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Evolution of coeloconic sensilla in the peripheral olfactory system of *Drosophila mojavensis*

Daniel C. Nemeth[1], Byrappa Ammagarahalli[1], John E. Layne, Stephanie M. Rollmann*

Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221, USA

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ABSTRACT

Populations inhabiting habitats with different environmental conditions, such as climate, resource availability, predation, competition, can undergo selection for traits that are adaptive in one habitat and not the other, leading to divergence between populations. Changes in the olfactory systems of insects that rely on different host plants, for example, can occur in response to differences in sensory stimuli between habitats. In this study, we investigate the evolution of host preference by characterizing the coeloconic sensilla in *Drosophila mojavensis,* a species that breeds on different necrotic cacti across its geographic range. These cactus species differ in the volatile chemicals they emit, a primary sensory cue for host plant discrimination. Analysis of odor-evoked responses identified four coeloconic sensilla that were qualitatively similar to those of *Drosophila melanogaster*, but varied in the breadth and strength of their olfactory sensory neuron responses to some acids and amines. Variation in responses to certain odorants among *D. mojavensis* populations was also observed. Compared to *D. melanogaster*, there was a lack of sensitivity of antennal coeloconic type 3 (ac3) sensilla to primary ligands of OR35a across all populations. Consistent with this result was a lack of detectable *Or35a* gene expression. Using a comparative approach, we then examined odor specificity of ac3 sensilla for seven additional *Drosophila* species, and found that OR35a-like sensitivity may be limited to the melanogaster subgroup. The variation in specificity that was observed among species is not clearly attributable to the degree of ecological specialization, nor to the ecological niche.

1. Introduction

Different populations of a single species may encounter habitats that differ in abiotic and/or biotic conditions. Differences in these conditions can lead, by natural- or habitat-dependent sexual selection, to adaptations that are beneficial in one habitat but not the other, causing divergent evolution. Over time this can lead, even in the presence of some gene flow between populations (e.g., Nosil et al., 2005), to reproductive isolation (Schluter, 2001; Linn et al., 2003; Rundle and Nosil, 2005; Schulter and Conte, 2009), and hence to new species (Dobzhansky, 1937; Mayr, 1949).

A key characteristic of any habitat is the sensory information available, a habitat being novel, in part, because it contains novel sensory stimuli. Therefore, adaptation to different habitats can manifest as alterations in sensory organs. As natural examples, specifically by changes in sensory behavior, the interactions of phytophagous insects with their host plants have proved to be favorable models (Via, 1999; Pophof et al., 2005; Date et al., 2013; Nishida, 2014). Host plant recognition and preference behavior, driven by oviposition and feeding needs, involves olfactory cues (Kopp et al., 2008; Hussain et al., 2016). Preferences for potential new host plants, which emit a novel suite of odorants, can contribute to population divergence. Mate choice in some phytophagous insects is centered on the host plant, which therefore creates a barrier to gene flow between the populations, based primarily on their olfactory preference for the different host plant volatiles (Linn et al., 2003; Dekker et al., 2006; Olsson et al., 2006a,b). Indeed, insects have been shown to shift preference to an alternative host plant under new environmental conditions, leading to differential adaptation and divergent evolution of their sensory systems, two well-known examples being *Drosophila sechellia* (Stensmyr et al., 2003a; Dekker et al., 2006; McBride and Arguello, 2007; Mansourian and Stensmyr, 2015; Prieto-Godino et al., 2017) and *Rhagoletis pomonella* (Linn et al., 2003; Linn et al., 2005; Olsson et al., 2006a,b). In both cases the behavioral shift was accompanied by physiological differences among species/populations in the sensitivity and firing patterns of their olfactory sensory neurons (OSNs) to host volatiles. Here, we present findings of a similar

* Corresponding author at: Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221-0006, USA.
E-mail address: stephanie.rollmann@uc.edu (S.M. Rollmann).
[1] D.C.N. and B.A. contributed equally to this work.

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phenomenon among populations of a cactophilic fly, *Drosophila mojavensis*.

The olfactory system in drosophilids is composed of two olfactory organs, the third antennal segment, and the maxillary palp. Both organs are covered by tiny, porous sensory hairs called sensilla, of which there are three morphological types: basiconic, trichoid, and coeloconic sensilla; the third antennal segment possesses all three types, while the maxillary palp contains only basiconic sensilla (Stocker, 1994; Reddy et al., 1997; Clyne et al., 1999; Yao et al., 2005; Benton et al., 2009). Sensillar types are also distinct in that they are tuned to different classes of chemical volatiles; basiconics are hypothesized to detect basic food odors such as alcohols, esters, and ketones, trichoids aid in mate recognition by detecting different classes of pheromone molecules, and coeloconics are tuned to the detection of acids and amines, signaling overly ripe fruit or bacterial degradation of protein-rich substrates, respectively (Stocker, 1994; van der Goes van Naters and Carlson, 2007; Kopp et al., 2008; Benton et al., 2009; Ai et al., 2010; Mansourian and Stensmyr, 2015). The sensilla contain the dendrites of up to four olfactory sensory neurons, depending on the sensillar type (Hallem and Carlson, 2004; Vosshall and Stocker, 2007; Abuin et al., 2011; Silbering et al., 2011; Ai et al., 2013). Localized to the dendrites of OSNs are transmembrane protein complexes – olfactory receptors (ORs) – which bind odorant molecules that enter the sensillar pores (Stocker, 1994; Vosshall et al., 1999), leading to membrane depolarization and the propagation of action potentials toward distinct glomeruli in the antennal lobe, which is analogous to the olfactory bulb in mammals (Stocker, 1994; Clyne et al., 1999; Vosshall et al., 1999; Vosshall et al., 2000; Hallem and Carlson, 2004; Vosshall and Stocker, 2007; Min et al., 2013; Rytz et al., 2013; Bell and Wilson, 2016). At least, that is the case for OSNs innervating basiconic and trichoid sensilla, which generally express one odorant receptor, along with a co-receptor, ORCO. The OSNs innervating coeloconic sensilla, on the other hand, express instead a family of olfactory receptors called ionotropic receptors (IRs; with one exception, see below) (Vosshall et al., 2000; Yao et al., 2005; Vosshall and Stocker, 2007; Benton, 2007; Benton et al., 2009; Min et al., 2013; Rytz et al., 2013). The IRs are believed to be ancestrally derived from ionotropic glutamate receptors (Rytz et al., 2013). In *D. melanogaster,* four classes of coeloconic sensilla have been identified (ac1 - ac4), each containing dendrites from two or three IR-expressing OSNs (denoted a - c, respectively), with the exception of the OSN ac3b, which expresses OR35a (Couto et al., 2005). There are a total of sixteen *Ir* genes expressed in the antenna of *D. melanogaster*, ten of which are expressed in the OSNs that innervate the coeloconic sensilla. Two of the IRs, IR8a and IR25a, are particularly broadly expressed, and act as co-receptors for acid-sensitive and amine-sensitive IRs, respectively, and are necessary for proper receptor function (Benton et al., 2009; Abuin et al., 2011; Silbering et al., 2011; Ai et al., 2013; Min et al., 2013; Rytz et al., 2013). The IR olfactory subsystem is relatively conserved across all insects as these antennal chemosensors are also present in mosquitos, moths, and bees (Liu et al., 2010; Croset et al., 2010; Bengtsson et al., 2012).

