Antennal Proteome of the *Solenopsis invicta* (Hymenoptera: Formicidae): Caste Differences in Olfactory Receptors and Chemosensory Support Proteins

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Abstract

Little is known about the expression pattern of odorant and pheromone transporters, receptors, and deactivation enzymes in the antennae of ants carrying out different tasks. In order to begin filling in this information gap, we compared the proteomes of the antennae of workers and males of the red fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Male ants do not perform any colony work, and their only activity is to leave the nest on a mating flight. Previous studies showed that male ants express fewer types of odorant receptors than workers. Thus, we expected to find large differences between male and worker antennae for expression of receptors, transporters, and deactivators of signaling chemicals. We found that the abundance of receptors was consistent with the expected caste-specific signaling complexity, but the numbers of different antenna-specific transporters and deactivating enzymes in males and workers were similar. It is possible that some of these proteins have antenna-specific functions that are unrelated to chemosensory reception. Alternatively, the similar complexity could be a vestige of ant progenitors that had more behaviorally active males. As the reduced behavior of male ants evolved, the selection process may have favored a complex repertoire of transporters and deactivating enzymes alongside a limited repertoire of odorant receptors.

Key words: Solenopsis, proteomics, olfactory receptors, odorant degradation, male caste evolution

Graphical Abstract

The diversity of olfactory support proteins is about the same in worker and male fire ants, in contrast to the diversity of olfactory receptors, which is much higher in workers.
Social behavior in ant colonies depends, in part, on chemical signaling between individual colony members. The complexity of these interactions is mirrored by ant genomes, which code for large numbers of odorant receptors (ORs). For example, recent analyses (McKenzie et al. 2016, Cohanim et al. 2018) identified about 470 different OR sequences in the genome of the red fire ant, Solenopsis invicta Buren. The physiological process of chemical signal reception may also require, in the case of substances with low water-solubility, various hydrophobic ligand-binding proteins, such as odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). Some of these proteins have been proposed as transporters of signaling molecules to the ORs (Pelosi et al. 2014). In the S. invicta genome, 24 OBPs (Gotzek et al. 2011, Pracana et al. 2017) and 21 CSPs (Kulmini et al. 2013) have been identified. In addition to transporters and receptors, insect chemosensory reception requires enzymes that deactivate the semiochemicals (Kaisling 2009, Kaisling 2013). These enzymes have not often been studied at the genomic level in social insects, but there are 100 or more sequences in some insect genomes, of which an unknown number are involved in signaling molecule deactivation (Feyereisen 2012, Yonous et al. 2014). Little is known about the expression pattern of transporters, receptors, and deactivation enzymes in the antennae of ants carrying out different tasks, such as foraging, brood care, and reproduction. In order to begin filling in this information gap, we analyzed the proteomes of the antennae of S. invicta workers and males. Male ants do not perform any colony work, and their only activity is to leave the nest on a mating flight, after which they die. Previous studies showed that male ants are tuned to a narrower range of chemicals than females. For example, male ant antennae have fewer types of sensillae than workers (Renthal et al. 2003, McKenzie et al. 2016). Also, male ant antennae express fewer ORs than workers and their brain antennal lobes have fewer glomeruli (Zube and Rossler 2008, Nakanishi et al. 2010, McKenzie et al. 2016). Thus, we expected to find large differences between male and worker antennae for expression of receptors, transporters, and deactivators of signaling chemicals. As described in this paper, we found that the abundance of receptors showed the expected correlation with caste-specific signaling complexity, but the numbers of different antenna-specific transporters and deactivating enzymes in males and workers were similar. This similar complexity may be a vestige of ant progenitors that had more behaviorally active males. As the reduced behavior of male ants evolved, the selection process appears to have favored a complex repertoire of transporters and deactivating enzymes alongside a limited repertoire of ORs.

Materials and Methods

Collection of Ant Colonies
Polygynine S. invicta colonies were collected in Bexar County, Texas, using the floating method (Jouvenaz et al. 1977), and maintained in a controlled environment in plastic trays containing Petri dish nests. The ant colonies were fed with water, 10 % sucrose solution, and beef liver.

Dissection and Protein Extraction
Prior to dissection, the ants were anesthetized with CO2 and then cooled to 4°C for ~1 hr. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), antennae from workers (50 workers, 100 antennae), males (115 males, 230 antennae) and the tibiae from worker legs (13 workers, 78 tibiae) were dissected on a −20°C cold plate, and the appendages were held at 0°C until the extraction step. The proteins were extracted from pooled worker or male antennae or pooled worker tibia by grinding the tissue at 23°C in ~ 60 µl of 3% SDS solution using a 3 cm diameter ceramic mortar and a small ceramic pestle. The ant proteins were separated from insoluble material by centrifugation in a Sorvall MC 12V minicentrifuge at 10,000 rpm for 5 min. This extraction method probably extracts relatively more water-soluble and membrane proteins and fewer fibrous and cuticular proteins. One biological replicate was analyzed from each pool.

For blue native polyacrylamide gel electrophoresis analysis (BN–PAGE), the antennae of workers (103 workers, 206 antennae) or male antennae (400 antennae) and males (67 males, 134 antennae) were extracted by grinding at 23°C in ~80 µl of extraction solution consisting of 78 mM dodecyl maltoside, 49.8 mM Bis-tris, 16 mM HCl and 14% of 1X complete Mini protease inhibitor cocktail (Millipore Sigma, St. Louis, MO). The extract was centrifuged at 10,000 rpm for 5 min. In one experiment, the extracted proteins were concentrated using a 100 kDa Amicon Ultra centrifugal ultrafiltration unit (Millipore Sigma) at 14,000 rpm for 5 min to a volume of 13 µl. Two biological replicates of pooled worker antennae were analyzed, and one biological replicate of pooled male antennae was analyzed.

