Task-specific patterns of odorant receptor expression in worker antennae indicates a sensory filter regulating division of labor in ants

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Abstract

Division of labor (DOL) is a characteristic trait of insect societies, where tasks are generally performed by groups of specialized individuals. In social insects, young workers perform duties within the safety of the nest (e.g., brood care), while older ones undertake riskier tasks (e.g., foraging for food). This DOL remains dynamic, and workers may switch back and forth when colony needs require. Theoretical models propose that workers differ in their thresholds to take on certain tasks when confronted to task-related stimuli, resulting in variation in their response to such stimuli, task-specialization, and thus DOL. Such models assume that workers differ in how they respond to task-related information rather than in how they perceive such information. Here, we test the hypothesis that DOL rather stems from workers differing in their efficiency to detect task-related stimuli. We used transcriptomics to compare gene expression in the antennae and in the brain between nurses and foragers in the ant *Temnothorax longispinosus*. We found that seven times as many genes were differentially expressed between the behavioral phenotypes in the antennae compared to the brain. Moreover, nearly half of all odorant receptors genes were differentially expressed, with an overrepresentation of the 9-exon gene subfamily upregulated in the antennae of nurses. These findings indicate that nurses and foragers differ in how they perceive their olfactory environment, and task-related signals. The results of this study support the hypothesis that a sensory filter in the antennae predisposes workers to specialize in specific tasks, and may improve our understanding of DOL in insect societies.

Significance Statement

We tested the hypothesis that behavioral variation and task specialization in insect societies are regulated by differences in olfactory perception. We show that physiological differences between the most distinct behavioral phenotypes of nurses and foragers are less pronounced in the central nervous system than in the peripheral sensory organs. Nurses mostly activate odorant receptors from the 9-exon subfamily in the antenna that are predicted to be responsible for perception of chemical signals from nestmates, while foragers overexpress odorant receptors from diverse subfamilies. Our results demonstrate that workers performing different tasks also live in different sensory environments, suggesting that a sensory filter may play an important role in regulating division of labor in insect societies.

Main Text

Introduction

Division of labor (DOL) is an important organizing principle of complex biological systems that arose independently during three of the major evolutionary transitions (1). DOL was originally formulated in the context of the production process in human societies (2), but specialization to specific tasks is also found within cells and across many organisms (1). Examples range from bacteria, where clonal populations are divided into subpopulations focusing on different activities (3–6) to multicellular organisms with differentiation of cells into different tissues and organs, and individuals performing specific roles in animal societies (7, 8). To understand the evolution of complex life it is therefore essential to investigate the mechanisms that underlie DOL.

DOL in insect societies results from individuals specializing in the performance of specific tasks. In addition to the reproductive DOL between fertile queens and functionally sterile workers, there is a behavioral DOL among workers that specialize in tasks such as brood care, foraging, nest building, and defense (9–11). Several factors can affect task specialization, including age (12, 13), nutrition (14, 15), morphology (16), genotype (17–19), experience (20), and colony size (21–23). In most social insect species, workers display an age polyethism by which younger individuals tend to perform intranidal tasks, while older individuals are more likely to undertake activities such as nest-defense and foraging outside the nest (24, 25). Yet, such specialization among workers remains flexible, as foragers can revert to perform brood care when needed (26, 27).

Several molecular mechanisms have been implicated in the regulation of DOL. Task specialization is associated with transcriptional changes in the worker brain (28–33). Molecular pathways such as the insulin/insulin-like signaling (IIS), vitellogenin (Vg) and juvenile hormone (JH) pathways are involved in the regulation of worker behavior (34–39). Functional manipulations have confirmed that the expression of key genes in the worker brain controls task specialization (40–42). Behavioral variation among workers is also associated with signaling of biogenic amines (e.g., dopamine, octopamine, tyramine, serotonin), which act as neurotransmitters or neuromodulators involved in the modulation of the responsiveness to task-associated stimuli (43–46).

84 The self-organization and collective behavior in insect societies are maintained via the exchange
85 of chemical information (47). Social insects communicate primarily through glandular pheromones and
86 complex mixtures of long-chain hydrocarbons on their cuticle. These cuticular hydrocarbons (CHC) facilitate
87 recognition of nestmates, developmental stages, castes, sexes, and species (48, 49). Social insects
88 perceive chemical information via different types of sensilla on their antennae (50–52). Decoding the
89 identities of chemical compounds relies on odorant receptors (OR) located within each sensillum (53, 54).
90 OR are transmembrane proteins expressed in the dendrites of olfactory receptor neurons (ORN). The
91 largely conserved OR coreceptor (Orco) of insects (55) is required for odorant recognition in the dendritic
92 membrane: it forms an ion channel with specific OR, which determines the sensitivity and specificity of the
93 ORN (56). Odorant molecules penetrate through the antenna cuticular pores and are transported by
94 odorant-binding proteins to the ORN membrane, where they interact with receptors, leading to the
95 generation of action potentials (57, 58). ORN axons relay signals from the sensilla to the glomeruli of the
96 antennal lobes in the insect brain, which are the first processing unit for olfactory information. Then, ORN
97 make synaptic contact with the projection neurons and local neurons, which transfer information to the
98 central brain (59–61).

99 Several lines of evidence indicate that OR genes play central roles in the regulation of social life of
100 insects. First, social insect species typically harbor large numbers of OR genes (62–65). Second, some OR
101 gene families have specifically expanded during social evolution, such as the 9-exon subfamily in ants,
102 which appears to serve an important function in the perception of CHC (64, 66–70). Third, species that
103 evolved socially parasitic strategies resulting in reduced behavioral repertoires show a strong and
104 convergent reduction in the number of OR genes (71). Finally, experimentally produced mutant ants that
105 lack the *Orco* gene (coding for the co-receptor necessary for OR to properly function) show impaired social
106 behavior (72, 73).

107 Current DOL models bring together the chemical nature of social insect communication and
108 variation among workers in their response to chemical cues. They posit that flexible response thresholds
109 to task-related chemical stimuli serve as regulators of worker specialization (74). Workers take on a
110 particular task when the stimulus intensity exceeds their individual threshold for this task. Therefore,
111 individuals with lower thresholds for a given task are more likely to perform it than those with higher

112 thresholds (75). Individual response decisions and task performance are modulated via numerous
113 parameters on different time scales (7, 76–78), and despite extensive research on DOL and task allocation,
114 these mechanisms are not fully understood.