To gain further insight into the evolution of adaptive traits, it is most edifying to study a species with clear phenotypic divergence among conspecific populations. *D. mojavensis* is an example of such, as it consists of four separate populations, each specializing on a different host plant. Endemic to the arid deserts of North America, *D. mojavensis* uses the fermenting necrotic cactus tissue for food and oviposition sites (Fogleman and Abril, 1990; Newby and Etges, 1998). The four populations of *D. mojavensis* are largely isolated from each other, which has allowed divergence in their odor-guided host plant preferences. The populations endemic to Baja California feed and breed on pitaya agria (*Stenocereus gummosus*), the Sonora population uses organ pipe cactus (*Stenocereus thurberi*), on Santa Catalina Island the flies use prickly pear cactus (*Opuntia* spp), and in the Mojave Desert they use barrel cactus (*Ferocactus cylindraceus*) (Newby and Etges, 1998; Smith et al., 2012; Date et al., 2013; Date et al., 2017). Measurements of the chemistry of the host plants have revealed that each species of cactus emits a unique suite of volatile odorants, which differ in the types and/or relative amounts of chemical compounds. For instance, butyric, acetic, and propionic acid are present in variable amounts amongst agria and organ pipe rots (Fogleman and Abril, 1990). A more recent study on the volatiles from all four host cacti revealed differences in the types of volatiles emitted between stages of rot, which is of particular note as *D. mojavensis* flies are more attracted to the early stages of fermentation (Date et al., 2013). Both the naturally occurring “early rot” of organ pipe and the natural necrotic cactus tissue of prickly pear were dominated by carboxylic acids: butyric, hexanoic, octanoic, and nonanoic acid for organ pipe, and isobutyric, butyric, and hexanoic acid for prickly pear (Wright and Setzer, 2014). The sources of these carboxylic acids are most likely the resulting byproducts of fermenting yeast and bacteria that are infecting the rots (Wright and Setzer, 2014; Mansourian and Stensmyr, 2015). In barrel cacti there is little evidence of these acids being emitted from rots; however, little is known about this species (Date et al., 2013). Thus, there are chemical differences between cactus rots that serve as a primary sensory cue for host plant identification.

Insect olfactory systems have been studied extensively and much work has been conducted on the OSNs expressing ORs to understand the neural-sensory contributions to population and species differences (Stensmyr et al., 2003a; Dekker et al., 2006; Olsson et al., 2006; Kopp et al., 2008; Stensmyr et al., 2012; Crowley-Gall et al., 2016). Previous work on the olfactory system of *D. mojavensis* populations has revealed divergence in the electroantennogram (EAG) responses to host plant volatiles (Date et al., 2013); and this work was further refined to reveal cell-specific differences in OSN specificity, sensitivity, and abundance (Crowley-Gall et al., 2016). These studies have expanded knowledge of OR-based olfactory circuitry and its role in population divergence. However; the importance of acid and amine content as a potential environmental cue, coupled with the IR-expressing OSNs detecting them as a prime contributor to population divergence, warrants examination. Here, we examine *D. mojavensis* to further elucidate the mechanisms underlying population divergence in the acid- and amine-sensitive IR-expressing OSNs contained within coeloconic sensilla.

2. Materials and methods

2.1. Fly stocks

The *D. mojavensis* Mojave (A997b: Providence Mountain, CA, USA), Baja (SQ59a: San Quintin, Baja California, MX), and Sonora population (OPNM9: Organ Pipe National Monument, Arizona, USA) stocks were a gift from Dr. William Etges. The following stocks were from the *Drosophila* Species Stock Center (San Diego, CA, USA): *D. ananassae* (stock number 14024-0371.13), *D. arizonae* (15081-1271.33), *D. erecta* (14021-0224.01), *D. mojavensis* S. Catalina population (15081-1352.22), *D. persimilis* (14011-0111.49), *D. virilis* (15010-1051.87), and *D. yakuba* (14021-0261.01). The *D. melanogaster* stock (Canton-S: stock number 64349) was obtained from the Bloomington *Drosophila* stock center (Bloomington, IN, USA). The cactophilic *Drosophila* stocks (*D. mojavensis* and *D. arizonae*) were maintained on a standard banana cactus medium and the remaining stocks on a standard cornmeal food medium. All stocks were reared at 25 °C on a 12 h light/dark cycle.

2.2. Electrophysiology

2.2.1. Odorant stimuli

Chemical compounds were purchased at high purity and were diluted to 1 % in paraffin oil or distilled H_2O, as appropriate (chemical details in Table 1). Twenty microliters of odorant were loaded on a filter paper (15 × 10 mm) inserted into a 14.6 cm Pasteur pipette. Each odorant cartridge was used a maximum of two times during a recording period. Odorants were selected based on their presence in fermenting

Table 1
Chemical compounds used in the study.

Chemical compounds	CAS Number	Purity (%)	Company	Chemical class
butyric acid	107-92-6	≥99.5	Fluka	acid
propionic acid*	79-09-4	≥99.5	Sigma-Aldrich	acid
valeric acid	109-52-4	≥99	Sigma-Aldrich	acid
hexanoic acid	142-62-1	≥99.5	Sigma-Aldrich	acid
octanoic acid	124-07-2	≥98	Sigma-Aldrich	acid
isobutyric acid	79-31-2	99	Sigma-Aldrich	acid
2-oxopentanoic acid	1821-02-9	≥98	Sigma-Aldrich	acid
3-octanol	589-98-0	≥99	Sigma-Aldrich	alcohol
Isoamyl alcohol	123-51-3	≥99	Sigma-Aldrich	alcohol
1-pentanol	71-41-0	≥99	Sigma-Aldrich	alcohol
1-butanol	71-36-3	≥99.9	Sigma-Aldrich	alcohol
1-heptanol	111-70-6	≥98	Sigma-Aldrich	alcohol
(E)-2-hexenol	928-95-0	≥96	Sigma-Aldrich	alcohol
1-hexanol	111-27-3	98	Sigma-Aldrich	alcohol
linalool	78-70-6	≥97	Sigma-Aldrich	alcohol
isopropanol	67-63-0	≥99.7	Sigma-Aldrich	alcohol
butyraldehyde	123-72-8	≥99.5	Sigma-Aldrich	aldehyde
phenylacetaldehyde	122-78-1	≥95	Sigma-Aldrich	aldehyde
hexenal	66-25-1	≥98	Sigma-Aldrich	aldehyde
heptanal	111-71-7	≥92	Sigma-Aldrich	aldehyde
(E)-2-hexenal	6728-26-3	≥98	Sigma-Aldrich	aldehyde
benzaldehyde	100-52-7	≥99	Sigma-Aldrich	aldehyde
ethanolamine	141-43-5	≥99	Sigma-Aldrich	amine
1,4-diaminobutane*	110-60-1	99	Sigma-Aldrich	amine
pyridine	110-86-1	≥99.8	Sigma-Aldrich	amine
cadaverine	462-94-2	≥97	Sigma-Aldrich	amine
spermidine	124-20-9	≥99	Sigma-Aldrich	amine
phenethylamine	64-04-0	99	Sigma-Aldrich	amine
4-ethylguaiacol	2785-89-9	≥98	Sigma-Aldrich	phenol
p-cresol	106-44-5	≥99	Sigma-Aldrich	phenol
2-methoxy-4-propylphenol	2785-87-7	≥99	Sigma-Aldrich	phenol
phenol	108-95-2	90.8	Fisher	phenol
2-phenethyl acetate	103-45-7	99	Sigma-Aldrich	ester
ethyl benzoate	93-89-0	≥99	Sigma-Aldrich	ester
hexyl acetate	142-92-7	99	Sigma-Aldrich	ester
ethyl hexanoate	123-66-0	≥99	Sigma-Aldrich	ester
propyl acetate	109-60-4	99.5	Sigma-Aldrich	ester
ethyl butyrate	105-54-4	99	Sigma-Aldrich	ester
isoamyl acetate	123-92-2	≥99	Sigma-Aldrich	ester
phenethyl propionate	122-70-3	≥98	Sigma-Aldrich	ester
propyl hexanoate	626-77-7	≥98	Sigma-Aldrich	ester
isoamyl butyrate	106-27-4	≥98	Sigma-Aldrich	ester
ethyl octanoate	106-32-1	≥99	Sigma-Aldrich	ester
2-nonanone	821-55-6	≥99	Sigma-Aldrich	ketone
acetone	67-64-1	≥99.9	Fisher	ketone
2-butanone	78-93-3	≥99	Sigma-Aldrich	ketone
acetoin	513-86-0	≥96	Sigma-Aldrich	ketone
2-heptanone	110-43-0	≥99	Sigma-Aldrich	ketone
6-methyl-5-hepten-2-one	110-93-0	99	Sigma-Aldrich	ketone
acetophenone	98-86-2	99	Sigma-Aldrich	ketone
benzyl cyanide	140-29-4	≥98	Fluka	nitrile
benzonitrile	100-47-0	99	Sigma-Aldrich	nitrile
formamide	75-12-7	99.9	Fisher	amide
2-pentylfuran	3777-69-3	≥98	Sigma-Aldrich	furan

