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 suggest 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.

Keywords: social insects | transcriptomics | behavioral specialization | odorant perception

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

65 older individuals are more likely to undertake activities such as nest-defense and foraging
66 outside the nest [24,25]. Yet, such specialization among workers remains flexible, as
67 foragers can revert to perform brood care when needed [26,27].

68 Several molecular mechanisms have been implicated in the regulation of DOL.
69 Task specialization is associated with transcriptional changes in the worker brain [28–33].
70 Molecular pathways such as the insulin/insulin-like signaling (IIS), vitellogenin (Vg) and
71 juvenile hormone (JH) pathways are involved in the regulation of worker behavior [34–
72 39]. Functional manipulations have confirmed that the expression of key genes in the
73 worker brain controls task specialization [40–42]. Behavioral variation among workers is
74 also associated with signaling of biogenic amines (e.g., dopamine, octopamine, tyramine,
75 serotonin), which act as neurotransmitters or neuromodulators involved in the modulation
76 of the responsiveness to task-associated stimuli [43–46].

77 The self-organization and collective behavior in insect societies are maintained via
78 the exchange of chemical information [47]. Social insects communicate primarily through
79 glandular pheromones and complex mixtures of long-chain hydrocarbons on their cuticle.
80 These cuticular hydrocarbons (CHC) facilitate recognition of nestmates, developmental
81 stages, castes, sexes, and species [48,49]. Social insects perceive chemical information
82 via different types of sensilla on their antennae [50–52]. Decoding the identities of
83 chemical compounds relies on odorant receptors (OR) located within each sensillum
84 [53,54]. OR are transmembrane proteins expressed in the dendrites of olfactory receptor
85 neurons (ORN). The largely conserved OR coreceptor (Orco) of insects [55] is required
86 for odorant recognition in the dendritic membrane: it forms an ion channel with specific
87 OR, which determines the sensitivity and specificity of the ORN [56]. Odorant molecules

88 penetrate through the antenna cuticular pores and are transported by odorant-binding
89 proteins to the ORN membrane, where they interact with receptors, leading to the
90 generation of action potentials [57,58]. ORN axons relay signals from the sensilla to the
91 glomeruli of the antennal lobes in the insect brain, which are the first processing unit for
92 olfactory information. Then, ORN make synaptic contact with the projection neurons and
93 local neurons, which transfer information to the central brain [59–61].

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

103 Current DOL models bring together the chemical nature of social insect
104 communication and variation among workers in their response to chemical cues. They
105 posit that flexible response thresholds to task-related chemical stimuli serve as regulators
106 of worker specialization [74]. Workers take on a particular task when the stimulus intensity
107 exceeds their individual threshold for this task. Therefore, individuals with lower
108 thresholds for a given task are more likely to perform it than those with higher thresholds
109 [75]. Individual response decisions and task performance are modulated via numerous

110 parameters on different time scales [7,76–78], and despite extensive research on DOL
111 and task allocation, these mechanisms are not fully understood.

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

Results

Identification of *Temnothorax longispinosus* behavioral phenotypes

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

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

To investigate transcriptomic variation between nurses and foragers, we used RNA-seq to generate seven nurse and seven forager brain and antenna samples, each consisting of pooled tissue from seven workers of a single colony. Of the 14,837 protein-coding genes annotated in the *T. longispinosus* genome, we found that 91% (13,494) and 92% (13,683) were expressed (FPKM > 0) in the brain and the antennae, respectively. Using an adjusted p-value of 0.05 as significance threshold, we detected 333 differentially

154 expressed genes (DEG) in the brain (217 upregulated in nurses, 116 in foragers), and
155 2,254 in the antennae (1,234 upregulated in nurses, 1,020 in foragers; Dataset S1; Fig
156 1*B*). We found an overlap of 162 DEG between the two tissues, including 143 DEG that
157 showed differences in the same direction across tissues. Principal component analysis
158 (PCA) plots indicate that both brain and antenna samples cluster by behavioral phenotype
159 rather than the colony of origin (Fig 1 *C*-*D*).
160

