Sexual dimorphism in visual and olfactory brain centers in the perfume-collecting orchid bee *Euglossa dilemma* (Hymenoptera, Apidae)

Philipp Brand | Virginie Larcher | Antoine Couto | Jean-Christophe Sandoz | Santiago R. Ramírez

1Department of Evolution and Ecology, Center for Population Biology, University of California, Davis, California
2Evolution Genomes Behavior and Ecology, Centre National de la Recherche Scientifique, Université Paris-Sud, IRD, Université Paris Saclay, Gif-sur-Yvette, France

Correspondence
Philipp Brand, Department of Evolution and Ecology, Center for Population Biology, University of California, One Shields Avenue, Davis, CA 95616.
Email: pbrand@ucdavis.edu

Funding Information
National Science Foundation, Grant/Award Number: DEB-1457753 (to SRR); CNRS and ANR, Grant/Award Number: 17-CE20-0003 (to JCS); European Society for Evolutionary Biology Godfrey Hewitt Mobility Award (to PB); Center for Population Biology Research Award (to PB)

Abstract
Insect mating behavior is controlled by a diverse array of sex-specific traits and strategies that evolved to maximize mating success. Orchid bees exhibit a unique suite of perfume-mediated mating behaviors. Male bees collect volatile compounds from their environment to concoct species-specific perfume mixtures that are presumably used to attract conspecific females. Despite a growing understanding of the ecology and evolution of chemical signaling in orchid bees, many aspects of the functional adaptations involved, in particular regarding sensory systems, remain unknown. Here we investigated male and female brain morphology in the common orchid bee *Euglossa dilemma* Bembé & Eltz. Males exhibited increased relative volumes of the Medulla, a visual brain region, which correlated with larger compound eye size (area). While the overall volume of olfactory brain regions was similar between sexes, the antennal lobes exhibited several sex-specific structures including one male-specific macroglomerulus. These findings reveal sexual dimorphism in both the visual and the olfactory system of orchid bees. It highlights the tendency of an increased investment in the male visual system similar to that observed in other bee lineages, and suggests that visual input may play a more important role in orchid bee male mating behavior than previously thought. Furthermore, our results suggest that the evolution of perfume communication in orchid bees did not involve drastic changes in olfactory brain morphology compared to other bee lineages.

KEYWORDS
antennal lobe, brain morphology, *Euglossa*, macroglomerulus, olfaction, orchid bees, sexual dimorphism, vision, RRID: SCR_007353

1 INTRODUCTION

Insect mating behavior involves the detection and processing of a variety of intraspecific stimuli. Mate recognition, mate finding, and mate choice are often mediated by specialized chemical and visual traits such as pheromones and body coloration (Andersson, 1994; Smith & Harper, 2003; Wyatt, 2003). Depending on the type and direction of sexual communication, such traits can be correlated with sex-specific adaptations of the sensory system. Brain regions underlying olfactory detection, for example, often show enlarged structures in the sex that uses pheromone signals emitted by the opposite sex to identify or locate mates (Arnold, Masson, & Budharugsa, 1985; Arnold, Budharugsa, & Masson, 1988; Couto, Lapeyre, Thiéry, & Sandoz, 2016; Koontz & Schneider, 1987; Rospars & Hildebrand, 2000; Steinzer, Kelber, Pfabigan, Kleineidam, & Spaethe, 2013). Similarly, brain regions where visual stimuli are integrated are often enlarged in the sex that relies on visual cues to locate mates (Steinzer, Brockmann, Nagaraja, & Spaethe, 2013; Steinzer & Spaethe, 2014). Such patterns of sexual dimorphism in
neural morphology are often observed across insect lineages and likely evolved in response to lineage-specific sensory adaptation (Hansson & Anton, 2000).

Some of the most prominent examples of sexual dimorphism in the insect olfactory pathway are found in the primary olfactory center of the insect brain, the antennal lobe (AL). This structure is innervated by odor-detecting olfactory sensory neurons (OSNs) that project from each antenna via the antennal nerve. The AL comprises tens to hundreds of globular structures called glomeruli (Baumann, Oland, & Tolbert, 1996; Shepherd, 1974). Each glomerulus is innervated by axons from all OSNs that express the same type of odorant receptor (Vosshall, Wong, & Axel, 2000). Within each glomerulus, OSNs form numerous synaptic contacts with local interneurons (mostly inhibitory) and projection neurons, forming local networks responsible for primary odor processing (Fonta, Sun, & Masson, 1993; Homberg, Montague, & Hildebrand, 1988; Kanzaki, Sø, Seki, & Wada, 2003; Rybak et al., 2016). Projection neurons convey olfactory information to higher-order integration centers, the mushroom body and the lateral horn (Abel, Rybak, & Menzel, 2001; Arbas, Strausfeld, & Hildebrand, 1989; Hildebrand, 1996; Kanzaki, Malun, Waldow, Kraus, & Boeckh, 1993; Kanzaki et al., 2003; Watanabe, Nishino, Mizunami, & Yokohari, 2017; Zube, Kleineidam, Kirschner, Neef, & Rössler, 2008).

