Comparative analysis of behavioral and transcriptional variation underlying CO_2 sensory neuron function and development in *Drosophila*

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ABSTRACT

Carbon dioxide is an important environmental cue for many insects, regulating many behaviors including some that have direct human impacts. To further improve our understanding of how this system varies among closely related insect species, we examined both the behavioral response to CO_2 as well as the transcriptional profile of key developmental regulators of CO_2 sensory neurons in the olfactory system across the *Drosophila* genus. We found that CO_2 generally evokes repulsive behavior across most of the Drosophilids we examined, but this behavior has been lost or reduced in several lineages. Comparisons of transcriptional profiles from the developing and adult antennae for subset these species suggest that behavioral differences in some species may be due to differences in the expression of the CO_2 co-receptor *Gr63a*. Furthermore, these differences in *Gr63a* expression are correlated with changes in the expression of a few genes known to be involved in the development of the CO_2 circuit, namely *dac*, an important regulator of sensilla fate for sensilla that house CO_2 ORNs, and *mip120*, a member of the MMB/dREAM epigenetic regulatory complex that regulates CO_2 receptor expression. In contrast, most of the other known structural, molecular, and developmental components of the peripheral *Drosophila* CO_2 olfactory system seem to be well-conserved across all examined lineages. These findings suggest that certain components of CO_2 sensory ORN development may be more evolutionarily labile, and may contribute to differences in CO_2-evoked behavioral responses across species.

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Introduction

Odor-guided behaviors are important for the survival of many different species and can vary dramatically, even among closely related taxa. During evolution, different sets of ecological constraints can create divergent behavioral responses to certain chemicals among closely-related species by reconfiguring the structure and function of the olfactory system underlying these behaviors.[1,2] However, how evolution modifies the genetic and developmental mechanisms underlying these behavioral differences remains poorly understood.[3]

Carbon dioxide (CO_2) is an important environmental cue for many insects, regulating many behaviors including some that have direct human impacts. For mosquitoes and other blood-feeding insects, CO_2 is a highly attractive cue and an important component of host-seeking behavior.[4] On the other hand, CO_2 is a repulsive cue that elicits a robust aversive response for the ubiquitous laboratory model species *D. melanogaster*.[5] Previous studies have suggested that these behavioral differences are associated with changes in the components of the CO_2 receptors, as well as changes in the organization of the CO_2 circuits.[6-8]

One advantage of studying the insect olfactory system is that the structure and development of the system is fairly well-understood, especially in *D. melanogaster*. Studies on *D. melanogaster* have shown that fruit flies detect volatile chemicals via olfactory receptors expressed on olfactory sensory neurons (ORNs) in 2 olfactory appendages, the antenna and the maxillary palps. These appendages are covered with fine sensory hairs called sensilla that house a cluster of between 1 and 4 ORNs. Sensilla are categorized both by morphology and by the combinations of ORN classes that they house.[9,10] Additionally,

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each ORN class connects to a unique position on the antennal lobe of the fruit fly brain, called a glomerulus, where it forms synapses with higher order neurons.[1,11]

In the antennae, each ORN cluster arises through asymmetric divisions of a single multipotent precursor cell. The identity of each precursor cell determines the number and the identity of the ORNs in each sensillum and is defined over 3 stages during the development of the antennae. First, the combinatorial expression of several cross-regulatory pre-patterning transcription factors patterns the antennal disk along the proximodistal and anteroposterior axis, and generates zones with unique differentiation potentials.[12,13] Next, selection of sensory organ precursors occurs within each zone through the expression of the proneural genes *atonal* and *amos*.[14,15] Finally, once a multipotent precursor is selected, it undergoes asymmetric cell divisions, regulated by Notch signaling and terminal selector transcription factors, to generate the ORN clusters for each sensilla subtype.[16-18] Transcription factors that regulate ORN expression in the maxillary palps have also been identified.[19] Changes in the transcriptional profiles of the regulatory modules change these developmental programs, which restructures the olfactory circuits ultimately leading to changes in odor-guided behaviors.[20]

In *D. melanogaster*, the CO_2 receptor is a heterodimer of 2 proteins: Gr21a and Gr63a.[5] This receptor is expressed in the ab1 sensilla subtype from the large basiconic class of sensilla. The CO_2 ORNs that house this receptor connect to the ventrally-located V-glomerulus in the antennal lobe to mediate repulsive behavior.[6] Developmentally, several of the transcription factors that define the ab1 sensilla have been identified.[12] Additionally, the expression of *Gr21a* and *Gr63a* in *Drosophila* species has been shown to be regulated in part by the MyB-MuvB/dREAM complex through the histone methyltransferase su(Var)3–9.[21]Previous studies have found that several proteins of the complex, such as MyB and mip130, are direct regulators of Gr63a expression, while others like mip120 and E2f2 are negative regulators. Interestingly, in *D. melanogaster* the microRNA *miR-279* and the transcription factor *prospero* are required to inhibit the generation of CO_2 ORNs in the maxillary palps. Mutations in these genes generate ectopic addition of CO_2 ORNs to the maxillary palps in addition to the population in the antennae.[6] More recently, species-specific differences in CO_2 behaviors were reported for a few *Drosophila* species. *D. virilis* and *D. suzukii* show decreased repulsion from CO_2 compared with *D. melanogaster*, yet still showed physiologic antennal responses to CO_2, suggesting that receptor function is not the sole regulator of such behavioral differences.[22] Together, these studies suggest that transcriptional changes in the genetic and developmental programs that affect olfactory receptor expression, ORN development and connectivity all can affect olfactory coding, and might drive behavioral differences toward CO_2 between different *Drosophila* species.

In this study, we examined the behavioral response toward CO_2 for an expanded number of *Drosophila* species and asked how the molecular and developmental mechanisms underlying CO_2 olfactory receptor neurons have evolved across the genus using transcriptional profiles from the developing peripheral olfactory system of 6 of the species. We also examined the transcription profiles of genes known to be involved in the function and development of the CO_2 olfactory circuit to identify evolutionarily labile components as well as potential mechanisms that may be responsible for behavioral differences. We found that, as anticipated, CO_2 generally evokes repulsive behavior across most of the *Drosophila* species we examined. However, this behavior has been lost or reduced in a subset of species. Comparisons of transcriptional profiles from the developing and adult antennae for several these species suggest that these behavioral differences may be due to differences in the expression of the CO_2 co-receptor *Gr63a*. Furthermore, we found variations in the developmental expression of a few transcription factors known to be involved in the development of the CO_2 circuit, namely *dac* and *mip120*, which were previously shown to regulate *Gr63a* expression. In contrast, most of the other known molecular and developmental components of the peripheral CO_2 sensing ORNs show very stable transcriptional levels across all examined *Drosophila* lineages. These findings suggest that certain components of the CO_2 system may be more developmentally and evolutionarily labile, and may contribute to differences in CO_2-evoked behavioral responses across species.

Results

Behavioral response to carbon dioxide across the Drosophila *genus*

We investigated variation in carbon dioxide response across the genus. Previous studies have shown reduced

