E-Article

Social Behavior, Ovary Size, and Population of Origin Influence Cuticular Hydrocarbons in the Orchid Bee *Euglossa dilemma*

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Submitted September 11, 2020; Accepted April 6, 2021; Electronically published September 8, 2021

Online enhancements: appendixes, data. *Dryad data:* https://doi.org/10.25338/B8FH0P.

abstract: Cuticular hydrocarbons (CHCs) are waxy compounds on the surface of insects that prevent desiccation and frequently serve as chemical signals mediating social and mating behaviors. Although their function in eusocial species has been heavily investigated, little is known about the evolution of CHC-based communication in species with simpler forms of social organization lacking specialized castes. Here we investigate factors shaping CHC variation in the orchid bee *Euglossa dilemma*, which forms casteless social groups of two to three individuals. We first assess geographic variation, examining CHC profiles of males and females from three populations. We also consider CHC variation in the sister species, *Euglossa viridissima*, which occurs sympatrically with one population of *E. dilemma*. Next, we consider variation associated with female behavioral phases, to test the hypothesis that CHCs reflect ovary size and social dominance. We uncover a striking CHC polymorphism in *E. dilemma* spanning populations. In addition, we identify a separate set of CHCs that correlate with ovary size, social dominance, and expression of genes associated with social behavior, suggesting that CHCs convey reproductive and social information in *E. dilemma*. Together, our results reveal complex patterns of variation in which a subset of CHCs reflect the social and reproductive status of nestmates.

Keywords: fertility signals, cuticular hydrocarbons, bees, social signaling, chemical communication.

Introduction

Social insects exhibit some of the most sophisticated forms of social organization among animals, with some species displaying elaborate mechanisms of communication. In most species of social insects, chemical signals play a central role in mediating communication within and between colonies (Wilson 1965). Cuticular hydrocarbons (CHCs), which form a wax layer on the cuticular surface of all insects, are the most widespread signals that facilitate social insect communication and organization (Gibbs and Pomonis 1995; Oi et al. 2015).

The complete set of CHCs expressed on an insect—the CHC profile—may consist of multiple classes of linear and branched alkanes and alkenes, and their qualitative and quantitative variation can be highly dynamic and sensitive to a range of environmental, genetic, and physiological factors (Bonduriansky et al. 2015; Walsh et al. 2020). The expression of some CHC compounds is directly linked to pathways that regulate reproduction in insects, therefore enabling CHCs to potentially reflect honest information about individual physiological conditions (Kuo et al. 2012; Makki et al. 2014). In contrast, the expression of some CHCs may vary little with physiology and instead covary primarily with environmental variables, population of origin, or geographic distance between colonies (Dronnet et al. 2006; Bonelli and Lorenzi 2014; Otte et al. 2018).

Because CHC profiles are complex mixtures of compounds, different CHC components can convey different types of information, including social role, nestmate identity, mate quality, and species identity. In the primitively eusocial wasp *Polistes dominula*, for instance, CHCs are implicated in mate choice, social fertility signaling, and nestmate recognition (Izzo et al. 2010; Bruschini et al. 2011; Cappa et al. 2013). In the termite *Reticulitermes virginicus*, the relative abundance of specific CHCs encode information

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about social caste, while the presence or absence of specific CHCs mediate species recognition behaviors with sympatric congeners (Howard et al. 1982).

In highly eusocial Hymenoptera, a robust association exists between ovary activation in reproductive queens and the correlated expression of CHCs that contrasts sharply with ovary inactivation in sterile workers (Smith and Liebig 2017). However, whether these molecules primarily function as fertility signals, communicating the queen's presence, or instead function to directly inhibit worker ovary development remains an open question (Smith and Liebig 2017; Holman 2018). These functions can be difficult to disentangle, as they both lead to inactive worker ovaries in the presence of the queen (Oldroyd 2018). Although there is support for the hypothesis that queen-associated CHCs have evolved from fertility-linked CHCs in the ancestors of eusocial species, a lack of data from solitary or facultatively social species has hindered comparative analysis (Oi et al. 2015; Oliveira et al. 2015).

Because most studies investigating how CHCs regulate reproductive and social behaviors have been restricted to highly eusocial insect species, such as honeybees, ants, or vespine wasps (Van Oystaeyen et al. 2014; Holman 2018), little is known about the types of information that CHCs convey in species with small social groups without specialized castes (Leonhardt et al. 2016). A better understanding of such species has the potential to provide unique insight into the reproductive and social factors influencing chemical variation (Steiger and Stökl 2018). The lack of fixed worker castes or the complex communication needs of large colonies, for example, provides a simpler background against which to assess the roles of behavior and physiology in structuring CHC variation.

Euglossine bees (>250 species) are the earliest-branching group within the monophyletic corbiculate bees and the only lineage within this clade that lacks obligate eusocial behavior, instead exhibiting solitary, communal, or facultatively social nesting strategies (Soucy et al. 2003; Solano-Brenes et al. 2018; Friedel et al. 2020). Some species, such as *Euglossa dilemma*, have distinct solitary and social phases of their life cycle, with differences in ovary size occurring across these phases, although all individuals in social nests are reproductive (Andrade-Silva and Nascimento 2012; Saleh and Ramírez 2019). In addition to these life history characteristics, *E. dilemma* is geographically widespread, with multiple native and nonnative populations (Brand et al. 2020).

Nests in *E. dilemma* are initiated by a single foundress that provisions an initial brood batch. After completing approximately 4 to 10 brood cells, the foundress ceases foraging and reproduction to guard her developing brood (Saleh and Ramírez 2019). When a foundress enters this "guard" phase, her oocytes reduce in size. Once offspring emerge, the foundress's oocytes increase in size and she becomes the social dominant, while one or two of her female offspring may remain as subordinate helpers. Other female offspring disperse to begin their own nests. Both dominant and subordinate females are mated and reproductive. However, the dominant female is responsible for all of the direct reproductive output of the nest, due to oophagy and replacement of all subordinate eggs. The dominant bee is usually the original foundress and thus the mother of the subordinate bees, although subordinate bees can take over the dominant position if the original foundress bee dies. In some species of *Euglossa*, females may also form social affiliations with unrelated bees (Andrade-Silva et al. 2016). Male bees do not participate in any nest activities and leave the nest shortly after emergence.

In this study, by sampling multiple populations across the geographic range of *E. dilemma*, we investigate patterns of CHC variation in relation to species identity and population of origin. We also investigate whether CHCs covary with social behavior, for which we obtained data from females sampled while performing different solitary and social behaviors. We begin by documenting CHC variation in *E. dilemma* males and females from Florida (nonnative), Costa Rica (native), and Mexico (native). Next, we consider how this relates to CHC variation in the recently diverged sister species, *Euglossa viridissima*, which occurs sympatrically with *E. dilemma* in Mexico (Eltz et al. 2011). Following this, we examine how social behavior, relatedness among nestmates, and ovary size may be reflected in CHC variation and investigate links between socially associated genes and CHC expression. Using these sources of data, we test the hypothesis that female CHCs honestly reflect social dominance and reproductive status, encoding information that may mediate nestmate interactions in *E. dilemma*'s casteless social groups.

