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Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(10)

ISSN

0027-8424

Authors

Goldman-Huertas, Benjamin
Mitchell, Robert F
Lapoint, Richard T
et al.

Publication Date

2015-03-10

DOI

10.1073/pnas.1424656112

Peer reviewed

Evolution of herbivory in Drosophilidae linked to loss of behaviors, antennal responses, odorant receptors, and ancestral diet

Benjamin Goldman-Huertas[a], Robert F. Mitchell[b,c], Richard T. Lapoint[a,c], Cécile P. Faucher[b,d], John G. Hildebrand[b,1], and Noah K. Whiteman[a,1]

Departments of [a]Ecology and Evolutionary Biology and [b]Neuroscience, and [c]Center for Insect Science, University of Arizona, Tucson, AZ 85721; and [d]Life Science Solutions, Thermo Fisher Scientific, 64293 Darmstadt, Germany

Contributed by John G. Hildebrand, January 4, 2015 (sent for review August 29, 2014; reviewed by Ewald Grosse-Wilde, Benjamin Prud'homme, and Michael G. Ritchie)

Herbivory is a key innovation in insects, yet has only evolved in one-third of living orders. The evolution of herbivory likely involves major behavioral changes mediated by remodeling of canonical chemosensory modules. Herbivorous flies in the genus *Scaptomyza* (Drosophilidae) are compelling species in which to study the genomic architecture linked to the transition to herbivory because they recently evolved from microbe-feeding ancestors and are closely related to *Drosophila melanogaster*. We found that *Scaptomyza flava*, a leaf-mining specialist on plants in the family (Brassicaceae), was not attracted to yeast volatiles in a four-field olfactometer assay, whereas *D. melanogaster* was strongly attracted to these volatiles. Yeast-associated volatiles, especially short-chain aliphatic esters, elicited strong antennal responses in *D. melanogaster*, but weak antennal responses in electroantennographic recordings from *S. flava*. We sequenced the genome of *S. flava* and characterized this species' odorant receptor repertoire. Orthologs of odorant receptors, which detect yeast volatiles in *D. melanogaster* and mediate critical host-choice behavior, were deleted or pseudogenized in the genome of *S. flava*. These genes were lost step-wise during the evolution of *Scaptomyza*. Additionally, *Scaptomyza* has experienced gene duplication and likely positive selection in paralogs of *Or67b* in *D. melanogaster*. Olfactory sensory neurons expressing Or67b are sensitive to green-leaf volatiles. Major trophic shifts in insects are associated with chemoreceptor gene loss as recently evolved ecologies shape sensory repertoires.

plant–herbivore interactions | gene loss | olfaction | *Drosophila melanogaster* | *Scaptomyza flava*

Understanding the origins and consequences of trophic shifts, especially the transition to herbivory, has been a central problem in evolutionary biology. The paleontological record suggests that evolutionary transitions to herbivory have been rare in insects (1), and the first transitions to herbivory in vertebrates occurred long after the colonization of land (2). However, species radiations result from herbivorous transitions in insects and vertebrates, suggesting that herbivory is a key innovation (3, 4). Identifying functional genomic changes associated with the evolutionary transition to herbivory could yield insight into the mechanisms that have driven their success. However, the origins of the most diverse clades of herbivorous insects are ancient and date to the Jurassic or earlier (5), limiting meaningful genomic comparisons. In contrast, herbivory has evolved more times in Diptera than in any other order (3). The Drosophilidae is an excellent system to study the evolution of herbivory from a functional genomic perspective because it includes several transitions to herbivory, and the genomic model *Drosophila melanogaster* (6, 7).

The transition to herbivory involves adaptations in physiology (8–10), morphology (11), and behavior (12). The evolution of sensory repertoires could reinforce or even precipitate these adaptations through adaptive loss or relaxation of functional constraint subsequent to a trophic shift (13). Adaptive loss of chemoreceptors has been rarely shown but occurs in nematodes, although their olfactory systems are distinct from insects (14). Families of mammalian olfactory receptor proteins have been remodeled during transitions to flight, aquatic lifestyles, and frugivory (15–18). Similarly, the evolution of diet specialization in *Drosophila* species correlates with chemoreceptor gene losses (19–21), and hematophagous flies have lost gustatory receptors that detect sweet compounds (22). More profound changes such as the evolution of new protein families are associated with major evolutionary transitions such as the evolution of flight in insects (23). Although gene loss is unlikely to be a driving force of innovation, loss-of-function mutations may be exaptations that allow novel behaviors to evolve by disrupting ancestral attractions. If detection of different chemical cues becomes selected in a novel niche, then loss through relaxed constraint may indicate which chemical cues have changed during a trophic shift.

The chemosensory repertoires of many drosophilid species have been functionally annotated. The genus *Drosophila* includes 23 species with published genome sequences (24–27), and *D. melanogaster* presents the most fully characterized insect olfactory system (28), allowing potential linkage of receptor remodeling to a mechanistic understanding of behavioral change.

