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Caste- and environment-associated differential expression of olfactory receptors in pest ants

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As human society continues to grow and evolve, so does the need for effective pest management strategies. Olfactory-mediated control methods, such as attractant and repellent compounds, are a proposed strategy for mitigating the damaging effects of some insect pests, most notably ants, that rely on olfaction for communication. To develop such compounds, it is first important to comprehensively understand the target species' olfactory transcriptome in order to guide future targeted functional characterization of relevant olfactory proteins. Here, we perform bulk RNA-seq analysis of antennae from three notable pest ant species, *Camponotus floridanus*, *Atta sexdens*, and *Atta cephalotes*. Specifically, we highlight the expression profiles of *olfactory receptor* genes, as they may serve as potential targets of future industry research and application. We find that the ant antennal transcriptome differs between each species' castes, potentially reflecting varying behaviors and tasks, and also appears to be influenced by the surrounding environment. Our findings suggest a general up-regulation of *olfactory receptor* genes amongst foraging castes, also demonstrating that, when comparing foraging ants from differing environments, olfactory-related genes exhibit considerable patterns of differential expression. These findings suggest variable olfactory sensitivity depending on the aforementioned factors, warranting further investigation into whether differing caste and environmental conditions may negatively influence the effectiveness of broad-range olfactory-mediated pest management strategies. Development of pest management tools that target specific groups of insects by environment or caste may lead to more effective control.

Keywords Olfactory receptor, Antenna, Transcriptome, Ant caste, Environmental plasticity, Pest management

Pest ants present a persistent challenge to human societies around the world, consistently impacting agriculture and infrastructure and thereby human livelihood as well. The natural activity of leafcutter ants, such as *Atta sexdens* and *Atta cephalotes*, causes significant agricultural losses, notably damaging crops such as citrus, cocoa, coffee, cotton, cassava, maize, and garden flowers[1–3]. This not only reduces agricultural resources but also harms the economies of nations reliant on agricultural exports. Carpenter ants, such as the Florida carpenter ant *Camponotus floridanus*, damage infrastructure, destroying wooden structures such as homes and telephone poles, and negatively impacting the timber and ornamental tree industries[4–6]. However, not all ants are harmful to human society; many play crucial roles in their habitats, acting as important ecosystem engineers[7,8]. Therefore, pest control methods developed to target pest ant species should be highly specific to ensure that only problematic insects are affected.

Ants primarily communicate and perceive their environment through chemosensation, utilizing olfactory cues such as pheromones to organize their colonies and maintain their social structure[9–11]. Volatile odorants bind to olfactory receptors (ORs), heterotetrameric protein complexes localized on the dendrites of olfactory sensory neurons (OSNs), mainly located in insect antennae. These protein complexes consist of a single tuning OR, which determines the receptor's specificity, and three Orco subunits, obligate co-receptor proteins that are

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a commonality amongst modern insect lineages[12,13]. When specific odorants bind to the tuning OR subunit, the complex operates as a ligand-gated ion channel, allowing the flow of ions, initiating neural activity, and transmitting signals from the antennae to the antennal lobes of the insect brain[14,15]. Through olfactory cues, ants can communicate readily with nestmates, ensuring efficiency in maintaining caste structure, as well as perceiving and responding to relevant environmental signals from outside the nest. Without a properly functioning olfactory system, ants display abnormal behavior, cannot function normally on both the individual and colony levels, and exhibit abnormal neural development[16–19].

The importance of olfaction to ants is further evidenced by the expansion of *olfactory receptor* (*Or*) genes that this lineage of insects has undergone. While insects such as *Drosophila melanogaster* have around 60 *Ors*, Hymenoptera (including wasps, bees, and ants) possess more, with ants displaying the highest numbers at 300–500 *Ors*, dramatically outnumbering those in other insect species[20–25]. This expanded *Or* repertoire may be related to a need for advanced chemosensory communication within these eusocial species for efficient colony activity[24–28].

Olfaction is a proposed strategy for mitigating the effects of pest insects[29–33] and it may be particularly effective amongst pest ants, as olfactory cues have the potential to disrupt social organization[16,17]. The development of attractants or repellants that specifically target certain species—or even specific castes within a species—could help reduce off-target effects, minimizing the impact on non-pest insects. Across various settings (for example, agricultural and urban), there is a universal desire to repel pest insects and either attract or have a neutral effect on non-pest or beneficial insects. Developing pest management strategies that fit these needs is of increasing importance as pesticide resistance and off-target effects continue to plague our fields and cities. Development of olfactory-mediated pest mitigation strategies that specifically target particular groups of insects (i.e., certain ant castes) could be an effective resolution to these persistent problems. To design chemical compounds with specificity for a particular ant species or caste, it is essential first to have a detailed understanding of the *Or* gene expression profile in the target organism. Once this gene expression profile is established, particular *Ors* of interest (e.g., those that are highly expressed in a particular caste) can be prioritized for deorphanization, the identification of the particular odorant ligands that bind to these ORs and induce a neuronal response.

To date, ant ORs have seen limited deorphanization[34,35], with no published studies focusing on ORs of pest ants (with the exception of one *C. floridanus* gene, *CfOr263*, which encodes an OR protein product that selectively responds to 2,4,5-trimethylethiazole[24]). Ant olfactory receptors show a high level of similarity across different species, but the functional characteristics of these homologous receptors may vary. Slight differences in amino acid sequence at certain sites of the OR protein, such as at the odorant-binding pocket, may result in critical conformational changes to the protein structure itself, thereby influencing ligand binding and the overall function and specificity of the receptor[12,13]. Therefore, it is critical to broaden our understanding of OR function across species, rather than relying on a few examples and assuming them to be representative of all ants. Here, we explore the *Or* expression profiles of three pest ant species, *A. sexdens, A. cephalotes,* and *C. floridanus*, analyzing various castes of these species through antennal bulk RNA-seq to identify potentially significant *Or* genes. Such *Ors* may be candidates for future deorphanization studies and could be utilized for species or caste-specific pest control in ants.