Sexual dimorphism in the olfactory pathway may involve sex-specific differences in the total number of glomeruli present in the AL or in the size of individual glomeruli (Hansson & Anton, 2000). Such morphological differences are often linked to sex-specific behavior during foraging, social interactions, or mating. Insect species that rely on pheromone communication often exhibit enlarged glomeruli in the sex that detects and encodes pheromones (Couto et al., 2016; Montgomery & Ott, 2015; Kuebler, Kelber, & Kleineidam, 2010; Zhao et al., 2016). Many of these so-called macrogglomeruli show neuronal activity toward single pheromone compounds and/or pheromone blends and likely represent sensory adaptations to pheromone communication in insects (Deisig et al., 2012; Galizia & Menzel, 2001; Hansson & Anton, 2000; Sandoz, 2004).

Insect sex pheromone signals are usually produced in highly specialized glands by one sex in order to attract the opposite sex (Prestwich & Blomquist, 2014; Wyatt, 2003). Accordingly, pheromone communication systems are classically separated into sender and receiver components, with often clearly identified sensory adaptations in the receiving sex. In orchid bees, however, this classification is less straightforward. Orchid bee males collect scents from floral and non-floral sources to concoct a complex species-specific chemical "perfume" blend (Dressler, 1982; Eltz, Whitten, Roubik, & Linsenmair, 1999; Roubik & Hanson, 2004; Weber, Mitko, Eltz, & Ramírez, 2016; Zimmermann, Ramírez, & Eltz, 2009). The perfume is subsequently released during a stereotypical display behavior at perching sites (Pokorny et al., 2017). It is only in combination with perfume display behavior that mating occurs (Dodson, 1966; Kimsey, 1980; Pokorny et al., 2017; Stern, 1991; Zimmermann, Roubik, & Eltz, 2006). Although the exact function of perfume bouquets is still unclear, they are most likely involved in sexual selection, presumably by enabling species-specific recognition or the discrimination of males within a species.

In orchid bees, both sexes need to detect and process perfume signals. Male bees exhibit a strong attraction to individual chemical compounds that are part of their perfume blend as well as to the entire perfume blend, which they readily collect when presented in the field (Dodson, Dressler, Hills, Adams, & Williams, 1969; Dressler, 1982; Roubik & Hanson, 2004; Zimmermann et al., 2006). Females on the other hand are attracted to display sites during active male perfume display, that is, when the complete blend is presented. Therefore, the male and female olfactory systems must ensure the detection and recognition of the chemical components present in the species-specific perfume, to produce and recognize the perfume blend in order to attract and find suitable mates. This scenario deviates substantially from classic pheromone communication systems in that here both sexes need to actively interact with the signal (i.e., perfume) and/or its individual chemical components at some point in their life. While the chemical ecology of perfume collection (Weber et al., 2016; Zimmermann et al., 2009) and the evolutionary genetics of olfactory gene families are well understood (Brand & Ramírez, 2017; Brand et al., 2015), the neurobiology of orchid bee olfaction remains unexplored.

Orchid bee mating behavior likely involves other sensory components in addition to olfactory cues (Kimsey, 1980). Male bees expose perfumes through a stereotypic behavior in which perfume is released during repetitive short hovering flights off perching sites (Dressler, 1982; Pokorny et al., 2017). In addition to perfume display, males engage in patrolling flights and male-male interactions consisting of fast flight routines including two or more "jousting" individuals (Roubik & Hanson, 2004). Although the prevalence of this behavior across the phylogeny of orchid bees remains unknown, males from species of multiple genera exhibit similar territorial behaviors (Kimsey, 1980; Roubik & Hanson, 2004). Both, male-male interactions and patrolling behavior likely require a powerful visual system. Thus, in addition to possible sensory adaptations related to perfume communication, orchid bee mating behavior may also exhibit sex-specific specializations of the visual system. To this day, the visual system of orchid bees remains mostly unexplored (but see Taylor et al., 2016).

Here we analyze the brain morphology of the orchid bee Euglossa dilemma to answer four related questions. (a) Do the brain regions associated with olfaction and vision exhibit different relative sizes between sexes? (b) Is the number of glomeruli in the AL sex-specific? (c) Are there macrogglomeruli in males and/or females? (d) Is the brain and the external head morphology in orchid bees substantially different relative to other bee species that lack perfume collection behavior?

### 2 | MATERIALS AND METHODS

#### 2.1 | Sampling

Males and females of *E. dilemma* were caught in the wild at the Fern Forest Natural Center in Broward County, FL, USA (26°13′45.8″N, 80°11′08.3″W) in August 2017. Whole bees or heads were placed in 95% ethanol immediately after collection for tissue preservation.