Most drosophilids feed on yeast and other microbes growing on decaying plant tissues (29). Adult female *D. melanogaster* and

Significance

The evolution of herbivory in animals is rare but has resulted in major adaptive radiations. Its rarity suggests that there are barriers to colonization of plants. Behavioral adaptations, involving host plant finding, are likely the first to evolve during the transition to herbivory. A recently evolved herbivorous fly species was derived from yeast-feeding ancestors. This herbivorous fly, unlike its yeast-feeding relatives, lost attraction to yeast volatiles, the ability to detect yeast volatiles, and three genes that encode olfactory receptors critical for detecting yeast volatiles in *Drosophila melanogaster*. Loss-of-function mutations may play a role in the transition to herbivory in insects, which account for nearly 25% of all species of life.

Author contributions: B.G.-H., R.F.M., R.T.L., C.P.F., J.G.H., and N.K.W. designed research; B.G.-H., R.F.M., R.T.L., and C.P.F. performed research; B.G.-H., R.F.M., R.T.L., J.G.H., and N.K.W. analyzed data; and B.G.-H., R.F.M., R.T.L., C.P.F., J.G.H., and N.K.W. wrote the paper.

Reviewers: E.G.-W., Max Planck Institute for Chemical Ecology; B.P., CNRS; and M.G.R., University of St. Andrews.

The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KM278522–KM278527, KM277412–KM277433). Odorant receptor (OR) sequences from the *S. flava* and *S. pallida* genomes are provided in Datasets S1 and S2.

See Commentary on page 2927.

[1]To whom correspondence may be addressed. Email: jhildebr@email.arizona.edu or whiteman@email.arizona.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1424656112/-/DCSupplemental.

distantly related species innately prefer yeast chemical cues to those produced by the fruit on which they oviposit (30, 31). *D. melanogaster* detects volatiles with chemoreceptors of several different protein families, but especially receptors from the odorant receptor (OR) gene family, some of which, such as *Or42b*, are highly conserved across species (31). *Or42b* is necessary for attraction and orientation to vinegar and aliphatic esters (32–34). Similar compounds activate *Or42b* across many *Drosophila* species (35), suggesting that volatile cues for yeast, and the associated receptors, are conserved across the Drosophilidae.

The ancestral feeding niche for the genus *Scaptomyza* (Drosophilidae) is microbe-feeding, but *Scaptomyza* use decaying leaves and stems rather than the fermenting fruit used by *D. melanogaster* and other members of the subgenus *Sophophora* (29, 36). The close association of *Scaptomyza* with decaying plant tissues may have precipitated the evolution of herbivory <20 MyBP (Fig. 1; ref. 36). Adult females of the species *S. flava* feed and oviposit on living leaves of many cruciferous plants (Brassicales) including *Arabidopsis thaliana*. Females puncture leaves with serrated ovipositors to create feeding and oviposition sites, and larvae mine and pupate within the living leaves (7).

Here, we use *Scaptomyza* as a model to test the hypothesis that functional loss of chemosensory genes has played a role in a major ecological transition to herbivory in insects. We hypothesized that the conserved detection of yeast volatiles would be lost in the herbivorous *Scaptomyza* lineage. We tested this loss by comparing *D. melanogaster* and *S. flava* at behavioral, physiological, and genetic levels. First, we hypothesized that gravid ovipositing *S. flava* females would not be attracted to yeast volatiles. Second, we hypothesized that the olfactory sensory organs of *S. flava* would have a decreased ability to detect individual yeast volatiles and volatile mixtures. Third, chemoreceptor genes from the OR gene family implicated in detection of yeast volatiles would be lost in the *S. flava* genome. Finally, we predict chemoreceptor genes potentially mediating detection of plant volatiles would show evidence of positive selection and possibly, neofunctionalization.

