.* 2007). The G29A polymorphism in *Obp99d* is of particular interest, as it contributes to 25.3% of the genotypic variance (Table 3). The G29A polymorphism leads to a cysteine to tyrosine substitution in the computationally predicted signal peptide (Bendtsen *et al.* 2004). Haplotype analysis shows that the haplotypes GTGG, GGGA, and GTAA in *Obp99d* show lower response scores than AGAA, which indicates that the replacement of cysteine (first G in GTGG, GGGA, GTAA haplotypes) by tyrosine (first A in the AGAA haplotype) critically affects the response to acetophenone. It is possible that phenotypic variation arising from the G29A polymorphism is due to reduced efficiency of protein folding or secretion of OBP99d.

SNPs in regulatory regions could contribute to phenotypic variation by modulating transcription, whereas nonsynonymous SNPs in coding regions can affect secondary mRNA structure (Kimchi-Sarfaty *et al.* 2007). We found that allelic polymorphisms in the downstream noncoding region of *Obp99a* are indeed correlated with differences in expression levels. Interestingly, greater expression of *Obp99a* correlated with lower responses to acetophenone and vice versa. Thus, it is possible that binding of acetophenone to Obp99a might prevent rather than facilitate access to odorant receptors.

It is of interest that the A610G polymorphism in the 3′-untranslated region of the *Obp99a* gene may affect its expression level without affecting variation in behavioral response to benzaldehyde, even though another SNP (C57G) in *Obp99a* has previously been found associated with variation in response to this odorant (Wang *et al.* 2007). The A610G and C57G polymorphic markers, however, are not in linkage disequilibrium. In addition, it is possible that differences in affinities of Obp99a for benzaldehyde and acetophenone may allow effects of small changes in transcript abundance to be manifest as behavioral variation in response to one, but not the other odorant.

Odorant-binding proteins and olfactory behavior: Drosophila odorant-binding proteins have been implicated as obligatory intermediates for pheromone recognition (Xu *et al.* 2005) and host plant selection (Matsuo *et al.* 2007). Yet, their general functions in olfaction remain poorly characterized. Electrophysiological studies have generated detailed response profiles of a large number of odorant receptors (de Bruyne *et al.* 2001). However, behavioral responses in flies missing a single odorant receptor could not be predicted from the physiological responses of this receptor (Keller and Vosshall 2007). Furthermore, responses to odorant receptors expressed in heterologous systems were elicited only at high odorant concentrations (Störtkuhl and Kettler 2001; Wetzel *et al.* 2001; Dobritsa *et al.* 2003). Our association analyses between polymorphisms in *Obp* genes and phenotypic variation in responses to two closely related odorants indicate that these odorants interact differently with overlapping odorant-binding proteins and suggest that modulation of odorant access to their cognate receptors by odorant-binding proteins enhances discriminatory ability of the Drosophila olfactory system.

We thank Akihiko Yamamoto for maintaining Drosophila lines and Steven A. West for excellent technical assistance. This work was supported by the National Institutes of Health grants GM059469 (to R.R.H.A.) and GM45146 to (T.F.C.M.).

LITERATURE CITED

Anholt, R. R. H., R. F. Lyman and T. F. C. Mackay, 1996 Effects of single P-element insertions on olfactory behavior in *Drosophila melanogaster*. Genetics **143:** 293–301.

Bendtsen, J. D., H. Nielsen, G. von Heijne and S. Brunak, 2004 Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:** 783–795.

de Bruyne, M., K. Foster and J. R. Carlson, 2001 Odor coding in the Drosophila antenna. Neuron **30:** 537–552.

Dobritsa, A. A., W. van der Goes van Naters, C. G. Warr, R. A. Steinbrecht and J. R. Carlson, 2003 Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. Neuron **37:** 827–841.

Falconer, D. S., and T. F. C. Mackay, 1996 *Introduction to Quantitative Genetics*, Ed. 4. Prentice Hall, Harlow, UK.

Gao, Q., B. Yuan and A. Chess, 2000 Convergent projections of *Drosophila* olfactory neurons to specific glomeruli in the antennal lobe. Nat. Neurosci. **3:** 780–785.

Ha, T. S., and D. P. Smith, 2006 A pheromone receptor mediates 11-cis-vaccenyl acetate-induced responses in *Drosophila*. J. Neurosci. **26:** 8727–8733.

Hekmat-Scafe, D. S., C. R. Scafe, A. J. McKinney and M. A. Tanouye, 2002 Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. Genome Res. **12:** 1357–1369.

