Population differences in olfaction accompany host shift in *Drosophila mojavensis*

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Evolutionary shifts in plant–herbivore interactions provide a model for understanding the link among the evolution of behaviour, ecological specialization and incipient speciation. *Drosophila mojavensis* uses different host cacti across its range, and volatile chemicals emitted by the host are the primary cue for host plant identification. In this study, we show that changes in host plant use between distinct *D. mojavensis* populations are accompanied by changes in the olfactory system. Specifically, we observe differences in olfactory receptor neuron specificity and sensitivity, as well as changes in sensillar subtype abundance, between populations. Additionally, RNA-seq analyses reveal differential gene expression between populations for members of the odorant receptor gene family. Hence, alterations in host preference are associated with changes in development, regulation and function at the olfactory periphery.

1. Introduction

An important component to understanding the formation of new species is identifying the proximate mechanisms of adaptation to different local environments. In many cases, shifts to alternative habitats are accompanied by changes in behaviour and also by alterations to the peripheral detection of sensory cues [1–3]. The olfactory system of insects, for example, has been shown to be critical for directing appropriate host plant identification [4–6], and changes in host preference behaviour for alternative hosts have been shown to contribute to divergence among populations and reproductive isolation [7,8].

Significant advances have been made in our understanding of how insects identify olfactory cues. Studies of the genetic model *Drosophila melanogaster* show that odours are detected by membrane receptor proteins expressed in primary sensory neurons that innervate distinct morphological types of sensilla: basiconic, intermediate, coeloconic and trichoid [9]. Sensilla are distributed on the third antennal segment and maxillary palp, and each contain up to four neurons in invariant combinations defined by the odour response specificity of their receptor [10]. In general, each odorant receptor neuron (ORN) expresses a single ligand binding receptor, and two receptor families have been identified. The odorant receptors (ORs) are expressed almost exclusively in basiconic, intermediate and trichoid sensilla, and are responsive to, respectively, food odours, odours promoting oviposition on citrus and pheromones [11–15]. The ionotropic receptors are expressed in coeloconic sensilla and function in the detection of acids and amines [16,17].

Several mechanisms within the insect olfactory system may directly contribute to adaptation to different environments. Previous studies comparing different *Drosophila* species observed a disparity in behavioural responses to host plant volatiles, and this disparity is thought to be reflected in alterations in the peripheral nervous system [1,3,18]. A shift by a select ORN in its
ligand affinity from ethyl to methyl hexanoate was found between generalist D. melanogaster and D. sechellia, a specialist on Morinda fruit, and it has been suggested that this shift may be due to an amino acid substitution in the ligand binding domain of the receptor [18]. In addition to potential changes in receptor protein structure and function, differences were found in the number of this ORN and its sensitivity to methyl hexanoate, the latter hypothesized to be caused by changes in the number of methyl hexanoate responsive neurons [1]. Finally, comparisons between D. melanogaster and D. erecta, which specializes on screw pine fruit (Pandanus spp.), revealed a greater sensitivity in D. erecta to a key Pandanus volatile as a consequence of a change in the ORN number [3]. Hence, differences in olfactory responses can occur if the same ORNs exhibit differences in one or more of the following: (i) receptor specificity, i.e. response profile to a set of odorants, (ii) general sensitivity, and (iii) relative proportion of ORN types.

Understanding the pathways by which reproductive isolation evolves, however, should optimally involve investigating these potential mechanisms during species formation [19]. Hence, we studied Drosophila mojavensis, a species that occupies different ecological environments across the southwestern USA and northwestern Mexico and is a model for studying factors underlying adaptive trait divergence as well as host plant preference [20]. The population inhabiting Baja California mates and feeds on fermenting agria cactus (Stenocereus gummosus) and is hypothesized to have diverged from the mainland Mexico population approximately 230–270 000 years ago with the rise of the Sea of Cortez [21]. Subsequent divergence of the mainland Mexico Sonoran desert population, which uses organ pipe cactus (S. thurberi), from the Mojave desert population, which uses barrel cactus (Ferocactus cylindraceus) in southern California, is estimated to have occurred about 117–135 000 years ago [21]. Finally, a population on Santa Catalina Island off the coast of Los Angeles, CA, feeds and breeds on prickly pear cactus (Opuntia spp. [22]).