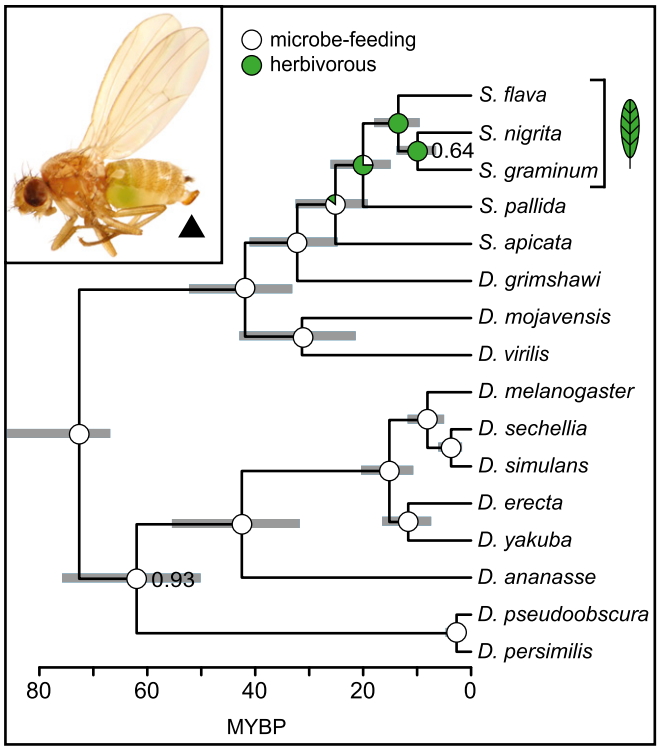

Fig. 1. Time-calibrated Bayesian phylogeny of *Drosophila* and *Scaptomyza* species with herbivorous taxa in dark green. *Scaptomyza* flies are nested phylogenetically within the *Drosophila* genus. (*Inset*) Adult female *S. flava* fly with green-pigmented abdomen after feeding on *Arabidopsis thaliana*. Arrowhead indicates serrated ovipositor used to create feeding and oviposition holes within leaf tissue. Node labels indicate posterior clade probability (PP). Unlabeled nodes have PP = 1. Error bars are 95% highest posterior density interval. Pie graphs indicate probability of change to herbivory (green) or microbe-feeding (white) traits reconstructed at each node. Herbivorous taxa are in brackets indicated by leaf.

Results and Discussion

Characterization of the Olfactory Capabilities of an Herbivorous Drosophilid. Olfaction is used by insects to find resources, mates, and oviposition substrates (37). We tested the hypothesis that *S. flava* is not attracted to yeast volatiles, whereas *D. melanogaster* is attracted to yeast volatiles. We used a four-field olfactometer assay (38, 39) in which filtered air blown through four corners of a diamond-shaped arena establishes four independent airfields. Two of the four fields were exposed to yeast volatiles from *Saccharomyces cerevisiae* cultures. The presence of gravid adult females of both species in either yeast or control fields was recorded every 6 s for 10 min. *D. melanogaster* flies spent 82.4 ± 18.2% SD of the assay time in yeast-volatile fields (Wilcoxon signed rank, $W = 295$, $P < 0.0001$, $n = 25$) and more time in yeast-volatile fields than *S. flava* (Mann–Whitney $U = 669$, $P < 0.0001$, $n = 25$, $n = 31$) (Fig. 2*A*). *S. flava* did not spend more time in yeast-volatile fields (Wilcoxon signed rank $W = 266.5$, $n = 31$, $P = 0.49$) and divided residence time evenly between yeast and control fields (51.5 ± 24.5% SD), consistent with a loss of attraction to yeast volatiles in *S. flava* flies.

Because *S. flava* flies failed to increase their residence time in olfactometer quadrants exposed to yeast volatiles, we hypothesized that *S. flava* antennal olfactory sensory neurons (OSNs) were deficient in their ability to detect yeast volatiles. We addressed this hypothesis by conducting electroantennogram (EAG) measurements in adult *D. melanogaster* and *S. flava* flies of both sexes 4–20 d after eclosion, exposed to the same yeast volatiles used in the olfactometer assays and to crushed rosette leaves of the host plant of *S. flava* flies in our laboratory colonies (*Arabidopsis thaliana* accession Col-0). EAG responses are driven by the aggregate depolarization of OSNs in the antennae and scale with the concentration and identity of stimulants (40). We found no difference between sexes and combined data for male and female flies. We recorded consistently lower EAG signals in *S. flava* flies compared with *D. melanogaster*, preventing interspecific comparisons of signal amplitude, possibly due to differences in electrical properties of antennae (40).

The antennae of *S. flava* were more strongly stimulated by *Arabidopsis* volatiles than by yeast ($P < 0.01$; Fig. 2*B*), whereas the antennae of *D. melanogaster* were more responsive to volatiles from yeast than those from *Arabidopsis* ($P < 0.0001$; Fig. 2*C*). We concurrently measured responses to a small panel of three volatiles associated with *A. thaliana* [(*Z*)-3-hexenol, myrcene, phenethyl isothiocyanate; ref. 41] and two with *S. cerevisiae* (2-phenylethanol, ethyl acetate; ref. 42). Antennae of both species detected all volatiles compared with a negative control (*S. flava*, $P < 0.0001$; *D. melanogaster*, $P < 0.0001$; Fig. 2 *B* and *C*). The antennae of *S. flava* were most responsive to (*Z*)-3-hexenol (Fig. 2*B*), a volatile produced by damaged leaves of many plant species (43), and were also highly attuned to phenethyl isothiocyanate, a hydrolyzed product of glucosinolates, which are the major defensive compound in host plants of *S. flava*. Responses to myrcene and 2-phenylethanol were not in the expected direction, although 2-phenylethanol, as a widespread floral volatile (44), may remain an important chemical cue for *Scaptomyza* adults.