Methods

Florida Euglossa dilemma *Collection*

Euglossa dilemma females were trap nested year-round between 2015 and 2019 using small wooden boxes (3.5 inches × 2.5 inches × 1.4 inches) placed on the eaves of buildings in Fort Lauderdale, Florida, where they were accidentally introduced and first detected in 2003 (Skov and Wiley 2005). Entrance holes (5/16-inch diameter) were drilled into the front of each box. Following colonization of the nest boxes, transparent red plexiglass lids were placed on the boxes to facilitate observation. Females were individually marked using numbered plastic disks glued to the thorax. Following daily nest observations that occurred for at least an hour per day (approximately 2–4 weeks per nest), we classified individuals into four distinct behavioral groups, sampling

individuals in these groups. Two of these four groups (foundress and guard) represent solitary behaviors that a female bee will transition through before the nest becomes social, containing dominant and subordinate bees. We assigned females to the foundress category ($n = 21$) if they were observed alone constructing a nest in a box that did not have prior bee activity. A solitary foundress next progresses to the guard phase once brood provisioning is complete. Guard bees ($n = 20$) showed little to no foraging and no brood cell construction, and they spent most of the day inside the nest with capped brood cells and a resin seal over the nest entrance during typical daytime foraging hours. After offspring emergence, nests could become social if emerged offspring remained in the nest. We assigned bees to dominant ($n = 32$) and subordinate ($n = 33$) behavioral groups based on differences in foraging behavior and oophagy in multifemale nests. Subordinate individuals performed regular foraging trips for pollen and resin and did not perform oophagy. Dominant individuals were not observed foraging for pollen and displayed consistent oophagy of subordinate eggs. Multifemale nests never had more than one dominant bee. After observation was complete, females were anesthetized by placing the entire nest box on dry ice, and then females were removed and frozen in liquid nitrogen and/or a −80° freezer until analysis. Our sampling and observations were done on existing nests and thus likely represent a variety of ages and genetic relationships among dominants and subordinates, including matrifilial and sororal affiliations as well as possible affiliations between nonrelatives, which are known to happen in *Euglossa* species (Andrade-Silva et al. 2016). Newly emerged females were sampled within 24 h of emergence from harvested trap nests maintained in a mesh enclosure in ambient conditions during the summer and fall of 2019 in Fort Lauderdale, Florida ($n = 17$). Male Florida *E. dilemma* ($n = 22$) were collected at chemical baits around Fort Lauderdale and stored in a −80°C freezer until further analysis.

Costa Rica E. dilemma *Collection*

Euglossa dilemma females were trap nested at Palo Verde Biological Station in Guanacaste, Costa Rica, in the spring of 2018 using small wooden boxes (as described above) placed around the station. Females were also caught from flowers found around the station. Because of logistical constraints, Costa Rica *E. dilemma* females ($n = 13$) were not assigned to the four behavioral groups described above. *Euglossa dilemma* males ($n = 20$) were collected at chemical baits placed at Palo Verde Biological Station. Males and females were frozen after collection until analysis of CHCs. Collection permits were obtained through the National System of Conservation Areas (SINAC; permit M-P-SINAC-PNI-ACAT-073-2019).

Mexico E. dilemma *and* E. viridissima *Collection*

CHC data on Mexican *E. dilemma* and *E. viridissima*, collected from the Yucatán Peninsula, were provided by the authors of Pokorny et al. (2015) and used for comparison to our data sets from Florida and Costa Rica. In addition to the samples from Pokorny et al. (2015), data from several unpublished *E. dilemma* and *E. viridissima* females from Mexico were provided. We discuss integration of our data sets with these published and previously unpublished data sets below.

CHC Extractions

We extracted CHCs from one set of forewings and hindwings adapting the protocol in Martin et al. (2009). In brief, we placed the wings in a glass scintillation vial with 100 μL of hexane, swirled the wings, and then waited 10 min before moving the wing extract to gas chromatography–mass spectrometry (GC-MS) vials, which were left overnight to evaporate in a closed fume hood. The next day, 30 μL of hexane was added to the sample, which was then run on a GC-MS instrument (Agilent 7890B GC, 5977A MS, HP-5MS Ultra Inert 30-m, 0.25-mm, 0.25-mm column). We extracted CHCs from the wings to keep our methods consistent with the wing extractions from Pokorny et al. (2015) used in this study. To evaluate the degree to which wing extractions provide an accurate representation of CHCs on the cuticular surface, we also compared extracts from the wings, abdomen, and the whole body from 16 individuals, with details provided in appendix 1 (apps. 1–4 are available online).

Extracts were run using a 1-μL splitless injection on the GC-MS instrument, modifying a program from Choe et al. (2012) that began at 100°C for 1 min and then was increased 15°C per minute until 300°C was reached, after which the program was held at 300°C for 3 min. Helium was used as the carrier gas.

CHC Data Analysis

Chromatograms generated using the GC-MS instrument were integrated to include all peaks with an area of least 0.1% of the largest peak. Compound identification was accomplished by comparison to previously published information for *E. dilemma* (Pokorny et al. 2014, 2015) and comparison to available mass spectral libraries and known mass indices. We excluded from the analysis peaks that were not consistently identified as CHCs (linear and branched alkenes and alkanes) across individuals. Following the removal of non-CHC peaks, we determined the relative

abundance of each CHC peak per sample to generate proportional data.

Visualization of chemical data sets was done on these proportional data using nonmetric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity implemented in the R package vegan (ver. 2.5-4; Oksanen et al. 2019). Data and code underlying all figures has been deposited in the Dryad Digital Repository (https://doi.org/10.25338/B8FH0P; Saleh et al. 2021). In addition, to find the relative contribution to dissimilarity of specific CHCs, we used SIMPER (similarity percentages) analysis, as implemented in the vegan package.

To find potential modules of covarying CHC peaks, we used the R package corrplot (Wei and Simko 2017) to perform hierarchical clustering of the peaks using the Ward.D2 clustering method, based on Spearman correlation coefficients among peaks.

We used one-way ANOVAs to test for differences in the relative abundances of specific CHCs across social behaviors. We adjusted for multiple comparisons using Tukey's honestly significant difference test. We also used Levene's test and the Shapiro-Wilk test to verify ANOVA assumptions of homogeneity of variances and normally distributed data, respectively. If the data violated either of these assumptions, the data were square root transformed. If transformed data still failed ANOVA assumptions, we used the Kruskal-Wallis test with the Steel-Dwass test for multiple comparisons (Douglas and Michael 1991). We plot untransformed data for clarity. To identify and test for correlations between specific CHC peaks and ovary size, we used linear regression with the Pearson coefficient when data sets were normally distributed and the Spearman coefficient when data deviated from normality.

One bee that appeared as an extreme outlier in NMDS analysis (fig. S1; figs. S1–S12 are available online) was removed from this and all subsequent statistical analysis but was included in the genotype data set (described below). This bee was observed entering a nest and usurping the dominant position. Data for this individual (N28_W85) are included in the supplemental material (available online).

Combining Data Matrices among Populations

Because Mexican *E. dilemma* and *E. viridissima* were sampled separately and run on a separate GC-MS instrument from the Florida and Costa Rica samples, detection of low-abundance CHCs may vary in a batch-specific manner. Consequently, to avoid including CHCs that could inflate group separation because of technical differences, in our combined population analysis we included only CHCs detected in >85% of samples across the entire data set. In addition, we removed four of the 40 samples from the Mexican *E. dilemma* and *E. viridisimma* population (one *E. viridissima* female and three *E. dilemma* females) because of missing or ambiguous peaks that could not be reconciled with our Costa Rica and Florida data sets. Primarily, this appears to be due to possible sample contamination or low quantities of extracted sample. We include these individuals in our supplemental data (labeled as "removed outliers"). In total, 36 individuals were included from the Mexico population, which includes data from Pokorny et al. (2015) as well as additional unpublished samples (*E. viridissima* females, $n = 8$; *E. viridissima* males, $n = 10$; *E. dilemma* females, $n = 8$; *E. dilemma* males, $n = 10$).

Ovary Size Measurement

An ovary size index was calculated on a subset of individuals that were available for dissection ($n = 67$) by taking the length of the longest basal oocyte divided by the intertegular distance to incorporate body size variation among individuals (Cane 1987). We use this metric to represent ovary size in *E. dilemma*.