All of these populations use volatiles emitted from fermenting cactus to identify their host plant [4,22–25]. Our previous work revealed divergence among populations in neurophysiological responses to these host plant volatiles [25]. Principal component analyses of electroantennogram (EAG) and electropalpogram (EPG) recordings showed that the Mojave population, in particular, was significantly different in its responses to host plant volatiles relative to the other three D. mojavensis populations, which grouped closer together. This work revealed that alterations to the peripheral olfactory system accompanied shifts in host plant use and to differences in behaviour in this system.

Here, we begin to determine more precisely the nature of the inter-population differences. We focus our efforts on the basiconic sensilla because they have been shown to respond to food odours and to the classes of chemical compounds that are the primary constituents detected in cactus headspace [25]. We characterize the distribution and number of basiconic sensilla subtypes, as well as odour-evoked ORN responses to cactus volatiles for the Mojave and S. Catalina populations. Differences include variation in the proportion of sensilla subtypes and in ORN sensitivity and specificity. Molecular mechanisms underlying this host shift were examined using RNA-seq, which revealed differential gene expression for members of the odorant receptor gene family.

2. Material and methods

(a) Fly stocks

Flies were raised at 25°C on banana cactus media on a 12 L:12 D cycle. The Mojave population stock (A997b; Providence Mountain, CA, USA) was a gift from Dr Bill Etges and the S. Catalina population (stock number 15081-1352.22) was from the Drosophila Species Stock Center (San Diego, CA, USA). Experiments were conducted on females because females show increased behavioural responses relative to males to individual compounds, synthetic mixtures or fermenting substrates [4,25].

(b) Single sensillum recordings

Single sensillum recordings (SSRs) for both the antennae and maxillary palps were conducted essentially as described by de Bruyn et al. [26]. In brief, flies were restrained in a pipet tip with their sensory organs exposed. Sensilla were visualized using an Olympus BX40 microscope. Action potentials were recorded by inserting a sharpened tungsten electrode into the base of a sensillum on the surface of the third antennal segment or maxillary palp. A tungsten reference electrode was inserted into the eye. Humidified air flowed continuously over the preparation. Chemical compounds were purchased at the highest purity available from Sigma-Aldrich (St Louis, MO, USA). They consisted of volatiles emitted consistently from different samples of the host cacti [25], SM Rollmann 2016, unpublished data), as well as an additional set of compounds used to aide in the identification of the recorded ORNs by similarity to known drosophilid ORN response profiles (e.g. [18,26–32]). Odorants were diluted (10⁻²) in paraffin oil and delivered in a 500 ms puff via a glass pipet at 30 s intervals. The signal was amplified using an IDAC4 (Syntech, The Netherlands) and the frequency of action potentials was counted for a 0.5 s period pre- and post-stimulations using AUTOSPIKE software (Syntech). Individual neuronal responses were calculated as the change in frequency of action potentials compared to the pre-stimulus frequency. For the majority of sensilla types a minimum of five recordings were made for each fly line (exceptions were ab6 (four recordings), ab9 (three) and abX (three), whose rarity slowed recording).

Homology between populations and to D. melanogaster was assigned based on conservation of ORN odour response properties, sensillar morphology, and was aided by the stereotypical ORN combination in each sensillar type [18,26–32]. These classifications were tested and confirmed by hierarchical cluster analysis, using Matlab (Mathworks, Natick, MA, USA). Distances between pairs of observations in odorant-response space was calculated using Euclidean geometry. These observations were the responses of 152 antennal D. mojavensis ORNs to 49 odorants and the responses of 60 palpal ORNs to 45 odorants. Clusters were formed using Ward’s minimum variance criterion (similar to [29]). Non-responding ORNs (less than 25 spikes s⁻¹) were excluded from the analyses. We statistically compared odour-evoked action potential frequencies for each odorant for flies from different populations using Student’s t-tests, correcting for multiple testing by controlling false discovery rate (FDR of 0.05 [33]). Finally, to assess differences in the spatial distribution of each ORN, we mapped the location of sensillar subtypes by photographing each surface of the antenna/palp and overlaying a fine x–y grid. The location of the recording was selected by pseudo-randomly generating a series of x–y coordinates, and visually estimating the basiconic sensillum closest to each location on the grid. Between 77 and 202 sensilla were recorded per surface (antenna: anterior, medial and posterior; palp: lateral and medial) of the olfactory organ.