115 As indicated by the name, “response threshold models” typically assume that individuals differ in
116 their response to specific signals, not necessarily in their ability or efficiency to detect such signals. This
117 implies that such thresholds and the associated responses are set in the central nervous system, possibly
118 via molecular pathways in the brain that correlate with behavioral variation (74, 79, 80). We propose that
119 DOL models would benefit from considering odor sensitivity as a potential upstream sensory filter that may
120 affect task specialization. Along these lines, we hypothesize that behavioral variation among workers may
121 also stem from their ability and/or efficiency in detecting different signals. For example, we propose that
122 individuals that specialize in brood care do so because they are better at detecting brood cues, rather than
123 (or in addition to) being more likely to respond to similar levels of brood cues. To test this hypothesis that a
124 sensory filter regulates inter-individual behavioral variation, and thus the DOL in social insects, we
125 investigated transcriptional differences in the brain and antennae of workers that specialize in either brood
126 care or foraging behavior in the ant *Temnothorax longispinosus*. We found that i) behavioral variation was
127 associated with important transcriptomic changes in the antennae, ii) these changes included a large
128 proportion of the OR gene repertoire, and iii) individuals specializing in brood care overexpressed an OR
129 gene family putatively involved in detecting social cues. These findings support our hypothesis that the
130 peripheral nervous system, acting as a sensory filter, plays an important role in regulating behavioral
131 differences between workers and thus in the DOL of social insects.

132 **Results**

133

134 ***Identification of* Temnothorax longispinosus *behavioral phenotypes***

135 Task specialization in the ant *T. longispinosus* is neither genetically fixed nor rigid, but changes with age
136 and in response to colony needs (38). We conducted behavioral observations of seven *T. longispinosus*
137 laboratory colonies to identify individuals that specialize in brood care behavior (hereafter referred to as
138 nurses), and others that specialize in foraging (hereafter referred to as foragers) (Fig. 1*A*; see Methods
139 section for more details). Individuals identified as nurses interacted with the brood in 54% ± 22% (mean ±
140 sd) of the observations, and were recorded outside the nest in 1% ± 3% of the observations. On the
141 contrary, foragers were found outside the nest in 42% ± 26% of the observations, but interacted with the
142 brood in only 2% ± 4% of the observations.

143

144 ***Larger task-associated transcriptomic changes in the antennae than in the brain***

145 To investigate transcriptomic variation between nurses and foragers, we used RNA-seq to generate seven
146 nurse and seven forager brain and antenna samples, each consisting of pooled tissue from seven workers
147 of a single colony. Of the 14,837 protein-coding genes annotated in the *T. longispinosus* genome, we found
148 that 91% (13,494) and 92% (13,683) were expressed (FPKM > 0) in the brain and the antennae,
149 respectively. Using an adjusted p-value of 0.05 as significance threshold, we detected 333 differentially
150 expressed genes (DEG) in the brain (217 upregulated in nurses, 116 in foragers), and 2,254 in the antennae
151 (1,234 upregulated in nurses, 1,020 in foragers; Dataset S1; Fig. 1*B*). We found an overlap of 162 DEG
152 between the two tissues, including 143 DEG that showed differences in the same direction across tissues.
153 Principal component analysis (PCA) plots indicate that both brain and antenna samples cluster by
154 behavioral phenotype rather than the colony of origin (Fig.1 *C-D*).

155

156 ***OR gene expression differs between antennae of nurses and foragers***

157 To investigate whether odorant perception differs between nurses and foragers, we focused our attention
158 on the expression of OR genes in the antennae. We found that all 419 OR genes in the genome of *T.*
159 *longispinosus* (71) were expressed in the antennae, and that 48% (202/419) of them were differentially

160 expressed between nurses and foragers. Specifically, 65 OR genes were upregulated in nurses (15% of all
161 OR genes), and 137 in foragers (32% of all ORs) (Fig. 2). Then, we studied which OR subfamilies were
162 preferentially expressed in nurses and foragers. The 65 OR genes overexpressed in nurses belonged to
163 three OR subfamilies, while the 137 OR genes upregulated in foragers were distributed among 19 OR
164 subfamilies. Foragers overexpressed 26, 8, and 8 OR genes from the L, P, and H subfamilies, respectively,
165 while no OR genes from these subfamilies were upregulated in nurses (Dataset S2). We found that 61%
166 (26/43) of the genes from the L subfamily were overexpressed in foragers, which represents a significant
167 overrepresentation (Fisher's test, odds ratio = 3.63, p-value < 0.001). For the P and H subfamilies we did
168 not find such an overrepresentation, likely due to lower gene numbers (P subfamily: Fisher's test, odds ratio
169 = 3.62, p-value = 0.06; H subfamily: Fisher's test, odds ratio = 1.87, p-value = 0.19). On the other hand, we
170 found that 82% (53/65) of the OR genes overexpressed in nurses belong to the 9-exon subfamily. This
171 results in an overrepresentation of the 9-exon subfamily in genes that were overexpressed in nurses
172 (Fisher's test, odds ratio = 20.98, p-value < 0.001), with 46% (53/114) of this subfamily being overexpressed
173 in nurses. In contrast, only 6% (8/137) of the 9-exon subfamily was overexpressed in foragers, which is
174 less than expected by chance (Fisher's test, odds ratio = 0.10, p-value < 0.001). We also found that nurses
175 overexpressed *Orco* compared to foragers (FDR p-value = 0.001).
176

177 ***Genes in biogenic amine pathways vary in expression between nurses and foragers***

178 Because biogenic amines are implicated in the regulation of behavior, and they may affect sensory
179 perception (45, 81–83), we screened our lists of DEG in the brain and antennae for genes in biogenic amine
180 pathways. We found five genes associated with biogenic amine signaling in the antennae, and none in the
181 brain. Genes in the serotonin (*5-hydroxytryptamine*, DBV15_11483), tyramine (*tyramine beta-hydroxylase*,
182 DBV15_00422) and octopamine (*octopamine receptor* LOC112465659) pathways were upregulated in
183 foragers, while nurses showed a higher expression of genes in the dopamine (*dopamine 1-like receptor 2*,
184 DBV15_07611) and octopamine (*octopamine beta2 receptor*, DBV15_10418) pathways (Fig. 3 *A-E*).
185

186 ***Association between behavioral variation and molecular pathways in brain and antennae***

187 Our analysis revealed expression differences between the brains of nurses and foragers for genes involved
188 in the regulation of task specialization in social insects. Previous studies have found that *Vg* genes and the
189 associated JH and IIS/TOR pathways are important endocrine networks that play central roles in the
190 regulation of lifespan, fertility and behavior in bees and ants (35, 84–89). Therefore, we searched our RNA-
191 seq data for task-associated expression of genes involved in the metabolism, biosynthesis, and regulation
192 of these pathways in the brain and antennae. We found evidence that multiple genes from these pathways
193 are differentially expressed between nurses and foragers (Table S1). Among the most interesting genes
194 upregulated in the brain of nurses were *conventional Vg* (LOC112466671), *Vg-like A* (named/classified as
195 per 27; DBV15_03138), *venom carboxylesterase-6-like* (DBV15_11528), and *protein takeout*
196 (DBV15_08771), while *allatostatin A-like* (LOC112454443) and *insulin-like growth factor I* (*IGF1*)
197 (LOC112454447) were overexpressed in the brain of foragers. Remarkably, *venom carboxylesterase-6-*
198 *like* and *IGF1* were overexpressed in the antennae of nurses and foragers respectively. Additionally, high
199 expression levels of the zinc-finger transcription factor *Krüppel homolog-1* (*Kr-h1*) were detected in the
200 antennae of foragers (DBV15_06330) (Fig. 3*F*). The expression of this gene has been correlated with caste
201 and behavioral differences in the brain of social insects (86, 90–93), but little is known about its function
202 and gene targets in the antennae.