repulsion toward CO_2 compared with *D. melanogaster* for a few *Drosophila* species, namely *D. virilis* and *D. suzukii*,[22] but the ancestral state and the extent of behavioral differences across the genus remains unknown. To investigate those questions, we measured the CO_2 response from 15 different species across the genus (Fig. 1A). The species we examined were primarily from the *melanogaster* (*D. melanogaster, D. simulans, D. sechellia, D. erecta*, and *D. ananassae*) and *nasuta* (*D. nasuta, D sulfurigaster, D. kohkoa, D. pallidifrons*, and *D. niveifrons*) subgroups, along with several additional taxa in between (*D. virilis, D. lummei, D. mojavensis, D. willistoni* and *Zaprionus indianus*). The approximate phylogenetic relationships of these taxa are shown in Figure 1A.[23-26] The behavioral response of each species toward CO_2 was measured using a T-maze assay, which measures the instantaneous preference or avoidance response of walking flies toward an odor vs. a control. The results of our T-maze assays, represented as a preference index (see Methods) suggest that the majority of the *Drosophila* species we examined had a robust aversive response toward CO_2, including all members of the *melanogaster* subgroup and members of the *virilis-repleta* radiation (Fig. 1B). However, this aversive response is either greatly reduced or not present in many species that we examined, particularly most members of the *nasuta* subgroup, *D. virilis*, and *D. wilistoni* (Fig. 1B). Using ANOVA followed by post-hoc Tukey's HSD, we found a significant effect for species, which we were able to group into 2 categories (avoidance vs. indifference) based on whether their behavioral responses to CO_2 were significantly different *($P < 0.05$)* from *D. melanogaster* or from *D. melanogaster Gr63a* mutant flies (Fig. 1B). Comparing these 2 categories to the *Drosophila* phylogenetic tree suggested that the robust aversive response toward CO_2 was the ancestral state for Drosophilids (fewest required derived characters), and this behavior has been independently lost in a few lineages, such has the ancestors of *D. virilis* and *D. wilistoni*, and the ancestors of the *nasuta* subgroup. Interestingly, if this hypothesis is true, it is possible that a member of the *nasuta* subgroup, *D. kohkoa*, independently re-acquired aversion toward CO_2. Overall, our results suggest that the behavioral response to CO_2 among Drosophilids is fairly diverse and evolutionarily labile.

Differential expression of CO_2 receptor components across the Drosophila *genus*

Previous studies have shown that physiologic antennal responses to CO_2 are conserved even in *Drosophila* species that have a reduced avoidance response toward

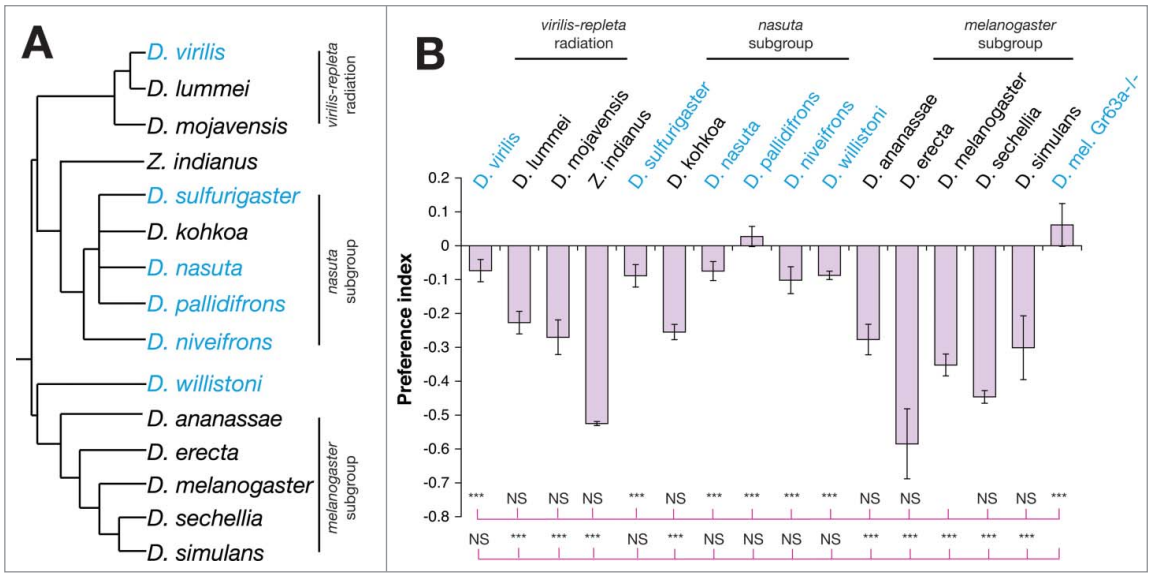

Figure 1. Behavioral response of different *Drosophila* species toward carbon dioxide. (A) Phylogenetic tree showing the phylogenetic relationships of all the *Drosophila* species examined in this study. Light blue colors indicate species that we found to have reduced aversion toward CO_2 relative to the other species. (B) Preference indices for different *Drosophila* species in response to a T-maze assay where flies are given a choice between regular air and 5% CO_2. The value given for each species is the average value over 3–10 trials of 5 runs (see Methods). Error bars indicate standard error of measure (SEM). Red lines indicate significant differences between each species and wild-type *D. melanogaster* (top line), and between each species and *D. melanogaster Gr63a* mutants (bottom line), shown as the results of an ANOVA followed by Tukey's post-hoc test for significance (NS = not significant, *** = $P < 0.001$).

CO_2.[22] This suggests that behavioral differences for CO_2 are likely due to differences in the amount or pattern of receptor expression or to changes in neural circuitry, rather than differences in receptor function. To look for differences in CO_2 receptor expression, we used a publicly available RNASeq data set (see Methods) which contains the antennal transcriptome profiles of 6 *Drosophila* species (*D. melanogaster, D. sechellia, D. simulans, D. erecta, D. ananassae*, and *D. virilis*) from 4 different time points during development (3rd instar larva, 8 h after puparium formation (APF), 40 h APF, and adults). We first looked for differences in gene expression in the adult stage for *Gr21a* and *Gr63a*, the 2 components of the CO_2 receptor in *D. melanogaster*. Of the 6 species included in this data set, only *D. virilis* has reduced aversive response toward CO_2 (Fig. 1B), and this correlates with reduced expression in *D. virilis* for *Gr63a* (Fig. 2A), but not *Gr21a* (Fig. 2B) in the adult antennae, using normalized transcript counts. The reduced expression for *Gr63a* in *D. virilis* compared with *D. melanogaster* was confirmed using quantitative RT-PCR on adult antennae, which showed a greater reduction in expression for *Gr63a* in *D. virilis* relative to *D. melanogaster* compared with *Gr21a* (Fig. 2C), a result that is comparable to RNASeq data that has been normalized (Fig. 2D).

Given the reduced Gr63a expression in *D. virilis*, which has reduced repulsion toward CO_2, we also tested the behavioral response of heterozygotic Gr63a mutant *D. melanogaster* which have only one functional copy of the *Gr63a* gene (and thus presumably reduced expression) toward CO_2 (Fig. 2E). However, the behavioral response of the *Gr63a* heterozygotes toward CO_2 was not significantly different compared with wild-type *D. melanogaster*, suggesting that a reduction in *Gr63a* transcription alone is not sufficient to cause behavioral differences, and that additional processes such as developmental or higher-order circuit changes must also be involved.