Antennae of *S. flava* were less responsive to yeast and the yeast-associated volatile ethyl acetate than to plant-related volatiles, but these relative comparisons were insufficient to prove that the detection threshold for yeast volatiles had decreased in

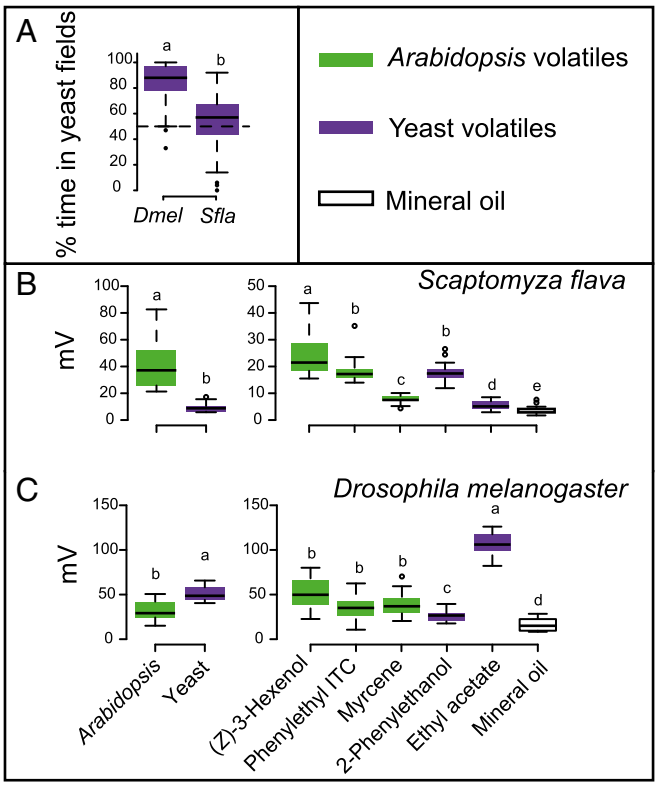

Fig. 2. Herbivorous *S. flava* have diminished behavioral and antennal responses to yeast volatiles, consistent with a loss of attraction to yeast. (*A*) Gravid adult female *S. flava* (Sfla) and *D. melanogaster* (Dmel) flies were introduced into a four-field olfactometer arena. Half the fields were exposed to volatiles from cultures of *Sa. cerevisiae* yeast or to a control solution. Boxplots of electroantennographic (EAG) recordings of adult *S. flava* (*B*) and *D. melanogaster* (*C*) flies exposed to volatiles of crushed *A. thaliana* leaves, yeast cultures, single compounds, and mineral oil controls. Green boxplots indicate *A. thaliana*-associated volatiles; purple are yeast-associated volatiles. Letters indicate significance of FDR-corrected post hoc Wilcoxon rank-sum test. Boxplot whiskers are 1.5× interquartile range.

Scaptomyza. We therefore tested the sensitivity of *S. flava* and *D. melanogaster* flies to this and other short-chain aliphatic esters by exposing females to half-log dilution series of ethyl acetate, ethyl propionate, and isobutyl acetate (*SI Appendix*, Fig. S1). We defined sensitivity as the first concentration increase that generated an increased antennal response. *S. flava* was insensitive to ethyl acetate at the concentrations tested ($P > 0.05$ in all comparisons, Wilcoxon rank sum, adjusted by false discovery rate). *S. flava* was also less responsive to ethyl propionate (minimum threshold $10^{-1.5}$; W = 2.2; $P < 0.05$) and isobutyl acetate (10^{-1}; W = 2.2, $P < 0.05$) compared with *D. melanogaster* (ethyl propionate at $10^{-2.5}$; W = 2.5; $P < 0.05$; isobutyl acetate at 10^{-2}; W = 2.3; $P < 0.05$). *Scaptomyza* is considerably less sensitive to short aliphatic esters, which may account for differences in signal strength in response to plant and yeast volatile mixtures and the lack of attraction to yeast volatiles by *S. flava*. This unresponsiveness is consistent with the fact that deficits in the production of aliphatic esters in a yeast strain decreased attractiveness to *D. melanogaster* flies (45).

OR Gene Annotation and Phylogenetics. The lack of attraction and minimal EAG response to yeast volatiles in *S. flava* suggested that chemosensory genes have been lost or changed in herbivorous *Scaptomyza* species. ORs are expressed in the dendrites of OSNs in the antennae and maxillary palps and are the primary receptors by which most neopteran insects detect odors in their environments (46). The OR family has been functionally annotated in *D. melanogaster* (reviewed in ref. 28), and members of subfamily H OR genes in particular (20) are highly conserved and enriched in receptors for aliphatic esters (35), a group of compounds *S. flava* detected poorly.