Results

Ant collection, RNA extraction, and RNA-sequencing

In order to better understand the *olfactory receptor* expression profiles amongst worker ants of our three target species, we constructed RNA-seq libraries from antennae of different castes of *C. floridanus*, *A. cephalotes*, and *A. sexdens*. For *C. floridanus*, we targeted majors and minors from a lab-reared setting and from two local wild settings (hereon described as Wild Spaces A and B). For *A. cephalotes*, we targeted lab-reared soldiers, foragers, dump workers, and gardeners/minima, and for *A. sexdens* we targeted only soldiers and foragers from several wild spaces across Brazil; we did not explore other castes of this species because the data was extremely challenging to analyze (see the results and subsequent discussion of *A. sexdens* soldiers and foragers as described below). Antennae were removed from the sampled ants, and total antennal RNA was extracted and used to construct libraries for sequencing. Libraries were sequenced using the NovaSeq6000 (Illumina) sequencing platform, and a total of approximately 3.3 billion reads were generated across all samples.

olfactory receptor gene expression profiles differ between castes

Through paired-end sequencing of 23 RNA-seq libraries using the NovaSeq 6000 (Illumina) sequencing platform, we were able to identify 248 *Or* genes in *C. floridanus*, including a single *orco* gene (Table S1). Samples clustered well on principal component analysis (PCA) plots and distance heatmaps by caste, with little exception (Fig. 1A). Of the 248 identified *Or* genes, 231 (93%) were expressed (with a baseMean of 10 or higher), and 207 *Ors* were significantly differentially expressed (defined as padj ≤ 0.05 here and in all subsequent results) between majors and minors as identified by DEseq2[36] (Table S1). Furthermore, all differentially expressed *Or* genes exhibited up-regulation in minors (Fig. 1B), suggesting that olfactory receptors may serve relevant roles in minor-specific tasks (i.e., nursing and foraging behaviors). This is not surprising, given that minors have been shown to exhibit higher olfactory sensitivity than majors, which is thought to reflect the larger repertoire of social behaviors that this caste exhibits[37]. However, further functional analysis (i.e., electrophysiological validation of OR function) will have to be performed in order to verify the role of *Or* upregulation in minor-specific behaviors. Overall, 2609 significantly differentially expressed genes (DEGs) were identified between *C. floridanus* majors and minors (Table S1) (padj ≤ 0.05) among over 12,500 identified genes, ~ 8% of these DEGs being *Ors*.

Unlike the relatively simple worker caste structure of *C. floridanus*, *A. cephalotes* features a wider variety of worker subcastes, the features of which we attempted to capture in our analysis. We sequenced 20 RNA-seq