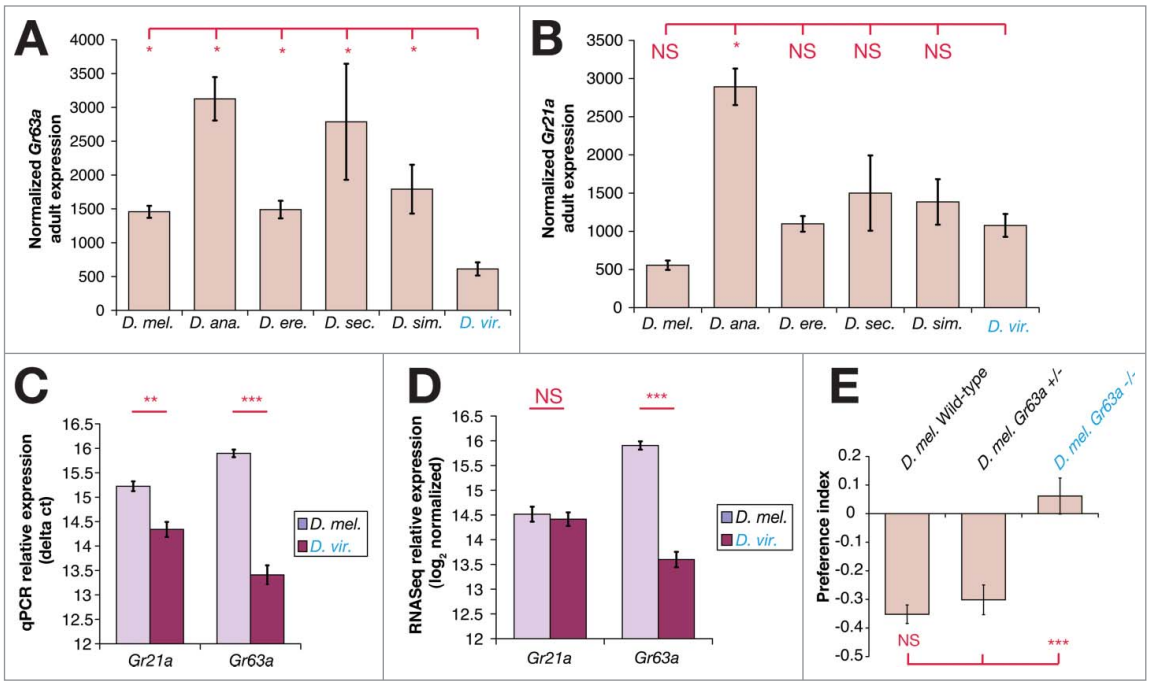

Figure 2. Differential expression of CO_2 receptors across *Drosophila*. (A, B) DESeq2-normalized transcript counts for Gr63a and Gr21a, the 2 CO2 co-receptors, in the adult antennae across 6 *Drosophila* species as determined by RNASeq analysis. Error bars indicate SEM. Significantly different comparisons, as determined by DESeq2 p-adjusted values using a stringent criteria ($P < 1^{-10}$), are indicated with an asterisk (NS = not significant). (C) Relative expression of *Gr21a* and *Gr63a* in the adult antenna for *D. virilis* relative to *D. melanogaster* as measured with real-time quantitative RT-PCR. Values shown indicate the mean over 3 runs normalized against Act5C (set to +18), and error bars indicate SEM. (D) To provide a direct RNASeq comparison to (C), this plot shows the relative expression of *Gr21a* and *Gr63a* in the adult antenna for *D. virilis* relative to *D. melanogaster* as measured with RNASeq, $\log_2$ normalized against Act5C (set to +18). Error bars indicate SEM. (E) Preference indices for *D. melanogaster* wild-type and *Gr63a* heterozygous and homozygous mutants in response to a T-maze assay where flies are given a choice between regular air and 5% CO_2. The value given for each species is the average value over 6–10 trials of 5 runs (see Methods). Error bars indicate SEM. Significant differences shown are the results of an ANOVA followed by Tukey's HSD (NS = not significant, *** = $P < 0.001$).

Conservation of genes regulating the development of CO_2 receptor expression across Drosophila

The availability of the RNASeq data set with the transcriptome profiles for adult and developing antennae across multiple *Drosophila* species also allowed us to investigate the broader question on how conserved the molecular and development programs of the CO_2 olfactory system are across species. To answer this question, we compiled RNASeq data for all of the genes known to be involved in the development of CO_2 ORNs, including factors that regulate the development of the ab1 sensillum and factors that regulate CO_2 receptor expression.[6,12,17,21,27,28] We calculated the absolute log fold change (ALFC) in gene expression for each gene across each pairwise interspecies comparison. The ALFC value has been used in previous studies as a simple proxy for the variability of a gene across evolutionary time scales,[29] with higher ALFC values indicating that a gene has greater transcriptional variability, and lower values indicating that gene expression is conserved. We first plotted our results as a boxplot of median and quartile ALFC values for each gene (Fig. 3A), which showed that the genes regulating the development of CO_2 ORNs can be roughly divided into 2 categories, with some genes having similar transcription levels across species and others having more variable levels of transcription. However, it is important to note that the genes with higher variability are not unusually variable; the variability they present is consistent with the average across the transcriptome (Fig. 3A). Instead, the difference between the 2 groups seems to be due to the group with conserved gene expression being remarkably well conserved — that is, far below the transcriptome average. Interestingly, most of the genes belonging to the MMB/dREAM complex have highly conserved expression, while the members of *mir-279* pathway and the transcription factors that specify ab1 identity have greater transcriptional variability across species. In addition, we also represented our results as a heatmap showing each individual species comparison (pairwise ALFC), along with a clustering analysis of the ALFC values for each gene using hierarchical clustering (Fig. 3B). The clustering analysis also supported the distribution of genes regulating the development of CO_2 ORNs into 2 groups with good bootstrap support, with one group in the upper part of the figure having high transcriptional variability across species, and the other group in the lower part of the figure being more transcriptionally conserved. Again, most of the genes belonging to the MMB/dREAM complex appear to have highly conserved levels of gene expression. In contrast, the members of the *mir-279* pathway and the transcription factors that specify ab1 identity, along with *Gr21a* and *Gr63a*, have relatively higher transcriptional variability across species, comparable to the variability of the housekeeping genes *Act5C* and *Gapdh2* (in other words, having average variability). Furthermore, the heatmap indicated that the transcriptional variability in the developmental genes was not restricted to any one species, nor did it seem to be primarily related to phylogenetic distance between species as large differences were observed even among closely related species. Overall, our results suggest that some components of the regulatory mechanisms underlying CO_2 receptor expression have very similar transcription levels across species while other components have transcription that is more evolutionarily labile.

Next, we hypothesized that the changes in *Gr63a* expression may result from changes in the developmental programs underlying receptor expression and/or sensilla identity. To investigate this, we used the same RNASeq data set to compare changes in adult *Gr63a* expression across 6 *Drosophila* species with changes in the transcription of genes known to be involved in regulating *Gr63a* expression or ab1 sensilla identity during development (such as pre-patterning transcription factors and the MMB/dREAM complex) to identify developmental genes that may potentially underlie behavioral differences toward CO_2. Regression analysis identified *mip120* and *dac* as the 2 developmental genes with changes across the 6 species that most strongly correlate with changes in *Gr63a* expression (Fig. 3C, see also Fig. S1 for a combined figure with other developmental genes). *Mip120* is part of the MMB/dREAM complex, an epigenetic regulator known to repress *Gr63a* expression,[21,30] while *dac* is a pre-patterning transcription factor known to be involved in the specification of large basiconic sensilla.[28] RNASeq data shows that *mip120* has increased transcription in *D. virilis* compared other species, while *dac* has increased transcription in species with higher *Gr63a* expression such as *D. ananassae* and *D. sechellia* (Fig. 3D). Our data are consistent with prior work showing that *mip120* and *Gr63a* expression are inversely correlated.[21] Overall, our results suggest