#### 2.2 | Head morphology measurements

Head morphology was measured based on photographs taken from pinned and dried specimens. Photographs of the head were taken...
from the frontal, lateral, and ventral view with a Leica S6D microscope at 6x magnification for the frontal and lateral as well as 12x for the ventral view. We measured a total of eight distance parameters (Table 1) in 10 individuals of each sex using ImageJ (Schneider, Rasband, & Eliceiri, 2012). Pixels were transformed into mm in ImageJ using the photograph of a ruler placed next to the samples. In addition to these measurements, we calculated the frontal eye width by subtracting the frontal inter eye span from the head width. Eye surface area was measured from replicas made with nail polish (Ribi, Engels, & Engels, 1989). Replicas were flattened by small incisions with a micro scalpel and photographed using a Leica S6D microscope. Absolute areas were estimated using ImageJ. We measured both eyes in 5 of the 10 individuals of each sex and calculated the mean eye area per individual, which was then used in all analyses. In order to correct for potential body size differences between males and females, we measured the intertegula span of all individuals, which is considered an accurate estimator of body size in bees (Cane, 1987). Photographs of the dorsal thorax were taken at 6x magnification and distance measurements were made in ImageJ. To correct for body size, we divided all parameters by the intertegula span for all individuals. We performed non-parametric Mann-Whitney U tests to test for differences between males and females for each size-corrected measurement.

2.3 Brain preparation

Brains were fixed in 4% paraformaldehyde (in 0.1M phosphate-buffered saline (PBS); pH 7.2) for 3 days at 4 °C. Brains were dissected from the head capsule either before or after fixation. After fixation and dissection, brains were washed in PBS (3 x 10 min) and stained in 10% neutral red solution (Neutral Red Solution, Buffered; Sigma-Aldrich) for three hours at room temperature. Afterwards the brains were washed in PBS (4 x 10 min) and dehydrated in an ethanol series (50%, 70%, 90%, 95%, 3 x 100%, 10 min each). The dehydrated brains were then cleared in pure methyl salicylate (Sigma-Aldrich) at 4 °C for at least three days.

2.4 Confocal microscopy

Brains were mounted on custom aluminum slides with a central hole covered by coverslips on both sides. The mounts were imaged using a confocal laser-scanning microscope (LSM700; Carl Zeiss, Jena, Germany) with a solid-state 555 nm laser beam. Whole-mount brain scans were performed with a W N-Achromat 10x 0.3 NA objective and 2x2 tile-scanning at 3 μm intervals (2x line average), creating ~2,000 x 2,000 pixel stacks used for 3D reconstruction. ALs were scanned with a W Plan-Apochromat 20x/1.0 NA DIC objective at 1 μm intervals (2x line average), creating confocal stacks of 1,024 x 1,024 pixels.

2.5 3D reconstruction

We used the image stacks to perform 3D reconstructions of whole brains (N = 4 for each sex) and ALs (N = 5 for each sex). For whole brain reconstructions, six neuropils were reconstructed by segmentation and interpolation or wrapping in Amira 5.4.3 (FEI, Berlin, Germany; RRID: SCR_007353). These included three visual neuropils (medulla, lobula, and the anterior optic tubercle) as well as the AL, central complex, and MB (as single volume including the calyces, peduncle, and horizontal and vertical lobes). Paired structures (all but the central complex) were always both measured and the total volume was used in all analyses. The total volume of all selected neuropils was considered as an estimate of total brain volume. ALs were reconstructed based on the AL stacks by outlining all glomeruli in three planes (xy, xz, yz) and building 3D models of each glomerulus with the wrap feature in Amira. Absolute volumes of each neuropil were estimated using Amira. Relative volumes were computed by dividing the volume of each neuropil by the total brain volume for each individual. A non-parametric Mann-Whitney U-test was used to test for differences between volumes. Relative volumes were arcsine transformed before testing following Streinzer, Kelber, et al. (2013b).

2.6 Antennal lobe organization

To identify possible differences in AL organization, we compared the number and size-distribution of glomeruli between sexes. Non-parametric Mann-Whitney U tests were used to test for significant differences in glomeruli number and total glomerular volume, defined as the sum of all individual glomerular volumes. To identify potential macroglomeruli, we analyzed the size distribution of relative glomerular volumes for outliers in each sex. To test if conspicuous glomeruli represent outliers relative to the overall distribution of glomerular volumes, we performed the K statistic, a recently developed

| TABLE 1 | Male and female head morphology corrected for body size |
|----------|---------------------------------------------------------|
|          | Face width (FW) | Face length (FL) | Inter-eye span anterior (IEa) | Inter-eye span dorsal (IEd) | Eye length (EL) |
| Males    | 1.36 (±0.03)    | 0.91 (±0.04)    | 0.85 (±0.02) | 0.62 (±0.02) | 0.88 (±0.02) |
| Females  | 1.37 (±0.03)    | 0.94 (±0.04)    | 0.88 (±0.02) | 0.65 (±0.03) | 0.92 (±0.01) |
| p-value  | 0.7394          | 0.05243         | 0.01469      | 0.003886      | 1.08E-05      |
|          | Eye width anterior (EWA) | Eye width lateral (EWL) | Eye area | Central Ocellus diameter (OD1) | Lateral Ocellus diameter (OD2) |
| Males    | 0.40 (±0.02)    | 0.50 (±0.03)    | 1.79 (±0.05) | 0.09 (±0.004) | 0.08 (±0.007) |
| Females  | 0.41 (±0.02)    | 0.49 (±0.03)    | 1.65 (±0.05) | 0.1 (±0.006)  | 0.09 (±0.004) |
| p-value  | 0.2475          | 0.4359          | 0.007937      | 0.6305         | 0.1051         |