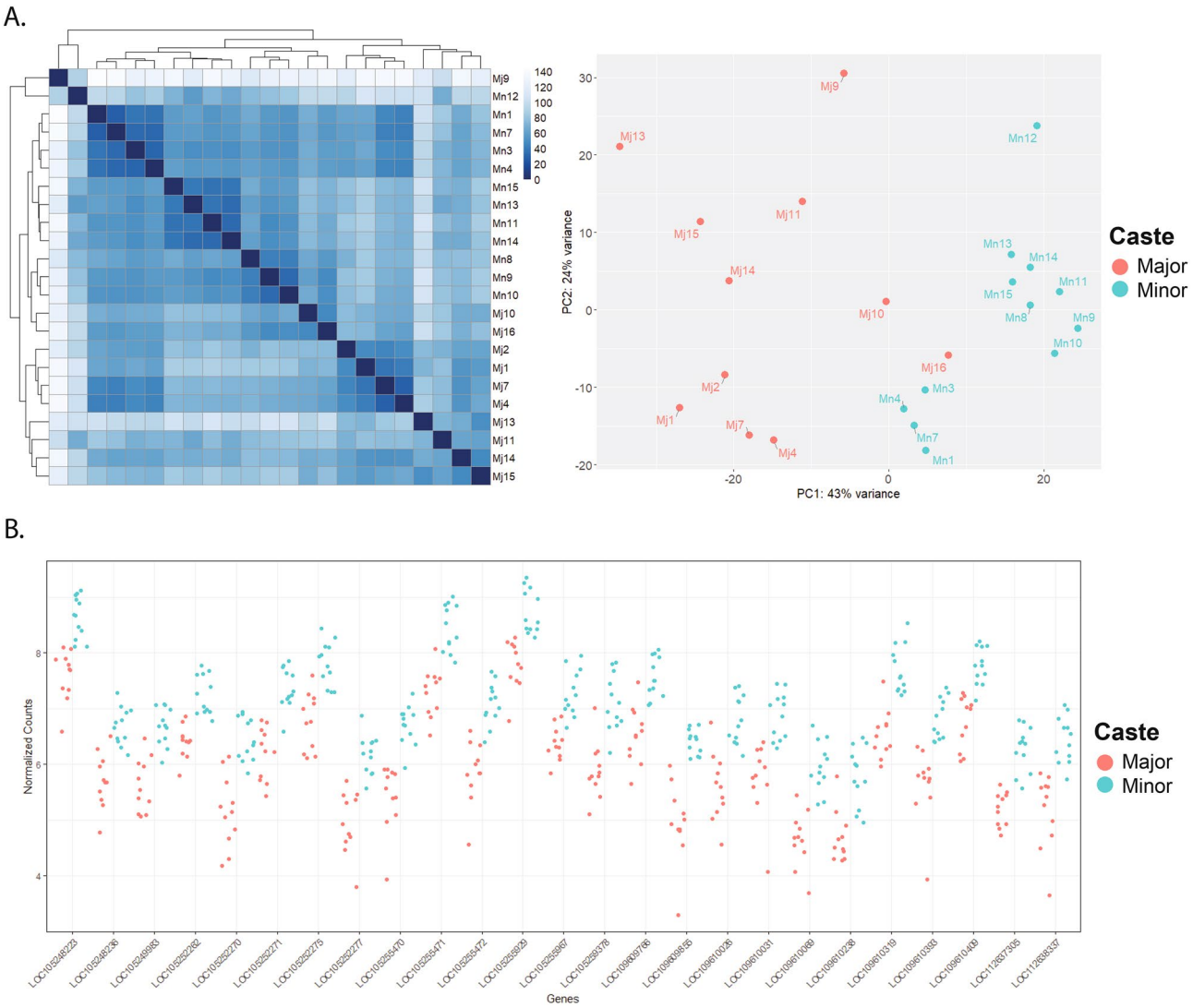

Fig. 1. *Camponotus floridanus* transcriptome profiles separate by caste. **(A)** Sample distance heatmap and PCA analysis of our *C. floridanus* antennal transcriptome data. The sample distance heatmap (left) illustrates automated sample clustering and relationships between samples, with distance being reflected by the intensity of the colored squares. Caste is indicated by Mj (Major) and Mn (Minor), each sample being identified by a unique combination of caste and number. The PCA plot (right) demonstrates that a large proportion of variance (PC1, 43% of observed variance) is explained by caste (indicated by Condition), with Major (red) and Minor (blue) samples occupying different regions of the plot. **(B)** Normalized counts of the top 25 differentially-expressed *olfactory receptor* genes plotted to demonstrate the difference in expression pattern between Majors (red) and Minors (blue). In general, minors exhibit a trend of higher gene expression.

libraries (5 replicates per worker subcaste) to explore differential gene expression amongst this more complex caste system.

Differential expression analysis between castes revealed substantial transcriptome differences, and similarly to *C. floridanus* samples clustered relatively well on PCA plots and heatmaps by caste (Fig. 2A). The highest numbers of significant DEGs (padj ≤ 0.05) were found between foragers and minima, soldiers and minima, and foragers and dump workers (Table S2). Notably, only 623 significant DEGs were identified between soldiers and foragers. This is an interesting finding considering that over 2,600 genes were identified between *C. floridanus* majors and minors, which share common behavioral phenotypes with *A. cephalotes* soldiers and foragers.

We were able to identify some differentially expressed *Ors* within each *A. cephalotes* caste comparison, their proportion ranging between 1.0–3.3% of all significant DEGs (padj ≤ 0.05). Dump workers vs. minima, soldiers vs. dump workers, and soldiers vs. minima had the highest proportions of *Or* genes compared to non-*Or* genes within their differential expression datasets. Overall, we identified 194 *Or* genes in our *A. cephalotes* datasets, including one *orco* gene, and all of them (100%) are expressed in these workers.

Unlike *C. floridanus* and *A. cephalotes*, there is no publicly available reference genome for *A. sexdens*. Therefore, we performed de novo transcriptome assembly using Trinity[38,39], identified candidate coding regions