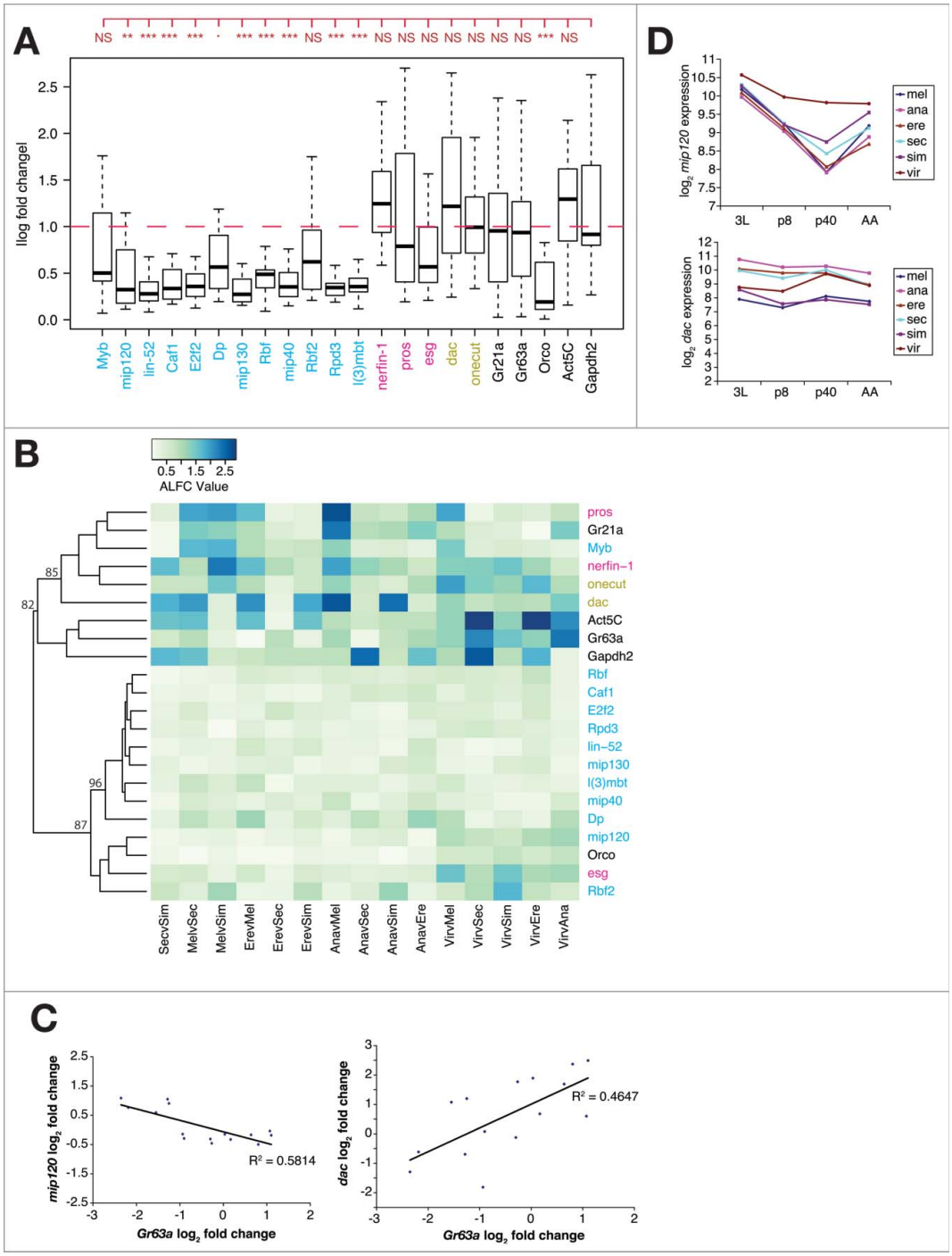

Figure 3. Transcriptional variability of developmental regulators of CO_2 receptors across 6 *Drosophila* species. (A) Boxplot and (B) heatmap plotting the absolute log fold change (ALFC) in the expression of known developmental regulators of CO_2 receptors across all pairwise interspecies comparisons and across 4 developmental time points (3rd instar larvae, 8 h APF, 40 h APF, adult). Color coding of genes is as follows: cyan – MMB/dREAM complex; magenta – *miR-279* pathway; yellow – ORN/sensilla identity. The boxplot shows combined values for each transcription factor across development (n = 60), with the mean ALFC value for the antennal transcriptome shown as a red line for comparison. ALFC values for *Orco, Gr63a* and *Gr21a* during the adult stage are also included (n = 15). Statistical comparisons show results for ANOVA + Tukey's HSD (. = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, NS = not significant). In the heatmap, ALFC values shown are the mean ALFC value across all 4 developmental time points for each specific pairwise comparison, except for *Orco, Gr63a* and *Gr21a* which show values only from adults. Numbers on nodes indicate bootstrap support for the clustering analysis. (C) Plots of interspecies pairwise log fold change for *Gr63a* against interspecies pairwise log fold change for genes known to be involved in *Gr63a* expression, from RNASeq data (n = 15). Solid lines indicate linear regression. See also Figure S1. (D) DESeq2 and log-normalized RNASeq transcript counts for *mip120* and *dac* across 6 species (see Methods) and 4 developmental time points (3L – 3rd instar larvae, p8 – 8 h APF, p40 – 40 h APF, AA – adult antennae). Error bars omitted for visual clarity.

that the indifference of *D. virilis* toward CO_2 compared with members of the *melanogaster* subgroup may be due to a reduction in *Gr63a* expression, which in turn may be due to transcriptional changes in certain developmental regulatory genes.

Changes in miR-279 expression in D. virilis *maxillary palps*

Previously, *miR-279* and its transcriptional regulator *prospero* were shown to repress the generation of ectopic CO_2 ORNs in *D. melanogaster* maxillary palps.[6,27] We next asked if there were differences in the *miR-279* regulatory pathway that may be involved in behavioral differences between species. The RNA-Seq data set is from antennal samples and therefore does not include microRNA expression, which means we cannot directly determine differences in antennal *miR-279* expression in other *Drosophila* species. Instead, we decided to compare the maxillary palp expression of the *D. melanogaster* (a species that is repelled by CO_2) *miR-279* promoter to the *D. virilis* (a species that has reduced aversive response to CO_2) *miR-279* promoter by generating transgenic reporters of *miR-279* expression in *D. melanogaster*. In a previous study, we showed that a 3 kb *miR-279* genomic construct that contains only 2 kb of upstream sequences and 1 kb downstream of the transcriptional start site was 100% efficient at rescuing the *miR-279* mutant phenotype in the olfactory system.[6] This 2 kb upstream sequence is also capable of expressing *miR-279* in the maxillary palps.[6] Additionally, a bioinformatics interrogation of *miR-279* promoter in different Drosophilids found that Prospero binding sites upstream of *mir-279* critical for its expression in *melanogaster* maxillary palp precursors,[27] are modified in *D. virilis* (Fig. 4A). These data suggest that the regulatory elements that are required for the developmental and tissue-specific expression of *miR-279* are harbored within this 2 kb sequence, including the Prospero binding sites. From the sequence analysis, we also observed that the same Prospero binding sequence is changed from "nnnTAAGACAnnn" in *D. melanogaster* to "nnnTGCGCGAnnn" in *D. virilis*. We generated GAL4 reporter transgenes driven by the 3 kb *D. virilis miR-279* promoter, and compared UAS-CD8GFP expression in the developing maxillary palps at 50 h after puparium formation to *D. melanogaster* (Fig. 4B). Interestingly, we found that *D. melanogaster* flies containing *D. virilis mir-279-gal*4 UAS-cd8GFP had less GFP expression in the developing maxillary palps compared with *D. melanogaster* flies containing the *D. melanogaster* equivalent, suggesting that *mir-279* expression in *D. virilis* may be reduced (Fig. 4B-C). However, a comparison of *Gr21a* and *Gr63a* expression in the maxillary palps of *D. melanogaster* and *D. virilis* using quantitative RT-PCR (Fig. 4D), along with fluorescent RNA *in situ* hybridization experiments for Gr21a in *D. virilis* (data not shown), showed no indication of any ectopic expression of the CO_2 receptor in the *D. virilis* maxillary palps. As such, we believe that it is unlikely that the *mir-279* regulatory pathway ultimately contributes to any reduction in the aversive behavioral response toward CO_2, at least for *D. virilis*.

Conservation of CO_2-sensing antennal structures and the target glomerulus in the antennal lobe

Finally, we asked if there were any differences in the CO_2-sensing antennal structures or the CO_2 circuitry in other *Drosophila* species and how conserved these are across the genus. To investigate this, we used different imaging methods to determine the existence and spatial location of *Gr21a*-expressing ORNs, large basiconic sensilla, and the V-glomerulus in a subset of the species that we examined for CO_2 behavioral response. First, using fluorescent whole-mount RNA *in situ* hybridization, we found *Gr21a*-expressing ORNs in all the species we examined (*D. melanogaster, D. sechellia, D. simulans, D. virilis*), which was unsurprising given our previous findings but also revealed that the spatial location of the CO_2 receptor in the proximal-medial region of the antennae is conserved (Fig. 5A). Quantification of this data indicated that the number of *Gr21a*-expressing cells in the antennae is conserved across the 4 species as well (Fig. 5B). Similarly, scanning electron micrographs of the antennae from *D. melanogaster, D. sechellia, D. erecta*, and *D. virilis* indicated that the presence and spatial distribution of large basiconic sensilla in the proximal-medial region of the antennae is also conserved across multiple *Drosophila* species (Fig. 5C). In addition, antibody stainings of the antennal lobe for the nc82 protein, a neuronal marker, in *D. melanogaster, D. sechellia, D. willistoni*, and *D. virilis* indicated that the V-glomerulus is present and distinct even in species with reduced aversion toward CO_2 (Fig. 5D). Interestingly, 3D reconstruction of the