Electrophoresis
SDS-PAGE of the extracted proteins was performed using NuPAGE 4–12% acrylamide Bis-tris gels (ThermoFisher, Carlsbad, CA). BN-PAGE of the extracted proteins was performed using NativePAGE Novex 4–16% Bis-Tris gels (ThermoFisher). A native PAGE unstained protein standard (ThermoFisher) was used as soluble protein standard. A membrane protein standard was prepared by following the protocol of Wittig et al. (Wittig et al. 2010).

In-Gel Trypsin Digestion
For the subsequent mass spectrometry analysis, proteins were digested by trypsin using an in-gel protocol (Renthal et al. 2017). The SDS–PAGE gels, which had been run approximately 4 cm, were divided into four roughly equal segments. For the BN-PAGE gels, segments were cut to collect Orco-OR complexes in the molecular weight range expected for tetramers to octamers. In order to select regions of the native gels that would contain olfactory receptor tetramers, we calculated the average OR molecular weight as 44 kD for 111 sequences of S. invicta odor-specific olfactory receptors listed in NCBI above 35 kD (assuming that sequences of ORs with molecular weights less than 35 kD may be incomplete).

Mass Spectrometry
Tryptic peptides obtained from in-gel trypsin digestion of antennal and tibial protein extracts were analyzed by a nano-reverse phase liquid chromatography (LC) electrospray ionization tandem mass spectrometry (MS) system, consisting of an UltraMate 3000 Nano LC system and an LTQ-Orbitrap Elite mass spectrometer (ThermoFisher, San Jose, CA), using instrument settings similar to those described previously (Renthal et al. 2017). The mass spectra of the peptide sequences were compared with the protein sequences of S. invicta in the National Center for Biotecnology Information (NCBI) protein database (2018 data) by using Mascot software (version 2.6; Matrix Science, Boston, MA). Protein hits were counted if the Mascot ions scores were above the threshold of statistical significance for identity (P < 0.05) (Perkins et al. 1999). The peptide false discovery rate was 1%, determined using the same database with reversed sequences. For protein hits carrying headers in the NCBI protein database such as ‘uncharacterized’ or ‘unknown’, the ‘Features’ section of the NCBI data file was examined.
and often annotations that identified the proteins were found. In the absence of further annotation, uncharacterized sequences were subjected to BLAST searches against Arthropoda, and also Swiss-Model was used for three-dimensional homology searches. In many cases, these searches yielded tentative identifications of uncharacterized or unknown sequences.

Label-free quantification was done using the exponentially multiplied protein abundance index (emPAI). For secreted proteins (identified by SignalP software, cbs.dtu.dk) emPAI was calculated by the method of Ishihama et al. (Ishihama et al. 2005) as previously described (Renthal et al. 2017). For cytoplasmic proteins, emPAI was calculated by Mascot software. The emPAI values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In order to account for variability in the volumes of samples extracted from gel slices that were analyzed on the LTQ-Orbitrap Elite, the emPAI values were corrected for variations in the amount of trypsin autolysis products detected by mass spectrometry in each sample, assumed to be inversely proportional to relative sample volume. For each gel slice analyzed, emPAI was calculated for porcine trypsin. In addition, the relative amount of trypsin was calculated using the Top3 method of Silva et al. (Silva et al. 2006), in which the intensities of the three highest intensity ions are averaged. These intensities were obtained by converting the Thermo RAW files to mgf format, using MConvert software (ProteoWizard), and then collecting the precursor ion intensities of the trypsin autolysis MS² ions using custom software. The variation in trypsin between samples, measured by averaging the Top3 and emPAI values, was taken as a ratio to the section containing GAPDH, and this was used to correct the GAPDH-normalized emPAI values of each protein. The average sample volume error factor, calculated by this method, was 1.2 ± 0.4.

Cytochrome P450 sequences that were identified in S. invicta antennae and tibiae were compared with all S. invicta P450 sequences found in the NCBI database and with a published list of Nasonia vitripennis P450s (Oakeshott et al. 2010) by generating a maximum likelihood phylogenetic tree from ClustalO-aligned sequences using RAxML software (Stamatakis 2014) run on CIPRES 3.1. The tree output was displayed in CLC Sequence Viewer 8 software (Qiagen).

A gene ontology (GO) analysis was done using g:Profiler software (Raudvere et al. 2019), with a significance threshold of 0.05.

Results

Antennal and Tibial Proteomes

The worker antennal proteome was compared with the proteomes of male antennae and worker tibiae. We assume that proteins common to all three appendages are unlikely to be chemoreception-specific. Tibia are known to have chemosensory sensilla (Mitchell et al. 1999, Newland et al. 2000). However, the chemosensory functions of the tibia are probably limited to gustatory activity. Consistent with this, in S. invicta tibiae we could not detect any odorant receptor coreceptor (Orco) or sensory neuron membrane protein (SNMP), in contrast to antennae (see below). Proteins such as OBPs or P450s belong to families having some members with chemoreception-related functions and others with non-chemoreception functions. When members of these families were detected in both antennae and tibiae, we considered them less likely to be involved in chemoreception than proteins detected exclusively in the antennae.