All values are corrected measurements for individual intertegula span ± SD of 10 individuals per sex with the exception of eye area, which is the mean of both eyes in five individuals per sex. Significant p-values under a Mann-Whitney-U test (p ≤ 0.05) are indicated in bold. Abbreviations correspond to measurements in Figure 1. See Supporting Information Table S1 for raw measurements.
nonparametric test that determines the relative distance of glomeruli from the main distribution between the 10th and 90th percentile (Kuebler et al., 2010; Streinzer, Kelber, et al., 2013) using the following formula:

\[ K = \frac{V_G - V_{10}}{V_{90} - V_G} \]

Where \( V_G \) is the volume of the focal glomerulus, \( V_{10} \) the 10th percentile, and \( V_{90} \) the 90th percentile of glomerular volume distribution. Following Roselino, Hrncir, da Cruz Landim, Giurfa, and Sandoz (2015), glomeruli with a \( K \) value between 1.5 and 3 were defined as mild outliers and glomeruli with a \( K \geq 3 \) were defined as extreme outliers. Only extreme outliers were classified as macroglomeruli. \( K \) values for conspicuous glomeruli were calculated for each individual. All statistical analyses were performed in base R (R Core Team, 2010).

3 | RESULTS

3.1 | Head morphology

Head morphology in \( E. \) dilemma males and females was similar to the naked eye (Figure 1). However, precise measurements revealed that eye area was increased in males compared to females (Table 1; Supporting Information Table S1). Furthermore, relative eye length (EL) and both anterior and dorsal inter-eye span (IEa and IEd) were larger in females than in males. While these measurements were statistically significant, the differences of the mean values for IEa, IEd, and EL were small (between 0.03 and 0.04 cm; Supporting Information Table S1), and should be interpreted with caution. All comparisons were corrected for body size, which indicates that female eyes have a tendency to be relatively longer while male eyes show a tendency to be wider and of increased area. All other head measurements showed no differences between sexes.

3.2 | Whole-brain reconstructions

In agreement with the similarity in head size observed between sexes, there were no differences in the estimated total volume of all neuropils between males and females (Males: \( 5.03 \times 10^8 \pm 3.1 \times 10^7 \) \( \mu \)m\(^3\), Females: \( 4.8 \times 10^8 \pm 7.0 \times 10^7 \) \( \mu \)m\(^3\), \( p = 0.7 \), Mann-Whitney-U test). Similarly, the different neuropils did not exhibit any absolute size differences (\( p > 0.2 \), Mann-Whitney-U test; Figure 2b), but the relative volume of the medulla was higher in males and the relative volume of the mushroom bodies was increased in females (\( p < 0.05 \), Mann-Whitney-U test; Figure 2c). The remaining neuropils exhibited similar relative volumes in both sexes (\( p \geq 0.05 \), Mann-Whitney-U test).

3.3 | Antennal lobes

Individual glomeruli were clearly discernible in all ALs allowing for complete 3D reconstructions. We found a significantly higher number of glomeruli in the female than in the male AL (females: 162 ± 1.6; males: 141.6 ± 2.3; Student’s \( t \)-test: \( t = -17.13, p < 0.001 \)). Close examination of the ALs in both sexes showed that this higher number in females originated from the existence of a female-specific cluster of 17 to 20 small glomeruli (mean: \( 18.2 \pm 1.1 \) glomeruli) on the caudo-dorsal side of the antennal nerve with slightly medial orientation.

FIGURE 1  Morphological differences between \( E. \) dilemma males and females. (a + b): Lateral view of pinned male (a) and female (b) individual. (c + d): Frontal view of male (c) and female (d) heads (note that the tongue is clipped here). Overall, head morphology is similar between sexes. (e + f): Lateral (e) and frontal (f) head measurements in red. Anterior eye width (EWa) indicated in orange was calculated as the difference between face width (FW) and anterior inter-eye width (IEa). Letters in (e + f) correspond to measurements listed in Table 1 [Color figure can be viewed at wileyonlinelibrary.com]
This cluster was absent in males. The number of glomeruli in the female brain was similar to the 169 functional odorant receptor genes encoded in the *E. dilemma* genome (Brand & Ramírez, 2017), thus further supporting the 1:1 rule of ORs to glomeruli in insects (Vosshall, Amrein, Morozov, Rzhetsky, & Axel, 1999; Vosshall et al., 2000).