161 OR gene expression differs between antennae of nurses and 162 foragers

163 To investigate whether odorant perception differs between nurses and foragers, we
164 focused our attention on the expression of OR genes in the antennae. We found that all
165 419 OR genes in the genome of *T. longispinosus* [71] were expressed in the antennae,
166 and that 48% (202/419) of them were differentially expressed between nurses and
167 foragers. Specifically, 65 OR genes were upregulated in nurses (15% of all OR genes),
168 and 137 in foragers (32% of all ORs) (Fig 2). Then, we studied which OR subfamilies
169 were preferentially expressed in nurses and foragers. The 65 OR genes overexpressed
170 in nurses belonged to three OR subfamilies, while the 137 OR genes upregulated in
171 foragers were distributed among 19 OR subfamilies. Foragers overexpressed 26, 8, and
172 8 OR genes from the L, P, and H subfamilies, respectively, while no OR genes from these
173 subfamilies were upregulated in nurses (Dataset S2). We found that 61% (26/43) of the
174 genes from the L subfamily were overexpressed in foragers, which represents a
175 significant overrepresentation (Fisher's test, odds ratio = 3.63, p-value < 0.001). For the
176 P and H subfamilies we did not find such an overrepresentation, likely due to lower gene

177 numbers (P subfamily: Fisher's test, odds ratio = 3.62, p-value = 0.06; H subfamily:
178 Fisher's test, odds ratio = 1.87, p-value = 0.19). On the other hand, we found that 82%
179 (53/65) of the OR genes overexpressed in nurses belong to the 9-exon subfamily. This
180 results in an overrepresentation of the 9-exon subfamily in genes that were
181 overexpressed in nurses (Fisher's test, odds ratio = 20.98, p-value < 0.001), with 46%
182 (53/114) of this subfamily being overexpressed in nurses. In contrast, only 6% (8/137) of
183 the 9-exon subfamily was overexpressed in foragers, which is less than expected by
184 chance (Fisher's test, odds ratio = 0.10, p-value < 0.001). We also found that nurses
185 overexpressed *Orco* compared to foragers (FDR p-value = 0.001).
186

187 Genes in biogenic amine pathways vary in expression
188 between nurses and foragers

189 Because biogenic amines are implicated in the regulation of behavior, and they may affect
190 sensory perception [45,81–83], we screened our lists of DEG in the brain and antennae
191 for genes in biogenic amine pathways. We found five genes associated with biogenic
192 amine signaling in the antennae, and none in the brain. Genes in the serotonin (*5-*
193 *hydroxytryptamine*, DBV15_11483), tyramine (*tyramine beta-hydroxylase*,
194 DBV15_00422) and octopamine (*octopamine receptor* LOC112465659) pathways were
195 upregulated in foragers, while nurses showed a higher expression of genes in the
196 dopamine (*dopamine 1-like receptor 2*, DBV15_07611) and octopamine (*octopamine*
197 *beta2 receptor*, DBV15_10418) pathways (Fig 3 *A*-*E*).
198

199 **Association between behavioral variation and molecular**
200 **pathways in brain and antennae**

201 Our analysis revealed expression differences between the brains of nurses and foragers
202 for genes involved in the regulation of task specialization in social insects. Previous
203 studies have found that *Vg* genes and the associated JH and IIS/TOR pathways are
204 important endocrine networks that play central roles in the regulation of lifespan, fertility
205 and behavior in bees and ants [35,84–89]. Therefore, we searched our RNA-seq data for
206 task-associated expression of genes involved in the metabolism, biosynthesis, and
207 regulation of these pathways in the brain and antennae. We found evidence that multiple
208 genes from these pathways are differentially expressed between nurses and foragers
209 (Table S1). Among the most interesting genes upregulated in the brain of nurses were
210 *conventional Vg* (LOC112466671), *Vg-like A* (named/classified as per [42];
211 DBV15_03138), *venom carboxylesterase-6-like* (DBV15_11528), and *protein takeout*
212 (DBV15_08771), while *allatostatin A-like* (LOC112454443) and *insulin-like growth factor*
213 *I* (*IGF1*) (LOC112454447) were overexpressed in the brain of foragers. Remarkably,
214 *venom carboxylesterase-6-like* and *IGF1* were overexpressed in the antennae of nurses
215 and foragers respectively. Additionally, high expression levels of the zinc-finger
216 transcription factor *Krüppel homolog-1* (*Kr-h1*) were detected in the antennae of foragers
217 (DBV15_06330) (Fig 3*F*). The expression of this gene has been correlated with caste and
218 behavioral differences in the brain of social insects [86,90–93], but little is known about
219 its function and gene targets in the antennae.