Transcriptomic Correlates with CHCs

We examined the expression of genes previously found to be associated with social behavior in *E. dilemma* to assess possible links among social behavior, reproductive physiology, and CHC expression. Specifically, we quantified the correlation between the relative abundance of specific CHCs and differentially expressed genes (DEGs) previously identified between dominants and subordinates (Saleh and Ramírez 2019). Using ovary transcriptomic data ($n = 27$ individuals), we examined correlations between CHCs of interest and the 10 genes found to be differentially expressed between the ovaries of dominants and subordinates. From brain transcriptomic data ($n = 31$ individuals), we examined correlations between CHCs of interest and the 204 genes found to be differentially expressed between the brains of dominant and subordinates. The preexisting transcriptomic data used here were generated and processed as detailed in Saleh and Ramírez (2019; data are available in the National Center for Biotechnology Information [NCBI] BioProject database, accession no. prjna523381) along with CHCs taken from the same individual bees. In addition to examining correlations among genes and CHCs, we also assessed relationships between the expression of these genes and ovary size, which was not previously explored in this data set. We note that the brain and ovary tissues used here do not directly express CHCs, which instead are secreted by the oenocytes located on the cuticular surface (Makki et al. 2014). However, assessing correlations among CHCs and expression data from these tissues may provide insight into the pathways or genes that could mediate links among CHC expression, social behavior, and

reproductive physiology. One foundress individual from the original transcriptomic data set failed CHC extraction and was not included in the analysis. Expression data consist of the $\log_2$-normalized counts per million transcripts, relative to the average value across the entire set of individuals. Correlations between CHCs of interest were performed using the Spearman correlation test. False discovery rate (FDR) *P* value adjustment (<.05) was used independently on the brain (204 genes) and ovary (10 genes) data sets to correct for the large number of correlation tests conducted.

DNA Extractions for Relatedness Genotyping

We genotyped individual bees from a subset of nests to investigate the association between relatedness and CHC variation. DNA extractions were carried out using the Qiagen DNeasy Blood and Tissue kit (product ID: 69504), in accordance with the manufacturer's protocol, using half of the thorax, ground and incubated with proteinase K. DNA concentration was assessed using a Qubit prior to library preparation. Genotype-by-sequencing (GBS) libraries were prepared from 55 individuals from 21 nests using 50 ng of DNA per individual to generate single-nucleotide polymorphisms (SNPs), following the protocol published in Elshire et al. (2011). We sequenced all adult females found in our collected nests as well as emerging offspring from several controls (discussed further in app. 2). We used the enzyme ApeKI, as previous experience indicated its high efficiency in *E. dilemma*. During enzyme digestion, we incubated the samples for 2 h at 37°C, followed by 20 min at 65°C. Finally, we optimized the polymerase chain reaction protocol to include 20 cycles. The GBS library was then sent to the Vincent J. Coates Genomics Laboratory in Berkeley, California, for one lane of 100-bp single-end sequencing on an Illumina HiSeq 4000 instrument. Raw, demultiplexed reads are available for all individuals in the NCBI BioProject database (accession no. prjna623571).

SNP Calling and Relatedness Estimation

We used the program STACKS (Catchen et al. 2013) to demultiplex pooled sequences and BWA-MEM (Li 2013) to align reads to the *E. dilemma* genome (Brand et al. 2017) before running STACKS again to call SNPs. We varied STACKS parameters so that the relationships of control individuals were optimized (for more information, see app. 2). For these controls, we had four nests composed of individuals of known relationships (three nests with a mother and daughter or daughters and a nest with two unrelated individuals). We also included two samples of the same individual as an additional control. Ultimately, we found that setting strict parameters in STACKS gave us the best resolution for resolving relationships among our controls. We counted only SNPs found across all individuals in the data set, and we set maximum heterozygosity levels at 0.7 with the minor allele frequency set at 0.1. Using this strict filtering approach, we generated 669 SNPs found across all individuals that were used to estimate relatedness.

We used the program COANCESTRY (Wang 2011) to generate relatedness values based on our SNP set. COANCESTRY estimates relatedness between pairs of individuals (or dyads) relative to background allele frequencies calculated from the provided samples. We used the Wang estimator statistic, which should be less biased by relatively small sample sizes (tens instead of hundreds of individuals), such as in our study (Wang 2017). We note that our final relatedness estimates are much lower than typical values for Hymenoptera (fig. S5; table S1; tables S1, S2 are available online). However, underestimation of relatedness values is expected when calculating relatedness estimates with SNPs from genomic data sets that have a relatively low sample size and contain inbred individuals (Wang 2014, 2017). Despite this, we expect our data set to provide internally consistent estimates that can be used for relative comparisons among our samples. We used our control samples of known relationship to calibrate the approximate relatedness values between mothers, daughters, sisters, and nonkin (fig. S5, app. 2).

Results

Euglossa dilemma *Shows CHC Dimorphism across Populations*

We identified 11 CHC compounds that were present across all populations and were present in >85% of all individuals in the data set (see fig. S2 for example chromatograms and table S3 for summary statistics). These CHCs captured most of the CHC variation present across samples. In Mexican *E. dilemma* and *E. viridissima*, these 11 CHCs made up an average of 95.4% of the total peak area of all CHCs detected. In Florida and Costa Rica individuals, which were processed together, these 11 CHCs made up an average of 97.6% of the total peak area of all CHCs detected. Plotting the three populations of *E. dilemma* and one population of the sister species *E. viridissima* using NMDS revealed a discrete polymorphism in the CHCs from *E. dilemma*, with individuals falling into two clusters, which we hereafter refer to as chemotype A and chemotype B (figs. 1, S2). *Euglossa dilemma* females from all three populations appear to fall into both chemotypes, while male *E. dilemma* appear to occupy one chemotype or the other depending on the population of origin. SIMPER analysis revealed that these chemotypes are overwhelmingly driven by differences in the relative abundances of two alkenes, 9-heptacosene (9-C27:1) and 9-pentacosene (9-C25:1), which account for 80.75% of the