To characterize changes in the OR gene repertoire in *S. flava* associated with the olfactory phenotypes, we sequenced the genome of *S. flava* and annotated OR genes by using reciprocal tBLASTn searches (47) of previously annotated *Drosophila* OR protein sequences (*SI Appendix*, Table S1) against this de novo *S. flava* genome assembly. All significant BLAST hits and their homology assignments are listed in *SI Appendix*, Table S2 and *S. flava* sequences are in Dataset S1. We found 65 full-length ORFs for OR genes in *S. flava* (Dataset S2). Consistent with previous OR gene-naming conventions (48–50), ORs were named after the *D. melanogaster* ortholog or the most closely related gene, with the exception of *OrN1* and *OrN2* orthologs, which are not present in *D. melanogaster* (50).

Protein translations of *S. flava* genes were included in a phylogeny of *D. melanogaster*, *Drosophila virilis*, *Drosophila mojavensis,* and *Drosophila grimshawi* OR protein sequences to assess homology (*SI Appendix*, Fig. S2). The latter three species are the closest relatives of *Scaptomyza* with fully sequenced genomes (Fig. 1).

S. flava retains duplicates of *Or42a*, *Or67a*, *Or74a*, *Or83c*, *Or98a*, and *OrN2* found in other sequenced *Drosophila* species (*SI Appendix*, Fig. S2). *Scaptomyza* also has duplications not shared with close relatives, although nine of these genes are pseudogenized. The majority of paralogs (56%) were found on the same scaffold in tandem arrays (*SI Appendix*, Table S2). The functional significance of these gene duplications is not yet clear, but it is suggestive that *Or67b*, with three copies in *S. flava*, is in single copy in nearly all sequenced *Drosophila*. In *D. melanogaster*, neurons expressing *Or67b* respond to green leaf volatiles such as (*Z*)-3-hexenol (51), to which *S. flava* also has a robust antennal response (Fig. 2*B*).

Only four widely conserved ORs were uniquely lost (*Or22a* and *Or85d*) or pseudogenized (*Or9a*, *Or42b*) in the *Scaptomyza* lineage (Table 1). Syntenic regions flanking OR losses were recovered in the genome assembly (Fig. 3 *B*–*E*). Orthologs of *Or9a*, *Or22a*, and *Or42b* are intact in 23 *Drosophila* species with genome sequences, and *Or85d* is missing only in the *Drosophila albomicans* and *Drosophila rhopaloa* genome assemblies. As predicted, orthologs of ORs that persist in microbe-feeding *Drosophila* species and are lost in *S. flava*, function in yeast-volatile detection (Table 1). Or42b is highly conserved in sequence (31) and receptor function (35) among *Drosophila* species, and the receptor is highly attuned to aliphatic esters at low concentrations (32). Knockouts of *Or42b* in adult *D. melanogaster* result in failure to orient in flight toward aliphatic ester odor plumes (33), and rescuing these neurons restores attraction to yeast volatiles (34). Similarly, no sequences similar to *Or22a* were present in the *S. flava* assembly, although we recovered conserved intergenic regions in *S. flava* that flank *Or22a* in other *Drosophila* species (Fig. 3*B*). Or22a also detects aliphatic esters (56) and in the specialist species *Drosophila erecta* and *Drosophila sechellia*, Or22a detects volatiles produced by host fruit (57, 58). Both Or22a and Or42b are activated by floral volatiles of *Arum palestinum*, which mimics yeast fermentation volatiles and attracts a diversity of drosophilids (31). Finally, *Or85d* orthologs were not detected in the *S. flava* genome by BLAST or by inspection of genome regions flanking *Or85d* in other species (Fig. 3*E*). *Or85d* is expressed in the maxillary palps (59) and in *D. melanogaster* is responsive to the yeast metabolites 2-heptanol, ethyl acetate, and isoamyl acetate (59). Or85d is highly sensitive to phenethyl acetate, a common volatile of many yeast species (54). In *D. melanogaster*, Or9a is activated by a broad range of ketone-, alcohol-, and carboxylic acid-containing ligands (60). Some of these ligands, such as acetoin, are common yeast volatiles and strong attractants (30). The consequences of *Or9a* pseudogenization will require further study.

Table 1. OR genes lost or with signatures of positive selection in *S. flava* lineage

OR gene	OR genes with significant branch-site test*	Ortholog expression pattern in *D. melanogaster* (52, 53)	Known ligands of *D. melanogaster* orthologs (51)
Or9a pseudogene	NA	Antennal basiconic 8B OSNs	3-hydroxy-2-butanone, 2,3-butanediol
Or22a deleted	NA	Antennal basiconic 3A OSNs	Ethyl hexanoate, Methyl hexanoate, Ethyl butyrate
Or42b pseudogene	NA	Antennal basiconic 1A OSNs Larval dorsal organ	Ethyl acetate, Ethyl butyrate
Or67b-1,2[†]	$\omega_2 = \infty$[‡] (FDR $P = 0.023$)[§]	Antennal basiconic 9B OSNs Larval dorsal organ	(*Z*)3-hexenol
Or67b-3	$\omega_2 = 8.79$ (FDR $P = 0.024$)	Antennal basiconic 9B OSNs Larval dorsal organ	(*Z*)3-hexenol
Or85d deleted	NA	Palp basiconic 9B OSNs	2-heptanone, isoamyl acetate, phenethyl acetate (54)
Or88a	$\omega_2 = 8.80$ (FDR $P = 0.006$)	Antennal trichoid 4C OSNs	Female and male conspecific volatiles (55)