A total of 2,949 proteins were identified by Mascot software as statistically significant (P < 0.05). Of these, 664 were found in both male and worker antennae and worker tibiae, and 690 were found in both worker and male antennae but not in tibiae. In addition, 74 proteins were in both worker antennae and tibiae, and 182 proteins were in both male antennae and worker tibiae. Worker antennae had 401 proteins not found in male antennae or worker tibiae, and male antennae had 724 proteins not found in worker antennae or worker tibiae. Worker tibiae had 214 proteins not found in worker or male antennae. A Venn diagram is shown in Fig. 1, and a list of the most abundant proteins identified is shown in Supp Table S1 (online only). Among the most abundant antennal proteins are hydrophobic ligand-binding proteins and hydrophobic ligand deactivation enzymes, which are likely to be involved in chemosensation, as discussed below.

Through GO analysis, the proteins identified in male antennae, worker antennae, and worker tibia, were associated with 202 GO terms for biological processes, of which 35 were in worker and male antennae but not in worker tibiae, 7 in worker tibiae but not male or worker antennae, 41 only in male antennae, 5 only in worker antennae, 10 in male antennae and worker tibiae but not in worker antennae, and 2 in worker antennae and worker tibiae but not in male antennae (Supp Table 2 [online only]). The largest group of over-represented GO terms in antennae compared with tibiae are related to actin or other fiber polymerization and/or depolymerization: 21 were GO terms in male antennae and 6 were GO terms in worker antennae, but none were in tibiae.

Olfaction-Related Hydrophobic Ligand-Binding/Transport Proteins

Odorant-Binding Proteins

Mass spectra matching 11 of the 24 known S. invicta OBPs (Gotzek et al. 2011, Pracana et al. 2017) were detected in antennae and tibiae (Table 1). Ten were detected in worker antennae. Six of the same proteins were detected in the male antennae, and four were detected in the tibiae. One additional OBP was detected only in the tibiae. The four tibial OBPs that were also found in antennae probably are not involved in chemoreception or are multi-functional proteins. The six worker and four male OBPs that are exclusively antennal potentially could be involved in odorant reception. Three of the OBPs detected in the antennae are expressed from genes on the social chromosome.

![Fig. 1. Venn diagram of identified proteins in S. invicta worker antenna (W), male antenna (M) and worker tibia (T).](https://academic.oup.com/jinsectscience/article/20/5/29/5937575/Downloaded-from-https://academic.oup.com/jinsectscience/article-lookup/2015/05/6357575)
In addition to OBPs and CSPs, three other protein families with potential ligand-binding/transport activity related to chemical signal detection were identified. We found ten tubular lipid-binding (TULIP) proteins (Kopec et al. 2011, Wong and Levine 2017) (Table 3), using either genome annotations (‘juvenile hormone-binding protein’ or ‘circadian clock-controlled protein’), or 3D homology modeling of sequences marked ‘uncharacterized protein’. Five different TULIP proteins were found in worker antennae, seven in male antennae, and two in worker tibiae. Of these proteins, three were uniquely expressed in worker antennae and four were uniquely expressed in male antennae. We also found three lipocalin sequences, identified either by annotation (‘apolipoprotein D’) or by 3D homology modeling (Pracana et al. 2017). The antennae and tibiae each had two different lipocalin sequences, but only the worker antennae expressed a sequence not marked ‘uncharacterized protein’. Five different TULIP proteins were found in worker antennae, seven in male antennae, and two in worker tibiae. Of these proteins, three were uniquely expressed in worker antennae and four were uniquely expressed in male antennae. We also found three lipocalin sequences, identified either by annotation (‘apolipoprotein D’) or by 3D homology modeling. The antennae and tibiae each had two different lipocalin sequences, but only the worker antennae expressed a sequence not found in tibiae. Finally, two Niemann–Pick C2 sequences were found, one expressed in all three appendages and one expressed only in worker antennae.

### Odorant/Pheromone Degrading Enzymes

Cytochrome P450

Twenty-nine different cytochrome P450 sequences were detected in extracts from worker antennae, and 23 P450 sequences were detected in male antennae (Table 4). Twenty-five of these P450 sequences were antennal and were not found in worker tibiae: 10 exclusively in worker antennae, five exclusively in male antennae, and 10 in both. These 25 P450s are likely to include enzymes involved in deactivation of odorant and pheromone molecules. In addition, 10 P450s were found in both antennae and tibiae, and 6 were found exclusively in tibiae. These 16 P450s are likely to include enzymes involved in metabolic reactions unrelated to olfaction. Of the 10 most abundant P450s in worker antennae, six were also found in tibia; and of the 10 most abundant P450s in male antennae, seven were also found in tibiae. Thus, it appears that generally, the P450s involved in chemosensory deactivation are expressed at lower levels than metabolic P450s.

Four sequences were detected that correspond to genes coding for tandem P450 sequences (i.e., two coding sequences connected by a short length of nucleotides lacking a stop codon and a new start codon).
Two of the proteins were found in both worker and male antennae but not in tibiae, and two were found in tibiae but not antennae. The most abundantly expressed tandem P450, XP_025986982.1, was found in antennae. For this protein in workers, 13 out of 15 tryptic peptides detected by mass spectrometry were in the N-terminal half of the sequence, and in males 9 of 12 detected peptides were in the N-terminal half. This strongly suggests that the two halves correspond to separate proteins. Between the two halves, there may be some missing genomic nucleotides that contain termination and start codons, or there could be transcriptional, post-transcriptional or post-translational processing that results in separate proteins. For the other three tandem P450s, the number of detected peptides was too small to draw conclusions about whether the halves were separate.