The relative size distribution of individual glomerular volumes was generally similar between sexes with the exception of a peak at 5% relative volume in males, which was absent in females (Figure 4). This peak corresponded to one very large glomerulus that was found in all male ALs but not in females, located on the laterodorsal side of the AL (Figure 3). In addition to this glomerulus (G1), we identified a second conspicuously large glomerulus (G2), located adjacent to G1 on the ventral side (Figure 3). The glomeruli G1 and G2 had a mean relative size of 5.8% and 2.7%, respectively, relative to the total AL volume. Due to the larger size of these glomeruli we were not able to unambiguously identify putatively homologous glomeruli in the female AL. We used the K statistic in order to test if these glomeruli qualify as macroglomeruli. This analysis revealed that G1 was an extreme outlier (*K* = 4 ± 0.45) and thus qualifies as a macroglomerulus (i.e., MG1).

G2 on the other hand was only a mild outlier (*K* = 1.7 ± 0.25). None of the female glomeruli were outliers.

### 4 | DISCUSSION

#### 4.1 | Sex-specific investment in the visual system suggests increased visual processing capabilities in males

Our analyses of the *E. dilemma* head morphology revealed that eye size tends to be larger in males than in females. In accordance, the Medulla, a visual neuropil, showed a similar tendency to be enlarged in males. This is a common pattern in sexual dimorphism of Hymenopteran head and brain morphology and is usually related to a higher number and/or a larger size of the ommatidia in males (Narendra et al., 2011; Ribi et al., 1989; Streinzer, Brockmann, et al., 2013; Streinzer & Spaethe, 2014). The visual system in bees is involved in a multitude of behaviors including foraging, predator evasion, and mating. Both male and female orchid bees forage for floral resources such
as nectar and pollen, and thus are likely to have similar visual requirements to locate and discriminate floral resources. In addition, males and females are likely to be exposed to predators equally during scent collection and foraging. Thus, a higher investment in the male visual system is unlikely to be driven by foraging behavior alone. In contrast, male-specific behaviors, such as mating display, may underlie a greater investment in the visual system in males.

During perfume display, male bees establish territories around perching sites used for perfume release and mating (Kimsey, 1980; Stern, 1991). Between bouts of active perfume display, males engage in patrolling flights as well as in male-male jousting—fast flight chasing routines involving two or more males of the same species. The exact sequence and importance of specific movements during jousting remain unknown. Furthermore, it is unclear to what extent these behaviors are part of mating behavior or mate choice. However, it is possible that male-male interactions are important for establishing dominance over high-quality perching sites (i.e., territories) thereby facilitating mate discrimination and mate choice. Accordingly, it is plausible that a greater investment in the male visual system corresponds to male-specific display behaviors.

Fast flight and the ability to detect fast flying conspecifics is a part of mating behavior in many bee species, especially corbiculate bees,
which include honey bees, bumble bees, stingless bees, and orchid bees. In most bee species analyzed to date, a higher investment in visual neuropils has been detected in males (Roselino et al., 2015; Streinzer, Kelber, et al., 2013). Remarkably in corbiculate bees, males often rely on visual cues to spot and catch females during flight since mating can be airborne and often includes scrambling competition. (Eickwort & Ginsberg, 1980; Ruttn, 1956; Zmarlicki & Morse, 1963). Such behavior likely requires a specialized male visual system. However, orchid bees exhibit mating behaviors that are unique among bees. In order to mate, a female approaches a perching site from downwind and lands on the tree where she copulates with the displaying male (Pokorny et al., 2017; Stern, 1991; Zimmermann et al., 2006). Although vision likely plays an important role in orchid bee mating behavior, there is no indication of male scrambling competition or the need to catch a female in flight in order to mate. Therefore, in contrast to most bee lineages, it is more likely that the observed sexual dimorphism in the orchid bee visual system correlates with pre-mating competition rather than active mate detection. A higher investment in the visual system in male bees may underlie different aspects of male mating behavior across different bee lineages.

4.2 | Sexual dimorphism in the antennal lobe suggests sex-specific olfactory adaptations

Our whole-brain reconstructions revealed similar volumes of the antennal lobes between sexes, while the size of the mushroom body relative to the overall brain size was higher in females. While this supports previous findings on sex-specific differences in multimodal sensory integration and memory in Hymenoptera, it shows that investments in first-order olfactory processing neuropils are similar between sexes in *E. dilemma*, which is uncommon in ants and bees (Ehmer & Gronenberg, 2004; Kuebler et al., 2010; Roselino et al., 2015; Streinzer, Kelber, et al., 2013b).