203 We performed a GO enrichment analysis to gain a deeper understanding of the biological
204 processes represented in the lists of DEG. This analysis detected many enrichments based on single one
205 or few genes, and we mention below a few interesting processes with the number of genes driving the
206 enrichment in parentheses (see Table S2 for complete list). Genes that were upregulated in the brain of
207 nurses were enriched for biological processes such as *translation* (19 genes), *cellular iron ion homeostasis*
208 (2 genes), and *catabolic process* (6 genes); while *translation* (22 genes), *endocytosis* (6 genes), *glucose*
209 *metabolic process* (4 genes), and *regulation of cell cycle* (3 genes) were enriched in the antennae. In the
210 list of genes that were overexpressed in the brain of foragers, we found that enriched processes included
211 *peptide metabolic process* (4 genes), *methionyl-tRNA aminoacylation* (1 gene), *positive regulation of type*
212 *I interferon production* (1 gene), and *cell redox homeostasis* (1 gene); while *phosphatidylinositol*
213 *phosphorylation* (4 genes), *inositol phosphate dephosphorylation* (3 genes), *carbohydrate metabolic*

214 *process* (17 genes), *innate immune response* (2 genes), and *nucleotide catabolic process* (3 genes) were

215 enriched in the antennae.

216 **Discussion**

217 In this study, we tested the hypothesis that variation among social insect workers in their ability to detect
218 different chemical signals regulates task specialization, and thus DOL in insect societies. To do so, we
219 compared the brain and antenna transcriptomes of nurses and foragers in the ant *T. longispinosus.* We
220 report several lines of evidence that support our hypothesis. First, we found almost seven times as many
221 genes to be differentially expressed between nurses and foragers in the antennae as in the brain, indicating
222 that peripheral sensory organs may be more important than the central nervous system in the task
223 specialization process of social insect workers. Second, we found half the OR genes in the *T. longispinosus*
224 genomes are differentially expressed between nurse and forager antennae, suggesting that behavioral
225 specialization is associated with different sensory filters that result in specific perceptions of the chemical
226 environment. Third, our analyses revealed that nurses and foragers upregulated distinct families of OR
227 genes, indicating that their sensory filters may target different types of chemical cues, possibly adjusted to
228 the tasks they perform. Finally, we detected several genes in multiple biogenic pathways to be differentially
229 expressed between the brains of nurses and foragers, potentially involved in fine-tuning the sensitivity of
230 the odorant filters.

231 Many organisms have evolved sensory filters to focus on only a subset of all environmental cues.
232 In insects, the peripheral olfactory filtering system enables individuals to detect and discriminate odors that
233 convey ecologically relevant information used to mediate important behaviors such as courtship, locomotion
234 and navigation to avoid predators and locate food or nesting sites (94). For example, the mosquito
235 *Anopheles gambiae* strongly responds to odorant components of its vertebrate hosts that provide meals
236 (95). Sensory filters have been selected for because they limit the amount of information perceived by the
237 organism, and thus the energy and time required by the brain to process it. Within insect colonies,
238 individuals typically exhibit many morphological and physiological traits associated with increased efficiency
239 in task specialization. They can be understood as adaptations that allow individuals to become more
240 proficient at their task due to learning, training, or the perceiving valuable task-related information, allowing
241 the colony to avoid the cost of task switching (96). In this context, it is interesting to find that *T. longispinosus*
242 workers may have different sensory filters that would serve as a basis for their task specialization, as

243 workers would mostly perceive chemical cues that pertain to their tasks. Our findings indicate that the
244 sensory filter of ant workers is dynamic, and its changes may underlie their behavioral maturation.

245 Age, genetic background, social environment, and individual experience produce behavioral
246 variation among workers that results in division of labor (13, 20, 23, 97). In our study, age in particular likely
247 differed between nurses and foragers. We propose that the factors known to regulate task specialization
248 actually drive different OR expression patterns, which in turn produce behavioral variation via contrasting
249 abilities to detect different sets of odors. This hypothesis is supported by several studies (reviewed in 97)
250 showing that the physiological condition of an animal can influence the level of receptor expression,
251 including mating status, oviposition, feeding, circadian rhythm, experience, and aging. Alternatively, we
252 cannot exclude that the exposure to different odors that is associated with performing different tasks may
253 at least in part have affected OR gene expression in the antennae. However, such an effect is unlikely to
254 explain the large-scale variation in gene expression, as there is very limited evidence that the mere
255 exposure to odors influences the expression of the gene coding for the OR that binds this odor (99, 100).

256 According to our sensory filter hypothesis, ant workers would be expected to primarily detect
257 chemical cues that correspond to their tasks. We found that the 9-exon subfamily of OR was
258 overrepresented in genes that were upregulated in the antennae of nurses. Interestingly, recent studies
259 have shown a rapid expansion of the 9-exon subfamily in ants, and several lines of evidence indicate that
260 OR genes from this subfamily mediate complex social interactions in ant colonies (65–67, 69). First,
261 comparisons of antennal transcriptomes revealed that 9-exon OR genes are expressed more frequently in
262 workers than in males, suggesting a role in social communication among workers (68, 69). Second,
263 representative OR from the 9-exon subfamily can detect CHC extracts from several castes (70). According
264 to McKenzie et al. (69), 9-exon OR genes were first expressed in solitary ancestors of aculeate wasps and
265 facilitated CHC discrimination, likely for prey or mate recognition, with a lineage giving rise to the ancestors
266 of ants. Moreover, OR genes of the 9-exon subfamily were convergently lost in socially parasitic ant species
267 that lost the ability to perform brood care or foraging (71), suggesting that they are essential for the
268 performance of these worker tasks. Our finding of an overexpression of 9-exon OR genes in the antennae
269 of nurses is in line with these previous studies, and suggests that many of these receptors have important
270 functions within the nest, such as sensing chemical cues from the larvae, queen, or other workers, and/or

271 that they are less important for sensing task-related stimuli or other signals outside the nest. Among the OR
272 genes overexpressed in nurses was also the co-receptor *Orco*, which is widely expressed in olfactory
273 sensory neurons and nearly unchanged in sequence in distant insect taxa (101–103). The overexpression
274 of *Orco* may indicate a generally more active odorant sensory system in the antennae of nurses compared
275 to foragers.