220 We performed a GO enrichment analysis to gain a deeper understanding of the
221 biological processes represented in the lists of DEG. This analysis detected many

222 enrichments based on single one or few genes, and we mention below a few interesting
223 processes with the number of genes driving the enrichment in parentheses (see Table S2
224 for complete list). Genes that were upregulated in the brain of nurses were enriched for
225 biological processes such as *translation* (19 genes), *cellular iron ion homeostasis* (2
226 genes), and *catabolic process* (6 genes); while *translation* (22 genes), *endocytosis* (6
227 genes), *glucose metabolic process* (4 genes), and *regulation of cell cycle* (3 genes) were
228 enriched in the antennae. In the list of genes that were overexpressed in the brain of
229 foragers, we found that enriched processes included *peptide metabolic process* (4
230 genes), *methionyl-tRNA aminoacylation* (1 gene), *positive regulation of type I interferon*
231 *production* (1 gene), and *cell redox homeostasis* (1 gene); while *phosphatidylinositol*
232 *phosphorylation* (4 genes), *inositol phosphate dephosphorylation* (3 genes), *carbohydrate*
233 *metabolic process* (17 genes), *innate immune response* (2 genes), and *nucleotide*
234 *catabolic process* (3 genes) were enriched in the antennae.

235 # Discussion

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

252 Many organisms have evolved sensory filters to focus on only a subset of all
253 environmental cues. In insects, the peripheral olfactory filtering system enables
254 individuals to detect and discriminate odors that convey ecologically relevant information
255 used to mediate important behaviors such as courtship, locomotion and navigation to
256 avoid predators and locate food or nesting sites [94]. For example, the mosquito
257 *Anopheles gambiae* strongly responds to odorant components of its vertebrate hosts that

258 provide meals [95]. Sensory filters have been selected for because they limit the amount
259 of information perceived by the organism, and thus the energy and time required by the
260 brain to process it. Within insect colonies, individuals typically exhibit many morphological
261 and physiological traits associated with increased efficiency in task specialization. They
262 can be understood as adaptations that allow individuals to become more proficient at their
263 task due to learning, training, or the perceiving valuable task-related information, allowing
264 the colony to avoid the cost of task switching [96]. In this context, it is interesting to find
265 that *T. longispinosus* workers may have different sensory filters that would serve as a
266 basis for their task specialization, as workers would mostly perceive chemical cues that
267 pertain to their tasks. Our findings indicate that the sensory filter of ant workers is
268 dynamic, and its changes may underlie their behavioral maturation.

269 Age, genetic background, social environment, and individual experience produce
270 behavioral variation among workers that results in division of labor [13,20,23,97]. In our
271 study, age in particular likely differed between nurses and foragers. However, previous
272 experiments designed to disentangle gene expression associated with behavioral
273 specialization, age and fertility showed that behavioral specialization is more strongly
274 associated with gene expression than age and fertility in *T. longispinosus* [38]. We
275 propose that the factors known to regulate task specialization could drive different OR
276 expression patterns, which in turn would produce behavioral variation via contrasting
277 abilities to detect different sets of odors. This hypothesis is supported by several studies
278 (reviewed in [98]) showing that the physiological condition of an animal can influence the
279 level of receptor expression, including mating status, oviposition, feeding, circadian
280 rhythm, experience, and aging. Alternatively, we cannot exclude that the exposure to

281 different odors that is associated with performing different tasks may at least in part have
282 affected OR gene expression in the antennae. However, such an effect is unlikely to
283 explain the large-scale variation in gene expression, as there is very limited evidence that
284 the mere exposure to odors influences the expression of the gene coding for the OR that
285 binds this odor [99,100].

286 According to our sensory filter hypothesis, ant workers would be expected to
287 primarily detect chemical cues that correspond to their tasks. We found that the 9-exon
288 subfamily of OR was overrepresented in genes that were upregulated in the antennae of
289 nurses. Interestingly, recent studies have shown a rapid expansion of the 9-exon
290 subfamily in ants, and several lines of evidence indicate that OR genes from this subfamily
291 mediate complex social interactions in ant colonies [65–67,69]. First, comparisons of
292 antennal transcriptomes revealed that 9-exon OR genes are expressed more frequently
293 in workers than in males, suggesting a role in social communication among workers
294 [68,69]. Second, representative OR from the 9-exon subfamily can detect CHC extracts
295 from several castes [70]. According to McKenzie et al. [69], 9-exon OR genes were first
296 expressed in solitary ancestors of aculeate wasps and facilitated CHC discrimination,
297 likely for prey or mate recognition, with a lineage giving rise to the ancestors of ants.
298 Moreover, OR genes of the 9-exon subfamily were convergently lost in socially parasitic
299 ant species that lost the ability to perform brood care or foraging [71], suggesting that they
300 are essential for the performance of these worker tasks. Our finding of an overexpression
301 of 9-exon OR genes in the antennae of nurses is in line with these previous studies, and
302 suggests that many of these receptors have important functions within the nest, such as
303 sensing chemical cues from the larvae, queen, or other workers, and/or that they are less