The most profound difference between the male and female olfactory brain regions we analyzed was in the number and morphology of glomeruli in the ALs. Such differences are usually hypothesized to be related to the olfactory capabilities of each sex and are common in bees and other Hymenopterans (Arnold et al., 1985; Hansson & Anton, 2000; Roselino et al., 2015; Streinzer, Kelber, et al., 2013). On average, *E. dilemma* females had 21 additional glomeruli relative to males. An increased number of glomeruli in females is commonly observed in bees, although the differential can vary substantially (103 vs. 160 in the honey bee [−36%]; 159 vs. 200 in the stingless bee *Melipona scutellaris* [−21%]; 98 vs. 133 in the long horned bee *Eucera berlandi* [−27%]; Roselino et al., 2015; Streinzer, Kelber, et al., 2013). Interestingly, the difference between sexes in *E. dilemma* was the lowest measured for any bee species so far (141 vs. 162, −13%), which may indicate that male and female orchid bees have more similar olfactory capabilities compared to other bee lineages. Interestingly, the higher number of glomeruli in *E. dilemma* females was mainly due to the presence of a single cluster of ~18 small glomeruli that is entirely absent in males (Figures 3 and 4b). From its position in the AL and the relative smaller size of its glomeruli, this cluster is reminiscent of the T8 cluster found across Hymenoptera (see Couto et al., 2016 for homogenization of cluster names). T8, described in hornets (Couto et al., 2016; 2017), is thought to be homologous to the female-specific clusters T3b in the honey bee (Galizia, Sachse, Rappert, & Menzel, 1999; Kirschner et al., 2006; Kropf, Kelber, Bieringer, & Rössler, 2014) or T6 in ants (Kelber, Rössler, & Kleiniedadam, 2010; Mysore, Shyamala, & Rodrigues, 2010; Nakamichi, Nishino, Watanabe, Yokohari, & Nishikawa, 2009). The staining used in this study does not allow to unequivocally reconstruct glomerular innervation patterns, therefore tract numbers cannot be attributed in orchid bees at present. It has been hypothesized that the T8 cluster in Hymenoptera could be involved in the detection of social pheromones such as cuticular hydrocarbons (ants: McKenzy et al. 2016; hornets: Couto et al. 2017; but see d’Ettorre et al. 2017). Indeed, similar to its sister species *E. viridissima*, *E. dilemma* is facultatively eusocial (Ramirez pers. obs.; Pech, May-Itza, Medina Medina, & Quezada-Euan, 2008) and thus may use social pheromones to organize nest structure. Future neurophysiological analyses of this putative T8 cluster will reveal the role of this pathway in olfactory communication and its potential role in social behavior.

Besides the T8 cluster, male and female *E. dilemma* ALs exhibited similar numbers of glomeruli (141 vs. 144). This suggests that non-T8 based olfactory processing is similar in both sexes. In most bee species, males exhibit lower behavioral complexity relative to females, mainly concentrating on mating and nectar foraging behavior, the latter of which is even absent in some eusocial species such as honey bees (Gould & Gould, 1995; Michener, 2007). It has been hypothesized that this correlates with a reduction in neural olfactory processing capabilities, as well as a specialization in the detection of a few sex pheromone compounds in male bees (Ayasse, Paxton, & Tengö, 2001; Hansson & Anton, 2000). However, in orchid bees such a unisexual specialization on pheromone compounds is unlikely, since males and females are thought to interact with the perfume at some time in their life cycle. Furthermore, most compounds collected by orchid bee males are terpenes and aromatic compounds, which are also generally found in floral scents and resins that female orchid bees collect during foraging (Dodson et al., 1969; Eltz et al., 1999). Accordingly, the similarity in the number of glomeruli between sexes (aside from the T8) may represent usage of similar chemical information during foraging and mating behavior in males and females.

While males had fewer glomeruli in the ALs, our volume estimates detected two enlarged glomeruli, one of which qualified as a macroglomerulus according to the volume distribution. These glomeruli were male-specific, which suggest a male-specific olfactory specialization in *E. dilemma*. Macrogglomeruli generally represent specialization of the olfactory system in the detection of crucial chemical information such as major pheromone compounds (Arnold et al., 1985; Hansson, Christensen, & Hildebrand, 1991; Nishino, Iwasaki, Kamimura & Mizunami, 2012; Sandoz, 2006; Nishikawa et al., 2008). Macrogglomeruli are often found in the pheromone-detecting sex (usually males) and show neural activation following pheromone exposure (Hansson & Anton, 2000; Sandoz, 2006). It is possible that the male olfactory system in orchid bees is also tuned to the recognition of important compounds present in perfume mixtures. However, it is similarly possible that the macrogglomerulus is involved in the detection of other chemical signals such as unknown pheromones.
In conclusion, we found sex-specific differences in *E. dilemma* with regard to AL glomerular arrangement (i.e., Td) and the size of particular glomeruli. Interestingly, the glomerular numbers are more similar between sexes than expected from previous analyses of other bee species. These sex-specific specializations of the olfactory system might be linked to differences in social and perfume behavior between male and female orchid bees.