(c) Gene expression analyses

We constructed six (i.e. two populations × three replicates) indexed Illumina Tru-seq RNA-seq libraries from adult heads.
We hand dissected 200 heads from live individuals of the Mojave and S. Catalina populations, and flash froze them in liquid nitrogen. We isolated total RNA and confirmed RNA quality using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), and we constructed RNA-seq sequencing libraries following Illumina’s Tru-Seq protocol (Illumina, San Diego, CA, USA). We confirmed library quality using the Agilent 2100 Bioanalyzer. We collected 101nt single-end reads from the pooled libraries on a single Illumina HiSeq 2000 flowcell at the Princeton University sequencing facility (Princeton, NJ, USA). Reads corresponding to each of the six samples were parsed according to Illumina index using barcode_splitter.py (https://gist.github.com/dgtrtwo/3725741) and quality trimmed to remove low-quality bases (Phred quality score <20) and reads with fewer than 30 nucleotides of contiguous high-quality bases using a custom python script. Total read counts and percentage of reads uniquely mapped for each sample are tabulated in the electronic supplementary material, table S1.

The D. mojavensis genome, annotations (.gff and .gtf) and predicted transcripts (v. r.1.04) were downloaded from Flybase (ftp://ftp.flybase.net/genomes/). Quality trimmed reads were mapped to the D. mojavensis genome with .gff annotation using STAR v. 2.4.2a [34]. The expression counts data for each gene were obtained using the quantMode GeneCounts flag option in STAR for uniquely mapped reads using the default settings, and the counts outputs for unstranded RNA-seq were used in downstream analysis.

To validate a list of Or gene targets to be used, we started with a list of Or orthologues in the D. mojavensis genome tabulated by Guo & Kim [35]. We first used blastn (http://blast.ncbi.nlm.nih.gov/) to identify the best-hit FBgn among predicted transcripts from the D. mojavensis genome. To further verify the assignment of the target sequences to gene names, all Or sequences were then blasted against the genome reference, referencing the annotation file to validate the chromosomal positions of each gene. In two cases (Or98a-1 and OrN2-1), this yielded best hits with low per cent identity and incomplete matches, indicating an inconsistency with the available predicted transcripts file. Thus, for these two cases the best-hit FBgn from genome reference and its .gff annotation were used instead.

We analysed expression using the exact test model in edgeR v. 3.8.6 [36]. The analysis included all genes annotated in the D. mojavensis genome. Genes with fewer than 10 counts summed across all samples were removed from the analysis. Given 52 Ors in our analysis, we corrected for multiple testing by controlling the FDR using the p.adjust function of the ‘stats’ package in R, method = ’fdr’. A description of the pipeline used has been posted at https://github.com/clarihan/DmojSSRsupplementalCode.

(d) In situ hybridization

In situ hybridizations were performed as described in Goldman et al. [37] To brief, sense and antisense digoxigenin-labeled RNA probes were generated for each odorant receptor gene using a Roche DIG RNA labelling kit (Indianapolis, IN, USA) according to manufacturer’s instructions. RNA probes were hydrolysed (60 mM Na2CO3, 40 mM NaHCO3, pH 10.2) at 60 °C for 1 h, ethanol precipitated and stored in formamide at −20 °C with the appropriate RNA probe. Tissue was then washed 5 × 2 h in Hyb buffer at 55 °C, with a list of proceeding overnight. A wash for 20 min in Hyb buffer at 55 °C, followed by 3 × 10 min in PBST at room temperature was conducted prior to incubation for 3 h in 1 : 500 anti-digoxigenin-alkaline phosphatase or POD (in PBST and 1X BSA). Three 10 min washes in PBST preceded incubation with either FastRed or TSA® Plus Cyanine 5 (Cy5) (Roche, Indianapolis, IN, USA and Perkin Elmer, Waltham, MA, USA, respectively).