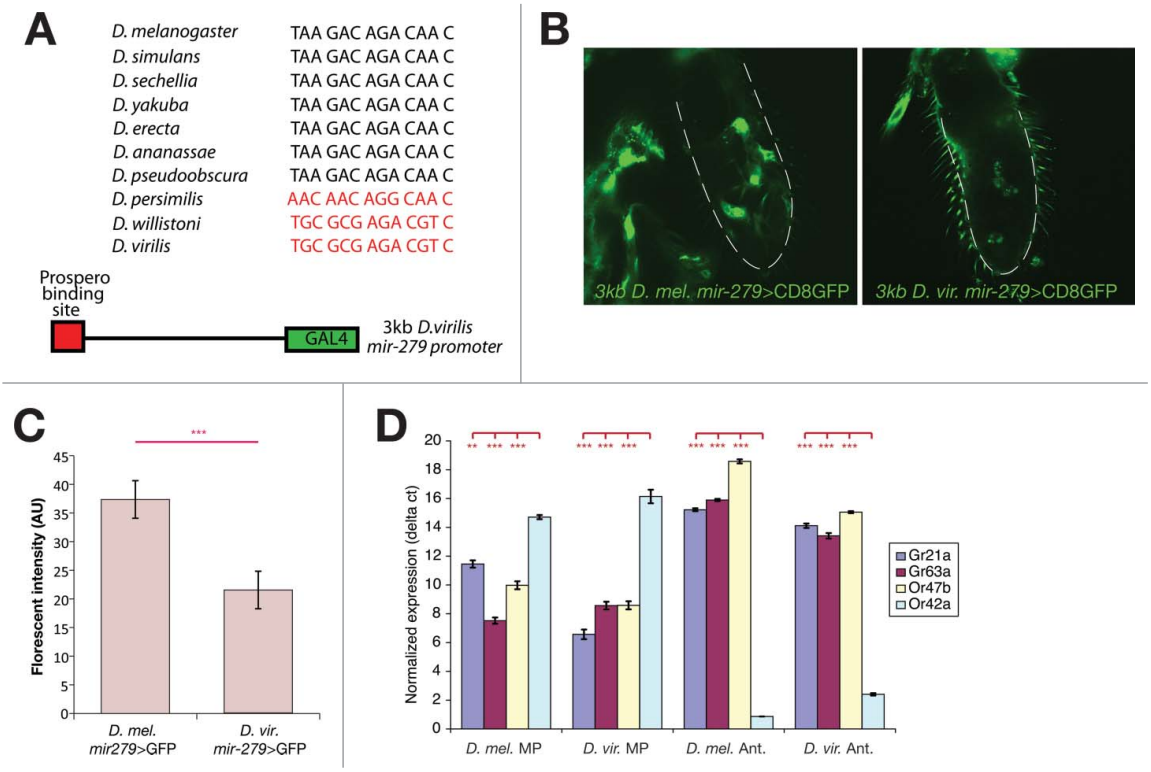

Figure 4. Conservation of the mir-279 regulatory system in *D. virilis*. (A) Comparisons of a *prospero* binding site in the promoter region of *miR-279* across multiple *Drosophila* species. (B) Confocal images of maxillary palps from transgenic *D. melanogaster* flies with the *D. virilis mir279*-Gal4 UAS-cd8GFP promoter-fusion construct at 50 h APF during development (left), and equivalent *D. melanogaster* flies with a *D. melanogaster mir279*-Gal4 UAS-cd8GFP promoter-fusion construct (right). (C) Quantification of (B) as the difference in the florescent intensity of GFP expression by *miR-279* promoters from *D. mel.* and *D. vir.* Asterisks indicate significant difference as determined by an unpaired t-test ($n = 6$, $P < 0.001$). (D) Relative expression of *Gr21a, Gr63a, Or42a* (positive control for maxillary palps) and *Or47b* (positive control for antennae) in maxillary palps (MP) and antennae (Ant.) for *D. virilis* and *D. melanogaster* as measured with real-time quantitative RT-PCR. Values shown indicate the mean over 3 runs normalized against Act5C (set to +18), and error bars indicate SEM.

V-glomerulus for *D. melanogaster* and *D. virilis* indicated that the *D. virilis* V-glomerulus has a smaller volume relative to the rest of the antennal lobe compared with *D. melanogaster* (Fig. 5E). Given that the number of *Gr21a* ORNs appear to be comparable in both species (Fig. 5C), it is possible that the smaller volume reflects a reduced number of interneurons or projection neurons (PNs) connecting to the *D. virilis* V-glomerulus compared with *D. melanogaster*. Overall, our results suggest that the presence and spatial location of CO_2-sensing antennal structures and circuits are mostly conserved across the *Drosophila* genus, but higher order neuronal connections may potentially be different in species with reduced repulsion toward CO_2.

Discussion

Here we investigated the relationship between changes in carbon dioxide response, which is critical for regulating many behaviors, and changes in the abundance and expression patterns of the genes giving rise the to CO_2 sensory system. We examined the behavioral response of various *Drosophila* species toward CO_2, and, for a subset of those species, we examined the transcription profiles of developmental regulators of CO_2-sensing ORNs, as well as the conservation of CO_2 sensory structures. We found that some *Drosophila* lineages strongly avoided CO_2 while others were indifferent, and our examination of transcription profiles suggests that these differences are accompanied by molecular and transcriptional changes in the expression of the *Gr63a* co-receptor and some of its developmental and transcriptional regulators.

Our results parallel a previous study examining the CO_2 response in Drosophilids[22] that also found a reduced aversive response for *D. virilis*. Further, we showed that reduced aversion toward CO_2 is common among *Drosophila* species, and that the behavioral response toward CO_2 independently