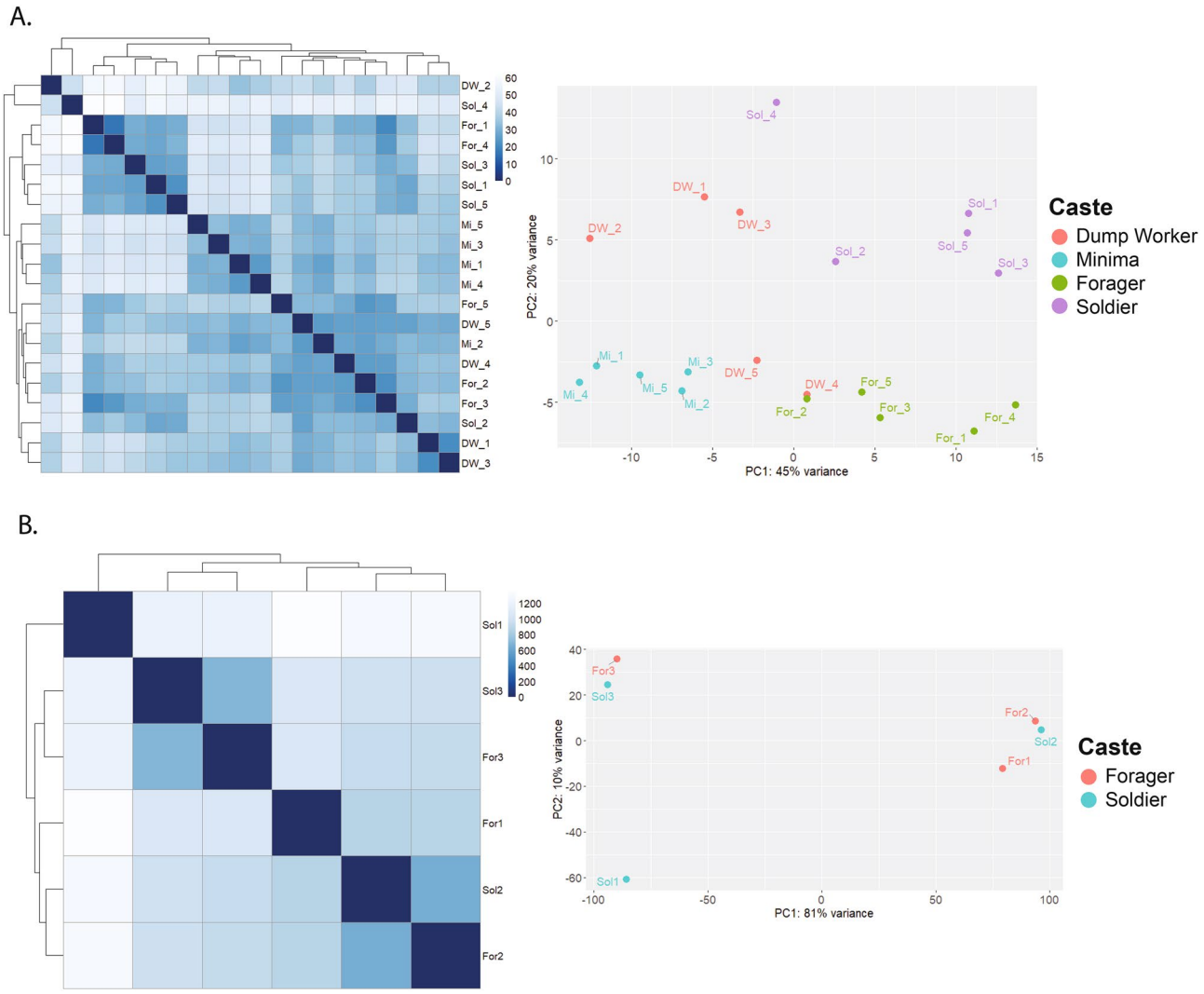

Fig. 2. Distance heatmaps and PCAs of our *Atta cephalotes* and *A. sexdens* antennal transcriptome data. **(A)** The sample distance heatmap (left) illustrates automated sample clustering and relationships between *A. cephalotes* samples, and the PCA plot (right) demonstrates that samples cluster by caste (indicated by Condition) across different regions of the plot. Caste is abbreviated for labeling purposes (DW = Dump Worker, For = Forager, Mi = Minima, Sol = Soldier), with each sample being identified by a unique combination of caste and number. **(B)** The sample distance heatmap (left) and the PCA plot (right) demonstrate poor clustering between *A. sexdens* samples or castes. As in (A), caste is abbreviated for labeling purposes (For = Forager, Sol = Soldier), with each sample being identified by a unique combination of caste and number.

via TransDecoder[40], and annotated them via Trinotate[41], followed by differential expression analysis using the same pipeline applied to the two aforementioned species. Through these methods, we identified only two significant DEGs between soldiers and foragers of this species (Table S3) (padj ≤ 0.05), with none of these genes being *Ors*. Nevertheless, annotation via Trinotate revealed over 2,700 transcripts resembling *Ors* across the entire transcriptome dataset (Table S4), including several transcripts resembling *orco* genes from other insect species, and only about 50% are expressed in worker ants.

Perhaps as a result of our challenging collection and analysis strategies, in the *A. sexdens* dataset, castes do not cluster well on a PCA plot (Fig. 2B), unlike the castes of the other two species we examined. Furthermore, de novo assembly added complexity to this analysis. These factors and others pertaining to *A. sexdens* will be covered thoroughly in the Discussion.

olfactory receptor gene expression profiles differ between environments

After exploring patterns of gene expression from the perspective of caste, we turned our attention to potential environmental influence on the expression of *Or* genes. Given that all the *A. cephalotes* samples came from a single lab environment and the aforementioned limitations of the *A. sexdens* analysis, we chose to explore this through the lens of *C. floridanus*. The *C. floridanus* samples came from two wild environments (referred to as Wild Spaces A and B, as indicated above) and from a colony that had been maintained in a lab environment

from a single mated female. Initially, we noted pronounced differences between samples coming from a wild setting and samples coming from a lab setting; samples cluster roughly on PCA plots by location, separating even between the two wild locations (Fig. 3A). This is also reflected in our *C. floridanus* sample distance heatmap (Fig. 1A). A total of 837 DEGs were identified between ants of wild and lab-reared settings, 43 of them being *Ors* (Table S5). We chose to further explore these apparent transcriptomic differences by comparing Wild Spaces A and B to one another, excluding ants from the lab-reared setting. Strikingly, 1,554 DEGs were identified between ants of the two different wild environments, 79 of them being *Ors* (Table S5). However, when plotting only *Ors* on PCAs, we find that samples separate primarily by caste (71% of observed variance), and only 8% of the observed variance can be explained by environmental differences (Fig. 3B).

Minor-specific environmental DEGs are enriched for olfactory-related GO terms

Following our findings of environmental influence on gene expression, we subsetted our environmental analysis by caste, looking for how the environment may be influencing gene expression within a single caste rather than across both *C. floridanus* castes. Comparison of wild minors with lab-reared minors resulted in 2,210 DEGs, 192 being *Ors*, and comparing minors of only Wild Spaces A and B resulted in 2,296 DEGs, including 29 *Ors*. Doing the same analysis for majors resulted in 256 DEGs between lab-reared and wild ants, 8 of these being *Ors*. Comparing majors from Wild Spaces A and B resulted in 1020 DEGs, including 8 *Ors* (these being different from the 8 mentioned previously). Overall, minors seemed to differ much more than majors in their