* Odorants diluted in distilled water.

cacti and/or use as diagnostics of sensillum identity, based on work in *D. melanogaster* (Downing, 1985; Fogleman and Abril, 1990; Yao et al., 2005; Benton et al., 2009; Silbering et al., 2011; Date et al., 2013; Wright and Setzer, 2014).

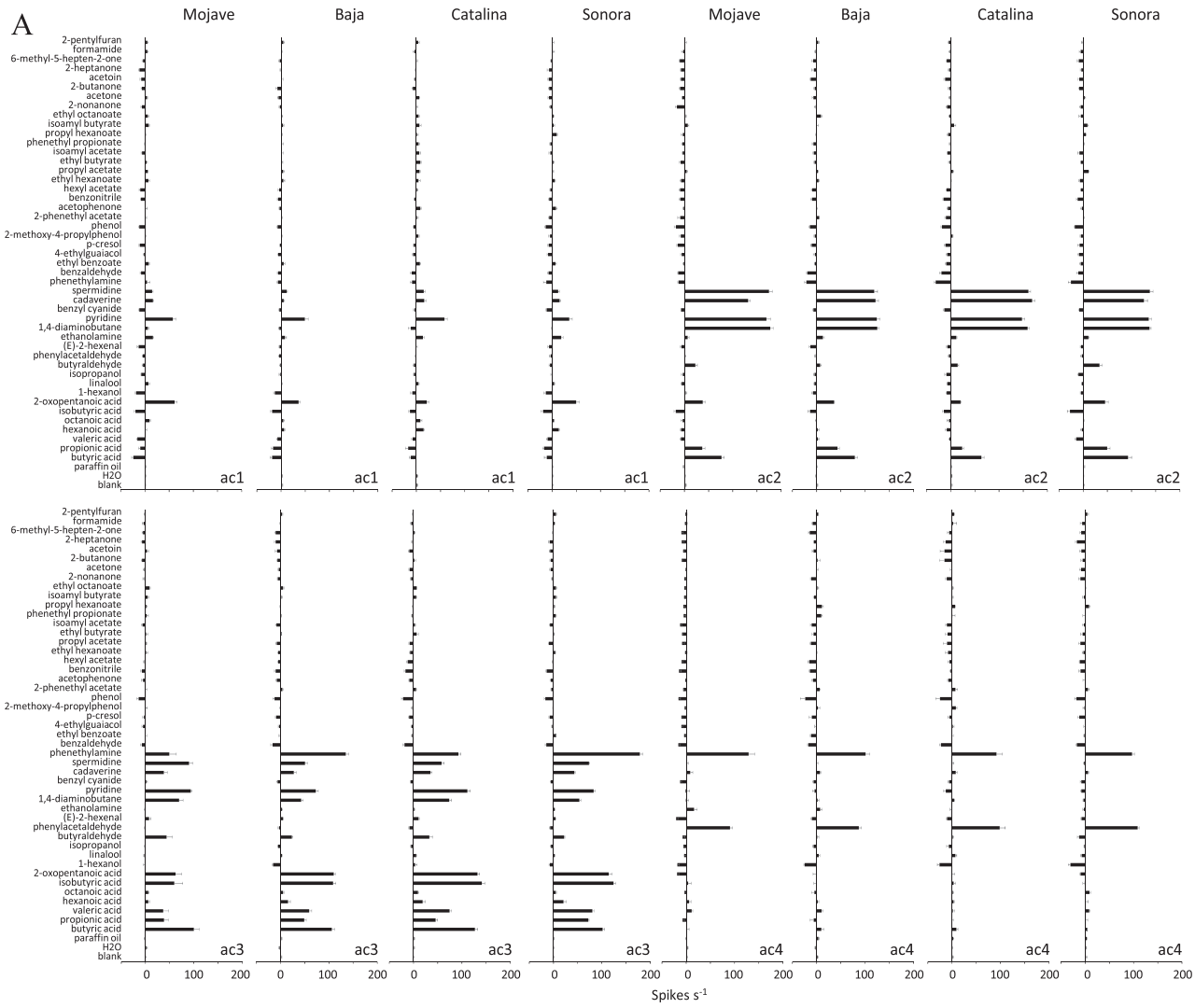
2.2.2. Single sensillum recordings (SSR)

Single sensillum recordings were performed as described in Crowley-Gall et al. (Crowley-Gall et al., 2016). A mated female fly (*D. mojavensis*; *D. virilis* and *D. arizonae* were 9 - 12 days old, all other species were 2 - 10 days old) was restrained in a pipette tip with its antenna exposed, and humidified air flowed continuously over the preparation. Electrolytically sharpened (in 1 M KOH) tungsten electrodes were inserted into the eye (reference) and into the base of a sensillum (recording). Each odorant stimulus was delivered for 500 ms with an inter-stimulus interval of at least 30 s. Signals were then amplified using an IDAC-4 (Syntech, Kirchzarten, Germany), and recorded and analyzed using Autospike software (Autospike v.3.9 Syntech, Kirchzarten, Germany). Due to the difficulty of reliably distinguishing the action potentials of individual neurons, all spikes for a given sensillum were counted without designation (Benton et al., 2009; Silbering et al., 2011; Prieto-Godino et al., 2017). For a given individual a single recording was obtained per sensillum type. Responses were calculated as the difference in spike number between 0.5 s prior to, and 0.5 s after, the onset of the response, which was identified visually for each stimulus/response. This difference in spike number was subsequently doubled to obtain the odor-evoked spikes per second. Differences in electrophysiological responses between populations/species were determined using a one-way analysis of variance (ANOVA) followed by a Tukey HSD *post-hoc* analysis and corrected for multiple testing by controlling false discovery rate (FDR = 0.05; Benjamini et al., 2006). All data included in the analysis were tested and found to meet the assumptions of ANOVA. Minor odor-evoked responses (−15 to 25 spikes/s) were excluded from the analyses.