### Other Oxidases, Esterases, and Transferases

Other oxidases, esterases, and transferases were detected that may be involved in odorant or pheromone deactivation. Worker antennae had 22 of these enzymes (Table 5), of which 14 were also present in male antennae and 11 in worker tibiae. Male antennae had three additional enzymes in this group that were not in worker antennae, two of which were also present in worker tibiae, and there were nine of the enzymes detected only in worker tibiae. Twelve of the deactivation enzymes were detected only in antennae, including five that were found in both worker and male antennae, one found only in male antennae, and six that were found only in worker antennae. The antenna-specific enzymes included glutathione S-transferases, UDP-glucuronosyltransferases, retinol dehydrogenase, carboxylesterases, and xanthine dehydrogenase. The sequences in Table 5 annotated ‘xanthine dehydrogenase’ are probably aldehyde reductases, because they lack the conserved tyrosine at the NAD+ binding site in xanthine dehydrogenase (Merlin et al. 2005). The sequences annotated ‘retinol dehydrogenase’ are probably short-chain dehydrogenases, and they may perform functions similar to the previously described antennal aldehyde reductases (Guo et al. 2014).

### Olfactory Receptors

No odor-specific olfactory receptors (ORs), ionotropic glutamate-type olfactory receptors (IRs), or gustatory receptors (GRs) were detected in the antennae, presumably because the expression levels of these receptors are below the mass spectrometer detection threshold. In the case of ORs, the receptors are heteromers in which each odor-specific receptor subunit is paired 1:1 with a common co-receptor subunit known as Orco (Larsson et al. 2004, Vosshall and Hansson 2011, Butterwick et al. 2018). Thus, the amount of Orco should equal the total amount of all odor-specific receptors expressed in the antenna, and it can be used as a proxy for the OR
Orco was detected in worker and male antennae, but not in tibiae. The ratio of the abundance of Orco in the worker antenna to the male antenna was approximately 5:1 (Table 6). Two IR subunits (named after *Drosophila melanogaster* IR8a and IR25a) have been described as having coreceptor-like function when combined with other IRs (Aubin et al. 2011), and sequences corresponding to these are known in S. invicta (XP_025995684.1 and XP_011156270.1; both are annotated as IR25a-like, but XP_025995684.1 appears to be the IR8a-like sequence). However, the total number of annotated IR genes in S. invicta is low: fewer than 40 IRs, compared with more than 200 annotated ORs. At this low level, even the proxy coreceptors are not detectable by mass spectrometry. Similarly, fewer than 30 *S. invicta* GR sequences have been annotated, and GRs are not known to have a coreceptor.

Another possible olfactory receptor subunit is sensory neuron membrane protein (SNMP) (Jin et al. 2008, Vogt et al. 2009, German et al. 2013, Gomez-Diaz et al. 2016). SNMP1 was detected in both worker and male antennae, but not in tibiae. The worker:male ratio in antennae was approximately 1:1 (Table 6). Because of the much larger amount of Orco in workers, the SNMP:Orco ratios were about 2:1 in workers and 10:1 in males (Table 6). This difference may be related to the involvement of SNMP in setting the olfactory signal response rate (Discussion).

In order to identify possible olfactory receptor complexes, we also analyzed dodecyl maltoside extracts of worker antennae by blue native gel electrophoresis. The extraction conditions were selected to preserve native oligomeric complexes of integral membrane proteins (Wittig and Schagger 2009, Wittig et al. 2010). We analyzed proteins found in two regions of the electrophoresis gels: approximately 200–350 kD and 400–600 kD. The lower molecular weight region would contain heteromers of Orco and OR subunits (“tetramers”), containing two subunits of each (Orco2OR2). The higher molecular weight region would contain higher molecular weight heteromers.