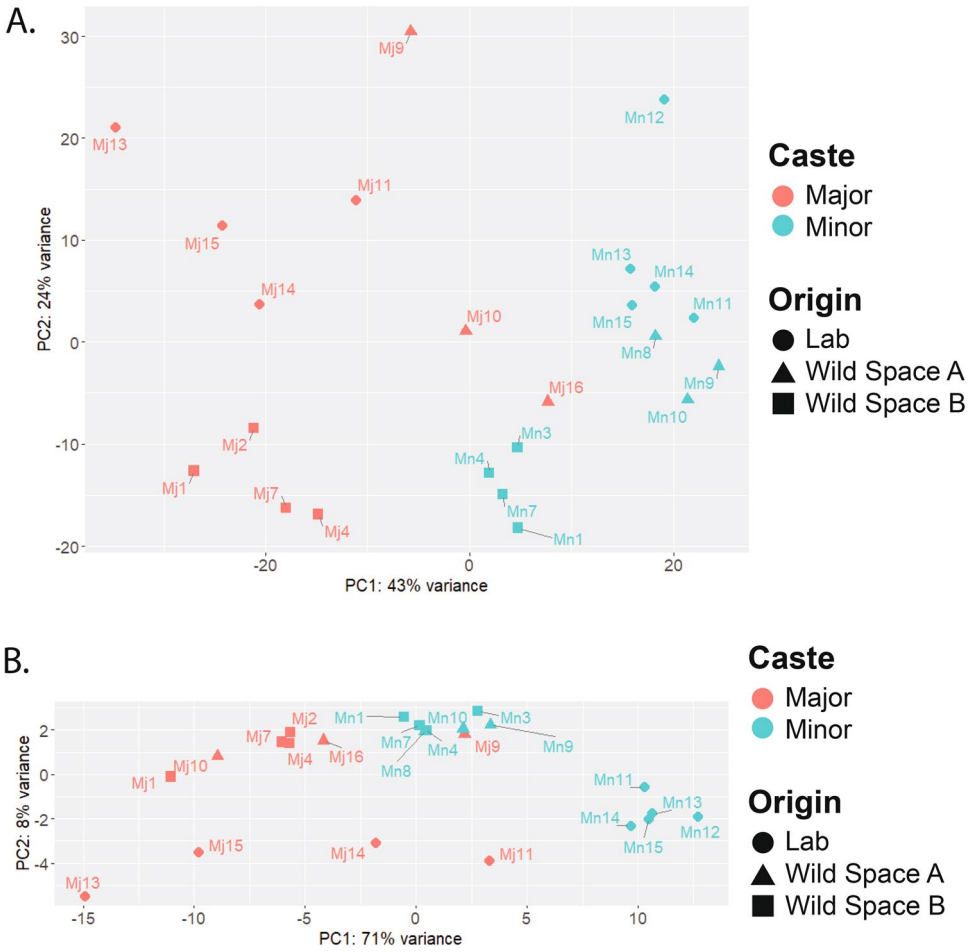

Fig. 3. Overall *Camponotus floridanus* transcription profiles are a product of both caste and environment. **(A)** PCA of all expressed genes from our *C. floridanus* samples. The first principal component (PC1) accounts for 43% of the observed variance and separates samples by Major (red) and Minor (blue) castes (indicated by Condition). The second principal component (PC2) accounts for 24% of the observed variance and generally separates samples into lab-reared (circles) and wild (triangles and squares) environmental origins (indicated by Type). However, there is also visible separation between Wild Space A (triangles) and Wild Space B (squares), indicating that transcription profiles differ even between ants of different wild environments. **(B)** Similar to (A), except this is a PCA of only *Or* genes (rather than all expressed genes, as in (**A**)) from our *C. floridanus* samples. There is still separation by caste (PC1, explaining 71% of observed variance), and some separation between lab-reared and wild-originating samples (PC2, explaining 8% of observed variance). However, the two wild spaces now cluster together, rather than having the separation observed in (A), indicating that *Or* genes are not the primary genes responsible for the wild environmental separation observed in (**A**).