Hierarchical cluster analysis was performed using MATLAB software (MathWorks, Natick, MA, USA). The distance between pairs of observations in response space was computed by Euclidean geometry, and the cluster tree formed using 'average' type linkage. Two different cluster analyses were performed. First, to objectively identify and compare the similarity of the four putative coeloconic sensillar types, the analysis was done with five recordings each of the four coeloconic sensillar types, from each of the four populations to 23 of the 46 odorants shown in Fig. 1, plus mean responses of the four sensillar types in *D. melanogaster* (this subset of 23 was selected because *D. melanogaster* responses were reported in the literature; Supplementary Table S1; Yao et al., 2005; Marshall et al., 2010; Silbering et al., 2011; Prieto-Godino et al., 2017). A second cluster analysis was done on the ac3 sensilla from eight *Drosophila* species in response to 17 (subset of tested odorants previously reported in *D. melanogaster*; Supplementary Table S2) of the 26 odorants presented in Fig. 5. The *D. mojavensis* representative was from the Baja population. Responses gleaned from the literature were from studies with essentially the same methodology as our own.

2.3. Gene expression: RT-PCR

Third antennal segments (500 - 600) from 10 to 11 day old female flies were dissected for each *D. mojavensis* population. Antennae were dissected directly into 700 µl of chilled TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and either extracted immediately or stored at −80 °C. The tissue was then homogenized, and chloroform was added followed by 15 s of vigorous shaking. After a 150 s incubation period at room temperature, the samples were centrifuged at 13,200 rpm at 4 °C for 15 min. The supernatant was removed and mixed with 70% ethanol and subsequently isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Two biological RNA replicates were isolated per population. cDNA was synthesized from 100 ng of RNA using an AccuScript High Fidelity 1st strand cDNA Synthesis Kit (Agilent, Santa Clara, CA, USA) according to manufacturer instructions. A negative control (without reverse transcriptase (RT)) was also generated for each sample. Samples prepared with cDNA template (RT) and negative control (no RT) were amplified with gene-specific primers using standard PCR methods. Each gene was also separately amplified using genomic DNA to validate primer effectiveness. Gene expression was examined for *Or35a* as well as two positive controls, *Ir75c_GI13610* and the housekeeping gene, *Actin 5C* (*Act5C*). The



B

	Mojave ac1	Baja ac1	Catalina ac1	Sonora ac1	Mojave ac2	Baja ac2	Catalina ac2	Sonora ac2	Mojave ac3	Baja ac3	Catalina ac3	Sonora ac3
2-nonanone					A	B	AB	AB				
phenethylamine					B	AB	A	AB	D	B	C	A
spermidine					A	C	AB	BC	A	C	BC	AB
cadaverine					B	B	A	B				
pyridine					A	B	AB	B	AB	C	A	BC
1,4-diaminobutane					A	C	B	C	A	B	A	AB
butyraldehyde					AB	C	BC	A				
1-hexanol									B	A	B	AB
2-oxopentanoic acid	A	BC	C	AB	AB	AB	B	A	B	A	A	A
isobutyric acid									B	A	A	A
valeric acid									B	AB	A	A
propionic acid					AB	AB	B	A	B	B	B	A
butyric acid					AB	AB	B	A				

Fig. 1. Response profiles of four antennal coeloconic sensillum types (ac1 – 4) among *D. mojavensis* populations. (A) Odor-evoked responses of each sensillar type (mean ± SEM) to tested odorants for all four *D. mojavensis* populations. Five recordings for each sensillum type and fly line were obtained. (B) Statistical differences in electrophysiological responses among the four *D. mojavensis* populations. Letters indicate significant population differences in odor-evoked responses, with greatest-to-least responses in alphabetical order.

primers are: *Act5C* F:5′ CCTCACTGAGCGTGGCTACT 3′ and R: 5′ ACC GATGGTAATGACCTGAC 3′; *Ir75c_GI13610* F:5′ CTTGAACTGTGAGCA GAGCA 3′ and R: 5′ CACATAGGTCACATCCGCATT 3′; *Or35a* F: 5′ TCCACTAATGGCACAGCAG 3′ and R: 5′ TGTGAGCAGTTCTATTGTCT TGG 3′. PCR products were separated by gel electrophoresis on a 2 % agarose gel and visualized with ethidium bromide. Each PCR product was also sequenced at the Cincinnati Children's Hospital Medical Center DNA Sequencing and Genotyping Core (Cincinnati, OH, USA) to confirm appropriate gene-specific amplification.

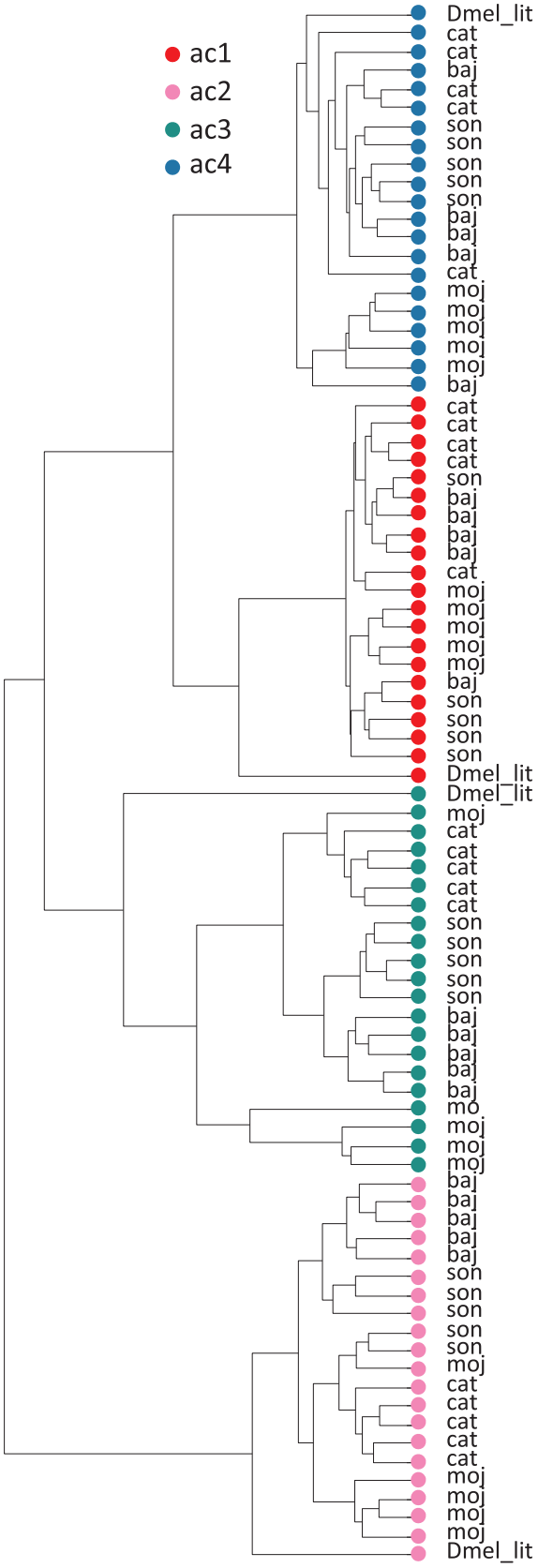


Fig. 2. Hierarchical cluster analysis of OSN response profiles for the four *D. mojavensis* populations and *D. melanogaster*.