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

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

Finding task-specific variation in OR gene expression raises the question as to which molecular mechanisms regulate those changes. Previous studies in insects

327 revealed that olfaction-guided behavior is mediated by biogenic amine receptors in the
328 antenna, and their expression is involved in fine-tuning the sensitivity of the olfactory
329 system [82,98]. For example, modified concentration cAMP and intracellular Ca^{2+} levels
330 due to octopamine-induced signal transduction in the moth *Manduca sexta* [106] activate
331 Orco, leading to changes in ORN sensitivity [107,108]. Our results suggest that the
332 biogenic amine signaling pathway may modulate the sensory filtering function of insect
333 antennae and alter sensitivity to various signals. We found that genes encoding tyramine
334 and its precursor, octopamine, are upregulated in forager antennae, similar to genes
335 involved in serotonin signaling. Tyramine and dopamine (which was upregulated in
336 nurses) have been implicated in modulating taste and olfactory receptor neurons, while
337 serotonin may serve as a neurotransmitter and neurohormone in antennal vessels and
338 mechanosensory organs [82]. Serotonin influences foraging activity [81] and regulates
339 food intake in many animals [109–111]. Dopamine signaling also plays an important role
340 in controlling the insect circadian clock and mediating clock-controlled behavioral
341 phenotypes such as locomotion [112,113]. In our focal species *T. longispinosus*, inside
342 workers were found to exhibit a stronger circadian rhythmicity than foragers, which may
343 be regulated via differences in the acetylation of histone proteins [114].

344 Changes in behavior and olfactory sensitivity in insects could be related to the
345 expression of genes involved in IIS, target of rapamycin (TOR), JH and Vg pathways,
346 according to age, circadian rhythm, mating and feeding status [98]. For example, appetite
347 state in *D. melanogaster* is signaled by insulin, which upregulates a peptide receptor on
348 the olfactory receptor cells that innervate the DM1 glomerulus. Activation of the DM1
349 glomerulus is enough to drive the fly to reach for food [115]. Recent studies have shown

350 that pheromone release and odor sensitivity appear to be under JH control in
351 *Schistocerca gregaria* and *Locusta migratoria*, which could lead to behavioral changes
352 [116–120]. Finally, an experimental downregulation of *Vg-like A* in *T. longispinosus*
353 workers resulted in decreased brood care behavior and a lower sensitivity to brood-
354 related chemical cues, suggesting changes in odor perception and olfactory-driven
355 decision making [42]. Our results reveal that genes associated with all these pathways
356 were differentially expressed in the brain and antennae between nurses and foragers,
357 predicting a link between their role in task-associated behavioral changes and the
358 regulation of odor perception. Given the central role of IIS, Vg, JH, and TOR pathways in
359 regulating division of labor in social insects, and our finding of task-associated patterns
360 of the antennal expression of genes from multiple biogenic amines, we hypothesize that
361 these modulators and hormones could be involved in the regulation of the olfactory filter.
362 How the detailed molecular mechanisms of these pathways in the brain are causally
363 linked to the complex changes in olfactory perception in the antennae should therefore
364 be investigated next.
365

366 # Conclusion

367 Our transcriptomic analyses of the brain and antennae of *T. longispinosus* nurses and
368 foragers provide support to our hypothesis that behavioral variation and task
369 specialization in ant workers are regulated via differences in olfactory perception. We
370 predict that antennal physiology acts as sensory filters that limit the type and amount of
371 chemical information passed to the brain. This would allow workers to target relevant
372 chemical information from the environment and discriminate signal from noise without

373 using energetically costly processing by the central nervous system. We argue that this
374 sensory filter is flexible and regulated through changes in physiological conditions such
375 as age, nutrition, and hormones. Variation among workers in their efficiency to detect
376 specific chemical cues would result in task specialization and division of labor. Our study
377 opens novel avenues of research to better understand the role of sensory filters in
378 controlling DOL in insect societies.