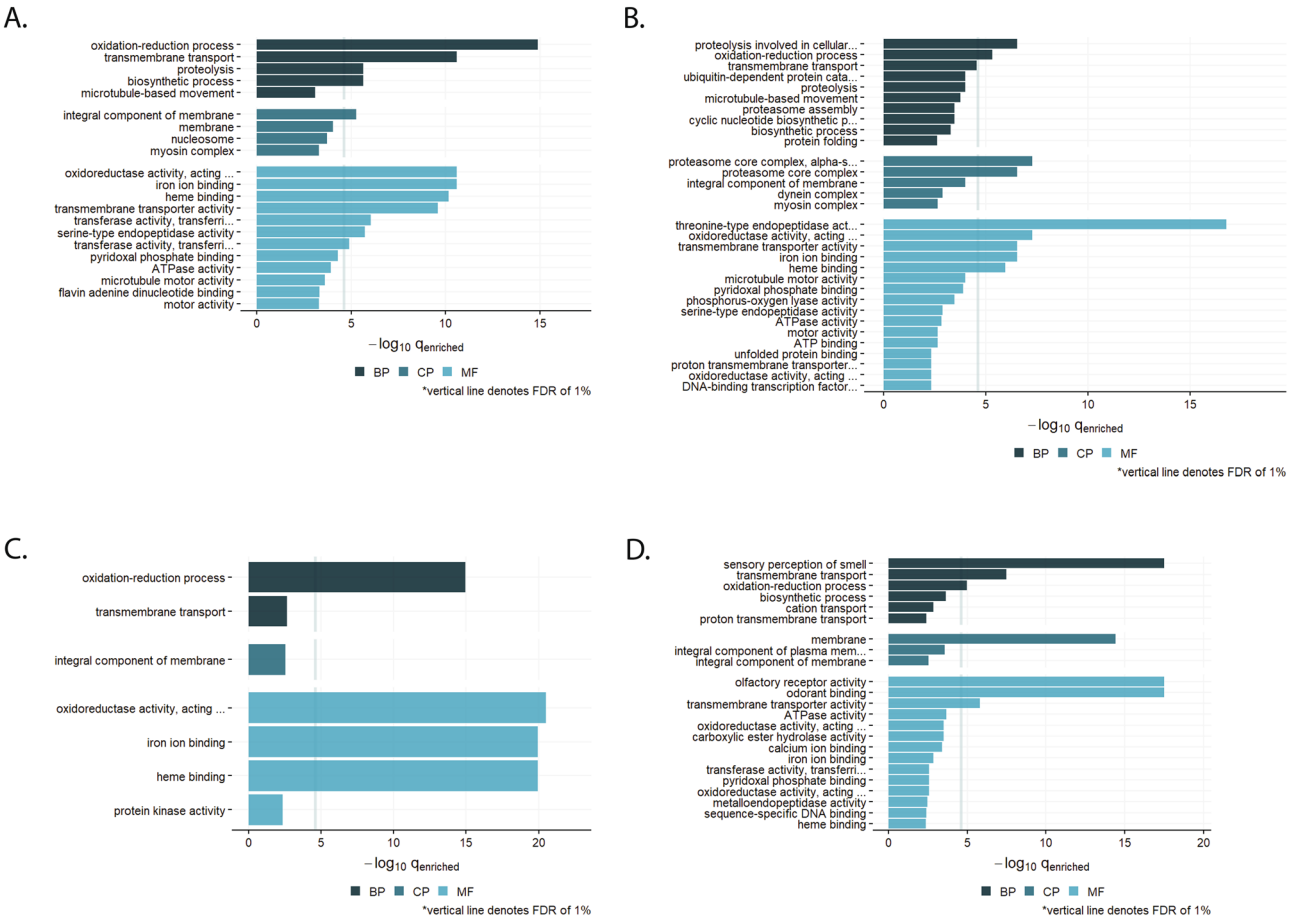

Fig. 4. Results of GO analysis of *Camponotus floridanus* DEGs. (**A**–**B**) GO analysis results of DEGs between majors originating from Wild Spaces A and B (**A**) and DEGs between minors originating from Wild Spaces A and B (**B**), with terms colored by biological process (BP, dark blue), cellular component (CP, medium blue), and molecular function (MF, light blue). Most of the terms are related to molecular function. This suggests that antennal transcriptomes of ants from various wild environments primarily differ in relation to the expression of genes with roles in molecular function. (**C**–**D**) GO analysis results of DEGs between wild and lab-reared majors (**C**) and DEGs between wild and lab-reared minors (**D**); terms are colorized as in (**A**–**B**). GO terms identified in (**C**) are similar (though fewer in number) to those identified in (**A**); however, GO terms identified in (**D**) are starkly different from those of (**B**), featuring several terms related to olfaction. These findings suggest there are substantial differences between lab-reared and wild minor antennal transcriptomes, particularly in relation to genes involved in olfaction, an entirely different result from the previous comparison of minors from two wild environments. However, this finding does not carry over to majors: DEGs between lab-reared and wild majors exhibit similar function to DEGs between majors of differing wild environments.

gene expression profiles between different wild environments and a lab setting, having many more DEGs across our various analyses. This may be expected because minors are involved in foraging and other tasks, as opposed to colony defense, the most important task for major workers[37] (though, as mentioned previously, functional analysis of ORs in minor workers should be performed in order to validate the significance of their respective gene expression patterns).

We next used Gene Ontology (GO)[42,43] in an attempt to better classify the genes that were differentially expressed between *C. floridanus* of different environmental conditions following the analysis pipeline previously established by Das and de Bekker[44]. Genes differentially expressed between majors of Wild Spaces A and B were particularly enriched for terms relating to oxidation–reduction and metabolic processes (Fig. 4A); genes differentially expressed between minors of the same conditions featured similar enrichment terms (Fig. 4B). When looking at genes differentially expressed between majors from both wild spaces and their lab-reared counterparts, we found similar results, with top terms being related to oxidation–reduction processes and iron binding in particular (Fig. 4C). However, when exploring genes differentially expressed between wild and lab-reared minors, we found notable new enrichment terms, mostly pertaining to olfactory perception and membrane functionality (Fig. 4D). Top terms included sensory perception of smell, membranes, olfactory receptor activity, and odorant binding, suggesting that lab-reared and wild minors differ strongly in their olfactory perception, apparently unlike their major counterparts.

Discussion

Pest ant species present profound challenges to agriculture and infrastructure around the world. Efficient and effective pest management strategies are essential to ensure that these species do not have a significant and lasting impact on human well-being and economic success.

Using antennal bulk RNA-seq, we have identified DEGs, especially differentially expressed *Or* genes between castes in three pest ant species, *C. floridanus*, *A. cephalotes*, and *A. sexdens*. 50–100% of *Or* genes are expressed in workers, among which many have higher expression in specific castes, which may serve as potential targets for caste-specific pest control in ants. In addition, we found that both caste and environment can effectively affect *Or* gene expression profiles. By harnessing olfactory perception, we would be able to effectively control pest ants in a specific manner without negatively affecting other insects and potentially targeting only castes with pest behaviors.

In order to develop a compound that triggers an olfactory-mediated response in pest ants, it is first necessary to understand the olfactory receptors of each species and their specific chemical ligands. Despite a high degree of cross-species OR homology, compounds that induce a meaningful behavioral response in one species may not have the same behavioral outcome in another[45]. Thus, we have started this study by exploring olfactory receptors in pest ant species, aiming to guide and inform future research on identifying relevant ligands for these receptors.