Keller, A., and L. B. Vosshall, 2007 Influence of odorant receptor repertoire on odor perception in humans and fruit flies. Proc. Natl. Acad. Sci. USA **104:** 5614–5619.

Keller, A., H. Zhuang, Q. Chi, L. B. Vosshall and H. Matsunami, 2007 Genetic variation in a human odorant receptor alters odour perception. Nature **449:** 468–472.

Kim, M. S., A. Repp and D. P. Smith, 1998 LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. Genetics **150:** 711–721.

Kimchi-Sarfaty, C., J. M. Oh, I. W. Kim, Z. E. Sauna, A. M. Calcagno *et al.*, 2007 A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science **315:** 525–528.

Kopp, A., O. Barmina, A. M. Hamilton, L. Higgins, L. M. McIntyre *et al.*, 2008 Evolution of gene expression in the *Drosophila* olfactory system. Mol. Biol. Evol. **25:** 1081–1092.

Kurtovic, A., A. Widmer and B. J. Dickson, 2007 A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature **446:** 542–546.

Laughlin, J. D., T. S. Ha, D. N. Jones and D. P. Smith, 2008 Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone-binding protein. Cell **133:** 1255–1265.

Malnic, B., J. Hirono, T. Sato and L. B. Buck, 1999 Combinatorial receptor codes for odors. Cell **96:** 713–723.

Matsuo, T., S. Sugaya, J. Yasukawa, T. Aigaki and Y. Fuyama, 2007 Odorant-binding proteins OBP57d and OBP57d affect taste perception and host-plant preference in *Drosophila sechellia*. PLoS Biol. **5:** e118.

McDonald, J. H., and M. Kreitman, 1991 Adaptive protein evolution at the *Adh* locus in *Drosophila*. Nature **351:** 652–654.

Nackley, A. G., S. A. Shabalina, I. E. Tchivileva, K. Satterfield, O. Korchynskyi *et al.*, 2006 Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. Science **314:** 1930–1933.

Nei, M., Y. Niimura and M. Nozawa, 2008 The evolution of animal chemosensory receptor gene repertoires: roles of chance and necessity. Nat. Rev. Genet. **9:** 951–963.

Nozawa, M., and M. Nei, 2007 Evolutionary dynamics of olfactory receptor genes in *Drosophila* species. Proc. Natl. Acad. Sci. USA **104:** 7122–7127.

Robertson, H. M., C. G. Warr and J. R. Carlson, 2003 Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **100** (Suppl. 2): 14537–14542.

Sakurai, T., T. Nakagawa, H. Mitsuno, H. Mori, Y. Endo *et al.*, 2004 Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. Proc. Natl. Acad. Sci. USA **101:** 16653–16658.

Steinbrecht, R. A., 1998 Odorant-binding proteins: expression and function. Ann. N Y Acad. Sci. **855:** 323–332.

Störtkuhl, K. F., and R. Kettler, 2001 Functional analysis of an olfactory receptor in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **98:** 9381–9385.

Stower, L., and D. W. Logan, 2008 LUSH shapes up for a starring role in olfaction. Cell **133:** 1137–1139.

Su, C.-Y., K. Menuz and J. R. Carlson, 2009 Olfactory perception: receptors, cells, and circuits. Cell **139:** 45–59.

Takahashi, A., and T. Takano-Shimizu, 2005 A high-frequency null mutant of an odorant-binding protein gene, *Obp57e*, in *Drosophila melanogaster*. Genetics **170:** 709–718.

Vosshall, L. B., A. M. Wong and R. Axel, 2000 An olfactory sensory map in the fly brain. Cell **102:** 147–159.

Wang, P., R. F. Lyman, S. A. Shabalina, T. F. C. Mackay and R. R. H. Anholt, 2007 Association of polymorphisms in odorant-binding protein genes with variation in olfactory response to benzaldehyde in Drosophila. Genetics **177:** 1655–1665.

Wetzel, C., H. J. Behrendt, G. Gisselmann, K. F. Störtkuhl, B. Hovemann *et al.*, 2001 Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. Proc. Natl. Acad. Sci. USA **98:** 9377–9380.

Wojtasek, H., and W. S. Leal, 1999 Conformational change in the pheromone-binding protein from *Bombyx mori* induced by pH and by interaction with membranes. J. Biol. Chem. **274:** 30950–30956.

Xu, P., R. Atkinson, D. N. Jones and D. P. Smith, 2005 *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron **45:** 193–200.

Yarmolinsky, D. A., C. S. Zuker and N. J. P. Ryba, 2009 Common sense about taste: from mammals to insects. Cell **139:** 234–244.

Communicating editor: G. Gibson