### Table 5. Other putative odorant/pheromone-degrading enzymes

| NCBI accession number | Enzyme                        | Worker antenna<sup>a</sup> | Male antenna<sup>b</sup> | Worker tibia<sup>b</sup> |
|-----------------------|-------------------------------|----------------------------|--------------------------|--------------------------|
| XP_011165644.1        | GST                           | 0.74                       | 0.39                     | 0.49                     |
| XP_025993326.1        | xanthine dehyd.<sup>d</sup>   | 0.63                       | 0.38                     | 0.017                    |
| XP_025992003.1        | GST                           | 0.57                       | 0.45                     | 0.055                    |
| XP_025996377.1        | GST 1-like                    | 0.55                       | 0.0                      | 0.069                    |
| XP_011158662.1        | GST 1                         | 0.29                       | 0.63                     | 0.21                     |
| XP_011165884.1        | xanthine dehyd. 1-like        | 0.36                       | 0.29                     | 0.017                    |
| XP_025989394.1        | UDP-GT<sup>e</sup> 2B31-like  | 0.40                       | 0.15                     | 0                        |
| XP_011159399.1        | retinol dehyd.<sup>f</sup> 12 | 0.25                       | 0.53                     | 0.10                     |
| XP_011159136.1        | GST theta-3 X1                | 0.20                       | 0.18                     | 0.075                    |
| ABA39530.1            | GST                           | 1.8                        | 0.98                     | 0.94                     |
| XP_025987486.1        | UDP-GT1-9                     | 0.25                       | 0.063                    | 0.062                    |
| XP_025990737.1        | UDP-GT 2B1-like X2            | 0.24                       | 0.11                     | 0                        |
| XP_025987060.1        | retinol dehyd. 12             | 0.16                       | 0.0                      | 0                        |
| XP_025995400.1        | UDP-GT 2B20                   | 0.20                       | 0.031                    | 0.024<sup>e</sup>        |
| XP_011160692.1        | esterase FE4 X2               | 0.16                       | 0.022                    | 0                        |
| XP_025995399.1        | UDP-GT 2C1-like               | 0.14<sup>f</sup>           | 0                        | 0                        |
| XP_025994825.1        | JH<sup>g</sup> esterase-like  | 0.097                      | 0                        | 0                        |
| XP_025991890.1        | UDP-GT 2B31                   | 0.11                       | 0                        | 0                        |
| XP_011163179.1        | esterase E4 X1                | 0.11                       | 0                        | 0                        |
| XP_011168182.1        | GST 1 X2                      | 0.052<sup>f</sup>          | 0.39                     | 0                        |
| XP_025989254.1        | xanthine dehyd. X1            | 0.0068<sup>f</sup>         | 0.0043                   | 0                        |
| XP_011164602.1        | esterase FE4-like             | 0.27                       | 0.10                     | 0.018<sup>e</sup>        |
| XP_011165643.1        | GST-like                      | 0.15                       | 0                        | 0                        |
| XP_011162023.1        | carboxylesterase-6            | 0.27                       | 0.13                     | 0                        |
| XP_025990047.1        | retinol dehyd. 1              | 0.27                       | 0.075                    | 0                        |
| XP_011165650.1        | GST-like                      | 0.0                       | 0                        | 0.045                    |
| XP_011162756.1        | retinol dehyd. 11             | 0.0                       | 0                        | 0.038                    |
| XP_011168216.2        | UDP-GT 2B31                   | 0.0                       | 0                        | 0.024<sup>e</sup>        |
| XP_011165649.2        | GST                           | 0.0                       | 0                        | 0.022<sup>e</sup>        |
| XP_011164603.1        | retinol dehyd. 14             | 0.0                       | 0                        | 0.022<sup>e</sup>        |
| XP_011170697.1        | retinol dehyd. 13             | 0.0                       | 0                        | 0.021<sup>e</sup>        |
| XP_011171057.1        | UDP-GT 2B31                   | 0.0                       | 0                        | 0.021<sup>e</sup>        |

<sup>a</sup>Assigned from annotation.

<sup>b</sup>Quantification from exponentially multiplied protein abundance index (emPAI), calculated from mass spectrometry data by Mascot and normalized to the emPAI values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

<sup>c</sup>GST, glutathione-S-transferase.

<sup>d</sup>Xanthine dehydrogenase.

<sup>e</sup>UDP-GT, UDP-glucuronosyltransferase.

<sup>f</sup>Retinol dehydrogenase.

<sup>g</sup>Only one peptide detected.

<sup>h</sup>JH, juvenile hormone.
regions, the relative amounts of SNMP increase in the concentrated protein samples, suggesting concentration-dependent aggregation of SNMP monomers or dimers into tetramers. We also detected some olfactory-related binding proteins and deactivation enzymes in the native gel segments corresponding to the Orco/OR ‘tetramers’ and ‘octamers’. Twenty P450 sequences were detected in these regions of the native gels. Of these proteins, 8 were sequenced and 2 were also found in sodium dodecyl sulfate (SDS) extracts of antennae but not tibiae. Only two were present in both the lower and higher molecular weight gel segments of both the concentrated and dilute samples: XP_011164432.1 and XP_025986987.1. The ratios to Orco, shown in Table 6, indicate that both are present at concentrations comparable to or higher than Orco. Whether they form complexes with Orco/OR cannot be determined from these data, in part because P450s can form homomeric complexes in the same molecular weight ranges as the gel segments we analyzed (Reed and Backes 2017). We also detected CSP19 (AAV91325.1) in both the low and high molecular weight regions of the dilute samples and in the low molecular weight region of the concentrated sample. The amounts detected were too low for meaningful calculations of stoichiometry. Although we previously found that CSP19 self-associates to form dimers (Gonzalez et al. 2009), it is very unlikely that this small protein could self-associate to form aggregates having molecular weights above 200 kD. Therefore, its presence in the 200–600 kD region of the native gels suggests the possible formation of strong associations with olfactory receptors or some other protein complex.

### Discussion

Chemical signaling is a significant communication channel in the social organization of ant colonies. Olfactory receptors and contact chemoreceptors are supported by a variety of chemical transporters and deactivation enzymes in chemosensory tissues. Transporters such as odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) and deactivation enzymes such as cytochrome P450s belong to large gene families involved in both chemosensory processes and the regulation of chemical signals.