Confocal Z stacks were taken per population and cell type (n = 10) using a Nikon A1Si inverted confocal microscope and ORNs were counted with aide from Imaris software (Bitplane, Concord, MA, USA). Flies of the S. Catalina population have smaller body size than the Mojave population, so the ORN numbers were adjusted for size. Specifically, the mean head width of the S. Catalina flies was measured to be 90% of the width of the Mojave flies. Assuming basiconic sensillum density is the same between populations, allometric scaling rules indicate that the Mojave population should have about 24% more of any given sensillar type simply based on its larger size, since sensillar number should be proportional to surface area. The assumption of similar density was tested by counting the total number of basiconic sensilla from SEM images. This showed a 27% advantage for Mojave (295 ± 15 (s.d.), n = 4) compared to S. Catalina (232 ± 17 (s.d.), n = 4), very close to the predicted value of 24% due simply to size. Thus, a significant deviation in the number, N_or, of ORNs in Mojave flies from N_or + 0.24N_or can be considered a developmental shift in ORN emphasis, and not merely an overall size difference.

3. Results

(a) Characterization of the response properties of Drosophila mojavensis odorant receptor neurons

Previous EAG and EPG responses to host plant volatiles revealed divergence among populations in electrophysiological responses to odorants, the most substantial of which was the Mojave population that uses barrel cactus [25]. To determine the mechanisms underlying these differences, we characterized the peripheral olfactory system of D. mojavensis and investigated whether the same ORNs in different populations exhibit differences in (i) response profile, i.e. relative receptor specificity to a set of odorants, (ii) general sensitivity and/or (iii) their proportion relative to total ORNs.

We conducted SSRs from the antennae and maxillary palps for two populations, Mojave and S. Catalina. We focused on the response of basiconic sensilla to a diverse set of volatile chemicals that are present in cactus headspace and/or are diagnostics for the known D. melanogaster sensillar subtypes. These subtypes are defined by the response properties of their ORNs. We identified 10 antennal and three palpal sensillar subtypes (figure 1a,b) electronic supplementary material, tables S2–S5). These subtypes varied in their ORN response profiles. Hierarchical cluster analysis confirmed our subjective within-population grouping of individual D. mojavensis recordings, and revealed that, irrespective of population, each subtype clusters together (electronic supplementary material, figure S1). Having established this within D. mojavensis, we used the mean responses of these subtypes in a cluster analysis with recordings of known D. melanogaster subtypes. This reveals that the three large antennal basiconic (ab) subtypes (Dmoj1b–3) were highly similar in their response properties to their D. melanogaster equivalent (figure 1a; electronic supplementary material, figure S2, [9,18,26,28]). Of the remaining seven antennal subtypes, many ORN responses were conserved.
The spike counts for ab1B and ab1C were combined because they could not be separated reliably and the ab1D response could not be consistently resolved from background. (Figure 1. p-0.05, **p<0.01, ***p<0.001.)

Figure 1. ORN response profiles for the Mojave and S. Catalina populations. (a) Response profiles of antennal ORNs for each of 10 sensillar basiconic subtypes. The spike counts for ab1B and ab1C were combined because they could not be separated reliably and the ab1D response could not be consistently resolved from background. (b) Response profiles of maxillary palp ORNs for each of three sensillar subtypes. In both panels, a superscript 1 denotes ORNs in which the corresponding receptor is not bioinformatically predicted to be present in D. mojavensis [35]. Chemical classes: blue, sulfur; orange, ketone; green, ester; black, aromatic; red, alcohol; pink, other. p-values: *p<0.05, **p<0.01, ***p<0.001.
and clustered tightly with known *D. melanogaster* ORNs. Other ORNs did not, and these responses fell into two categories: (i) ORNs previously shown to have high variability among drosophilid species [28,35,38,39] or (ii) ORNs in which the corresponding receptor is not bioinformatically predicted to be present in *D. mojavensis* [35]. The remaining three, *Dmoj*abX–Z, could not be unambiguously matched by their responses to known *D. melanogaster* subtypes. We did not identify a clear equivalent to *D. melanogaster* ab5, ab8 or ab10 despite orthologous receptors having been identified for at least one of their ORNs [35]. In the case of the maxillary palps, three subtypes (*Dmoj*ab1–3) were identified and their ORNs display overall similarities to those found in other drosophilid flies (figure 1b; electronic supplementary material, figure S1, [29]).