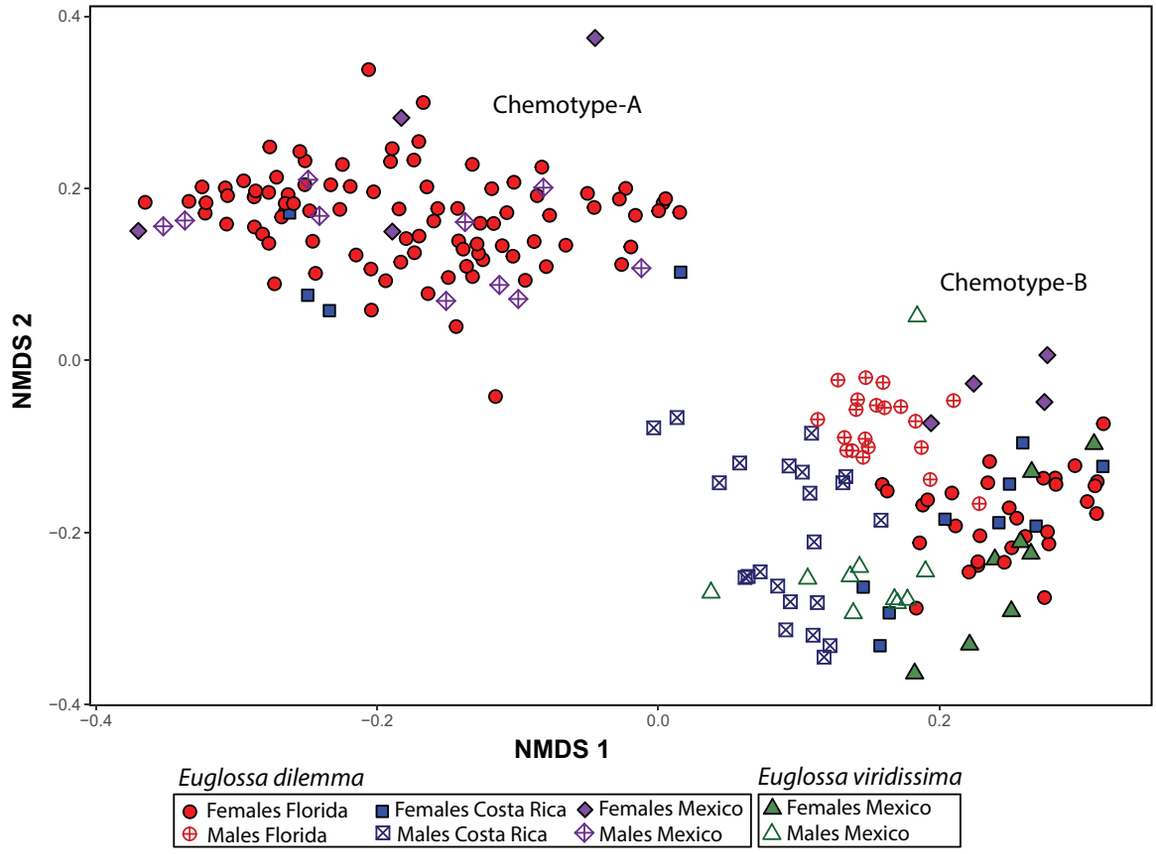

Figure 1: Nonmetric multidimensional scaling (NMDS) plot of cuticular hydrocarbon variation among 214 individuals across populations and sexes of *Euglossa dilemma* and *E. viridissima*. Each color and symbol combination represents individuals of one sex from one population. Individuals fall mostly into clusters, referred to here as chemotype A and chemotype B. Stress value for NMDS configuration = 0.052.

overall dissimilarity between the chemotypes. We note that the Mexican population of *E. dilemma* and sister species *E. viridissima*, which is the only sampled region where they are sympatric, shows consistent differences between males of each species. This pattern was first detected in Mexico by Pokorny et al. (2014), where it was suggested that these differences could be a useful tool in discerning species identity between males of *E. dilemma* and *E. viridissima* in this population.

Social Behaviors Are Not Correlated with Discrete Observed Chemotype Variation

We visualized CHC variation across social behaviors within the Florida population of *E. dilemma* using NMDS of all 17 detected CHCs in females in this population. We found representatives from all of the behavioral groups (foundresses, guards, dominants, subordinates, and newly emerged females) in each of the two chemotypes (fig. 2). In chemotype A, individuals are loosely clustered according to behavior, although this is less clear in chemotype B. In addition, we found that individuals within a nest can occupy either the same or different chemotypes, with seven of 21 genotyped nests containing individuals of both chemotypes (table S1).

Using our genotype data set, we specifically sought to assess whether kin within a nest can occupy the two different chemotypes. Alternatively, mixed chemotype nests may correspond to unrelated individuals, as *Euglossa* species are known to regularly form associations with nonkin (Andrade-Silva and Nascimento 2016). The presence of mixed chemotypes among kin nestmates would suggests that chemotype is probably not determined by early social interaction, homogenization of CHCs within a nest, or other environmental influences after eclosion. Furthermore, since *Euglossa* species have been shown to discriminate kin and nonkin (Andrade-Silva and Nascimento 2016), this may provide insight into whether chemotype variation encodes information about nestmate relatedness.

Of the 21 nests genotyped, seven showed mixed chemotypes among nestmates, and 14 showed the same chemotype among all nestmates (12 nests were all chemotype A, and

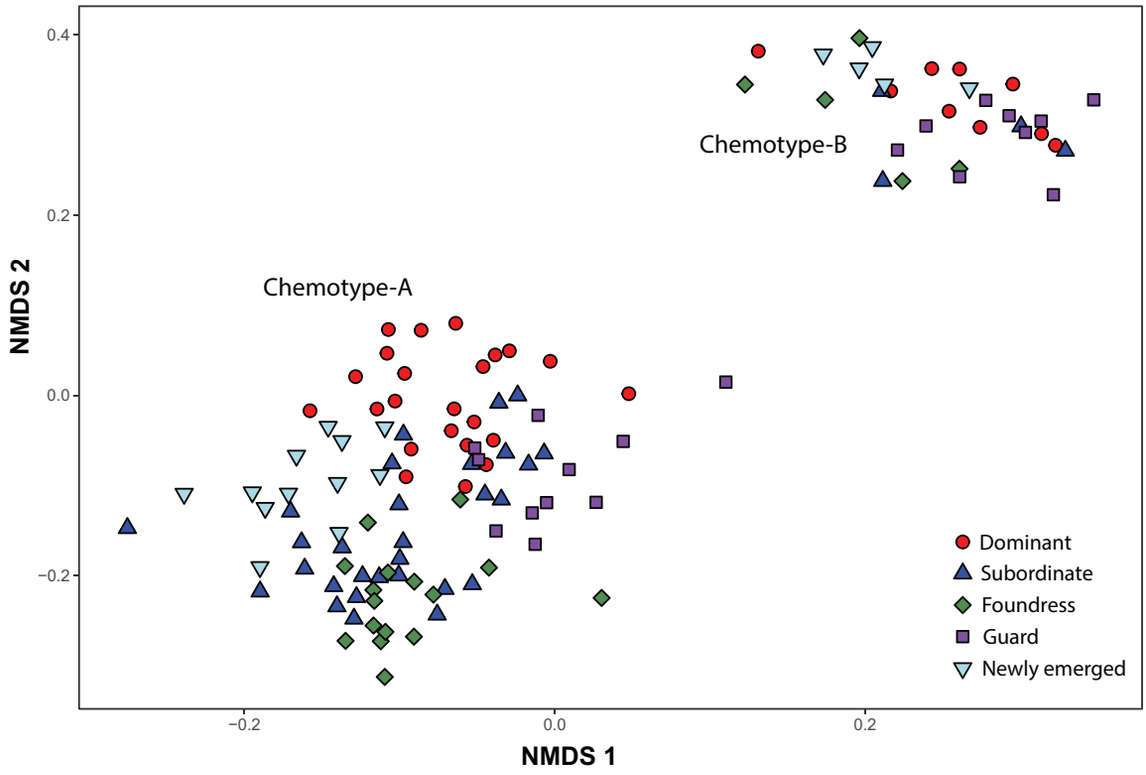

Figure 2: Nonmetric multidimensional scaling (NMDS) plot of cuticular hydrocarbon variation across *Euglossa dilemma* female behavioral groups in the Florida population. Unique color and symbol combinations represent the different behavioral groups. Individuals fall into clusters, referred to here as chemotype A and chemotype B. Stress value for NMDS configuration = 0.042.

two nests were all chemotype B). Of the seven mixed chemotype nests, three showed high relatedness values among nestmates expressing opposite chemotypes, indicating that they are likely kin (mothers, daughters, or sisters). Two of these seven nests showed low relatedness values indicating nonkin, and two of these seven nests showed intermediate relatedness values and could not be confidently assigned to kin/nonkin categories (app. 2, table S1). These results suggest that the kin/nonkin composition of nests does not consistently correlate with the presence of mixed chemotypes within a nest. Overall, kin relationships were clearly most common among all sampled individuals, with only two of 21 nests showing relatedness values indicative of nonkin nesting together. More detailed results and discussion can be found in appendix 2.