#### Table 6. Orco and Orco complexes

| Whole antenna | Orco<sup>a</sup> | W/M<sup>a</sup> ratio | SNMP<sup>b</sup> | W/M<sup>b</sup> ratio | SNP/Orcoratio |
|---------------|-----------------|------------------|-----------------|------------------|----------------|
| Worker, total | 3.1             | 4.8              | 5.0             | 0.78             | 1.6            |
| Male, total   | 0.64            | 4.8              | 6.4             | 10               |                |

| Native gel sections | Orco<sup>a</sup> | Hi/Lo<sup>c</sup> ratio | SNMP<sup>b</sup> | Hi/Lo<sup>c</sup> ratio | SNP/Orcoratio | P450<sup>d</sup> | P450/orcoratio |
|---------------------|-----------------|------------------|-----------------|------------------|----------------|----------------|----------------|
| 0.4–0.6 MDa dilute  | 0.40            | 1.3              | 0.91            | 2.3              | 1.4<sup>h</sup> | 1.4<sup>h</sup> | 3.5            |
| 0.2–0.35 MDa, dilute| 0.03            | 1.3              | 0.40            | 1.3              | 1.3<sup>h</sup> | 1.3<sup>h</sup> | 0.95           |
| 0.4–0.6 MDa, conc. | 0.51            | 1.2              | 1.6             | 3.2              | 1.8<sup>h</sup> | 1.8<sup>h</sup> | 3.5            |
| 0.2–0.35 MDa, conc. | 0.44            | 1.2              | 1.3             | 1.2              | 3.0            | 0.37<sup>h</sup> | 0.83           |

<sup>a</sup>Quantification from exponentially multiplied protein abundance index (emPAI), calculated from mass spectrometry data (Ishihama et al. 2005), normalized to the emPAI values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

<sup>b</sup>Quantification from emPAI, normalized to trypsin autolysis peptides.

<sup>c</sup>Ratio of Orco in workers to males.

<sup>d</sup>Ratio of Orco in 0.4–0.6 MDa gel section to 0.2–0.35 MDa gel section.

<sup>e</sup>Ratio of SNMP1 in workers to SNMP1 in males.

<sup>f</sup>Ratio of SNMP1 in 0.4–0.6 MDa gel section to 0.2–0.35 MDa gel section.

<sup>g</sup>Quantification from emPAI calculated from mass spectrometry data by Mascot, normalized to trypsin autolysis peptides.

<sup>h</sup>Data for XP_011164432.1.

<sup>i</sup>Data for XP_025986987.1.
and non-chemosensory activities. This complicates the study of chemosensory support protein function. We speculated that by comparing the antennal proteomes of fire ant (S. invicta) workers and males with the proteome of the tibia, a non-chemosensory appendage, that we would be able to identify probable non-chemosensory support proteins, thereby indicating which support proteins are likely to be essential for odorant transport and deactivation.

**GO Analysis**

Antennae were found to have many proteins classified with Biological Process GO terms related to actin polymerization/depolymerization, but these GO terms were not found in tibiae (Supp Table 2 [online only]). More GO terms related to actin polymerization/depolymerization were found in male antennae than in worker antennae. This difference may be related to the differences in sensilla morphology in males and workers. Males have many more sensillae on their antennae than workers, and they are mostly sensilla tricoda curvata (Renthal et al. 2003). One interpretation of the enrichment of GO terms relating to actin polymerization/depolymerization, but these GO terms were not found in tibiae (Supp Table 2 [online only]).

**Transport Proteins**

We found 16 antenna-specific transport proteins in workers and 13 in males (Tables 1–3). Although antennal OBPs have been assumed to be involved in olfaction, recent experiments indicate that many antennal OBPs are not necessary for odorant reception (Larter et al. 2016, Xiao et al. 2019). Also, one antennal OBP was recently suggested to be involved in carrying odorants away from receptors for deactivation (Scheuermann and Smith 2019). One protein we detected in both antennae and tibiae, OBP3 (also known as Gp-9), is present in hemolymph (Krieger and Ross 2002), so it is not possible from our whole-antenna analysis to know whether Gp-9 is also expressed in sensillar lymph. There is evidence that Gp-9 has a role in chemochemical reception. Gp-9 has two different alleles (Gp-9B and Gp-9b), and heterozygous workers can detect a transferable substance on the cuticle of homozygous Gp-9B queens (Keller and Ross 1998). We found Gp-9B at very high levels in tibiae, and also it was the most abundant OBP in antennae. Gp-9B was detected at very low levels in worker antennae (less than 1% of Gp-9B), and it was not detected in male antennae or tibiae. This is consistent with the very wide range of worker Gp-9b allele found in different polygynous colonies (Gotzek and Ross 2008, Leal and Ishida 2008). According to a detailed genotype analysis, the Gp-9b allele is frequently carried by male fire ants (Fritz et al. 2006). However, Gp-9 expression in males was reported to be 12-fold lower than workers (Liu and Zhang 2004), which is close to the 11-fold ratio for Gp-9b we found (Table 1). If Gp-9b were present at this level in male antennae from our colonies, it would not be detectable by mass spectrometry.

We detected venom proteins in the antennal and tibial proteomes. The ratios of the Sol 1 2 and Sol i 4 isoforms differ in male antennae and tibiae compared with venom sacs (Supp Table S2 [online only]). This raises the possibility that some of the venom proteins are expressed in these appendages as well as the venom sac. The three-dimensional structure of venom allergen 2 (Sol i 2) resembles OBPs (Borer et al. 2012). Sol i 4 has a similar sequence to Sol i 2, so it probably also has an OBP-like structure. It was suggested that these proteins may be involved in pheromone delivery (Borer et al. 2012, Das et al. 2018). Thus, it is possible that the Sol i 2 minor isoform and Sol i 4.02 are used as OBPs in chemochemical reception by male fire ants. Also, Sol i 4.01 could be involved in secretion from worker tibial glands (Billen 2009). Alternatively, the amounts of these minor forms expressed in venom sacs may be variable between colonies: the antennae, tibia, and venom sacs in the Supp Table S2 (online only) data were obtained from different colonies.