Ants interpret and respond to a wide array of olfactory stimuli as a primary mode of communication. Given that ants of different castes perform varying tasks, it is expected that their ability to detect and respond to olfactory cues varies likewise. Our data support this concept, as we see stark differences in *Or* gene expression between castes. Particularly in *C. floridanus*, there is a clear trend of *Or* gene expression being higher in foraging minors than in majors. One potential explanation for this is related to the need for minors to explore well outside the nest, foraging in constantly changing environments, and being sensitive to non-nest olfactory cues[37]. Majors, on the other hand, are less likely to venture far from the nest and therefore do not need to have as strong a response to the array of cues that minors must perceive and discriminate. Future studies should pursue electrophysiological or behavioral studies of different behavioral ant castes in order to validate the role of upregulated *Ors* in minor-specific behaviors.

Based on our data, *Or* expression differs not only between castes, but also between ants of different environments. Whether and how environmental factors affect social behavior are interesting questions to biologists. For example, previous studies have investigated the effect of diet on aggression and nestmate recognition in the Argentine ants, *Linepithema humile*[46,47]. From our *C. floridanus* data, we see clearly that differences in environment correspond with differences in gene expression, and minors in particular appear to be especially sensitive to this, further supporting our notion that minors have a strong need to respond to a wide array of cues external to the comparatively homogenous environment inside the nest. Notably, genes differentially expressed between minors of strongly varying environments (lab versus wild) are represented by GO terms pertaining to olfaction, suggesting the importance of olfactory perception to this caste along with its potential plasticity amongst individuals of varying environmental conditions. This result provides new evidence of environment-driven transcriptional plasticity, which should be considered by researchers aiming to develop universal pest management strategies: as environment is a defining factor in *Or* expression patterns, it may also influence how target pests respond to novel attractants, repellents, or other pest control strategies that rely on olfactory cues.

How might these findings be applied to real-world pest management? Attracting or repelling particular insects is of considerable importance in agricultural and urban environments, where harmful insects need to be removed without impacting beneficial insects (or even attracting beneficial insects, such as pollinators or natural predators of pests). In understanding *Or* expression patterns of different pest ant castes, an opportunity to develop attractants or repellents specific to particular ants arises. For example, developing a repellent that specifically targets a highly expressed *Or* protein in foraging leafcutter ants could successfully repel those ants while having a lesser effect on non-target species. Furthermore, in specifically targeting foraging leafcutters rather than targeting all leafcutter castes, the root of the problem is directly approached and is therefore more likely to have a stronger pest mitigation effect.

Additionally, we have shown that *Or* expression is environmentally influenced. Potentially, different olfactory-mediated pest mitigation strategies will have differing success rates depending on whether the organism is in an urban or agricultural setting (for example), as these different environments may have drastically changed the *Or* expression profile of the target pest. Such cases should be considered by industrial pest control manufacturers: should different pest management strategies be developed for different classes of environment to target pests in specific areas, or should pest management move in a more universal direction, aiming to develop broad-range methods for varying environments? Further study of environmental influences (e.g. microbial exposure, volatile chemicals, or diet differences) will be necessary to address these questions.

Unfortunately, we were only able to thoroughly explore *Or* gene expression in *C. floridanus* and *A. cephalotes* due to a variety of complications pertaining to our study of *A. sexdens*, likely stemming from our complicated collection strategy and need for de novo transcriptome assembly. Ideally, a reference genome should be established for *A. sexdens* and used to further explore *Or* gene expression in the castes of this species. The specific reasoning underlying our inconclusive results remains unclear, though several factors may have played a role. First, sample acquisition was challenging. Live ants were collected in Brazil, and their antennal RNA was extracted and shipped to the United States due to legislation preventing the transport of whole tissue samples. Additionally, de novo assembly presented its own unique challenges, with the resulting data yielding few DEGs. Whether this is an issue with the original RNA input or the assembly is unclear. With more complete information in the future (i.e., a well-annotated *A. sexdens* reference genome and access to fresh tissues), we may be able to address more complex questions about olfaction across different *Atta* pest species.

Overall, we have reported several lists of genes differentially expressed between different castes and species of pest ants, highlighting *Or* genes in particular. We show that *Or* gene expression patterns are products of not only species or caste, but also environment, features which should be considered in the development of future pest control strategies. Understanding the expression patterns of *Or* genes is a necessary step that will aid in the deorphanization of important receptors and the successful specific utilization of olfactory cues for the control of pest ant species.

Methods

Sample collection

Camponotus floridanus is a common wild species found throughout the state of Florida and through much of the southeastern United States[48,49]. Wild ants were collected from two urbanized locations in Gainesville, FL (29°39′5.8824″ N 82°19′29.3844″ W) in the Spring of 2020, one location being on the University of Florida campus, and the other being a suburban backyard. From each location, a single nest was excavated, so all collected individuals were from common colonies. Once brought back to the lab, they were frozen and stored immediately at − 80 °C. Lab-reared ants were acquired from Dr. Roberto Pereira of the Urban Entomology Lab at the University of Florida, which were also frozen and stored immediately at − 80 °C once brought to our lab. These ants had been raised in a lab setting for the entirety of their lives. Majors were identified by their larger body size and head width, while minors were selected based on a combination of size and observed foraging activity. Visually, these two castes are considerably distinct and unlikely to be confused with one another.