Female CHCs Form Three Covarying Modules

Since the two major chemotypes that we identified do not appear to be correlated with social behavior (varying even within a nest or among kin), it is unlikely that the CHC variation associated with chemotypes A and B reliably reflects ovary size or social hierarchy. Consequently, we sought to identify additional variation among CHCs that could potentially contain information on social status and/or reproductive physiology in multifemale nests. CHC profiles are often composed of groups of correlated peaks that may encode different sets of information (Martin and Drijfhout 2009). Therefore, we used hierarchical clustering of all CHCs among the four female behavioral groups sampled from naturally founded trap nests (foundress, guard, dominant, and subordinate) to identify groups of covarying peaks. Newly emerged females (<24 h after emergence) were excluded from this and further analysis, because of differences in their CHC profiles that were not representative of females sampled from established nests (app. 3, fig. S6). With our hierarchical clustering approach, we identified three groups of covarying peaks (fig. 3) that we hereafter refer to as CHC modules. The first two modules are composed of CHCs that correlate with the two chemotypes (A and B), with individuals in chemotype A showing a higher relative abundance of module 1 peaks than chemotype B individuals and vice versa. The first module is made up of alkenes and alkanes between 21 and 25 carbons long (diagnostic of chemotype A). The second module consists

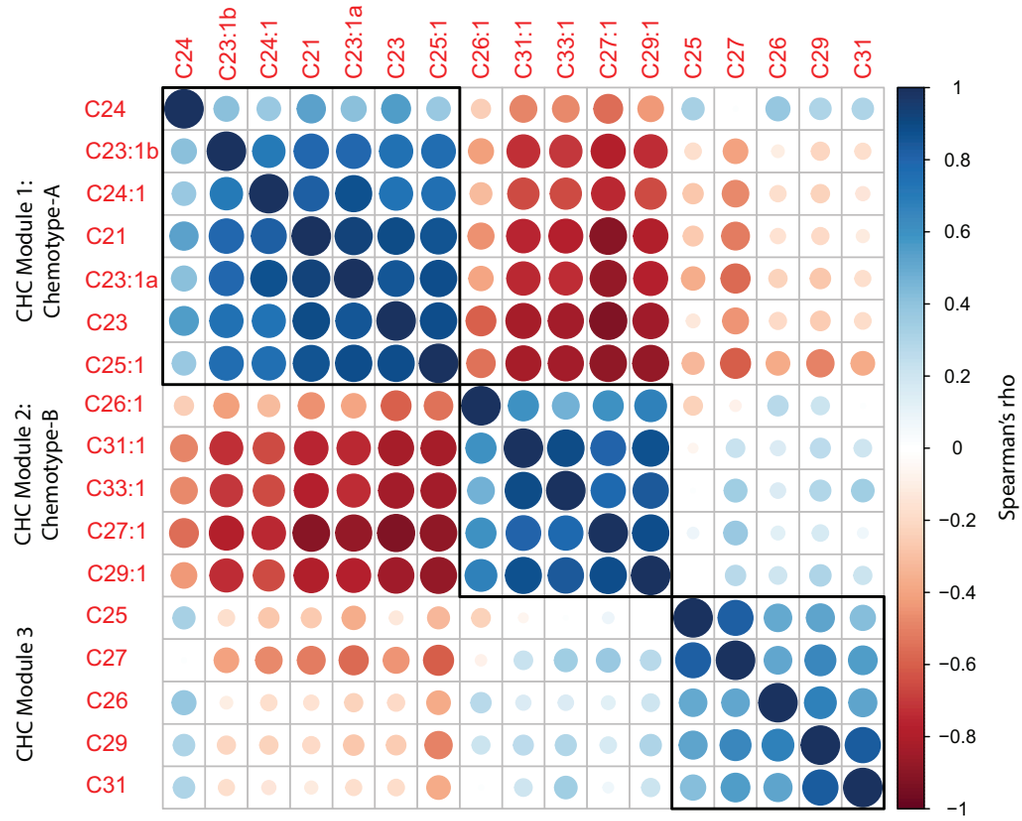

Figure 3: Correlation structure among cuticular hydrocarbons (CHCs) in *Euglossa dilemma* Florida females. The size and color of a circle indicate the strength and direction of the correlation between two CHCs, respectively. Larger circles indicate a higher Spearman's ρ value, dark blue represents a positive correlation, and dark red represents a negative correlation. CHCs have been ordered into three modules (black rectangles) using hierarchical clustering based on the Ward.D2 clustering method. CHCs labeled with a colon are alkenes, and those labeled with an "a" or "b" indicate that they are alkenes of the same carbon chain length with a different position for the double bond.

of alkenes between 26 and 33 carbons long (diagnostic of chemotype B). The third module is composed only of alkanes varying from 25 to 31 carbons. We further investigated correlations among behavior and physiology with this third set of peaks, hereafter referred to as module 3, to evaluate whether it could reliably reflect social information not represented in the discrete variation of the chemotype A and B peaks.

Module 3 CHCs Are Correlated with Social Dominance and Ovary Size

To assess whether the CHCs contained in module 3 reflect social dominance and ovary size, we first calculated the total relative abundance of all of the CHCs in this module and tested for differences among the four behavioral groups. Dominant bees showed a higher relative abundance of these CHCs than bees from other behavioral groups: foundresses, guards, and subordinates ($F_{3,102} = 27.25$, $P < .001$; fig. 4). We also inspected the relative abundance of this CHC module between dominants and subordinates taken from the same nest, as pairwise interactions within a nest may be particularly important for communicating social dominance. In 22 of 24 nests where we had data for both dominant and subordinate females, dominants showed higher relative expression of this CHC module compared with the subordinate or subordinates in that same nest. Furthermore, the module was significantly correlated with ovary size ($r = 0.47$, $P < .001$; fig. 5).

After performing these tests on the CHC module, we reran the same tests on the individual component CHCs, to assess whether the overall patterns were driven by a subset of the CHCs found in the module. Full results can be found in table S2. In summary, the individual CHCs in the module show consistent patterns, in which ovary size correlates with CHCs and behavioral differences in C25, C27, C29 and C31, with a nonsignificant relationship for C26, although patterns are in the same direction. We also assessed these