The ORN response profiles were diverse and varied in their breadth for both the palp and antenna (figure 1a,b; electronic supplementary material, figures S3 and S4). Most ORNs responded to a subset of the odour stimuli. The *D. mojavensis* ab1A neuron, for example, was much more broadly tuned than the ab7B neuron, which showed strong excitatory responses to a single test compound, ethyl lactate. Ethyl lactate has been speculated to be an indicator of pH and thus the suitability of a fermentation substrate [38]. On the other hand, seven neurons did not respond strongly to any tested compound (ab3B, ab4B, ab9A, abXA, abYB and pb2A). This is not unexpected in the case of ab4B, because it has previously been shown in several species, including in *D. mojavensis*, to have a conserved and exclusive activation by geosmin, an indicator of harmful microbes ([32], data not shown). Furthermore, *D. melanogaster* ab9A is responsive to citral, and pb2A to beta-ionone [40]—compounds not detected in characterizations of the volatile compositions of cacti in the *D. mojavensis* system [25]. Therefore, their function, as well as the function of ab3B, abXA and abYB, whose receptors are unknown in *D. mojavensis* [35], will require further study.

(b) Population differences in electrophysiological responses to volatiles

We next compared the response properties of the same ORN between the Mojave and S. Catalina populations. These comparisons revealed variation among ORNs in their degree of conservation. Seven ORNs showed no significant population differences in their response properties (figure 1a,b). This high level of conservation in five of the seven ORNs is in line with our understanding from *D. melanogaster* that they function as general detectors of habitat suitability, for instance, as a general yeast detector (ab1A, ab2A, ab7B, pb2B and pb3B [28,38,39]). The receptors of the remaining two ORNs are unknown.

By contrast, eight ORNs show high variability between populations and probably have roles in adaptation to host plants, with two main patterns emerging (figure 1a,b; electronic supplementary material, figures S3 and S4). First, we found odour specific sensitivity differences. There is a significant increase in sensitivity to a subset of odorants in one population and to a different subset of odorants in the other population, such as in ab2B, ab3A and pb3A neurons. The second pattern observed was general ORN sensitivity differences between populations. Six neurons exhibit generalized sensitivity differences to odour, all showing enhanced responses by the Mojave population (ab4A, ab7A, ab9B, abYA, abZB and pb1B). Of the three ORNs with known receptor orthologues, ab9B and pb1B are highly responsive to aromatics in *D. melanogaster* and in *D. mojavensis* [27,29,30,38,41]. This is in line with previous work in *D. mojavensis* in which the Mojave
population showed increased responses to aromatics and in which aromatics were found to be a dominant component of the headspace of barrel cactus, its host plant [25]. In *D. melano-
gaster*, the third neuron, ab7A, expresses Or98a and responds to several esters as well as aromatic esters such as ethyl and methyl benzoate [27]. In *D. mojavensis*, three predicted Or98a genes exist and ab7A responses to these aromatic esters were not observed [35].