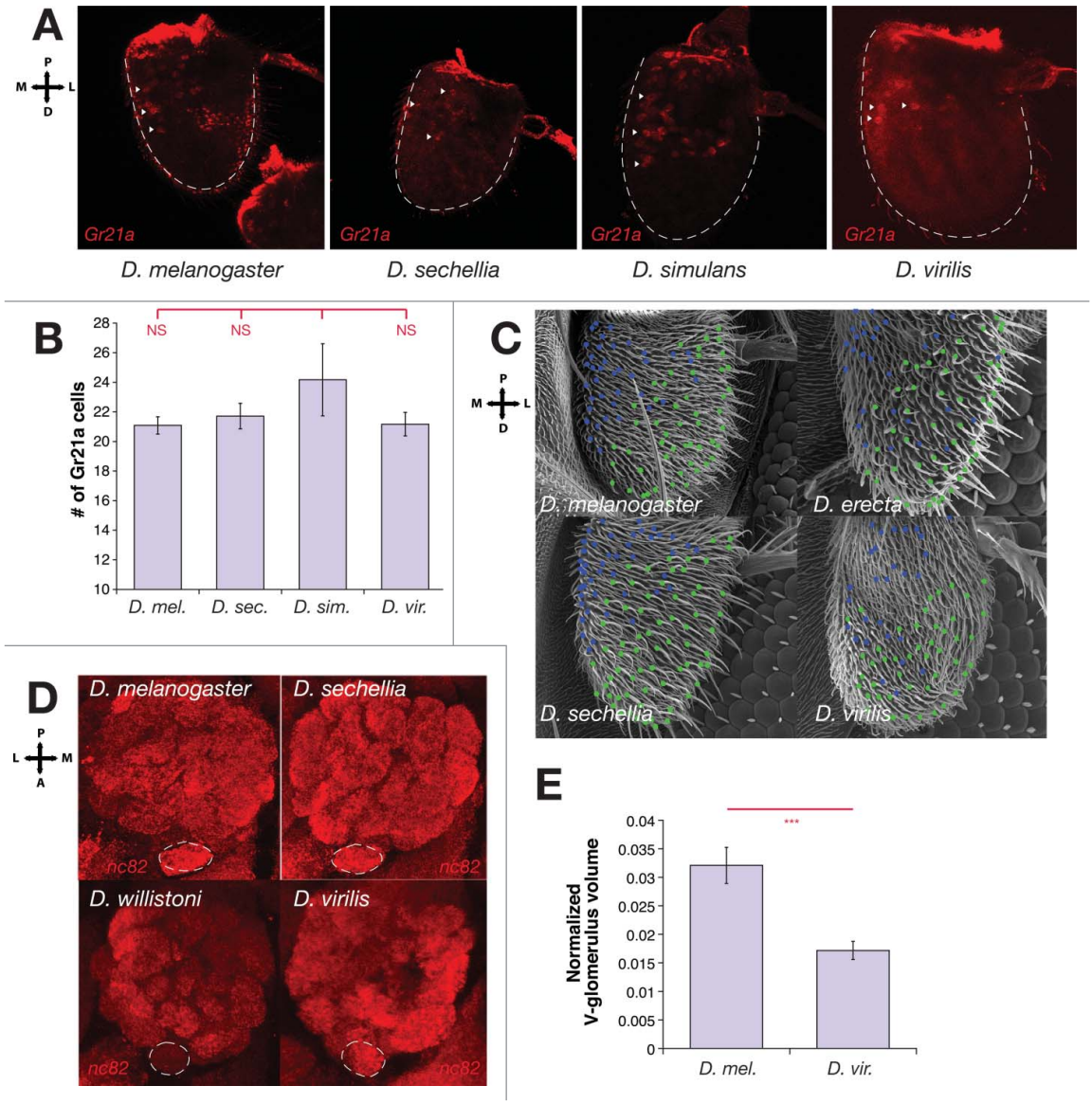

Figure 5. Conservation of CO_2-sensing antennal structures and the V-glomerulus. (A) RNA fluorescent *in situ* hybridization (FISH) of the adult antennae from multiple *Drosophila* species with a *Gr21a* antisense probe. (B) Quantification of RNA FISH results for *Gr21a* expression (n = 6–12). Error bars indicate SEM. Significance shown as determined by ANOVA + Tukey's HSD (NS = not significant). (C) Scanning electron micrographs of the adult antennae from multiple *Drosophila* species. Blue dots indicate the location of basiconic sensilla, orange dots indicate the location of trichoid sensilla. (D) Images of the antennal lobe from multiple *Drosophila* species, stained with an anti-nc82 antibody, a neuronal marker. The location of the V-glomerulus is highlighted in white. (E) Comparison of the volume of the V-glomerulus in *D. melanogaster* and *D. virilis*. To normalize for body size, values are shown as the ratio between the volume of the V-glomerulus and the volume of the entire antennal lobe. Significance indicated as determined by an unpaired t-test (n = 10, $P < 0.001$).

diverged in a few different *Drosophila* lineages such as the *virilis-repleta* radiation and the *nasuta* subgroup. The reason(s) for this behavioral divergence remains unclear, but likely relate to differences in each species ecological niche, as previously suggested.[22] For example, in species that are repulsed by CO_2, CO_2 may repel certain species from ripening fruit,[22] or function as an alarm pheromone[8]; in contrast, species that are indifferent toward CO_2 may not need those cues or use different odorants for those functions. Hypothetically, the loss of aversion toward CO_2 may allow such species to exploit new food sources that emit CO_2. For example, *D. virilis* is known to breed in slime fluxes on shedding trees[31] which is a known bacterial infection. With such ecological pressures, CO_2 produced by the fermentation of bacteria might have transformed CO_2 sensing circuit function to have a

positive attractive valence, which could lead to the weakening of the repulsive behaviors driven by the CO_2 sensing ORNs over time. Admittedly, our study tested behavioral response only at a specific concentration (5%); thus, it is very possible that the response of the species we examined may differ in non-laboratory conditions with different CO_2 concentrations. Previous studies have also found differences in the behavioral response toward CO_2 for *D. melanogaster* when the fly is in tethered flight as opposed to walking[32]; our study only examined the latter. Additionally, our study only examines the olfactory system, and does not examine gustatory responses toward dissolved CO_2 (in *D. melanogaster*, this attractive response is mediated by CO_2 sensing neurons in the labial palps[33]), which also may or may not be conserved between species. Nonetheless, we believe that our results are sufficient to suggest potentially interesting behavioral differences for an ecologically important odorant that warrants further investigation.

Our comparison of the gene expression profiles for CO_2-related genes across several *Drosophila* species found reduced *Gr63a* expression in the CO_2-indifferent *D. virilis* along with correlated changes in some *Gr63a* regulators during development, specifically *mip120*, a component of the MMB/dREAM complex,[21] and *dac*, an important developmental transcription factor.[28] Whether or not these differences in gene expression are the actual causative mechanism behind changes in behavior will require additional study, but our findings identify candidate genes for future experiments. In addition, as our comparisons of gene expression profiles included only 6 *Drosophila* species, with only one of those having reduced aversion toward CO_2, perhaps a more thorough analysis of the entire *Drosophila* genus would give us a more complete picture to identify the influence of developmental regulators on *Gr63a* expression and the resulting behavioral outcomes.

Similarly, our finding that certain components of the regulatory mechanisms underlying CO_2 receptor expression, specifically the members of the MMB/dREAM complex, are more transcriptionally conserved than others may be useful as a starting point for future experiments intending to manipulate the CO_2 behavioral response in insects. Among other reasons, it is possible that these components are parts of systems that more sensitive to perturbation, have more important functions, or have greater pleiotropy.[34] This explanation is consistent with our finding that the most conserved components are members of the MMB/dREAM complex that are key epigenetic modifiers with critical roles during development.[21]

Besides differences in gene expression, another factor that could contribute to behavioral differences between species is changes in CO_2-sensing neural circuits. Previous studies have suggested that CO_2 olfactory transduction to the antennal lobe is conserved across Drosophilids,[22] which is consistent with our findings regarding the general conservation of CO_2 sensing morphological structures. However, it is quite possible that that there are differences in neuronal connections in higher order circuits, such as projection neurons and local interneurons in the antennal lobe[35] that lead to a reduced CO_2 response in some species, as suggested by our limited comparisons of the V-glomerulus. There may also be higher order circuits in the mushroom body and lateral protocerebrum that could determine species-specific responses to CO_2. Further studies will be required to determine if these differences are present and play meaningful roles in the divergent behavioral responses of Drosophilids toward CO_2.

Materials and methods

Fly strains, rearing, and collections

The complete set of species genotypes used in our study is listed in Table S1. For our analyses of gene expression, the 6 *Drosophila* species genotypes examined were *D. ananassae* (14024–0371.00, *Drosophila* Species Stock Center (DSSC), University of California, San Diego), *D. erecta* (14021–0224.00, DSSC), *D. sechellia* (14021–0248.01, DSSC), *D. simulans* (14021–0251.165, DSSC), *D. virilis* (15010–1051.00, DSSC) and the *w118* strain of *D. melanogaster*. All flies used (for both behavioral experiments and RNASeq) were reared on cornmeal medium at room temperature.

T-maze CO_2 behavioral Assay

All experimental runs were conducted in the dark with the use of far red light. A vial of 30–40 flies (~7–10 d old) containing both males and females were loaded into transfer tubes and were then loaded into the T-maze elevator. Flies were left in the resting phase position for about 1 min. After the resting phase, 5%

CO_2 gas (which has previously been shown to elicit robust repulsive behavior[5,6]) was added to the CO_2 arm of the T-maze, and the flies were immediately moved to the choice point and allowed to decide between the CO_2 and air arms for 30 s. After 30 s, the flies in each arm were counted. Each vial of 30 flies counted as 1 trial, and each trial consisted of 5 runs through the T-maze protocol, with the final counts for each trial reported as the sum total of fly choices for each arm over the 5 runs. These counts were then used to determine the preference index value for each trial, calculated as the difference in the number of flies in the CO_2 arm vs. the air arm divided by the sum of flies in both arm.