### 4.2.1 What are the neural determinants of perfume communication in orchid bees?

It has been hypothesized that the collection of a species-specific perfume requires a high olfactory memory capacity in male orchid bees (Eltz, Roubik, & Lunau, 2005). Perfumes are species-specific not only in quality but also relative concentration of specific compounds (Weber et al., 2016; Zimmermann et al., 2009). Thus, male bees need to regulate the collection of single compounds in relation to the other compounds present in the perfume mixture. Indeed, Eltz and colleagues (2005) showed that males selectively avoid perfume compounds they collected previously, which suggests that perfume collection is regulated by prior experience and presumably memory formation. Moreover, based on the large number of compounds present in *E. dilemma* perfumes (22 compounds on average, Ramírez et al., 2010) it is likely that orchid bee males have a high capacity for olfactory learning and memory, usually a typical female feature in circulate bees (Hammer & Menzel, 1995). However, we found that the mushroom body, a neuropil involved in the formation and retrieval of olfactory memory, had a smaller relative volume in males compared to females. It must be noted, however that the hymenopteran mushroom bodies integrate multiple sensory modalities (the visual input in particular is massive, Mobbs, 1982,1984; Groenenberg, 1986) and that they are also responsible for higher-order cognitive tasks (Devaud et al., 2015; Giurfa, 2003; Heisenberg, 1998,2003). It is thus difficult to directly relate total mushroom body volume to a particular behavioral trait. In orchid bees, both males and females possess well-developed mushroom bodies (~25% of the reconstructed neuropils).

Similarities between males and females may have been integral to the evolution of perfume communication in orchid bees. It has been hypothesized that male collecting behavior has evolved from female pollen collecting behavior through a “feminization” process of male orchid bees (Kimsey, 1984). Both, males and females use similar movements to collect and store these external resources. Since locomotion is controlled by the nervous system it is likely that neural structures underlying these behaviors are similar between sexes. Our neuroanatomical observations make sense in light of the feminization hypothesis if the capabilities required for olfactory learning are more female-like in orchid bees in comparison to other bee species.

### ACKNOWLEDGMENTS

We thank two anonymous reviewers for comments on the manuscript. We thank Nicholas Saleh for providing heads of *E. dilemma* females, and Douglas Rowland and Florian Bastin for help with Amira software. We further thank Thomas Eltz for helpful discussions of the results. This project was made possible through an ESEB Godfrey Hewitt mobility award and a Center of Population Biology research award to PB. SRR received support from the David and Lucile Packard Foundation and the National Science Foundation (DEB-1457753). JCS received support from CNRS and ANR (17-CE20-0003).

### REFERENCES

Abel, R., Rybak, J., & Menzel, R. (2001). Structure and response patterns of olfactory interneurons in the honeybee, *Apis mellifera*. *The Journal of Comparative Neurology*, 437, 363–383.

Andersson, M. B. (1994). Sexual selection. Princeton, New Jersey: Princeton University Press. [Database]

Arnold, G., Budharugsa, S., & Masson, C. (1988). Organization of the antennal lobe in the queen honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). *International Journal of Insect Morphology and Embryology*, 17, 185–195.

Arnold, G., Masson, C., & Budharugsa, S. (1985). Comparative study of the antennal lobes and their afferent pathway in the worker bee and the drone (*Apis mellifera*). *Cell and Tissue Research*, 242, 593–605.

Ayasse, M., Paxton, R. J., & Tengö, J. (2001). Mating behavior and chemical communication in the order Hymenoptera. *Annual Review of Entomology*, 46, 31–78.

Baumann, P. M., Oland, L. A., & Tolbert, L. P. (1996). Glial cells stabilize axonal protoglomeruli in the developing olfactory lobe of the moth *Manduca sexta*. *The Journal of Comparative Neuroscience*, 373, 118–128.

Brand, P., & Ramírez, S. R. (2017). The evolutionary dynamics of the odorant receptor gene family in corbiculate bees. *Genome Biology and Evolution*, 9, 2023–2036.

Brand, P., Ramírez, S. R., Leese, F., Quezada-Euan, J. J., Tollrian, R., & Eltz, T. (2015). Rapid evolution of chemosensory receptor genes in a pair of sibling species of orchid bees (Apidae: Euglossini). * BMC Evolutionary Biology*, 15, 176.

Cane, J. H. (1987). Estimation of bee size using intertergular span (Apoidea.). *Journal of the Kansas Entomological Society*, 60, 145–147.

Corto, A., Lapeyre, B., Thiéry, D., & Sandø, J. C. (2016). Olfactory pathway of the hornet *Vespa velutina*: New insights into the evolution of the hymenopteran antennal lobe. *The Journal of Comparative Neurology*, 524, 2335–2359.

Deisig, N., Kropf, J., Vitecek, S., Pevergne, D., Rouyar, A., Sandø, J. C., … Barrozo, R. (2012). Differential interactions of sex pheromone and plant odour in the olfactory pathway of a male moth. *PLoS One*, 7, e32159.

Devaud, J.-M., Papouin, T., Carcaud, J., Sandø, J. C., Grünewald, B., & Giurfa, M. (2015). Neuronal substrate for higher-order learning in an insect: Mushroom bodies are necessary for configurual discriminations. *Proceedings of the National Academy of Sciences*, 112, E5854–E5862.