276 The behavioral transition from nursing to foraging may be triggered by a lower efficiency in detecting
277 brood cues via the downregulation of specific OR genes (e.g., from the 9-exon subfamily). This would result
278 in ants moving farther away from the brood, and this change in spatial location may trigger the behavioral
279 transition to outside tasks (104, 105). In addition to being less efficient at detecting brood cues, the sensory
280 filter of foragers may also become fine-tuned to detect a more diverse set of odors. Foragers overexpressed
281 a greater number of OR gene subfamilies compared to nurses (19 and 3 for foragers and nurses,
282 respectively), which may indicate that the olfactory system of foragers could be adjusted to the more diverse
283 chemical environment outside the nest. Similar to the 9-exon subfamily, the L subfamily has also been
284 expanded in social insects (62, 64, 65), and along with the P and H subfamilies, it has been lost in socially
285 parasitic ants (71). Interestingly, the OR genes from the L, P, and H subfamilies have been upregulated in
286 foragers, and thus may have a task-specific function, such as recognition of chemical cues related to
287 environmental perception or recruitment cues outside the nest. To date, however, there is no information
288 on which odors are recognized by these OR gene subfamilies.

289 Finding task-specific variation in OR gene expression raises the question as to which molecular
290 mechanisms regulate those changes. Previous studies in insects revealed that olfaction-guided behavior is
291 mediated by biogenic amine receptors in the antenna, and their expression is involved in fine-tuning the
292 sensitivity of the olfactory system (82, 98). For example, modified concentration cAMP and intracellular
293 Ca^{2+} levels due to octopamine-induced signal transduction in the moth *Manduca sexta* (106) activate Orco,
294 leading to changes in ORN sensitivity (107, 108). Our results suggest that the biogenic amine signaling
295 pathway may modulate the sensory filtering function of insect antennae and alter sensitivity to various
296 signals. We found that genes encoding tyramine and its precursor, octopamine, are upregulated in forager
297 antennae, similar to genes involved in serotonin signaling. Tyramine and dopamine (which was upregulated
298 in nurses) have been implicated in modulating taste and olfactory receptor neurons, while serotonin may

299 serve as a neurotransmitter and neurohormone in antennal vessels and mechanosensory organs (82).
300 Serotonin influences foraging activity (81) and regulates food intake in many animals (109–111). Dopamine
301 signaling also plays an important role in controlling the insect circadian clock and mediating clock-controlled
302 behavioral phenotypes such as locomotion (112, 113). In our focal species *T. longispinosus*, inside workers
303 were found to exhibit a stronger circadian rhythmicity than foragers, which may be regulated via differences
304 in the acetylation of histone proteins (114).

305 Changes in behavior and olfactory sensitivity in insects could be related to the expression of genes
306 involved in IIS, target of rapamycin (TOR), JH and Vg pathways, according to age, circadian rhythm, mating
307 and feeding status (98). For example, appetite state in *D. melanogaster* is signaled by insulin, which
308 upregulates a peptide receptor on the olfactory receptor cells that innervate the DM1 glomerulus. Activation
309 of the DM1 glomerulus is enough to drive the fly to reach for food (115). Recent studies have shown that
310 pheromone release and odor sensitivity appear to be under JH control in *Schistocerca gregaria* and *Locusta*
311 *migratoria*, which could lead to behavioral changes (116–120). Finally, an experimental downregulation of
312 *Vg-like A* in *T. longispinosus* workers resulted in decreased brood care behavior and a lower sensitivity to
313 brood-related chemical cues, suggesting changes in odor perception and olfactory-driven decision making
314 (42). Our results reveal that genes associated with all these pathways were differentially expressed in the
315 brain and antennae between nurses and foragers ,which confirms their role in task-associated behavioral
316 changes and regulation of odor perception. Given the central role of IIS, Vg, JH, and TOR pathways in
317 regulating division of labor in social insects, and our finding of task-associated patterns of the antennal
318 expression of genes from multiple biogenic amines, we hypothesize that these modulators and hormones
319 are involved in the regulation of the olfactory filter. How the detailed molecular mechanisms of these
320 pathways in the brain are causally linked to the complex changes in olfactory perception in the antennae
321 should therefore be investigated next.

322

323 **Conclusion**

324 Our transcriptomic analyses of the brain and antennae of *T. longispinosus* nurses and foragers provide
325 support to our hypothesis that behavioral variation and task specialization in ant workers are regulated via
326 differences in olfactory perception. We predict that antennal physiology acts as sensory filters that limit the

type and amount of chemical information passed to the brain. This would allow workers to target relevant chemical information from the environment and discriminate signal from noise without using energetically costly processing by the central nervous system. We argue that this sensory filter is flexible and regulated through changes in physiological conditions such as age, nutrition, and hormones. Variation among workers in their efficiency to detect specific chemical cues would result in task specialization and division of labor. Our study opens novel avenues of research to better understand the role of sensory filters in controlling DOL in insect societies.

334 **Materials and Methods**

335

336 ***Sample collection and behavioral determination***

337 A total of seven colonies of the ant *T. longispinosus* were selected with an average colony size of 110 ±
338 31.5 workers (mean ± SD, Dataset S3). The ants were collected in the forests of the Edmund Niles Huyck
339 Preserve, Renssellearville, NY, USA (42°31′41.0″N 74°09′38.8″W), in June of 2018 with permission. Upon
340 collection, we housed each colony in a plaster-floored nesting box (43 cm × 28 cm × 10 cm) divided into
341 three chambers containing a single slide nest, in which the colony relocated. A slide nest is an artificial
342 nesting site comprised of a small Plexiglas cavity sandwiched between two glass microscope slides.
343 Colonies were established at the Johannes Gutenberg University in Mainz, Germany, under a 14 h:10 h
344 light:dark photoperiod at 18°C to a 22°C temperature. We provided honey and water ad libitum and fed
345 crickets to the colony twice a week. To allow for visible behavioral division of labor between workers of the
346 two behavioral phenotypes, we marked, observed and recaptured ants from inside and outside the nest.
347 We defined foragers as workers that perform outside-nest tasks, including foraging for food, while nurses
348 remained inside the dark nest and cared for the ant brood. A total of 69 workers inside (from the brood pile)
349 and 76 workers outside the nest were marked with fine colored metal wires (0.02 mm Elektrisola,
350 Eckenhagen, Germany) between the petiole and post-petiole. We performed behavioral observations every
351 two hours, four times a day for five days (total = 20 scans), in which we noted down how many times an
352 individual spent performing brood care and foraging behavior and the position in the nest (Table S3). Based
353 on these behavioral observations, the marked individuals found outside the nest, exploring the surroundings
354 for food or water, were identified as foragers. These workers usually do not care for the brood and do not
355 frequently reside on brood piles, as ant colonies organize themselves spatially in a way that reduces contact
356 between foragers and brood (121). We identified nurses as workers that remain inside the nest in direct
357 contact with brood and were unlikely to leave the nest. Foragers were found in 42% ± SD 26% of the
358 observations outside of the nest, whereas nurses spend only 1% ± SD 3% outside. In contrast, nurses were
359 interacting with the brood in 54% ± SD 22% of the observations, whereas we found that foragers only did
360 this only in 2% ± SD 4% of the observations. We focused in this study on individuals highly specialized on
361 either foraging or brood care. Workers that performed both tasks regularly were not included in this study.