3. Results

3.1. Characterization of OSN response profiles for coeloconic sensilla

Electrophysiological recordings from coeloconic sensilla on the third antennal segment of *D. mojavensis* were conducted for all four populations. Four distinct antennal coeloconic sensillar types were identified (ac1 - ac4) and categorized based on their odor-evoked response properties (Fig. 1A). Hierarchical cluster analysis of *D. mojavensis* OSN responses with those of *D. melanogaster* further supports the presence of four homologous sensillar types (Fig. 2).

The OSN response properties for each of these types were in most cases qualitatively similar to those of *D. melanogaster* but varied in the breadth and strength of their responses to the tested odorants (Yao et al., 2005; Benton et al., 2009; Silbering et al., 2011; Prieto-Godino et al., 2017). In the case of ac1 and ac4, excitatory responses were most notable to pyridine and 2-oxopentanoic acid, or phenethylamine and phenylacetaldehyde, respectively. A greater breadth of response was observed for ac2 and ac3 sensillar types, with responses primarily to a wider suite of acids and amines. Within a given sensillar type, differences relative to *D. melanogaster* were often in the ranked strength of responses to the tested odorants. For instance, in ac2 sensilla, greater excitatory responses to butyric acid relative to propionic acid were observed in *D. mojavensis* (Fig. 1A), and the opposite pattern was found in *D. melanogaster* (Silbering et al., 2011; Prieto-Godino et al., 2017). Additionally, the odor-evoked response properties of ac3 sensilla differed from *D. melanogaster* in several notable ways. As hypothesized, strong excitatory responses were observed to the ac3 diagnostic butyric acid (agonist for ac3A receptor IR75a,b,c in *D. melanogaster* (Prieto-Godino et al., 2017)), and an examination of ac3 OSN spontaneous activity supports the presence of at least two neurons, as defined by their spike amplitudes (Fig. 3A and B). However, there was little to no response to hexyl acetate, (E)-2-hexenal, and 1-hexanol (Figs. 1 and 3C). These are all odorants which elicit strong excitatory responses from OR35a-expressing OSNs located within *D. melanogaster* ac3 sensilla (Fig. 3D; Yao et al., 2005; Benton et al., 2009; Prieto-Godino et al., 2017). This suggests that *D. mojavensis* does not express OR35a in any of the OSNs of this sensillum, specifically in ac3B. It should be noted, however, that a moderate excitatory response to 1,4-diaminobutane was found in *D. mojavensis* ac3 sensilla (Fig. 3E), a response that in *D. melanogaster* appears to be dependent on the presence of OR35a, as evidenced by a lack of response to this odorant in an OR35a mutant line (Yao et al., 2005; Silbering et al., 2011). Odor-evoked responses to spermidine, cadaverine, and pyridine were also large relative to *D. melanogaster* (Silbering et al., 2011; Prieto-Godino et al., 2017).

3.2. Electrophysiological differences among D. mojavensis populations

Odor-evoked OSN responses were significantly different between the four *D. mojavensis* populations, with the exception of those of ac4 sensilla (Fig. 1B; Supplementary Table S3). More specifically, looking at the ac2 and ac3 responses, significant differences were observed to select acids and amines among populations. Qualitatively, the Sonora population most frequently showed the greatest excitatory responses relative to the other populations. Three amines also elicited greater excitatory responses in ac2 sensilla of the Mojave population relative to other populations.

As noted previously the designation of ac1 - 4 to the *D. mojavensis* sensillar responses, as homologous to *D. melanogaster*, was born out by cluster analysis. Next, focusing on the relationships among the *D. mojavensis* populations, we found that the Baja and Sonora OSN profiles most consistently clustered together, and the Mojave population was most likely to both stand apart from the other three, and to be among the most variable, especially within the higher responding ac2 and ac3 (Fig. 2). The latter pattern is likely due to a few factors, among them the

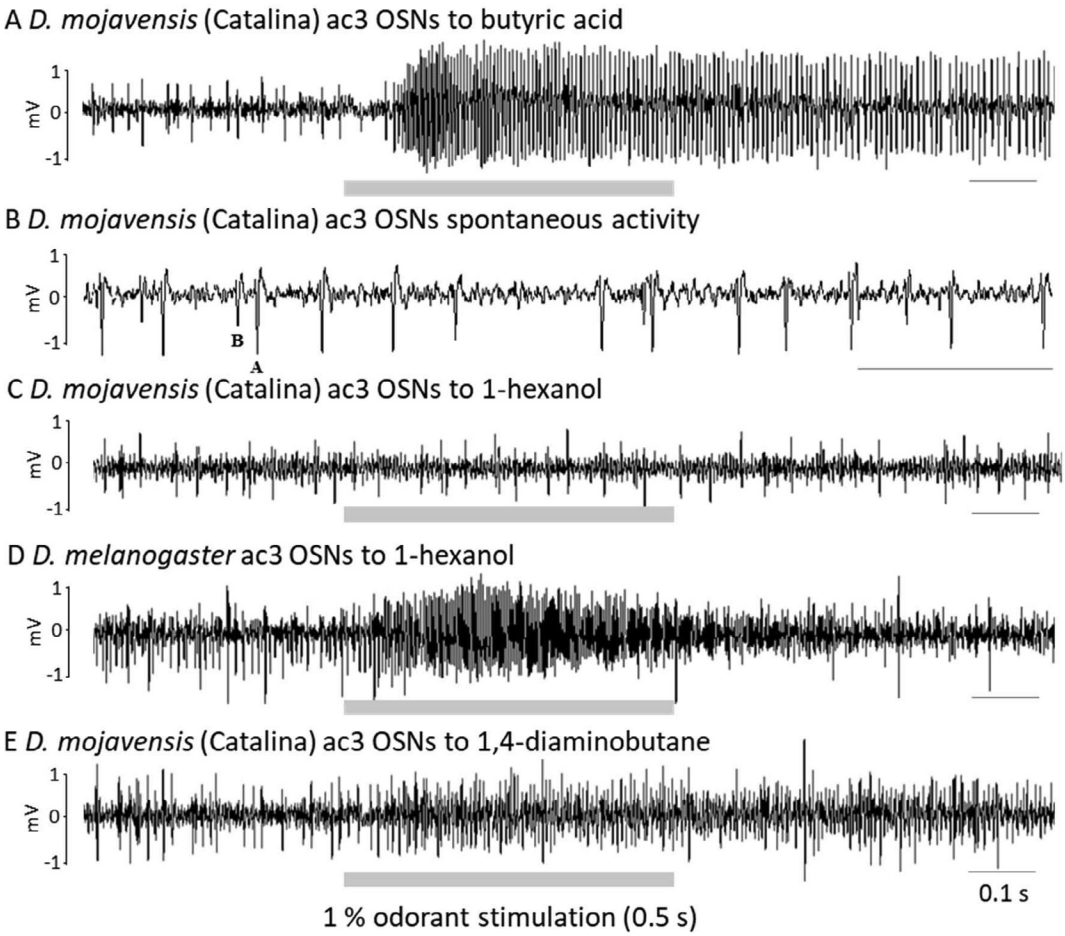


Fig. 3. Representative traces of an extracellular recording from the ac3 sensillum. Shown are: (A) *D. mojavensis* OSN responses to butyric acid (Catalina population); (B) spontaneous firing of ac3. The action potentials of two different neurons, labeled 'A' and 'B' are noted; (C) *D. mojavensis* OSN responses to the OR35a diagnostic odorant 1-hexanol; (D) *D. melanogaster* OSN responses to 1-hexanol; and (E) *D. mojavensis* OSN responses to 1,4-diaminobutane.