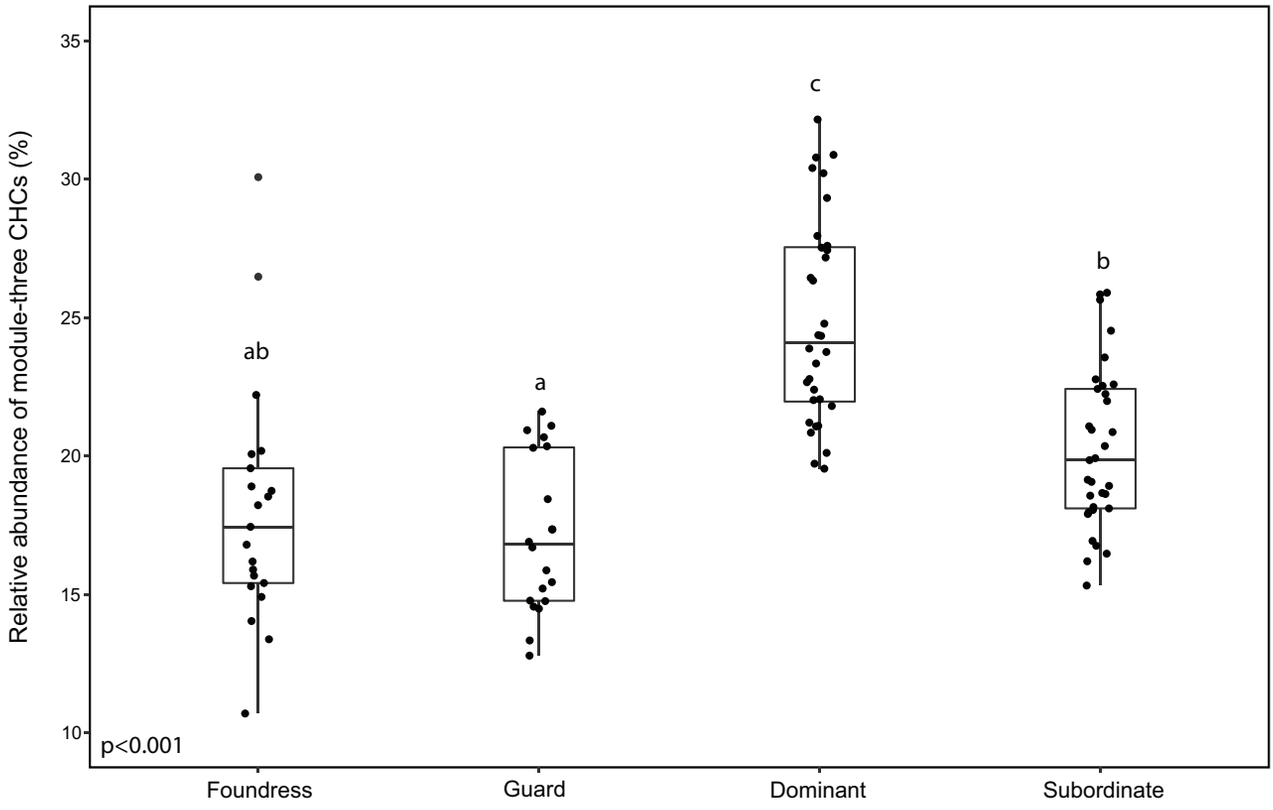

Figure 4: Relative abundance of module 3 CHCs (C25, C26, C27, C29, C31) across behaviors. Letters denote statistical groupings determined by the Tukey honestly significant difference test, and the *P* value was calculated using a one-way ANOVA. The boxplots show the mean value in each group.

patterns in CHC modules 1 and 2 (chemotype-associated peaks) to test whether the module 3 peaks are uniquely correlated with ovary size and dominance status (Cotton et al. 2004). We find that the chemotype-associated modules do not show significant correlations with ovary size and they are not overrepresented in dominant individuals (details and figures are provided in app. 4).

Correlations between Gene Expression and Module 3 CHCs

Next, we assessed relationships between the relative abundance of the module 3 CHCs and the expression of known DEGs between dominants and subordinates. In addition, we also assessed ovary size correlations with these DEGs to identify overlap in DEGs correlated with both ovary size and CHC module 3. These analyses may provide additional evidence for links (or lack thereof) among CHCs, reproductive physiology, and gene expression. This can allow for a better understanding of the possible information content of the module 3 CHCs, although correlation analysis cannot demonstrate whether any identified genes directly influence CHC expression or ovary size. Full results of the correlation analysis for the brain and ovary data with gene annotations, original *P* values, and FDR-adjusted *P* values can be found in tables S4 and S5. In the ovaries, five of the 10 previously identified DEGs between dominants and subordinates were significantly correlated with CHC module 3 following FDR correction. Of these five genes, three were also significantly correlated with ovary size. These three genes are facilitated trehalose transporter Tret 1-like (fig. 6), inositol oxygenase (fig. S7), and UDP-glucuronosyltransferase 1–8 (fig. S8), the expression levels of which are highly correlated (fig. S9). Consequently, we show the correlation between module 3 CHCs and facilitated trehalose transporter Tret-1 expression (fig. 6) as a representative example, with the other plots and correlations found in the supplemental material (figs. S7, S8; table S2). In the brain, three of the previously identified 204 DEGs were significantly correlated with CHC module 3 peaks following FDR correction. None of these genes show correlated expression with ovary size, and none

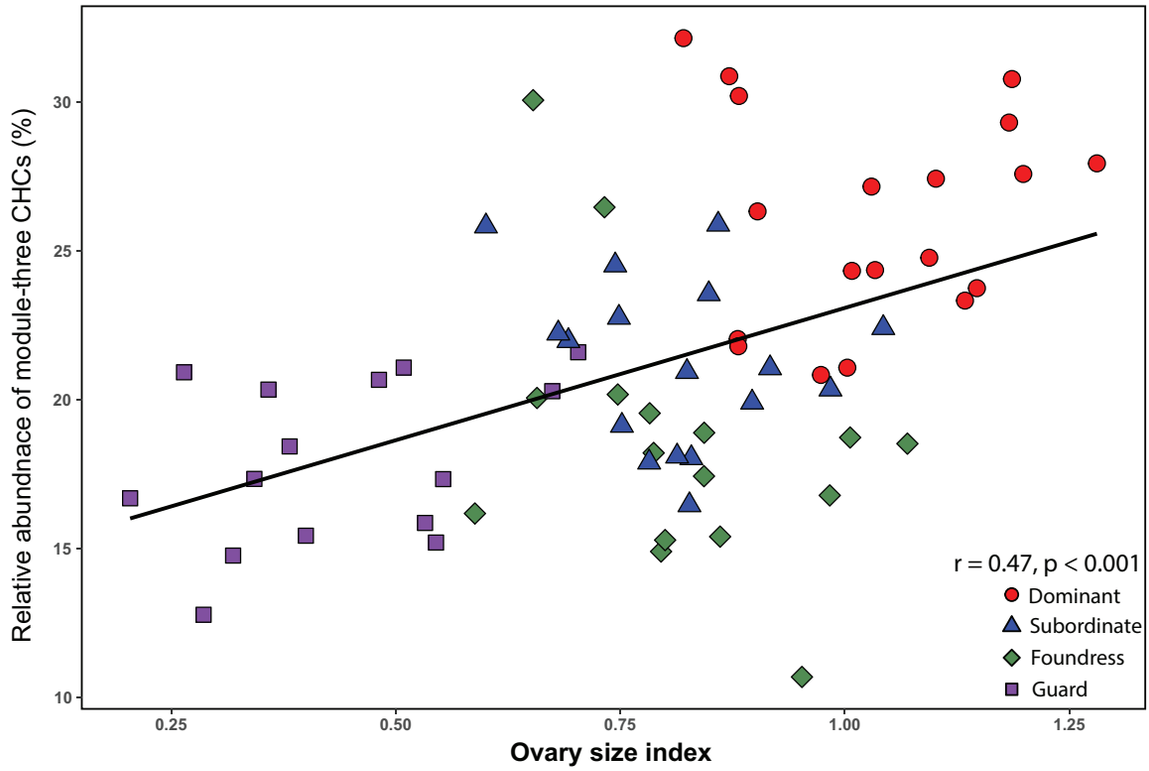

Figure 5: Correlation between ovary size and the module 3 (C25, C26, C27, C29, C31) cuticular hydrocarbon relative abundances across behaviors. Unique shape and color combinations correspond to the four sampled behavioral groups. The correlation coefficient and *P* value were calculated with Pearson's *r*.

of these three genes have functional annotation information available (see table S5 for *E. dilemma* gene IDs and correlation results).

Discussion

In this study, we examine CHC variation across species, populations, and social behaviors with an emphasis on *Euglossa dilemma*, finding that population and ovary size/dominance status correlate most strongly with separate components of the CHC profile. We identify a CHC polymorphism that exists across at least three populations, showing sex-specific patterns of expression that are distinct when *E. dilemma* is in sympatry with *Euglossa viridissima*. In addition, we identify a subset of CHCs not primarily associated with this polymorphism that reflect social dominance and ovary size. These CHCs have higher relative abundance in dominant bees than in subordinate bees, are correlated with ovary size, and show higher relative abundance in dominant bees compared with other behavioral groups. Furthermore, these CHCs are also correlated with the expression of several DEGs previously identified between dominants and subordinates. Taken together, these data provide an example of how a complex chemical phenotype may be parsed into multiple components, each potentially containing different types of information useful for communication. Furthermore, these results highlight the ability of CHCs to reflect social and reproductive information in an insect species with small social groups lacking an obligate reproductive division of labor.