362 After all observations were completed, the marked nurses and foragers were collected, directly frozen in
363 liquid nitrogen and stored at -80 °C until further processing for dissection and pooling according to
364 behavioral state and colony.
365
366 ***RNA extraction and sequencing***
367 For RNA extraction, we removed both antennae and stored them in a 1.5 ml Eppendorf tube containing 50
368 µl TRIzol (Invitrogen), cut the head off and fixed it on a slide with melted dental wax. We then made an
369 incision around the head with a surgical scalpel and removed the head capsule with forceps to expose the
370 intact brain. Finally, we carefully pulled the brain out of the head capsule and removed the remains of other
371 tissues that were connected to it. The dissected brain was transferred to a 1.5 ml Eppendorf tube containing
372 20µl PBS. Each dissection was completed in less than 5 minutes to prevent RNA degradation. We dissected
373 brain and antennae tissues from 48 nurses and 49 foragers. We pooled the brains and antennae from
374 seven workers from each behavioral state and colony. The only exception was the "GO" colony (NY18
375 E110), for which we pooled only six brains and twelve antennae from six nurses (Dataset S3) due to the
376 loss of one sample during the dissection process. Immediately after dissection of each brain and antennae,
377 the Eppendorf tubes were kept on dry ice while we dissected the remaining individuals. Brain and antennae
378 tissues were homogenized with a pestle. Sample brains were transferred separately to a 1.5 ml Eppendorf
379 tube containing 50µl of TRIzol. We added 50µl chloroform to each brain and antenna samples, cautiously
380 inverted for 30 s and then centrifuged samples at 12,000 g for 15 min at 4°C. We collected the resulting
381 supernatant and precipitated RNA with 25µl 70% ethanol. We conducted the subsequent RNA extraction
382 with the RNeasy Mini Kit (Qiagen), following the manufacturer's instruction. The resulting 28 samples (14
383 brains and 14 antennae) were stored at -80°C until library preparation.
384 RNA-seq libraries were prepared by Novogene Company Limited, Cambridge, UK, using the
385 NEBNext Ultra RNA Library Prep Kit for Illumina according to the manufacturer's protocol. After amplification
386 and purification, 28 libraries were sequenced on an Illumina NovaSeq 6000 S4 flow cell platform using a
387 paired-end 150 bp. Approximately 43 million raw reads were generated from each library (Dataset S3).
388
389 ***Gene expression analyses***

390 Raw data obtained from Novogene were checked using FastQC v.0.11.9 (122), and Illumina adapters were
391 removed using Trimmomatic v.0.36 (123). The protein-coding genes of *T. longispinosus*
392 (GCA_004794745.1; (124) and the congener *T. curvispinosus* (GCA_003070985.1) were retrieved from the
393 NCBI database and transferred to the recently published *T. longispinosus* genome (71) using the Liftoff
394 v.1.6.1 tool (125). In total, 10,029 of 13,061 (~77%) annotated protein-coding genes were transferred from
395 the original *T. longispinosus* assembly (genes identified as "DBV15") and 4,808 were transferred from *T.*
396 *curvispinosus* (genes identified as "LOC"), for a total of 14,837. For gene expression analysis, reads were
397 mapped to our *T. longispinosus* genome assembly, and the read counts table was generated using STAR
398 2.7.0 (126) with default settings. Detailed mapping statistics for each sample is available in Dataset S3. We
399 used the deseq2 v1.16.1 package to identify differentially expressed genes (127). To avoid biased results
400 due to low read counts, we removed from the counting matrix those genes for which less than 10 of the
401 reads mapped to at least 6 of our 14 samples (n - 1 of the smallest sample size). Then, we conducted a
402 differential gene expression analysis with DESeq2 (128). We began with comparisons between nurses and
403 foragers using the ~Colony+Task model, followed by a likelihood ratio test (LRT) approach, with colony ID
404 as a fixed factor. Genes were considered differentially expressed if the false discovery rate (FDR), using
405 Benjamini-Hochberg procedure, had an adjusted p-value of ≤ 0.05. The resulting lists of DEG refer to genes
406 that are overexpressed and underexpressed in foragers compared to nurses. We used the online tool Venny
407 v.2.1 (https://bioinfogp.cnb.csic.es/tools/venny) to generate a Venn diagram containing the DEG associated
408 with task and tissues. Separation of differentially expressed genes by task was visualized by performing
409 principal component analysis (PCA) on transformed reads of filtered transcriptomes from all contigs using
410 the plotPCA function provided by DESeq2. Finally, ORs genes that were upregulated in each behavioral
411 phenotype was visualized in a volcano plot using the EnhancedVolcano package in R.
412
413 ***Identification of behavior candidate genes and odorant receptors***
414 We used gene annotations based on a BlastX search of the *T. longispinosus* transcriptome compared to a
415 list of different invertebrate proteomes (i.e., *Acromyrmex echinatior, Apis mellifera, Camponotus floridanus,*
416 *Drosophila melanogaster, Harpegnathos saltator, Odontomachus brunneus, Temnothorax curvispinosus*)
417 downloaded from the NCBI database with an E-value of 1e-5 and below. Clusters containing more than one

sequence match per species were reduced to a single specimen based on the highest blast score. We constructed orthogroups across all of the above species using OrthoFinder (129), including amino acid sequences from the *T. longispinosus* proteome (124), and retained orthogroups containing caste DEG (Dataset S4) to again compare potential behavioral candidate genes previously identified as involved in regulating the division of labor in social insects (38, 41, 87, 93, 120, 130–133). GO enrichment analysis was performed with TopGO v.2.44.0 using a Fisher's exact test for the different gene sets compared to the whole genome with the weight01 algorithm (134). Only annotated GO terms with an adjusted p-value of ≤ 0.05 were considered significantly enriched. All enrichment analyses were performed in RStudio v.1.4.1106 (135).