*Additional parameters for all OR gene branch-site models listed in Table S7. NA, not applicable.
[†]Branch ancestral to *S. flava Or67b-1* and *Or67b-2* paralogs.
[‡]Estimated ratio in foreground branch for codons with dN/dS > 1 consistent with episodic positive selection.
[§]False discovery rate corrected *P* value.

Timing of the Evolution of Herbivory in *Scaptomyza*. A time-calibrated phylogeny of the family Drosophilidae suggests that herbivory evolved in *Scaptomyza* ca.13.5 million years ago (95% highest posterior density 10.02–17.48 million years ago), overlapping with age ranges inferred from previous analyses (36, 61) (Fig. 1). Ancestral state reconstructions were performed in the APE package (62) by using an equal rates model. This analysis indicated that microbe feeding is ancestral in *Drosophila* and *Scaptomyza* (99.7% probability) and that herbivory evolved once within the genus *Scaptomyza*.

We hypothesized that OR gene losses would coincide with the evolution of herbivory. We developed and used degenerate PCR primers (*SI Appendix*, Table S3) from genomes of multiple *Scaptomyza* and *Drosophila* species that targeted exonic sequences of *Or22a* and *Or9a* (Fig. 3 *B* and *C*), and conserved, flanking, intergenic sequences of *Or42b* and *Or85d* (Fig. 3 *D* and *E* and *SI Appendix*, Table S4). Sequences were deposited in GenBank (KM277412–KM277433).

Gene losses in *S. flava* were confirmed by PCR screen in three natural populations (*SI Appendix*, Table S4), with the exception of SflaOr9a-1, which appeared to be present in a functional copy in a population from Arizona. A preliminary genome assembly of *Scaptomyza pallida* was consistent with PCR screening results for OR loss patterns in this species (Dataset S1). We reconstructed the presence/absence of *S. flava* gene losses along ancestral nodes and found that three of the four OR gene losses in *S. flava* (*Or22a*, *Or85d*, *Or42b*) coincided with or preceded the evolution of herbivory in *Scaptomyza*. Losses were shared by herbivorous congeners (Fig. 3*A*). *Or22a*, while lost in *S. flava*, is intact in the microbe-feeding species *Scaptomyza apicata* and *S. pallida* and is also lost in two other herbivorous species, *Scaptomyza nigrita* and *Scaptomyza graminum*.

Specialist, microbe-feeding *Drosophila* species, such as *D. sechellia* and *D. erecta* have an accelerated rate of chemoreceptor gene loss (19, 20), but this pattern could also be due to nearly neutral processes (49, 63). *S. flava* feeds almost exclusively on plants within the Brassicales, and we hypothesized that this species has experienced an accelerated rate of chemosensory gene loss compared with other microbe-feeding *Drosophila* species. We tested this hypothesis by coding homologous groups of ORs as present or absent in *S. flava*, *D. virilis*, *D. mojavensis* and *D. grimshawi* (the closest *Drosophila* relatives of *Scaptomyza*; Fig. 1 and *SI Appendix*, Table S5), and inferred two models of gene loss in the Brownie software package (64). We found no evidence for the alternative model of increased rate of loss in *Scaptomyza* (AICc = 2.14411), but cannot rule out that there were insufficient loss events to parameterize the more complex model or that other chemoreceptor gene families have undergone accelerated loss in *S. flava*. Also, *S. flava* is oligophagous, feeding on many plant species in the Brassicales, and it is less specialized than *D. sechellia* and *D. erecta*.

Evidence for Episodic-Positive Selection in *S. flava*. Because the shift to herbivory in *Scaptomyza* likely involved many changes in olfactory