RNASeq data analysis

Our RNASeq analysis used a publicly available data set of antennal transcriptomes from 6 *Drosophila* species. This data set includes the antennal transcriptomes from 4 developmental stages: 3rd instar larvae, 8 h after puparium formation (APF), 40 h APF, and adults. The gender ratio for all adult samples was 1:1. The raw sequence data and metadata are available for download from the NCBI Gene Expression Omnibus (GEO) database using the accession numbers GSE85239 and GSE75986, and the normalized count tables are also available in the GSE85239 series. Differential expression of genes between species was analyzed using the DESeq2 suite in R. In our analyses, the conservation of a gene was determined by calculating the mean absolute log fold change for all pairwise species comparisons. Absolute log fold change (ALFC) values were calculated by comparing DESeq-normalized transcript counts for each species to every other species in a pairwise fashion, and taking the absolute of the log fold change in transcript counts to provide a non-directional estimate of the difference in gene expression between each pairwise species comparison for every gene.

Heatmaps and cluster analysis

Heatmaps were created in R using the heatmap.2 function from the gplot package (v3.0.1). Cluster analysis was done with the default clustering options from the heatmap.2 function that uses hierarchical clustering with the distance and cluster methods set as "euclidean" and "complete," respectively. Bootstrap analysis of clusters was done using the pvclust package in R (v2.0–0) for 10,000 iterations.

Genetics

To compare the function of the *D. melanogaster* and *D. virilis mir-279* promoters, we created transgenic *D. melanogaster* flies containing promoter-Gal4 fusion constructs for the *mir-279* promoter region from *D. virilis*. To create the *D. virilis mir-279* promoter-Gal4 construct, we cloned a 3 kb region upstream of the orthologous *D. virilis mir-279* gene from genomic *D. virilis* DNA into a pCasperAUG-GAL4-X vector[11,36] using the custom primers 5′-CGCCACATTTCTACTCAGTTTC and 5′-AGTACGCATATTCGATCCACTC. From there, injection of the construct into *D. melanogaster* w^{1118} eggs and screening for transgenic flies followed standard protocol.[37] Transgenic flies containing *mir-279 gal*4 constructs were crossed to flies containing UAS-cd8GFP[12] before imaging.

Real-time RT-PCR

Antennae or antennal discs from approximately 100 flies or 50 larvae, respectively, were dissected and analyzed for each species. RNA was extracted with an RNeasy kit (Qiagen), treated with on-column DNase digestion (Qiagen), and then reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). qPCR was performed with the FastStart Universal SYBR Green Master Mix (Roche) using standard protocol. Expression for each gene was analyzed in triplicate. RNA concentration was standardized to 15 ng/μl before reverse transcription, and cDNA was diluted 1:32 before use. Ct values were normalized to each species' Actin 5C (Act5c) expression. Primers used in the reactions are listed in Table S2.

Immunohistochemistry

Samples were fixed with 4% paraformaldehyde, washed with phosphate buffer with 0.2% Triton X-100, and stained as described previously.[12] Primary and secondary antibodies were used in the following dilutions: rabbit anti-GFP 1:1000 (Invitrogen), mouse anti-nc82 1:20 (Developmental Studies Hybridoma Bank), Alexa 488 goat anti-rabbit 1:1000, goat anti-mouse-Cy3 1:100. Confocal images were taken by an Olympus Fluoview FV1000. 3D reconstruction and calculations of glomerular or antennal lobe volumes were done using Imaris software (v8.2).

Flourescent whole-mount RNA in situ *hybridization*

Digoxigenin RNA probes were made using a Roche DIG RNA labeling kit (Indianapolis, IN, USA). Primers used can be found in Table S3. *Drosophila* heads were dissected into cold fixative (4% paraformaldehyde, 0.05% Tween 20 in 1X PBS) and fixed for one hour. Heads were then washed 3 × 10 min in PBST (1X PBS, 0.1% Tween 20). The third antennal segment was dissected and fixed for an additional 30 min. Tissue samples were washed 5 × 5 min in PTX (1X PBS, 1% Triton X) and incubated in hybridization (Hyb) buffer (50% formamide, 5X SSC, 0.05 mg ml−1 heparin, 0.1% Tween 20) for 2 h at 55°C, before hybridization overnight at 55°C with DIG-labeled RNA probe. Tissue was then washed 5 × 20 min in Hyb buffer at 55°C, with the last wash proceeding overnight. Samples were then washed for 20 min in Hyb buffer at 55°C, followed by 5 × 5 min washes in PBST at room temperature before incubation for 3 h in 1: 500 anti-DIG-AP in PBST and 1X BSA. Next, samples were washed 5 × 5 min in PBST and incubated in FastRed solution (Roche, Indianapolis, IN, USA) for 30 min. Samples were washed for a final 5 × 5 min in PBST and stored overnight in mounting solution before imaging.

Scanning electron microscopy

Fly heads were fixed (4% paraformaldehyde, 0.05% Tween 20 in 1X PBS) immediately after dissection for 20 min, then rinsed in PBST (1X PBS, 0.1% Tween 20) twice for 15 min each. Samples were then dehydrated using an ethanol series at 30%, 50%, 70%, 90 and 100% ethanol, twice for 10 min at each dilution. Samples were allowed to dry for 1 h, then sputter-coated with Au using a Denton Vacuum Desk IV sputter unit. SEM images were taken with a FEI XL30 SEM-FEG (Duke Shared Materials Instrumentation Facility) immediately after.

Abbreviations

APF	After puparium formation
ALFC	Absolute log fold change
CO_2	Carbon dioxide
Gr	Gustatory receptor
ORN	Olfactory receptor neuron

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

Conceptualization, JWP PCV; Methodology, JWP CDJ PCV; Investigation, JWP JM HY CL PR SO AM SC; Writing – Original Draft, JWP PCV; Writing – Review & Editing, JWP PCV; Funding Acquisition, CDJ PCV; Supervision, CDJ PCV.

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References

[1] Vosshall LB, Stocker RF. Molecular architecture of smell and taste in Drosophila. Annu Rev Neurosci 2007; 30:505-33. Epub 2007/05/18; PMID:17506643; https://doi.org/10.1146/annurev.neuro.30.051606.094306

[2] Hansson BS, Stensmyr MC. Evolution of insect olfaction. Neuron 2011; 72(5):698-711. Epub 2011/12/14; PMID:22153368; https://doi.org/10.1016/j.neuron.2011.11.003

[3] Ramdya P, Benton R. Evolving olfactory systems on the fly. Trends Genet 2010; 26(7):307-16. Epub 2010/06/12; PMID:20537755; https://doi.org/10.1016/j.tig.2010.04.004

[4] McMeniman CJ, Corfas RA, Matthews BJ, Ritchie SA, Vosshall LB. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell 2014; 156(5):1060-71. Epub 2014/03/04; PMID:24581501; https://doi.org/10.1016/j.cell.2013.12.044

[5] Jones WD, Cayirlioglu P, Kadow IG, Vosshall LB. Two chemosensory receptors together mediate carbon dioxide detection in Drosophila. Nature 2007; 445(7123):86-90. Epub 2006/12/15; PMID:17167414; https://doi.org/10.1038/nature05466

[6] Cayirlioglu P, Kadow IG, Zhan X, Okamura K, Suh GS, Gunning D, Lai EC, Zipursky SL. Hybrid neurons in a microRNA mutant are putative evolutionary intermediates in insect CO2 sensory systems. Science 2008; 319(5867):1256-60. Epub 2008/03/01; PMID:18309086; https://doi.org/10.1126/science.1149483

[7] Jones WD. MicroRNA mutant turns back the evolutionary clock for fly olfaction. Bioessays 2008; 30(7):621-3. Epub 2008/06/07; PMID:18536029; https://doi.org/10.1002/bies.20780

[8] Turner SL, Ray A. Modification of CO2 avoidance behaviour in Drosophila by inhibitory odorants. Nature 2009; 461(7261):277-81. Epub 2009/08/28; PMID:19710651; https://doi.org/10.1038/nature08295

[9] Couto A, Alenius M, Dickson BJ. Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr Biol 2005; 15(17):1535-47; PMID:16139208; https://doi.org/10.1016/j.cub.2005.07.034

[10] Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R. A spatial map of olfactory receptor expression in the Drosophila antenna. Cell 1999; 96(5):725-36. Epub 1999/03/25; PMID:10089887; https://doi.org/10.1016/S0092-8674(00)80582-6