Odorant receptor (OR) protein sets were clustered across multiple ant species using OrthoFinder to derive orthologous groups and identify subfamilies for each OR in *T. longispinosus*. To associate orthogroups with previously identified OR subfamilies, we used OR annotation in *Atta cephalotes, Acromyrmex echinatior* from Engsontia et al. (65), and *Camponotus floridanus*, *Harpegnathos saltator*, and *Solenopsis invicta* from Zhou et al. (64, 68). Missing subfamily information was labeled as "unassigned" (Dataset S2).

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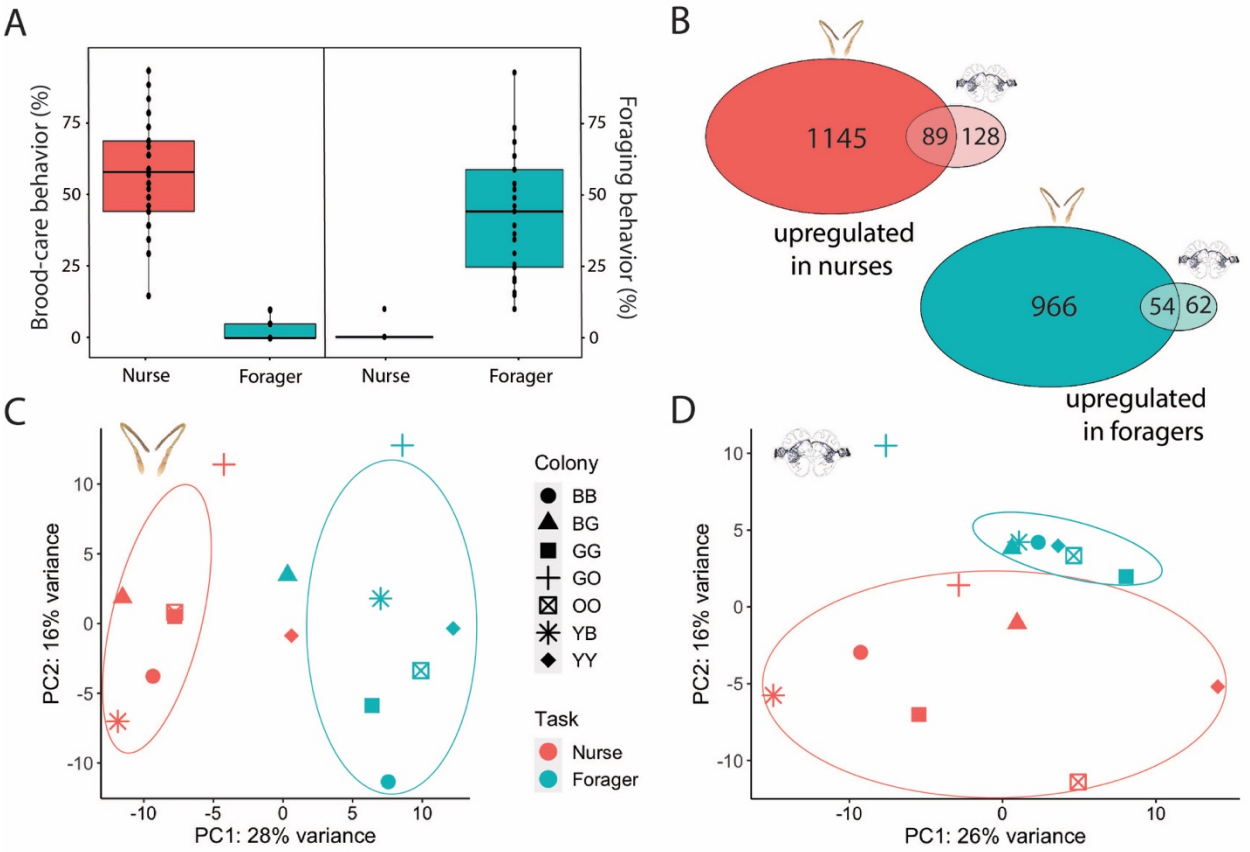
735 **Figures and Tables**



736

737 **Figure 1. Variation in behavior, brain and antenna gene expression between nurses and foragers.**
738 (*A*) Boxplot showing behavioral differences between ants selected for transcriptomic analysis. Each black
739 dots represent individual ants. For differential expression analysis, each sample contains the pooled RNA
740 of seven ants of the respective behavioral phenotype. (*B*) Venn diagrams showing the number of DEG that
741 were overexpressed in nurses (red) and foragers (turquoise) for both antenna and brain tissues. Principal
742 component analysis (PCA) plots based on all expressed genes for (*C*) antenna and (*D*) brain samples. The
743 color of each sample represents task (red = nurse, turquoise = forager), and the shape the colony of origin.
744 The ellipses were defined by a 95% confidence interval and show how similar/different the samples are
745 within task groups.