Mojave population exhibiting generally lower responses to tested acids, and generally higher responses to amines (with the exception, along with the Catalina population, of the ac2 response to phenethylamine). Finally, the Mojave ac3 responses were generally lower for the majority of odorants.

3.3. Olfactory receptor Or35a gene expression

The response profile of ac3 sensilla in *D. mojavensis* showed a marked difference to that of *D. melanogaster* in that it showed little to no response to known diagnostic odorants of OR35a-expressing OSNs (Fig. 1). In *D. melanogaster*, ac3 sensilla house two OSNs, ac3A which co-expresses IR75a, IR75b, and IR75c, and ac3B which expresses OR35a together with its OR co-receptor (ORCO) and IR76b. IR76b is co-expressed in one OSN in each of the four sensillar types and its role as a putative co-receptor and/or ligand detector remains to be determined (Abuin et al., 2011; Silbering et al., 2011). A lack of response in *D. mojavensis* to these odorants is unlikely to be due to a loss of the ac3B neuron because single sensillum recordings revealed the presence of at least two neurons (Fig. 3B). Therefore, we investigated whether *Or35a* gene expression can be detected in the *D. mojavensis* antenna. Expression was examined for *Or35a* as well as for two positive controls, Dmoj*Ir75c_GI13610* and *Actin 5C*. RT-PCR results revealed expression of the two positive controls in all four populations, but *Or35a* expression was not detected (Fig. 4). The lack of *Or35a* expression is consistent with the absence of ac3 responses to known diagnostics of OR35a-expressing neurons (Yao et al., 2005; Benton et al., 2009; Prieto-Godino et al., 2017). Additionally, in a RNA-seq study examining *D. mojavensis* population differences in odorant receptor expression, *Or35a* was not detected (Crowley-Gall et al., 2016).

3.4. Species differences in electrophysiological responses of the ac3 sensilla

To further investigate divergence in ac3 sensillar response profiles across species, we examined eight *Drosophila* species (Fig. 5A). We conducted single sensillum recordings of ac3 sensillar responses to 27 chemical compounds (Fig. 5B) that are known ac3 agonists, including diagnostic odorants for OR35a-expressing cells (Fig. 1; Benton et al., 2009; Silbering et al., 2011; Rytz et al., 2013; Prieto-Godino et al., 2017).

Differences in the sensitivity and specificity of OSN responses were found among species (Fig. 5B). For tested acids, qualitatively similar OSN responses to butyric and isobutyric acid were observed across species, with other tested acids eliciting more variable responses. Moreover, the noted increase in odor-evoked responses in *D. mojavensis* relative to *D. melanogaster* to spermidine, cadaverine, and pyridine were also found in *D. ananassae* and *D. persimilis*. Finally, as was the case in *D. mojavensis,* very few to no ac3 responses were observed in the melanogaster subgroup (Fig. 5A).

A second cluster analysis was performed, combining our ac1 - 4 recordings from *D. mojavensis* (Baja population) with *D. melanogaster* ac1 - 4 data gleaned from the literature (Yao et al., 2005; Marshall et al., 2010; Silbering et al., 2011; Prieto-Godino et al., 2017), since our recordings of *D. melanogaster* (Canton-S) differed slightly from previous reports (particularly for isobutyric acid and butyraldehyde). Also included were ac3 sensillar responses from the six additional *Drosophila*

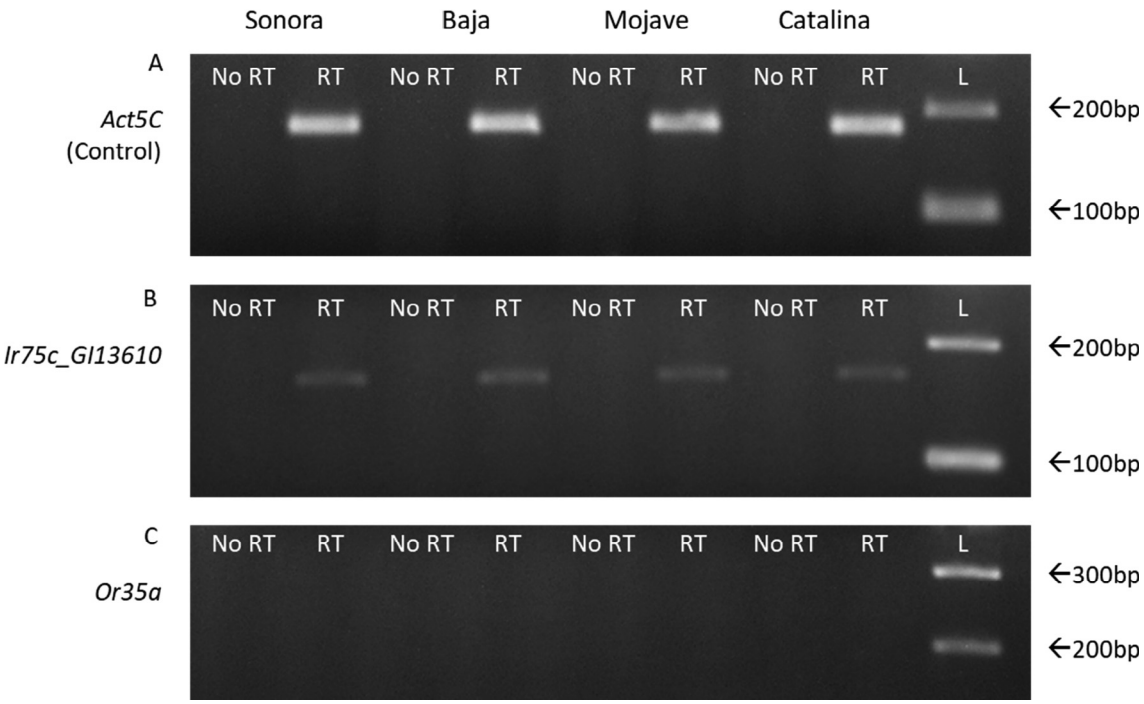


Fig. 4. RT-PCR analysis of gene expression in the antenna for all four *D. mojavensis* populations. For each population, expression was examined for (A) the housekeeping gene *Act5C*, (B) *Ir75c_GI13610*, and (C) *Or35a*. A negative control without reverse transcriptase (No RT) was also generated for each sample. L: 1 Kb HyLadder (Denville Scientific, Holliston, MA) is also shown.

species (Fig. 5B). The primary findings are that 1) as expected our *D. melanogaster* ac3 recordings formed a distinct branch with *D. erecta* and *D. yakuba*, which are all members of the melanogaster subgroup, and these differed, as mentioned, from *D. melanogaster* data from the literature (Fig. 6); 2) *D. mojavensis* ac1, ac2, and ac4 clustered with corresponding *D. melanogaster* data gleaned from the literature; 3) the cluster analysis does not necessarily recapitulate the phylogeny (Fig. 5A). For instance, *D. arizonae* clustered most closely with *D. virilis*, *D. persimilis* and *D. ananassae*, even though it is the sister group to *D. mojavensis*.