CSP19 previously was called SinvCSP (Lee and Ishida 2008) or SICSP1 (Gonzalez et al. 2009), and it was identified as the most abundant chemo sensory protein in the S. invicta antenna. Surprisingly, two other highly abundant antennal CSPs (CSP 7 and CSP16) were not detected in the earlier analyses. Although it is possible that these proteins were absent because of the variability between different colonies, the difference is more likely due to the methods used in the previous work. Mascot software could not have identified CSP7 in 2008 because its sequence was not in the NCBI database at that time. CSP7 and CSP16 probably could not have been detected by direct Edman sequencing without pyroglutamase treatment, because both have N-terminal glutamine, which likely would have cyclized into a blocked N-terminal pyroglutamate. We previously speculated that CSP19 carries nestmate recognition signals, by analogy with the major CSP in Camponotus japonicus antennae (Ozaki et al. 2005). However, we found that recombinant CSP19 does not bind to cuticular hydrocarbons (Gonzalez et al. 2009), raising the question of whether S. invicta might use signals other than cuticular hydrocarbons for nestmate recognition. Now that we have found that the S. invicta antenna contains CSP7, a close sequence homolog of the Camponotus japonicus cuticular hydrocarbon-binding CSP, the question of the S. invicta nestmate recognition signals should be further examined.

**Deactivation Enzymes**

We found 20 antenna-specific cytochrome P450 proteins in workers and 16 in males, suggesting a possible widespread role for P450s in fire ant olfaction (Table 4). Studies of specific odorant and pheromone deactivation enzymes previously showed the involvement of P450s (Wojtasek and Leal 1999, Maibeche-Coisne et al. 2004). However, a transcriptome analysis of 57 D. melanogaster P450s found that nearly all were expressed in other tissues (Younus et al. 2014), so it is not clear how many of the S. invicta P450s that are in antennae but not in tibiae are olfaction-related. For example, we detected AAQ90477.1 at low levels in worker and male antennae but not in tibiae, yet mRNA coding for this P450 (CYP4AB2) was previously found at very high levels in mRNA extracts of whole workers (Liu and Zhang 2004).

Previous studies of insect P450s showed large phylogenetic expansions of species-specific P450s (Feyereisen 2012). Thus, we examined a phylogenetic tree of hymenoptera P450 sequences from S. invicta compared with P450 sequences of the non-social hymenopteran, Nasonia vitripennis (Oakshott et al. 2010), to detect P450 sequences that may have expanded specifically to deactivate chemical signals related to social interactions. In a combined cladogram of 125 S. invicta P450s and 87 N. vitripennis P450s, we marked the locations of S. invicta P450s that were antennal and tibial proteins from Table 4 (Supp Fig. S1 [online only]). The sequences of mitochondrial and CYP clan 2 P450s showed mixing of S. invicta and N. vitripennis sequences within branches. In CYP clan 3, the 9e2 type proteins showed several separate S. invicta and N. vitripennis expansions, each containing 7 to 10 sequences. Within the S. invicta 9e2 expansions, eight of nine detected sequences were exclusively antennal, suggesting that these P450s might be used by fire ants to deactivate socially relevant chemical signals. In contrast, the 6k1 type proteins of CYP clan 3 show S. invicta and N. vitripennis branch mixing in the cladogram. Although all 5 of the detected sequences
We detected Orco on native gels at molecular weights between 200 and 350 kD (Table 6). This is consistent with a tetrameric structure for the olfactory receptor, as proposed by Butterwick et al. (Butterwick et al. 2018). Although the cryoelectron microscopy structure, cross-linking, and mutagenesis results of Butterwick et al. point to Orco as the active form of the receptor, the native gel data they reported appears to show an octamer, Orco, in reference to water-soluble calibration standards, but a tetramer in reference to one membrane protein standard. Our native gels were calibrated with four bovine mitochondrial membrane protein complexes, which were shown to give accurate molecular weights for integral membrane protein complexes. We also detected Orco between 400 and 600 kD, which is consistent with either an octamer (Orco-OR) or a complex between Orco/OR tetramers and additional subunits, such as SNMP.

A D. melanogaster Orco interactome was reported by Yu et al. (Yu et al. 2018), based on a pull-down method. No SNMP was detected, but several cytochrome P450s were identified. We found numerous P450s migrating in native gels in the same molecular weight range as Orco. However, it is not clear whether these P450s form complexes with olfactory receptors or are self-associating. The D. melanogaster Orco interactome also contained one OBP (Yu et al. 2018). We did not detect any OBPs in the same native gel regions as Orco, but we did find CSP19 (Table 6), indicating the possibility of a complex between it and ORs.