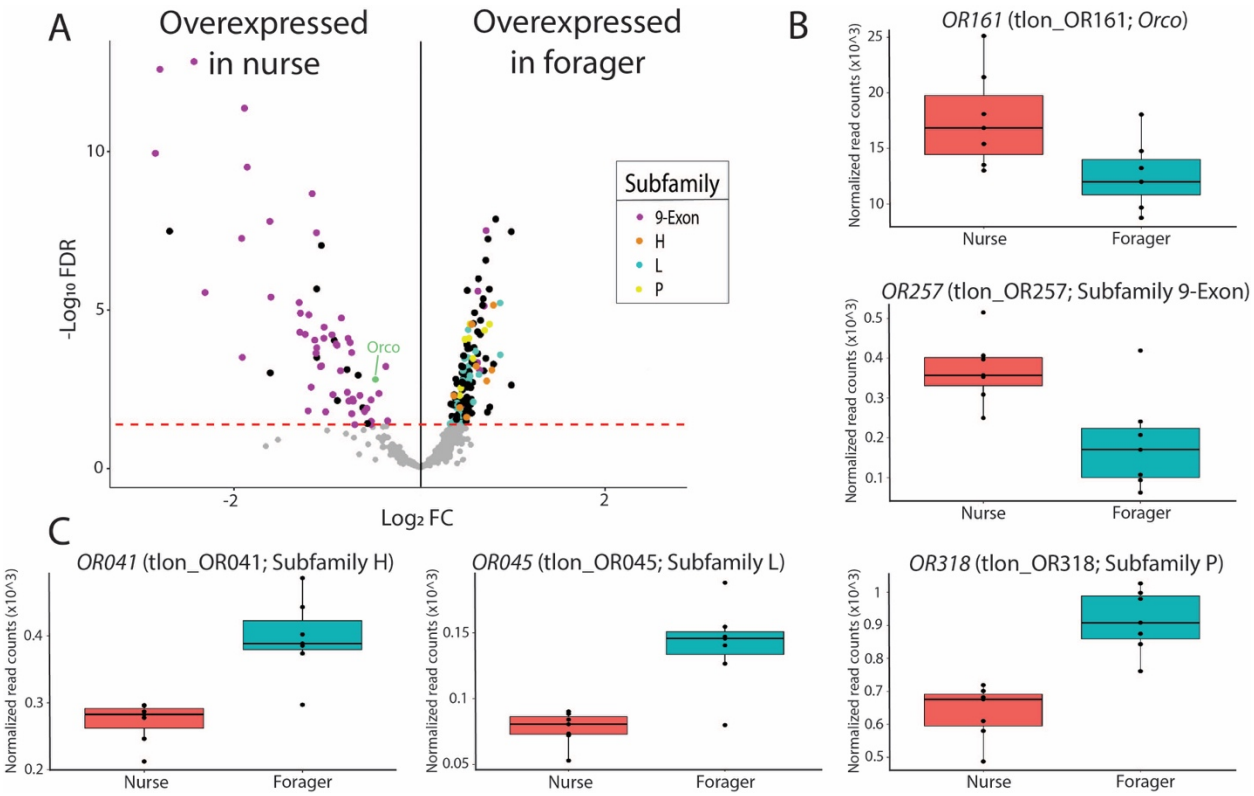
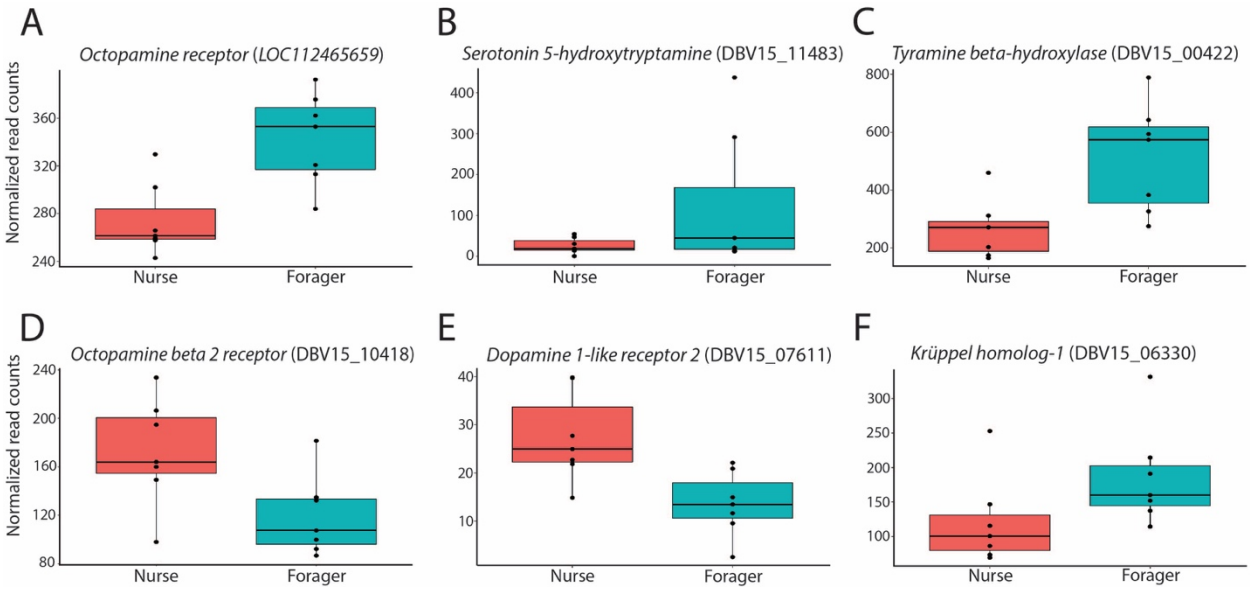


Figure 2. OR expression in the antennae differs between nurses and foragers. (*A*) Among the DEG overexpressed in nurses and foragers, the most represented OR subfamilies are indicated by different colors. The red dotted line represents the significance threshold of our differential expression analysis. (*B*,*C*). Boxplots representing the expression of (*B*) *Orco* and *OR257*, the gene with the highest expression difference in terms of $\log_2$FC in the 9-exon subfamily, and (*C*) *OR041*, *OR045* and *OR318*, the genes with the highest expression difference in terms of $\log_2$FC in the H, L and P subfamilies, respectively.



753

754 **Figure 3. Gene expression differences between nurse and forager antennae.** Boxplots representing
755 the expression of (*A*-*E*) selected genes in biogenic amines pathways, as well as (*F*) one behavioral
756 candidate gene (Supplementary Table S1). Gene identity is shown in parentheses.

Supporting Information

Table S1. List of candidate genes related to behavior phenotypes.

Gene Annotation	Locus (*T. longispinosus*)	Organism reference	Locus reference	Orthogroup	Task	Tissue	Pathways and genes	References
protein takeout isoform	DBV15_08771	*Camponotus floridanus*	LOC105257386	OG0007297	Nurse	Brain	JH: signalling	(1)
vitellogenin-1 (Vg conventional)	LOC112466671	*Harpegnathos saltator*	LOC105181726	OG0003549	Nurse	Brain	Vitellogenin	(1–4)
vitellogenin-2-like (Vg like-A)	DBV15_03138	*H. saltator*	LOC105191299	OG0009030	Nurse	Brain	Vitellogenin	(2, 3)
farnesyl pyrophosphate synthase	LOC112466955	*Drosophila melanogaster*	Fpps	OG0000401	Nurse	Brain	JH: biosynthesis	(5)
venom carboxylesterase-6 (Jhe1)	DBV15_11528	*H. saltator*	LOC112590013	OG0002262	Nurse	Brain/Antenna	JH: regulation of JH biosynthesis	(1, 3)
ribosomal protein S6	DBV15_00490	*D. melanogaster*	RpS6	OG0007546	Nurse	Brain/Antenna	IIS / TOR	(5)
dopamine 1-like receptor 2	DBV15_07611	*D. melanogaster*	Dop1R2	OG0001377	Nurse	Antenna	bioagenic amines	(6)
octopamine beta2 receptor	DBV15_10418	*D. melanogaster*	Octbeta2R	OG0000466	Nurse	Antenna	bioagenic amines	(6)