4. Discussion

Our examination of the OSN response profiles of antennal coeloconic sensillar types from the four *D. mojavensis* populations revealed electrophysiological differences among populations. Hierarchical cluster analysis showed that odor-evoked responses of the ac2 - ac4 sensilla of the Mojave population tended to be least similar to the other three populations. This is consistent with previous work by Date et al. (2013) in which principal component analyses of electroantennogram and electropalpogram responses indicated that the Mojave population was distinctly different in its neurophysiological responses to odor. Our study also reveals that populations differ in their responses to tested acids. The Mojave population exhibited reduced odor-evoked responses in ac3 sensilla to acids, and to date very few acids have been detected in fermenting barrel cactus (Date et al., 2013). In contrast the Sonora population shows greater odor-evoked response to several acids. Of particular note are the responses of ac2 sensilla to butyric acid and ac2 and ac3 sensilla to propionic acid. The Sonora population feeds and breeds on organ pipe cacti and a characterization of volatiles at different stages during necrosis found the presence of a high percentage of carboxylic acids in earlier stages (Wright and Setzer, 2014). The presence of butyric acid was also noted to be a dominant component in the Wright and Setzer study and was identified in several additional studies characterizing the volatile composition of laboratory and field necrotic cactus samples (Downing, 1985; Fogleman and Abril, 1990; Date et al., 2013). A handful of studies have also detected propionic acid in organ pipe cactus (Downing, 1985; Fogleman and Abril, 1990; Date et al., 2013).

The neurophysiological differences among populations raise the question of whether or not these odorants play a role in host discrimination. Increased physiological sensitivity in the Sonora population and diminished responses in the Mojave population to select acids may reflect the importance of these individual olfactory cues for the identification of appropriate necrotic cacti. Studies of other drosophilids have suggested that shifts in physiological sensitivity to individual volatiles reflect adaptation to a specific host plant. For example, *D. sechellia*, which specializes on *Morinda citrifolia* fruit, is hypothesized to have evolved sensitivity to hexanoic acid, a dominant component in ripe *Morinda* fruit (Prieto-Godino et al., 2017). Another drosophilid, *D. erecta* specializing on screw pine fruit (*Pandanus* spp.), is more sensitive to the screw pine volatile, 3-methyl-2-butenyl acetate, than three closely related *Drosophila* species (Linz et al., 2013). However, it is important to note in this system that volatile composition during necrosis is dynamic and the volatiles and their relative amounts vary depending on substrate and microbial community interactions (Date et al., 2013; Date et al., 2017). Moreover, the presence/absence of butyric acid and propionic acid is not restricted to organ pipe or barrel cacti. These acids were also found in other host plants (Fogleman and Foster, 1989; Fogleman and Abril, 1990; Date et al., 2013). Butyric acid, for instance, was found to be a volatile component of necrotic prickly pear (Wright and Setzer, 2014; Date et al., 2017). Yet, a corresponding increase in physiological sensitivity was not observed in the Catalina population. It showed the least excitatory responses to the butyric acid across populations. Thus, as in many insect systems, consideration should be given to the relative amount and combination of compounds and their contribution to host preference. Indeed, in the *D. mojavensis* Mojave population it is the combination of volatiles that is hypothesized to be important in host plant discrimination (Date et al., 2013). Moreover, characterization of the response spectra of basiconic sensilla of the Mojave and Catalina populations revealed differences in the sensitivity of several OR-expressing olfactory sensory neurons to host plant

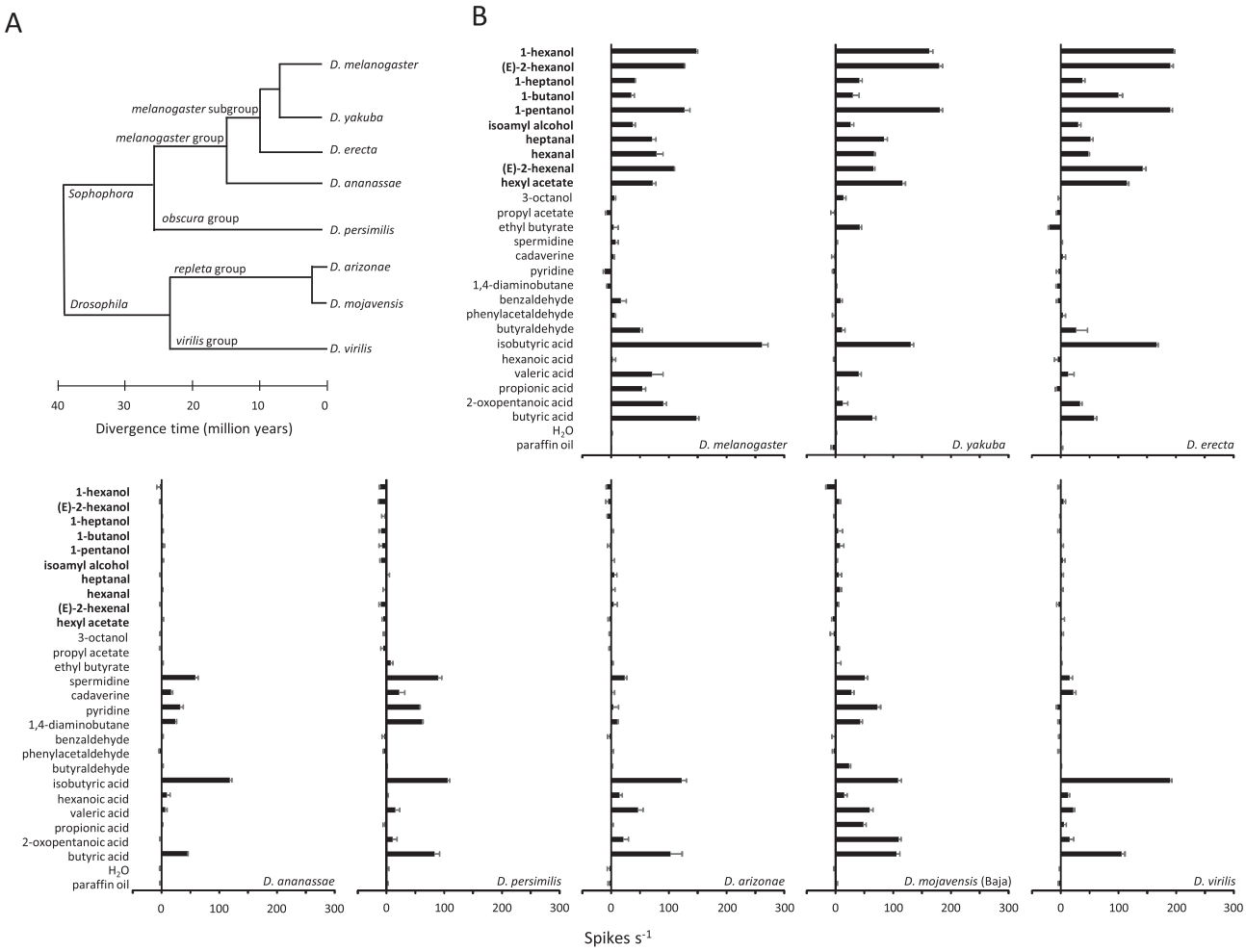


Fig. 5. Response profiles of OSNs in ac3 sensilla to selected odorants for eight *Drosophila* species. (A) Phylogenetic relationships among species (modified from Chin et al., 2014) (B) The summed odor-evoked responses (mean ± SEM) are shown for each species. The electrophysiological response of the *D. mojavensis* (Baja population) is data from Fig. 1. The first 10 odorants in bold are primary ligands of OR35a. For each fly line 3 - 5 recordings were obtained, except 2 recordings for *D. melanogaster*.

volatiles (Crowley-Gall et al., 2016). In sum, this study together with the broader characterization of sensillar types will set the stage for understanding the importance of individual volatiles and/or mixtures to host preference.