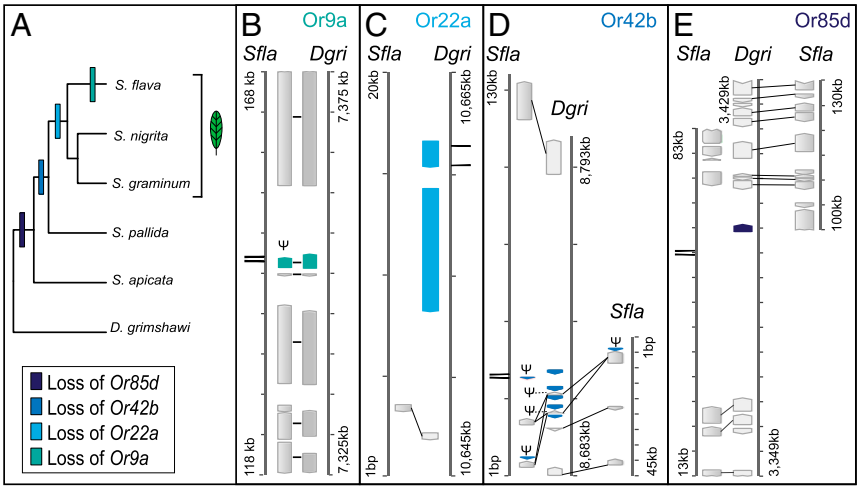

Fig. 3. OR genes critical for yeast volatile detection in *D. melanogaster* are lost in step-wise fashion in *Scaptomyza* phylogeny. *Or22a* loss is coincident with the evolution of herbivory. (*A*) OR gene losses mapped onto the *Scaptomyza* phylogeny using maximum likelihood ancestral state reconstruction based on PCR screens and genomic data. (*B–E*) Diagram of genes flanking Or9a (*B*), Or22a (*C*), Or42b (*D*), and Or85d (*E*) losses in *S. flava* (*B* and *C*, *Left*; *D* and *E*, *Left* and *Right*) and *D. grimshawi* (*B* and *C*, *Right*; *D* and *E*, *Center*). ORs colored as in *A*. Homologous genes are indicated by solid connecting lines, pseudogenes by Ψ in both species and the location of diagnostic primers by the long tick marks along the scale bar. bp, base pairs; kb, kilobase pairs. *Or22a* (*C*) is duplicated in the *D. grimshawi* genome, but deleted in *S. flava*. Two *S. flava* scaffolds show homology to each of the *Or42b* and *Or85d* regions in *D. grimshawi* (*D* and *E*). The *Or42b* region (*D*) in both *S. flava* and *D. grimshawi* is tandemly duplicated, whereas in *S. flava*, *Or42b* genes are pseudogenized while in *D. grimshawi* flanking *Or42a* genes are pseudogenized and *Or42b* intact.

cues, we hypothesized that some *S. flava* OR genes should bear signatures of episodic positive selection, as flies adapted to a novel environment. To test this hypothesis, we inferred null and alternative (branch-site) models in PAML 4.7a where subsets of codons in extant *S. flava* ORs could evolve under (*i*) purifying or neutral selection or (*ii*) purifying, neutral, or positive selection, relative to 12 *Drosophila* species (25). We used a phylogeny-aware alignment program, PRANK (65), to identify regions where indels were probable while minimizing sensitivity to alignment errors. Alignments where more than one taxon had an inferred indel in greater than two regions were trimmed by using Gblocks (66) to remove columns with ambiguous homology (*SI Appendix*, Table S6 and Dataset S3).

After correcting for false discovery (67), we found two ORs in which the branch-site model consistent with episodic positive selection was more likely than the null model (Table 1 and *SI Appendix*, Table S7). *Or88a* had the strongest statistical support for the branch-site model (FDR-adjusted $P < 0.01$). In *D. melanogaster*, Or88a functions in recognition of male and virgin female conspecifics (55). Two other branches among the *S. flava Or67b* paralogs also supported the branch-site model (FDR adjusted $P < 0.05$, $P < 0.05$): an ancestral branch preceding a *Scaptomyza*-specific duplication event and a branch leading to *Or67b-3*. Homologs of this gene in *D. melanogaster* encode ORs that respond to the green-leaf volatile (*Z*)-3-hexenol (51), one of the most salient ligands found in our EAG studies of *S. flava* (Fig. 2*B*). Experimental, functional, and population-based tests are needed to verify whether positive selection has fixed amino acid changes in the *Scaptomyza* lineage.

Conclusions

Trophic transitions in the history of animal life, such as herbivory, may be mediated by genetic changes in chemosensory repertoires. The majority of Drosophilidae feed on microbes (29), and distantly related drosophilid lineages are attracted by the same yeast-mimicking floral scent produced by *A. palestinum* (31). A subset of the ORs stimulated by this scent are highly conserved in other drosophilids, which may be part of a homologous and conserved olfactory circuit used to find fermenting host substrates across the family (31). We hypothesized that mutations disrupting the function of OR homologs in this conserved olfactory circuit could mediate the evolution of herbivory or other novel food preferences.

S. flava, an herbivorous drosophilid, has lost orthologs of ORs involved in this generalized yeast olfactory circuit. Consistent with these findings, *S. flava* did not respond to yeast volatiles in a behavioral assay. Antennae of *S. flava* were weakly activated by active yeast cultures and short-chain aliphatic esters, key compounds found in yeast volatile blends and known ligands of ORs in *D. melanogaster* lost in *S. flava*. However, retention of some ORs implicated in yeast-volatile detection, such as *Or92a* and *Or59b*, implies that *S. flava* may retain the ability to detect some untested yeast compounds (31).