aldehyde dehydrogenase	DBV15_01763	*D. melanogaster*	Aldh-III	OG0000175	Nurse	Antenna	JH: biosynthesis	(5)
cyclin G	DBV15_03888	*D. melanogaster*	CycG	OG0003395	Nurse	Antenna	IIS / TOR	(5)
happyhour	DBV15_11653	*D. melanogaster*	hppy	OG0000352	Nurse	Antenna	IIS / TOR	(5)
insulin receptor isoform (InRL)	DBV15_10224	*H. saltator*	LOC105183944	OG0001487	Nurse	Antenna	IIS	(3)
Pi3K21B	DBV15_03081	*D. melanogaster*	Pi3K21B	OG0002238	Nurse	Antenna	IIS	(5)
Ras oncogene at 64B	DBV15_02802	*D. melanogaster*	Ras64B	OG0007075	Nurse	Antenna	IIS / TOR	(5)
sarcoplasmic calcium-binding protein 2	DBV15_05072	*D. melanogaster*	Scp2	OG0005773	Nurse	Antenna	JH: metabolism	(5)
shaggy	DBV15_09601	*D. melanogaster*	sgg	OG0000404	Nurse	Antenna	IIS / TOR	(3, 5)
allatostatin A	LOC112454443	*D. melanogaster*	AstA	OG0007104	Forager	Brain	JH: regulation of JH biosynthesis	(5)
insulin-like growth factor I (IGF1)	LOC112454447	*H. saltator*	LOC105186969	OG0004678	Forager	Brain/Antenna	IIS	(3, 4)
5-hydroxytryptamine (serotonin) receptor 2A	DBV15_11483	*D. melanogaster*	5-HT2A	OG0000533	Forager	Antenna	bioagenic amines	(6)
octopamine receptor in mushroom bodies	LOC112465659	*D. melanogaster*	Oamb	OG0001501	Forager	Antenna	bioagenic amines	(6)

tyramine beta hydroxylase	DBV15_00422	*D. melanogaster*	Tbh	OG0006646	Forager	Antenna	bioagenic amines	(6)
density regulated protein	DBV15_10283	*D. melanogaster*	DENR	OG0004331	Forager	Antenna	IIS / TOR	(5)
dreadlocks	DBV15_03530	*D. melanogaster*	dock	OG0001746	Forager	Antenna	IIS / TOR	(5)
juvenile hormone-inducible protein 26	DBV15_02220	*D. melanogaster*	JhI-26	OG0000832	Forager	Antenna	JH: regulation of JH biosynthesis	(5)
Krueppel homolog 1-like	DBV15_06330	*H. saltator*	LOC112589664	OG0008125	Forager	Antenna	JH: signalling	(3, 4)
rapamycin-insensitive companion of Tor	DBV15_07973	*D. melanogaster*	rictor	OG0003860	Forager	Antenna	IIS / TOR	(5)
SAPK-interacting protein 1	DBV15_07343	*D. melanogaster*	Sin1	OG0005622	Forager	Antenna	IIS / TOR	(5)

Table S2. List of enriched GO biological process terms of the DEG in the brain and the antenna.

Gene Set (DEG)	Tissue	GO ID	Term	Annotated	Significant	Expected	Fisher (adjusted *p*-value)
Nurse	Brain	GO:0006412	translation	73	19	1.79	0.000
Nurse	Brain	GO:0006879	cellular iron ion homeostasis	5	2	0.12	0.006
Nurse	Brain	GO:0009056	catabolic process	155	6	3.79	0.024
Nurse	Brain	GO:0030155	regulation of cell adhesion	1	1	0.02	0.025
Nurse	Brain	GO:0010040	response to iron (II) ion	1	1	0.02	0.025
Nurse	Brain	GO:0030245	cellulose catabolic process	1	1	0.02	0.025
Nurse	Brain	GO:0009204	deoxyribonucleoside triphosphate catabolic process	1	1	0.02	0.025
Nurse	Brain	GO:0006559	L-phenylalanine catabolic process	2	1	0.05	0.048
Nurse	Brain	GO:0006572	tyrosine catabolic process	2	1	0.05	0.048
Nurse	Brain	GO:0007160	cell-matrix adhesion	2	1	0.05	0.048
Nurse	Brain	GO:0006546	glycine catabolic process	2	1	0.05	0.048
Nurse	Brain	GO:0019236	response to pheromone	2	1	0.05	0.048
Nurse	Brain	GO:0030334	regulation of cell migration	2	1	0.05	0.048
Forager	Brain	GO:0006518	peptide metabolic process	89	4	0.96	0.001

Forager	Brain	GO:0006431	methionyl-tRNA aminoacylation	1	1	0.01	0.011
Forager	Brain	GO:0032481	positive regulation of type I interferon production	1	1	0.01	0.011
Forager	Brain	GO:0045454	cell redox homeostasis	2	1	0.02	0.022
Forager	Brain	GO:0046855	inositol phosphate dephosphorylation	4	1	0.04	0.043
Nurse	Antenna	GO:0006412	translation	73	22	8.84	0.000
Nurse	Antenna	GO:0006897	endocytosis	15	6	1.82	0.006
Nurse	Antenna	GO:0006006	glucose metabolic process	10	4	1.21	0.006
Nurse	Antenna	GO:0051726	regulation of cell cycle	9	3	1.09	0.014
Nurse	Antenna	GO:0015012	heparan sulfate proteoglycan biosynthetic process	3	2	0.36	0.040
Nurse	Antenna	GO:0006886	intracellular protein transport	63	13	7.63	0.047
Forager	Antenna	GO:0046854	phosphatidylinositol phosphorylation	6	4	0.57	0.001
Forager	Antenna	GO:0046855	inositol phosphate dephosphorylation	4	3	0.38	0.003
Forager	Antenna	GO:0005975	carbohydrate metabolic process	81	17	7.74	0.004
Forager	Antenna	GO:0045087	innate immune response	2	2	0.19	0.009
Forager	Antenna	GO:0009166	nucleotide catabolic process	4	3	0.38	0.025
Forager	Antenna	GO:0007229	integrin-mediated signaling pathway	9	3	0.86	0.045

Forager	Antenna	GO:0006166	purine ribonucleoside salvage	4	2	0.38	0.047

Table S3. Behaviors and positions annotations during nest scans. Nursing was defined as the number of observations a worker antennating, grooming, feeding or carrying a brood item, and its position inside the nest (on the brood or near to the brood pile). Foraging was defined as the number of observations an individual was found outside the nest, collecting food or water.

Position	
Inside the nest	On the brood pile
	Near the brood pile
	Surroundings of the nest
	Near the entrance
Outside the nest	Chamber 1
	Chamber 2
	Chamber 3
Behavior	
Brood care	Antennation, grooming, feeding or carrying the brood
Nestmate care	Antennation, grooming, feeding or carrying a nestmate
Forager	Collecting food/drinking water outside the nest
Others	Resting
	Walking
	Being groomed or fed
	Grooming itself

Dataset S1 (separate file). Gene list with caste-differential expression, NCBI BlastX results, and gene read count of 28 RNA-seq samples.

Dataset S2 (separate file). OR subfamilies and orthogroups from the DEG lists between nurses and foragers in the antenna.

Dataset S3 (separate file). Summary of ant brain and antenna transcriptome data sets and mapping results.

Dataset S4 (separate file). List of orthogroups from the DEG list between nurses and foragers in *Temnothorax longispinosus*.

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