Whole-body specimens of lab-reared *Atta cephalotes* were provided by the Currie lab of the University of Wisconsin-Madison; samples were shipped to the University of Florida on dry ice, and stored long-term in our lab at − 80 °C. Caste was determined by a combination of head width, body size, and observed behavior (i.e., carrying plant material, maintaining fungus or brood, etc.). Soldiers were the largest, with head width and body size being both considerably larger than those of any other caste. They also demonstrate the highest defensive behavior. Foragers are a medium-sized caste, smaller than soldiers but larger than minima and dump workers, and are behaviorally distinct in that they actively harvest and carry plant material. Dump workers and minima are very small and visually similar, their major differences lying with behavior: dump workers were harvested based on active transport of materials to waste regions of the nest, while minima were harvested based on fungus garden and/or brood maintenance. All of these ants were lab-reared, having never been part of a wild colony.

Atta sexdens specimens were acquired in collaboration with Dr. José Mauricio Simões Bento of the University of São Paulo in Brazil and Drs. Fernando Ribeiro Sujimoto and Flávia Pereira Franco, members of his lab. Ants were collected by Dr. Sujimoto in the Spring of 2020 and Summer of 2021 from two rainforest regions of São Paulo state, Brazil: Piracicaba (22°45′45.5328″ S 47°50′33.8316″ W) and Paranapuã (20°6′2.4444″ S 50°35′27.4596″ W). Foragers were collected from a total of 6 mature nests, three replicates from each region of collection. Their caste was determined by behavior (i.e., carrying plant material) and head size. Soldiers were also collected from 3 mature nests, all from the Piracicaba region. Their caste was determined by aggression, body size, and head width, overall very similarly to how soldiers and foragers of *A. cephalotes* were identified. Ants were stored following collection at − 80 °C.

RNA extraction, library preparation, and sequencing

Total RNA extraction methods differed depending on species and caste. For all samples, irrespective of species and caste, antennae were removed from the head using microdissection scissors; antennae were cut at their base, where the most proximal portion meets the head.

Camponotus floridanus—Total RNA was extracted from the antennae of majors and minors, totaling n = 11 major replicates (4 lab-reared, 3 from Wild Space A, and 4 from Wild Space B) and n = 12 minor replicates (5 lab-reared, 3 from Wild Space A, and 4 from Wild Space B). A standard TRIzol protocol described previously[18] was used, followed by subsequent ethanol precipitation, deoxyribonuclease treatment, and a secondary round of TRIzol-chloroform and ethanol precipitation before dissolving in nuclease-free water.

Atta cephalotes—Total RNA was extracted from the antennae of 5 soldier replicates, 5 forager replicates, 5 dump worker replicates, and 5 minima replicates for a total of n = 20. The protocol for RNA extraction followed that which was used for *C. floridanus*.

Atta sexdens—Due to an inability to import whole tissue samples from Brazil, total RNA extraction was performed by Dr. Franco at the University of São Paulo. RNA extraction was performed using the RNeasy mini kit (Qiagen) according to instructions by the manufacturer, without DNase treatment. Samples were dried using RNAstable (Biomatrica) before being shipped to the University of Florida. Upon arrival, samples were eluted in nuclease-free water and treated with DNase. A total of 3 soldier and 3 forager replicates (total n = 6) were moved forward for sequencing and analysis.

Following total RNA extraction, libraries were generated using the NEBNext Ultra II RNA Library Prep Kit for Illumina with an input of 100 ng of RNA and 12 polymerase chain reaction (PCR) cycles, following manufacturer instructions, at the University of Florida. Libraries were quantified via 4150 TapeStation (Agilent) and qPCR, followed by sequencing in several rounds at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) using the NovaSeq6000 (Illumina) sequencing system.

Sequencing and data analysis

Approximately 3,258 billion reads were generated using the NovaSeq6000 (Illumina) platform across our multiple sequencing rounds. Mapping and alignment of reads to reference genomes were performed using Bowtie2 v2.2.9[50] and TopHat v2.1.1[51] in the cases of *C. floridanus* and *A. cephalotes*, as both species have publicly available reference genomes and annotations available publicly by the NCBI[52,53]. Differential gene expression analysis was performed using DEseq2 v1.38.3[36], with statistical significance being universally defined as padj ≤ 0.05.

Unlike the two aforementioned species, there is no publicly available reference genome for *A. sexdens* as of the preparation of this manuscript. Thus, the transcriptome had to be assembled de novo after sequencing. Raw reads first underwent quality filtration via Trimmomatic[54] following the recommended default settings. Following trimming, ~90% of our initial raw reads were retained for transcriptome assembly. de novo assembly was performed using Trinity v2.12.0 software[39], and TransDecoder 5.5.0[40] was used to identify candidate coding regions within our Trinity output. Following assembly, we performed annotation using Trinotate v3.0.1[41], estimated transcript expression values using Salmon v1.5.2[55], and performed differential gene expression analysis using DESeq2 (following the same pipeline as used for *C. floridanus* and *A. sexdens*). To calculate *Or* gene coverage (%) in each ant species, we considered baseMean (normalized reads in DESeq2) above 10 as significant expression levels.

Gene Ontology (GO)[42,43] analysis was performed using a pre-established pipeline published by Das and de Bekker[44].

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. RNA-seq data have been deposited at NCBI GEO (GSE288141).

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Author contributions

K.R.S. and H.Y. designed the experiments; K.R.S., M.S., N.N., and K.F. performed the experiments at UF; F.P.F. and F.R.S. performed the experiments at USP; K.R.S. analyzed bulk RNA-seq data; C.B.F., R.M.P., C.R.C., and J.M.S.B. provided aid in our ant acquisition; K.R.S. wrote the manuscript; H.Y. supervised the project.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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