Receptor and Support Protein Diversity
The ratio of Orco in worker antennae to male antennae is a measure of the relative numbers of olfactory receptors of the two castes. Using a label-free proteomics method, we estimate the ratio to be 5:1 (Table 6). This is in accord with what is known about the relative complexity of colony interactions of ant workers and males (Boomsma et al. 2005, Shik et al. 2013). The diversity of ORs does not necessarily parallel the amount of Orco, which may be skewed...
in one caste compared with another if the same odor-specific OR gene products are expressed at much higher levels in one caste, or if one caste has a higher level of self-associated Orco. The number of antennal lobe glomeruli is generally considered to be a good estimate for the number of different OR genes expressed. Typical social insect workers are known to have many more glomeruli than males (Arnold et al. 1985, Zube and Rossler 2008, Nakanishi et al. 2010, McKenzie et al. 2016), in contrast to non-social hymenoptera, where the numbers of antennal lobe glomeruli in males and females are about equal (Das and Fadamiro 2013). Considering the high worker-to-male Orco ratio that we measured, and the high worker-to-male antennal lobe glomeruli ratios previously measured in ants, it is surprising that the diversity ratio is so much lower for antenna-specific olfactory binding/transport proteins (Tables 1–3) and deactivation enzymes (Tables 4–5). The ratio of the number of different antennal-specific binding/transport/deactivation proteins in Tables 1–5 for workers compared with males is about 1:1. It is possible that this ratio is distorted by a classification artifact: many of the proteins we have classified as chemosensory support proteins might actually have antenna-specific functions that are unrelated to chemosensory reception. However, it is not clear why there would be more of these in males than workers. A second possible artifact could result from the limited lower range sensitivity of proteome detection by mass spectrometry. For example, workers might express low levels of many more proteins than we detected, or males might express many more non-OR odorant receptors (e.g., in the IR family) than we detected. Neither of these possibilities seems promising, considering the known limited number of additional IRs, OBPs, CSPs, and P450s that have been identified so far in the S. invicta genome. There is evidence supporting the similarity of worker and male OBPs expression down to low levels. A study of fire ant antennal OBP mRNA (Zhang et al. 2016) shows a similar pattern to our proteomics data in Table 1 for high-level transcripts, but no gap between workers and males appears in the low-level OBP transcripts, which are at about the same levels.

The molecular basis of OR specificity is not yet known, but physiological and behavioral studies show that ORs have a narrower selectivity than binding/transport proteins and deactivation enzymes (Wojtasek et al. 1998, Lautenschlager et al. 2007, Carey et al. 2010, Xu et al. 2012, Katti et al. 2013, Younus et al. 2014). Thus, hundreds of different ORs, each having high specificity for a particular chemical substance, can be supported by many-fold fewer binding/transport proteins and deactivation enzymes, which typically have a wider range of affinity for groups of chemically similar compounds, such as a homologous series of a particular organic functional group. Expression of more copies of a protein per mRNA has a lower fitness cost (Frumkin et al. 2017), so this might seem to favor the evolution of genes for broad-spectrum binding/transport proteins and deactivation enzymes. Presumably, as the worker caste evolved, much of the OR complexity was added to support the needs of social interactions. By the same reasoning, the evolution of the male ant caste might be expected to have decreased OR complexity, compared with the wasp-like progenitors of ants. The chemical detection capabilities needed by males in the wasp-like progenitors of ants are unknown, but they may have had behavioral traits similar to solitary or primatively social wasps. In some solitary wasps, male and female tasks overlap in work such as nest building and guarding (Brockmann 1992). For some primatively social wasps, both males and workers have similar needs to assess the quality of female egg-laying fitness: workers in selecting a queen to follow (West-Eberhard 1978), and males for their mating choice. Thus, similar ranges of olfactory receptor diversity may have been required in both males and females in ant progenitors. The chemicals constituting these signals probably covered a relatively wide range of organic functional groups, and this underlying diversity of the chemical repertoire thus would be retained in the

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![Fig. 3. Model for comparing of OR and olfactory support protein diversity. Each olfactory support protein can transport or deactivate groups of substances that belong to a homologous series of organic compounds. In contrast, ORs have much higher specificity, each binding to a very narrow range of substances. The total numbers of different ORs expressed in wasp-like ant progenitors, male ants and male workers are indicated, respectively, as Σp, Σm, and Σw. The total numbers of expressed olfactory support proteins are indicated, respectively, as Σi, Σj, and Σk. Thus, for the full set of chemicals detected by a species or caste, Σp > Σi, Σm > Σj, and Σw > Σk. Due to the reduced chemical signal reception needs of male ants, Σw > Σm. Surprisingly, we found that Σj = Σk. This may reflect the evolutionary origin of the male caste from wasp-like progenitors that performed similar work to females in nest-building, guarding, and queen assessment. Chemical signals with diverse organic functional groups may have been retained for signaling of male ant mating flights and male assessment of female alates, balancing higher protein expression costs against perhaps even higher costs required for evolution of an entirely new signaling scheme having a reduced repertoire of olfactory support proteins, Σj < Σk.](image-url)
expanded signals used by ant colonies as they evolved to higher levels of complexity, where the male caste does no colony work (Fig. 3). Apparently, it was less costly for male ants to retain and express ORs and olfactory support proteins encompassing a diverse set of chemical specificities than for ant colonies to evolve an entirely new chemical coding system having fewer organic functional groups to represent the limited information required by non-working male ants. We expect that the disparity between the diversity of male ant ORs and chemosensory support proteins is more extreme in S. invicta, which uses an aerial swarm mating system, than in ants that use a female-calling mating system (Boomsma et al. 2005), which may require more diverse male ORs.

Data Availability
Thermo raw mass spectra files and Mascot generic format peak lists are publically available in the MassIVE Repository: ftp://massive.ucsd.edu/MSV000085997/.

Supplementary Data
Supplementary data are available at Journal of Insect Science online.

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