Dodson, C. H. (1966). Ethology of some bees of the tribe Euglossini (Hymenoptera: Apidae.). *Journal of the Kansas Entomological Society*, 39, 607–629.

Dodson, C. H., Dressler, R. L., Hills, H. G., Adams, R. M., & Williams, N. H. (1969). Biologically active compounds in orchid fragrances. *Science (New York, N.Y.)*, 164, 1243–1249.

Dressler, R. L. (1982). Biology of the orchid bees (Euglossini). *Annual Review of Ecology and Systematics*, 13, 373–394.

Ehmer, B., & Groenenberg, W. (2004). Mushroom body volumes and visual interneurons in ants: Comparison between sexes and castes. *The Journal of Comparative Neurology*, 469, 198–213.

Eickwort, G. C., & Ginsberg, H. S. (1980). Foraging and mating-behavior in *Apoidea*. *Annual Review of Entomology*, 25, 421–446.

Eltz, T., Roubik, D. W., & Lunau, K. (2005). Experience-dependent choices in perfume communication in orchid bees. *Behavioral Ecology and Sociobiology*, 59, 149–156.

Eltz, T., Whitten, W. M., Roubik, D. W., & Linsemair, K. E. (1999). Fragrance collection, storage, and accumulation by individual male orchid bees. *Journal of Chemical Ecology*, 25, 157–176.

Fonta, C., Sun, X.-J., & Masson, C. (1993). Morphology and spatial distribution of bee antennal lobe interneurons responsive to odours. *Chemical Senses*, 18, 101–119.
Smith, J. M., & Harper, D. (2003). Animal signals. New York: Oxford University Press.
Stern, D. L. (1991). Male territoriality and alternative male-behaviors in the Euglossine bee, Eulaema meriana (Hymenoptera, Apidae). Journal of the Kansas Entomological Society, 64, 421–437.
Streinzer, M., Brockmann, A., Nagaraja, N., & Spaethe, J. (2013). Sex and caste-specific variation in compound eye morphology of five honeybee species. PLoS One, 8, e57702.
Streinzer, M., Kelber, C., Pfabigan, S., Kleineidam, C. J., & Spaethe, J. (2013b). Sexual dimorphism in the olfactory system of a solitary and a eusocial bee species. The Journal of Comparative Neurology, 521, 2742–2755.
Streinzer, M., & Spaethe, J. (2014). Functional morphology of the visual system and mating strategies in bumblebees (Hymenoptera, Apidae, Bombus). Zoological Journal of the Linnean Society, 170, 735–747.
Taylor, G. J., Ribi, W., Bech, M., Bodey, A. J., Rau, C., Steuwer, A., ... Baird, E. (2016). The dual function of orchid bee Ocelli as revealed by x-ray microtomography. Current Biology: CB, 26, 1319–1324.
Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., & Axel, R. (1999). A spatial map of olfactory receptor expression in the Drosophila antenna. Cell, 96, 725–736.
Vosshall, L. B., Wong, A. M., & Axel, R. (2000). An olfactory sensory map in the fly brain. Cell, 102, 147–159.
Watanabe, H., Nishino, H., Mizunami, M., & Yokohari, F. (2017). Two parallel olfactory pathways for processing general odors in a cockroach. Frontiers in Neural Circuits, 11, 549.
Weber, M. G., Mitko, L., Eltz, T., & Ramirez, S. R. (2016). Macroevolution of perfume signalling in orchid bees. Ecology Letters, 19, 1314–1323.
Wyatt, T. D. (2003). Pheromones and animal behaviour: Communication by smell and taste. Cambridge: Cambridge University Press.
Zhao, X.-C., Chen, Q. Y., Guo, P., Xie, G. Y., Tang, Q. B., Guo, X. R., & Berg, B. G. (2016). Glomerular identification in the antennal lobe of the male moth Helicoverpa armigera. The Journal of Comparative Neurology, 524, 2993–3013.
Zimmermann, Y., Ramirez, S. R., & Eltz, T. (2009). Chemical niche differentiation among sympatric species of orchid bees. Ecology, 90, 2994–3008.
Zimmermann, Y., Roubik, D. W., & Eltz, T. (2006). Species-specific attraction to pheromonal analogues in orchid bees. Behavioral Ecology and Sociobiology, 60, 833–843.
Zmarlicki, C., & Morse, R. A. (1963). Drone congregation areas. Journal of Apicultural Research, 2, 64–66.
Zube, C., Kleineidam, C. J., Kirschner, S., Neef, J., & Rössler, W. (2008). Organization of the olfactory pathway and odor processing in the antennal lobe of the ant Camponotus floridanus. The Journal of Comparative Neurology, 506, 425–441.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Brand P, Larcher V, Couto A, Sandoz J-C, Ramirez SR. Sexual dimorphism in visual and olfactory brain centers in the perfume-collecting orchid bee Euglossa dilemma (Hymenoptera, Apidae). J Comp Neurol. 2018:1–10. https://doi.org/10.1002/cne.24483