(c) Population differences in odorant receptor gene sequence and expression levels

Changes in the amino acid sequence of ORs or in gene expression levels could cause alterations in electrophysiological sensitivity. Odour specific alterations, such as for ab2A, ab3A and pb3A, are suggestive of amino acid sequence differences in their receptors. Two receptors have known orthologues: Or85a (ab2B) and OR22a (ab3A) [35]. Sequence comparison between the S. Catalina and Mojave populations revealed nine amino acid sequence differences in OR22a (H41N, L121S, F143Y, S163F, L217V, S238P, I305M, T347I and I369V). Interestingly, changes in ab3A neuron abundance and/or sensitivity have also been shown to occur in comparisons between the generalist *D. melanogaster* and host specialists *D. sechellia* and *D. erecta* [1,3]. In the case of Or85a, two genes are predicted with a T237N substitution in OR85a-1 and a L228I change in OR85a-2. Future studies will be needed to address whether one or several of these changes contribute to the observed shifts in affinity.

On the other hand, patterns of generalized sensitivity could be caused by amino acid sequence differences or, more likely, by changes in odorant receptor gene expression levels. To investigate the link between ORN sensitivity and *Or* gene expression levels, we used RNA-seq to examine the transcript abundance of the odorant receptor gene family in the Mojave and S. Catalina populations. We calculated fold-differences in transcript abundance of these genes and determined statistically significant differences in abundance between populations (figure 2; electronic supplementary material, table S6). We found considerable differences in *Or* gene expression levels between the two populations, with 18 *Ors* significantly differentially expressed (figure 2). Receptors classified as larval-specific in *D. melanogaster* were also identified [10]. The expression of such receptors is in accordance with results from other RNA-seq studies of the *D. melanogaster* antenna. In those studies, multiple genes previously identified by *in situ* hybridization or through reporter gene analyses to be only expressed in larvae or the maxillary palps were also detected in adults [42,43]. The functional significance of this, if any, remains to be determined.

We next asked whether the receptors for ORNs which showed generalized increases in sensitivity in the Mojave population were differentially expressed. Two of the three known receptors were significantly upregulated in the Mojave population: *Or67b* (ab9B) and *Or71a* (pb1B). Seven additional receptors known in *D. melanogaster* to be expressed in basiconic sensilla were also upregulated in the Mojave population. Of particular interest are four genes, *Or67b*, *Or71a*, *Or46a* and *Or67a-2*, which in *D. melanogaster* and/or...
here are strongly excited by aromatics [25, 27, 29, 30, 38, 41]. This sensitivity for aromatics has not been shown for the four basiconic receptors significantly upregulated in the S. Catalina population. These receptors have been shown to be sensitive to esters (figure 1a, b, [28]). Thus, the physiological differences between populations of this species appear to be reflected in, and likely derived from, the molecular machinery and reflective of their host plant use.

(d) Population differences in the proportion of select odorant receptor neurons

Changes in odorant receptor gene expression levels could be related to changes in the proportion of select ORNs. Therefore, we conducted whole-mount in situ hybridizations for differentially expressed ORs that label specific sensillar subtypes. Counts of ORN cell bodies revealed that the Mojave population has a greater number of Or67a-2-expressing neurons (figure 3). In S. Catalina, the increase was in ab1 sensilla (Or92a-expressing cells) and ab2 sensilla (Or59b). Finally, because of the relative simplicity of the maxillary palps, we examined all three palpal subtypes. The pb1 and pb2 sensilla (Or71a and Or46a) made up a greater proportion of total palpal sensilla in the Mojave population, and thus pb3 (Or85d) was reduced, compared to the S. Catalina flies. In short, we typically observed differences in the proportion of a specific ORN reflected in changes in expression of its odorant receptor.

This difference in the proportion of ORNs was also reflected in our mapping of the sensillar subtypes across the surface of the maxillary palp. We found a difference between populations in the spatial distribution of sensillar subtypes. We used a spatially random sampling regime and recorded from sensilla located at many pseudorandom x,y coordinates. We repeated this for multiple flies to attain good coverage—306 and 260 sensilla for the Mojave and S. Catalina flies, respectively (figure 4a). We discovered that the mean location of the dorsal pb3 sensilla is significantly shifted proximally in the Mojave flies relative to