Our examination also revealed differences in the specificity of odor-evoked responses, particularly variation in the response spectra of ac3 sensilla to known agonists of OR35a-expressing OSNs. In *D. melanogaster,* OR35a is expressed in one of the two OSNs housed within an ac3 sensillum. The OR35a receptor is broadly tuned, responding to multiple chemical classes, such as aldehydes, alcohols, and esters (Hallem and Carlson, 2006). Several of the compounds are emitted from unripe fruit and some primary ligands of OR35a, such as 1-hexanol, can elicit behavioral aversion in adult flies (Stensmyr et al., 2003b; Hallem and Carlson, 2006). Given its broad breath of response, OR35a may serve as a general detector of the presence or appropriateness of a food source (Yao et al., 2005; Mansourian and Stensmyr, 2015). In contrast, *D. mojavensis* ac3 sensilla showed little to no response to primary ligands of OR35a, with the exception of 1,4-diaminobutane, and further examination revealed a lack of detectable OR35a expression in this system. In *D. melanogaster*, ac3B exhibits mild excitatory responses to 1,4-diaminobutane and this response has been attributed to the OR35a receptor (Yao et al., 2005; Silbering et al., 2011). However, there is some dissimilarity between studies. In our recordings from *D. melanogaster* and in Marshall et al. (2010) ac3 sensilla did not respond to 1,4-diaminobutane, raising the question as to whether our mild response in *D. mojavensis* serve as evidence of the presence of OR35a. Hence, the mechanism underlying OSN responses to this odorant in *D. mojavensis* remains to be determined. It is unlikely that responses are via changes to IR76b, a putative co-receptor expressed in ac3B. IR76b is reported to be expressed in all four coeloconic types and ac1 and ac4 do not respond to the 1,4-diaminobutane (Benton et al., 2009). Expression of an as yet unknown receptor, or a shift in the ligand binding affinity of receptors expressed in ac3, could confer the altered responses. In fact, in a comparison among closely related drosophilids, a single amino acid substitution in IR75b was sufficient to alter ac3 specificity to hexanoic acid, increasing sensitivity to the odorant in *D. sechellia* (Prieto-Godino et al., 2017).

To further investigate whether this change in specificity to ligands of OR35a for ac3 sensilla is restricted to *D. mojavensis*, we examined responses across other *Drosophila* species. In Drosophilidae, alterations to the chemosensory gene repertoire have been reported in comparisons between generalist and specialist species as well as with shifts in ecological niche (McBride, 2007; Goldman-Huertas et al., 2015). Therefore, we examined odor-evoked response profiles of the ac3 sensilla of seven additional *Drosophila* that vary in their ecology (Markow and O'Grady, 2007). This comparative approach revealed that responses in the melanogaster subgroup may be a derived trait. The ac3 sensilla of members of this subgroup, the three tested in this study and *D. simulans* and *D. sechellia*, all responded to primary ligands of OR35a (Fig. 5; Prieto-Godino et al., 2017). The ac3 sensilla of the remaining five

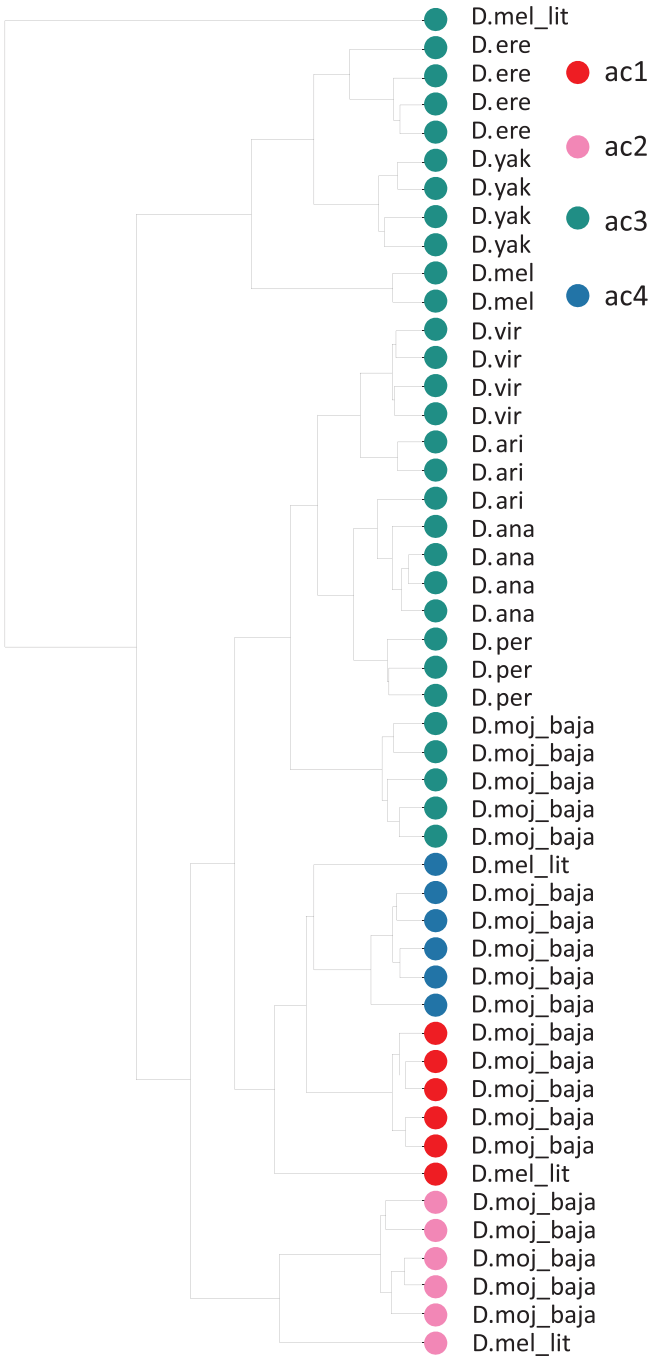


Fig. 6. Hierarchical cluster analysis of OSN response profiles for eight *Drosophila* species.

species we tested did not. This result suggests a lack of a one to one relationship between presence/absence of physiological sensitivity and ecological niche. Species responsive to OR35a ligands included specialists, such as *D. yakuba* and D. *erecta*, as well as non-specialists feeding on a variety of fermenting fruits. The same can be said for species that are unresponsive to these volatiles and also vary in their ecology. *D. mojavensis* and *D. arizonae* use cactus as a feeding and breeding substrate, while *D. ananassae* breeds on fruits. Little is known of the ecology of *D. persimilis,* and *D. virilis* is a human commensal that breeds on fruits and in nature on tree flux (Markow and O'Grady, 2007). Thus, while differences in physiological specificity to select odorants may serve to alter insect attraction or aversion and thereby impact microhabitat selection, the ecological importance of alterations in specificity of ac3 sensilla to primary ligands of OR35a within the genus *Drosophila* remains to be determined.

To conclude, characterization of odor-evoked response profiles of coeloconic sensilla revealed variation within and among *Drosophila* species. Differences in physiological sensitivity among *D. mojavensis* populations and between species may have been shaped by their distinct ecological niches. More broadly, this study provides a foundation on which to build our understanding of the complex mechanisms by which organisms adapt to diverse ecological habitats.

5. Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jinsphys.2018.08.003.

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