We hypothesized that OR genes would be intact in non-herbivorous *Scaptomyza* and gene losses would coincide with the transition to herbivory. *Or22a* loss did coincide with the evolution of herbivory, but losses of *Or42b* and *Or85d* likely predate the evolution of plant feeding. These more ancient losses of conserved yeast-volatile receptors suggest ancestral *Scaptomyza* may have already evolved novel olfactory pathways that were later co-opted by herbivorous lineages, and in fact, many *Scaptomyza* species feed on microbes living within decaying leaves or in leaf mines produced by other insects (68). Sister groups of many major herbivorous insect lineages also feed on detritus and fungi, suggesting that the transition from microbe feeding to herbivory may be common (5). The genetic changes that underlie host-finding remain to be identified, but recently duplicated ORs, such as the unique triplication of *Or67b* in *Scaptomyza*, are likely candidates for further functional study. Subtle, targeted remodeling of chemoreceptor repertoires may be a general mechanism driving changes in behavior, facilitating trophic shifts and ultimately diversification in animals.

Materials and Methods

Olfactometer Assay. Adult female *D. melanogaster* and *S. flava* were exposed to volatiles produced by *Sa. cerevisiae* yeast cultures in 1% sucrose in non-adjacent quadrants of a four-field olfactometer arena. Individual flies were introduced and allowed to walk throughout the arena and recorded for 10 min. Four independent airfields are created in the olfactometer arena by directing compressed air through flowmeters into the four corners of the arena and exiting through a central hole in the bottom of the device (*SI Appendix*, Fig. S3). Preference or aversion to yeast volatiles was determined by residence time in yeast-exposed quadrants over control quadrants. Assays were performed under infrared light in a dark room to remove visual cues. Details of the olfactometer assay are in *SI Appendix, Materials and Methods*.

Electroantennography. Headspace volatiles from the yeast cultures tested in the olfactometer assays and crushed leaves of *A. thaliana* host plants were used as olfactory stimuli in EAG analyses along with five compounds found in either yeast or *A. thaliana* headspace. A recording microelectrode was placed against the ventral base of the third antennomere of *S. flava* and *D. melanogaster* adult males and females, and a ground electrode was inserted into the eye. Each headspace sample and individual compound was tested 10 times, in seven flies for each species and sex. An additional EAG experiment was run on four *S. flava* and five *D. melanogaster* flies that tested a half-log dilution series ($10^{-3.5}$ to 10^{-1}) of three short-chain aliphatic esters. Details of EAG methods are in *SI Appendix, Materials and Methods*.

Gene Annotation. OR genes were annotated in *S. flava* by running a tBLASTn search with the full-length, OR protein sequences of multiple *Drosophila* species against the *S. flava* genome assembly, and manual curation. Orthology and paralogy were assigned by aligning translations all OR genes of *S. flava* and four other *Drosophila* species and inferring the evolutionary history of the gene family with ML and Bayesian methods in RAxML (69) and MrBayes (70), respectively. Details on annotation are in the *SI Appendix, Materials and Methods*.

Evolution of Olfactory Receptor Genes in *Scaptomyza*. PCR primers were developed targeting *S. flava* gene losses. Primers were developed for either exonic sequences or conserved sequences flanking genes and used to amplify fragments of yeast-feeding and herbivorous *Scaptomyza*, including laboratory and natural populations of *S. flava*. Gains and losses were mapped onto a time-calibrated *Drosophila* and *Scaptomyza* phylogeny by using maximum likelihood methods in Mesquite v2.75 (mesquiteproject.org). Tests of changes in gene loss rate were performed based on *S. flava* gene counts in Brownie (64) according to methods in McBride et al. (20). Finally, branch-site tests were performed on *S. flava* OR genes, with *S. flava* genes in foreground, and 12 *Drosophila* species in the background in PAML v4.7a (71). Details are in *SI Appendix, Materials and Methods*. Herbivory and microbe-feeding were reconstructed as ancestral states along the above species phylogeny by using the ace function in APE (62).

ACKNOWLEDGMENTS. We thank Sammy Dominguez, Daniel Papaj, and Lynne Oland for loan of materials and useful discussion on study design, and Carolina Reisenman for initial EAG tests. We acknowledge support from the National Science Foundation (NSF) through Graduate Research Fellowships Program DGE-0646147, Integrative Graduate Education and Research Traineeship DGE-0654435 (to B.G.-H.), and NSF DEB-1256758 (to N.K.W.); a University of Arizona Foundation Faculty Seed Grant (to N.K.W.) and University of Arizona Center for Insect Science Faculty Seed Grant (to N.K.W.); John Templeton Foundation Grant 41855 (to N.K.W.); National Institutes of Health (NIH) Postdoctoral Excellence in Research and Teaching Fellowship K12-631 GM000708 PERT (to R.F.M. and R.T.L.); and NIH Grant R01-DC-02751 (to J.G.H.).

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