Possible Mechanisms Underlying Chemotype Variation in E. dilemma

One possibility is that the discrete chemotype variation corresponds to a genetic polymorphism as opposed to an ontogenetic shift over an individual's lifetime. This scenario is supported by the finding that all behavioral groups, closely related females, and newly emerged females can exhibit both chemotypes (A and B). Furthermore, these chemotypes differ primarily in the relative abundance of two alkenes that are part of a homologous series (C27:1 and C29:1), so it is possible that the phenotypic expression of these chemotypes is the result of a relatively simple genetic polymorphism of a desaturase, elongase, or other fatty acid synthesis

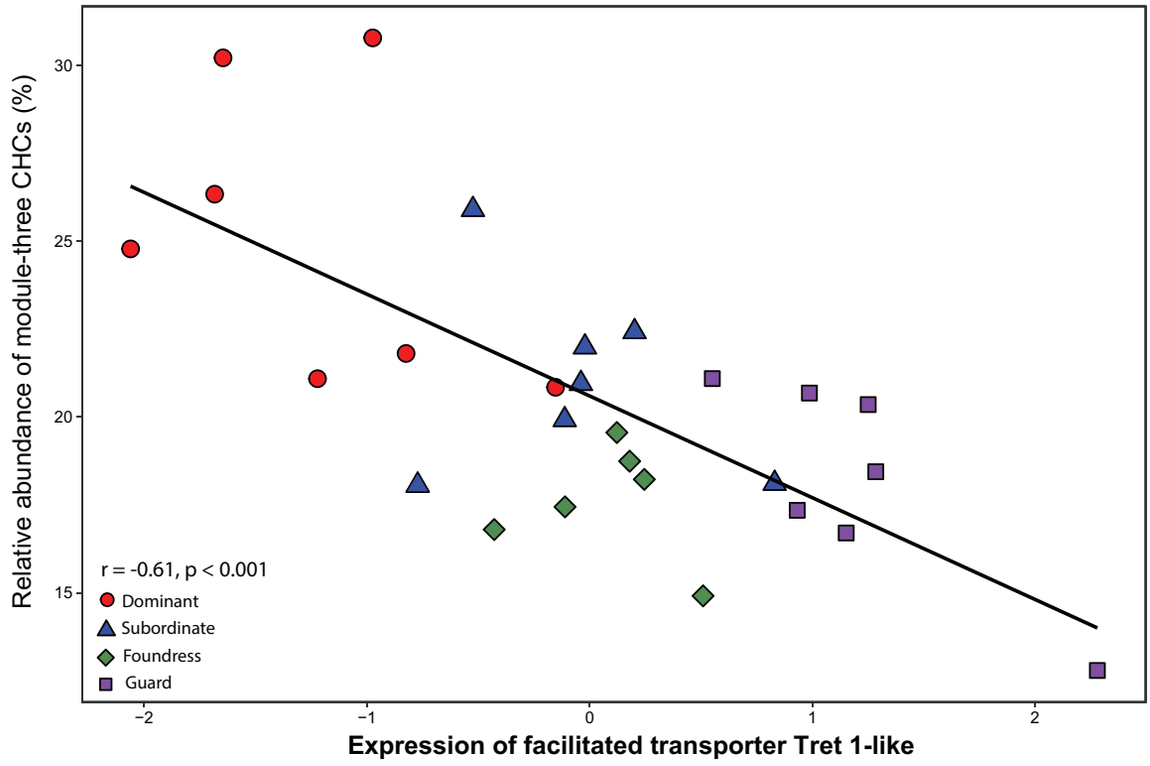

Figure 6: Correlation between ovary expression of facilitated transporter Tret 1-like and the relative abundances of the module 3 (C25, C26, C27, C29, C31) cuticular hydrocarbons across behavioral groups. The *X*-axis shows $\log_2$ expression levels for each individual relative to the mean value for that gene across individuals. Unique shape and color combinations correspond to the four sampled behavioral groups. The correlation coefficient and false discovery rate–adjusted *P* value were calculated with Spearman's ρ.

gene (Coyne et al. 1999; Luo et al. 2019). Alternatively, it could also be that differences in development primarily affect the expression of chemotype. In addition, the sex-specific expression pattern across populations that we see, where all female *E. dilemma* populations occupy both chemotypes, with males seeming to occupy either one or the other, does not lend itself to a clear interpretation of how chemotype is transmitted across generations.

Chemotypes Could Be Involved in Close-Range Interactions in Sympatric Populations

It has been suggested that orchid bee CHCs may be involved in close-range communication between males and females in mate choice and/or species recognition contexts (Pokorny et al. 2015). Although males of *E. dilemma* from Costa Rica and Florida occupy the same chemotype as *E. viridissima* males, we find that sympatric males of *E. dilemma* and *E. viridissima* from southern Mexico occur in opposite chemotypes. This could facilitate consistent identification of species by females, which, in most euglossine bees, typically approach males that are engaged in a sex-specific display behavior for mating opportunities (Pokorny et al. 2017). Future experimental manipulation of CHCs is a necessary next step to determine whether CHCs play a role in close-range mating behavior and in species recognition and reproductive isolation.

The pattern of differentiation that we observed among *E. dilemma* males in Mexico also aligns with population genetic data supporting two distinct lineages of *E. dilemma*, with a northern lineage reaching up through the Yucatán Peninsula and a southern lineage extending through Costa Rica and into Panama (Brand et al. 2020). The Florida population is believed to be derived from the southern *E. dilemma* lineage, which is consistent with our clustering of males from Costa Rica and Florida in the same chemotype.

Module 3 CHCs Reflect Dominance Status and Reproductive Physiology

Our data are consistent with the hypothesis that module 3 CHCs in *E. dilemma* could communicate dominance

and reproductive information in social groups, as these CHCs show significant associations with both. In contrast, the CHCs involved primarily in the differentiation of the two chemotypes are not correlated with ovary size and are not overrepresented in dominant bees.

These results are also consistent with the interpretation that module 3 CHC variation is driven by reproductive physiology and behavior as opposed to age, although age does generally influence CHC abundances (Gershman and Rundle 2016). Dominant bees and guard bees are, on average, closer together in age than dominant bees and subordinate bees or foundress bees, which are generally younger. Despite the similarities in age between dominant and guard bees, however, we see that ovary size differences between dominant and guard bees explains a substantial amount of variation in module 3 CHC relative abundances (fig. 5).

Transcriptomic Data Reveal Links among CHCs, Sociality, and Reproductive Physiology

Correlations between DEGs and CHCs in module 3, particularly from the ovary gene expression data, suggest robust links among social behavior, reproductive physiology, and CHC expression. While relatively few genes (10) were previously found to be differentially expressed between dominant and subordinate ovaries, five of these showed significant correlation with the CHC module 3 peaks, and three of these five genes were also significantly correlated with ovary size. All three of these genes are associated with either transporting or metabolizing sugars and show highly correlated expression (fig. S9). The first, facilitated trehalose transporter Tret1-like, is thought to regulate the tissue-specific uptake of trehalose, the primary "blood sugar" circulating in insect hemolymph (Shukla et al. 2015). The second gene, inositol oxygenase, is involved in metabolizing the sugar inositol (Parker et al. 2015). The expression of inositol oxygenase is strongly associated with caste behavior in *Polistes* wasps (Jandt et al. 2017) and is also associated with larval caste development and adult behavioral variation in honeybee workers (Hunt et al. 2010). Finally, UDP-glucuronosyltransferase 1–8 is an enzyme belonging to a gene family primarily known for detoxification, which is accomplished by catalysis of glycosidic reactions that increase the solubility of xenobiotics for excretion (Li et al. 2018). Nutrient-sensing pathways are broadly implicated in caste-associated physiology across social insects (Kapheim 2016). We note that our data set cannot address whether the expression of these genes has any direct influence on CHC expression. Especially given that behavior, ovary size, and CHCs are interrelated, it is not necessarily surprising that we also see correlated gene expression. Future work examining connections among social behavior, nutrition, and reproductive physiology will be necessary to understand how the subtle differences between dominant and subordinate physiology emerge.