[11] Vosshall LB, Wong AM, Axel R. An olfactory sensory map in the fly brain. Cell 2000; 102(2):147-59; PMID:10943836; https://doi.org/10.1016/S0092-8674(00)00021-0

[12] Li Q, Barish S, Okuwa S, Maciejewski A, Brandt AT, Reinhold D, Jones CD, Volkan PC. A Functionally Conserved Gene Regulatory Network Module Governing Olfactory Neuron Diversity. PLoS Genet 2016; 12(1): e1005780. Epub 2016/01/15; PMID:26765103; https://doi.org/10.1371/journal.pgen.1005780

[13] Li Q, Ha TS, Okuwa S, Wang Y, Wang Q, Millard SS, Smith DP, Volkan PC. Combinatorial rules of precursor specification underlying olfactory neuron diversity. Curr Biol 2013; 23(24):2481-90. Epub 2013/11/26; PMID:24268416; https://doi.org/10.1016/j.cub.2013.10.053

[14] Goulding SE, zur Lage P, Jarman AP. amos, a proneural gene for Drosophila olfactory sense organs that is regulated by lozenge. Neuron 2000; 25(1):69-78. Epub 2000/03/09; PMID:10707973; https://doi.org/10.1016/S0896-6273(00)80872-7

[15] Gupta BP, Rodrigues V. Atonal is a proneural gene for a subset of olfactory sense organs in Drosophila. Genes Cells 1997; 2(3):225-33. Epub 1997/03/01; PMID:9189759; https://doi.org/10.1046/j.1365-2443.1997.d01-312.x

[16] Endo K, Aoki T, Yoda Y, Kimura K, Hama C. Notch signal organizes the Drosophila olfactory circuitry by diversifying the sensory neuronal lineages. Nat Neurosci. 2007; 10(2):153-60. Epub 2007/01/16; PMID:17220884; https://doi.org/10.1038/nn1832

[17] Jafari S, Alkhori L, Schleiffer A, Brochtrup A, Hummel T, Alenius M. Combinatorial activation and repression by seven transcription factors specify Drosophila odorant receptor expression. PLoS Biol. 2012; 10(3):e1001280. Epub 2012/03/20; PMID:22427741; https://doi.org/10.1371/journal.pbio.1001280

[18] Endo K, Karim MR, Taniguchi H, Krejci A, Kinameri E, Siebert M, Ito K, Bray SJ, Moore AW. Chromatin modification of Notch targets in olfactory receptor neuron diversification. Nat Neurosci. 2012; 15(2):224-33. Epub 2011/12/27; PMID:22197833; https://doi.org/10.1038/nn.2998

[19] Ray A, van der Goes van Naters W, Carlson JR. A regulatory code for neuron-specific odor receptor expression. PLoS Biol 2008; 6(5):e125. Epub 2008/10/14; PMID:18846726; https://doi.org/10.1371/journal.pbio.0060125

[20] Ray A, van Naters WG, Shiraiwa T, Carlson JR. Mechanisms of odor receptor gene choice in Drosophila. Neuron 2007; 53(3):353-69; PMID:17270733; https://doi.org/10.1016/j.neuron.2006.12.010

[21] Sim CK, Perry S, Tharadra SK, Lipsick JS, Ray A. Epigenetic regulation of olfactory receptor gene expression by the Myb-MuvB/dREAM complex. Genes Dev 2012; 26 (22):2483-98. Epub 2012/10/30; PMID:23105004; https://doi.org/10.1101/gad.201665.112

[22] Krause Pham C, Ray A. Conservation of Olfactory Avoidance in Drosophila Species and Identification of Repellents for Drosophila suzukii. Sci Rep 2015; 5:11527. Epub 2015/06/23; PMID:26098542; https://doi.org/10.1038/srep11527

[23] Seetharam AS, Stuart GW. Whole genome phylogeny for 21 Drosophila species using predicted 2b-RAD fragments. PeerJ 2013; 1:e226. Epub 2014/01/17; PMID:24432193; https://doi.org/10.7717/peerj.226

[24] Bachtrog D. The speciation history of the Drosophila nasuta complex. Genet Res 2006; 88(1):13-26. Epub 2006/10/04; PMID:17014741; https://doi.org/10.1017/S0016672306008330

[25] Yu H, Wang W, Fang S, Zhang YP, Lin FJ, Geng ZC. Phylogeny and evolution of the Drosophila nasuta subgroup based on mitochondrial ND4 and ND4L gene sequences. Mol Phylogenet Evol 1999; 13(3):556-65. Epub 2000/01/06; PMID:10620413; https://doi.org/10.1006/mpev.1999.0667

[26] Yassin A, Da Lage JL, David JR, Kondo M, Madi-Ravazzi L, Prigent SR, Toda MJ. Polyphyly of the Zaprionus genus group (Diptera: Drosophilidae). Mol Phylogenet Evol 2010; 55(1):335-9. Epub 2009/09/19; PMID:19761854; https://doi.org/10.1016/j.ympev.2009.09.013

[27] Hartl M, Loschek LF, Stephan D, Siju KP, Knappmeyer C, Kadow IC. A new Prospero and microRNA-279 pathway restricts CO2 receptor neuron formation. J Neurosci 2011; 31(44):15660-73. Epub 2011/11/04; PMID:22049409; https://doi.org/10.1523/JNEUROSCI.2592-11.2011

[28] Song E, de Bivort B, Dan C, Kunes S. Determinants of the Drosophila odorant receptor pattern. Dev Cell 2012; 22 (2):363-76. Epub 2012/02/22; PMID:22340498; https://doi.org/10.1016/j.devcel.2011.12.015

[29] Enard W, Khaitovich P, Klose J, Zollner S, Heissig F, Giavalisco P, Nieselt-Struwe K, Muchmore E, Varki A, Ravid R, et al. Intra- and interspecific variation in primate gene expression patterns. Science 2002; 296 (5566):340-3. Epub 2002/04/16; PMID:11951044; https://doi.org/10.1126/science.1068996

[30] Lee H, Ragusano L, Martinez A, Gill J, Dimova DK. A dual role for the dREAM/MMB complex in the regulation of differentiation-specific E2F/RB target genes. Mol Cell Biol 2012; 32(11):2110-20. Epub 2012/03/28; PMID:22451490; https://doi.org/10.1128/MCB.06314-11

[31] Markow TA. The secret lives of Drosophila flies. eLife 2015; 4:e06793; PMID:26041333; https://doi.org/10.7554/eLife.06793

[32] Wasserman S, Salomon A, Frye MA. Drosophila tracks carbon dioxide in flight. Curr Biol 2013; 23(4):301-6. Epub 2013/01/29; PMID:23352695; https://doi.org/10.1016/j.cub.2012.12.038

[33] Fischler W, Kong P, Marella S, Scott K. The detection of carbonation by the Drosophila gustatory system. Nature 2007; 448(7157):1054-7. Epub 2007/08/31; PMID:17728758; https://doi.org/10.1038/nature06101

[34] Stern DL, Orgogozo V. Is genetic evolution predictable? Science 2009; 323(5915):746-51. Epub 2009/02/07; PMID:19197055; https://doi.org/10.1126/science.1158997

[35] Grabe V, Baschwitz A, Dweck HK, Lavista-Llanos S, Hansson BS, Sachse S. Elucidating the Neuronal Architecture of Olfactory Glomeruli in the Drosophila Antennal Lobe. Cell Rep 2016; 16(12):3401-13. Epub 2016/09/23; PMID:27653699; https://doi.org/10.1016/j.celrep.2016.08.063

[36] Fishilevich E, Vosshall LB. Genetic and functional subdivision of the Drosophila antennal lobe. Curr Biol 2005; 15(17):1548-53; PMID:16139209; https://doi.org/10.1016/j.cub.2005.07.066

[37] Li Q, Barish S, Okuwa S, Volkan PC. Examination of Endogenous Rotund Expression and Function in Developing Drosophila Olfactory System Using CRISPR-Cas9-Mediated Protein Tagging. G3 (Bethesda) 2015; 5(12):2809-16. Epub 2015/10/27; PMID:26497147; https://doi.org/10.1534/g3.115.021857