Figure 4. Distribution of sensillar subtypes. (a) Distribution across the surfaces of the maxillary palps and (b) the antenna for each population. For panel (a), the lines indicate the 50% and 75% isodensity clines. In panel (b), the numbers denote subtypes similar in their response profiles to D. melanogaster. The letters denote subtypes unable to be categorized based on known D. melanogaster ORN response properties.
the S. Catalina flies (approx. 10 μm). This is due to the fact that pb3 sensilla in the Mojave flies do not invade the distal tip of the palp as they do in the S. Catalina flies (figure 4a), where pb3s co-mingle with pb1s and pb2s, and the distal border of the pb3 range is over 20 μm more proximal in the Mojave flies than the S. Catalina flies. This difference in sensilla distribution in the maxillary palp coincides with a difference in relative numbers of sensilla subtypes, which could lead to a difference in sensitivity. No gross differences in sensilla distributions were found across the antenna (figure 4b). However, its size and the degree of sampling may preclude the detection of subtle population differences. Overall, the differential gene expression impact studies, in situ hybridizations and mapping of the maxillary palp are in close concordance.

4. Discussion

Alterations in olfactory electrophysiology and in the olfactory transcriptome were observed between populations that have diverged only a few hundred thousand years ago [21]. Of particular note is the Mojave population’s increase in both the sensitivity and proportion of ORNs responsive to aromatics, a primary constituent of its host plant. Moreover, the observed olfactory differences are reminiscent of the differences observed between D. sechellia and D. melanogaster [1,18]. This host-driven sensory augmentation has also been shown for other insects, such as Culex mosquitoes [44], but this is the first instance to our knowledge of such co-occurring differences between populations of a single species.

The mechanisms underlying divergence in olfaction within and between species is not well understood [45]. Multiple mechanisms underlie population differences in D. mojavensis. We identified the same ORNs in different populations exhibiting differences in response specificity, sensitivity and/or in their proportion relative to total ORNs. In vertebrates and invertebrates, variation in ORs has been associated with variation in perception and behaviour [46–48]. Changes in the amino acid sequence and in non-coding regions have been suggested to influence ligand binding affinity or receptor expression levels, resulting in shifts in odour specificity or sensitivity. Moreover, in our study we also identified between-population differences in ORN number in the antenna and maxillary palps. In the quantitatively simpler maxillary palp with only three sensilla subtypes, there was an increase in pb1 and pb2 sensilla in the Mojave population at the expense of pb3s. Studies between Drosophila species have identified the expansion of one sensillar subtype at the expense of others [1,3]. These developmental changes have been suggested to be due to differences in an as yet unknown regulatory factor(s) driving sensillar development [45]. We speculate that similar alterations are at play within the D. mojavensis system.

We observed multiple changes in multiple ORNs and receptors between populations. In a previous study on D. mojavensis, host-specific behavioural responses were observed to a mixture of host plant volatiles and not recapitulated with single compounds alone [25]. Moreover, in D. melanogaster variation in behavioural responses to single compounds are associated with polymorphisms in several receptors [47,48]. Therefore, it is likely that multiple receptors play a role in host preference behaviour in this system. Second, studies in D. melanogaster have suggested that many ORs relay distinct ecologically important information [38]. Changes in the olfactory system may influence not only attraction behaviour, but also processes such as oviposition and feeding behaviour. Olfactory sensory neurons in the maxillary palp, for example, have been suggested in D. melanogaster to function as proxy detectors of antioxidants and in taste enhancement [41,49].

Together our results highlight the fascinating complexities underlying adaptation to new ecological environments in the D. mojavensis system. This study provides new insight into the proximate mechanisms underlying divergence in olfactory preferences and the factors that contribute to phenotypic divergence and the evolution of host plant preference.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material. Data for RNA-seq libraries has been uploaded to SRA(SRP071645).

Authors’ contributions. A.C.G. and N.R. conducted the electrophysiology experiments, and J.E.L. and S.M.R. participated in the analysis of these data. A.C.G., S.M.R. and J.E.L. conducted in situ hybridization studies. P.D. performed the genomic experiments. P.D., C.H., S.M.R. and P.A. contributed to the design and analysis of the genomic experiments. All authors participated in the design of experiments, writing of the manuscript and gave final approval for publication.

Competing interests. We have no competing interests.

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