Comparison to Other Species

The linear alkanes that compose the module 3 CHCs in *E. dilemma* include several CHCs that are associated with the queen caste of multiple eusocial insects, including species of ants, wasps, and bees (Van Oystaeyen et al. 2014; Steitz et al. 2018). This is consistent with the idea that linear alkanes may be ancestrally linked to reproduction and have been co-opted for social communication independently across Hymenoptera, including in the corbiculate bees (Oi et al. 2015; Oliveira et al. 2015). The CHC with the highest relative abundance among the module 3 CHCs that we identify in *E. dilemma*, pentacosane (C25), shows evidence of queen signaling in multiple species of bumblebee and multiple species of stingless bees (Nunes et al. 2014; Amsalem et al. 2015; Oliveira et al. 2015). This may be an especially promising candidate for further investigation for its role in reproductive signaling in the evolutionary history of corbiculate bees, as it has now been identified as associated with reproductive physiology in species of three of the four tribes forming the corbiculate bees.

Within orchid bees, our findings both overlap and contrast in several ways with the only other orchid bee species where CHCs and social status have been investigated, *Euglossa melanotricha* (Andrade-Silva and Nascimento 2015). Four of the five linear alkanes we identify among the module 3 CHCs are also significantly differentiated between dominants and subordinates of *E. melanotricha* (C25, C27, C29, and C31), suggesting that their association with social behavior could be conserved in *Euglossa*.

Besides these chemical similarities, however, our results linking ovary size and CHCs in *E. dilemma* contrast with those from *E. melanotricha*, where no link between CHCs and ovary size was found. This could be due to differences in the biology of the two species, methodological differences between the studies, or some combination of the two. *Euglossa melanotricha* is distinct from *E. dilemma* in showing high levels of aggression between dominants and subordinates (Andrade-Silva and Nascimento 2012), while *E. dilemma* (and sister species *E. viridissima*) females show little aggression among nestmates (Cocom Pech et al. 2008; Saleh and Ramírez 2019). Given this, *E. dilemma* and *E. melanotricha* may have evolved different approaches to managing within-nest conflict and cooperation. Dominant bees in *E. melanotricha*, for instance, may rely on egg policing and aggression to reinforce chemical dominance, and *E. dilemma* dominant bees may rely on honest signaling of ovary size to encourage subordinate cooperation or retention in the nest. Alternatively, it is possible that our

inclusion of the solitary behavioral phases, which show the largest shifts in ovary size, increased our power to detect CHC and ovary size correlations. Examination of additional orchid bee species is necessary to determine how widespread the link between ovary size and CHC expression is across the phylogeny of orchid bees.

Are Module 3 CHCs Social Signals in E. dilemma*'s Casteless Social Groups?*

We identified CHCs that are correlated with social behavior and reproductive physiology in *E. dilemma*, although it is unknown whether these CHCs function as social signals. In some eusocial insects, exposure to queen signals can lead to the inactivation of worker ovaries (Smith and Liebig 2017). In addition, fertility signals can direct aggression toward egg-laying workers (Smith et al. 2009). In some primitively eusocial species, however, CHCs or other compounds may correlate with dominance and ovary size but lack the ability to regulate reproduction (Oi et al. 2019). In these cases, CHCs can still be relevant to social interactions, but they may function only within specific chemical and behavioral contexts (Mora-Kepfer 2014; Smith et al. 2015). In *E. dilemma*, both dominants and subordinates have activated ovaries, and aggression is rarely observed among nestmates. Furthermore, subordinate ovary size is equivalent to foundress ovary size, and dominant ovary size is slightly larger than either (Saleh and Ramírez 2019). This observation suggests that although dominant bees show elevated ovary size, it is unlikely that subordinate bees are suppressed physiologically via the production of pheromones emitted by dominant bees. In addition, oophagy of subordinate eggs by dominants is not selective in *E. dilemma*, with dominant bees appearing to eat all subordinate laid eggs (Saleh and Ramírez 2019).

Given these life history characteristics, if CHCs are indeed serving as social signals, one possibility is that they function more to advertise dominant quality than to control subordinate behavior. It is unknown what factors determine whether a newly emerged *E. dilemma* female will stay in the nest or disperse. CHCs could be used by newly emerged females to assess the dominant individual as a part of a disperse/stay decision-making process. Newly emerged *Polistes* wasps, for example, incorporate information about their nesting environment to inform dispersal decisions (Tibbetts 2007). If dominant bees have the largest ovary size on average, there could be a greater fitness advantage for newly emerged females to remain in the nest and raise sisters laid by a highly fecund mother rather than expending energy on nest initiation. Alternatively, aggression may be rare in *E. dilemma* social groups precisely because dominant females honestly signal larger ovary size. This appears to be the case in the primitively eusocial wasp *Ropalida marginata*, for instance, where a reduction in the quantity of queen-specific pheromones stimulates aggression between otherwise nonaggressive queens and workers (Saha et al. 2012). Manipulation of CHC profiles in dominants and subordinates could therefore reveal latent aggression. Further experiments are needed to evaluate these hypotheses.

Finally, it is also possible that the CHCs in module 3 do not function as signals but instead serve as cues that, while linked to behavior and physiology, do not elicit a behavioral response shaped by natural selection. If this is the case, it would strongly support a scenario in which social signals, particularly in the corbiculate bees, began as fertility-linked cues already sensitive to physiology and social environment that subsequently evolved into signaling molecules along with a more elaborate social organization.

Conclusions

In addition to documenting a CHC polymorphism across populations, we identify a set of CHCs largely independent of this polymorphism that correlate with ovary size and dominance status in *E. dilemma* social groups. Experimental manipulation of these CHCs in social settings is a necessary next step in assessing the role of CHCs in mediating conflict and cooperation in *E. dilemma* nests. However, these data show that even in small casteless social groups of two or three individuals, CHCs can reflect ovary size and dominance status in a similar fashion to the information encoded in highly eusocial Hymenoptera that display large social colonies with specialized castes. This is especially interesting considering the phylogenetic position of orchid bees as sister to the rest of the eusocial corbiculate bees. These data further motivate increased sampling of CHCs among orchid bees and the other corbiculate bees, to better understand the role of CHCs in the evolution of communication in social insects.

Acknowledgments

We thank the members of the Ramírez lab and Cheryl Dean for their help and advice throughout the project. We thank Nikki Hochberg, Ron Phenix, and all of the staff at the Fern Forest Nature Center for their support of this project. We thank Joe Patt and Aleena Tarshish Moreno for their help. We also thank the editors and reviewers for their insightful comments on the manuscript. The project was supported by the David and Lucile Packard Foundation (to S.R.R.), the National Science Foundation (to S.R.R.; DEB-1457753), and the Daphne and Ted Pengelley Award (University of California, Davis, to N.W.S.).

Statement of Authorship

N.W.S. and S.R.R. initially designed the project. All authors participated in sample collection. N.W.S. and K.H. performed sample processing and data analysis. All authors participated in writing and revising the manuscript.

Data and Code Availability

Data and R code required to reproduce all figures and analyses in this article are available in the Dryad Digital Repository (https://doi.org/10.25338/B8FH0P; Saleh et al. 2021). RNA sequence data are available in the National Center for Biotechnology Information (NCBI) BioProject database (accession no. prjna523381), as are DNA data for genotyping (accession no. prjna623571).

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Associate Editor: Sarah D. Kocher
Editor: Daniel I. Bolnick