Materials and Methods

Sample collection and behavioral determination

A total of seven colonies of the ant *T. longispinosus* were selected with an average colony size of 110 ± 31.5 workers (mean ± SD, Dataset S3). The ants were collected in the forests of the Edmund Niles Huyck Preserve, Renssellearville, NY, USA (42°31′41.0″N 74°09′38.8″W), in June of 2018 with permission. Upon collection, we housed each colony in a plaster-floored nesting box (43 cm × 28 cm × 10 cm) divided into three chambers containing a single slide nest, in which the colony relocated. A slide nest is an artificial nesting site comprised of a small Plexiglas cavity sandwiched between two glass microscope slides. Colonies were established at the Johannes Gutenberg University in Mainz, Germany, under a 14 h:10 h light:dark photoperiod at 18°C to a 22°C temperature. We provided honey and water ad libitum and fed crickets to the colony twice a week. To allow for visible behavioral division of labor between workers of the two behavioral phenotypes, we marked, observed and recaptured ants from inside and outside the nest. We defined foragers as workers that perform outside-nest tasks, including foraging for food, while nurses remained inside the dark nest and cared for the ant brood. A total of 69 workers inside (from the brood pile) and 76 workers outside the nest were marked with fine colored metal wires (0.02 mm Elektrisola, Eckenhagen, Germany) between the petiole and post-petiole. We performed behavioral observations every two hours, four times a day for five days (total = 20 scans), in which we noted down how many times an individual spent performing brood care and foraging behavior and the position in the nest (Table S3). Based on these behavioral observations, the marked individuals found outside the nest, exploring the surroundings for food or water, were identified as foragers.

402 These workers usually do not care for the brood and do not frequently reside on brood
403 piles, as ant colonies organize themselves spatially in a way that reduces contact
404 between foragers and brood [121]. We identified nurses as workers that remain inside the
405 nest in direct contact with brood and were unlikely to leave the nest. Foragers were found
406 in 42% ± SD 26% of the observations outside of the nest, whereas nurses spend only 1%
407 ± SD 3% outside. In contrast, nurses were interacting with the brood in 54% ± SD 22% of
408 the observations, whereas we found that foragers only did this only in 2% ± SD 4% of the
409 observations. We scanned the behavior of workers over 20 observations, albeit earlier
410 studies have shown that a single observation allows to group *T. longispinosus* and other
411 ants reliably into nurses and foragers that differ in behavior [121,122], gene expression
412 [38] and CHC composition [42]. Furthermore, spatial location can alone can predict
413 behavior in *Temnothorax* workers [123]. We focused in this study on individuals highly
414 specialized on either foraging or brood care. Workers that performed both tasks regularly
415 were not included in this study. After all observations were completed, the marked nurses
416 and foragers were collected, directly frozen in liquid nitrogen and stored at -80 °C until
417 further processing for dissection and pooling according to behavioral state and colony.
418

419 RNA extraction and sequencing

420 For RNA extraction, we removed both antennae and stored them in a 1.5 ml Eppendorf
421 tube containing 50 µl TRIzol (Invitrogen), cut the head off and fixed it on a slide with
422 melted dental wax. We then made an incision around the head with a surgical scalpel and
423 removed the head capsule with forceps to expose the intact brain. Finally, we carefully
424 pulled the brain out of the head capsule and removed the remains of other tissues that

425 were connected to it. The dissected brain was transferred to a 1.5 ml Eppendorf tube
426 containing 20µl PBS. Each dissection was completed in less than 5 minutes to prevent
427 RNA degradation. We dissected brain and antennae tissues from 48 nurses and 49
428 foragers. We pooled the brains and antennae from seven workers from each behavioral
429 state and colony. The only exception was the "GO" colony (NY18 E110), for which we
430 pooled only six brains and twelve antennae from six nurses (Dataset S3) due to the loss
431 of one sample during the dissection process. Immediately after dissection of each brain
432 and antennae, the Eppendorf tubes were kept on dry ice while we dissected the remaining
433 individuals. Brain and antennae tissues were homogenized with a pestle. Sample brains
434 were transferred separately to a 1.5 ml Eppendorf tube containing 50µl of TRIzol. We
435 added 50µl chloroform to each brain and antenna samples, cautiously inverted for 30 s
436 and then centrifuged samples at 12,000 g for 15 min at 4°C. We collected the resulting
437 supernatant and precipitated RNA with 25µl 70% ethanol. We conducted the subsequent
438 RNA extraction with the RNeasy Mini Kit (Qiagen), following the manufacturer's
439 instruction. The resulting 28 samples (14 brains and 14 antennae) were stored at -80°C
440 until library preparation.

441 RNA-seq libraries were prepared by Novogene Company Limited, Cambridge, UK,
442 using the NEBNext Ultra RNA Library Prep Kit for Illumina according to the manufacturer's
443 protocol. After amplification and purification, 28 libraries were sequenced on an Illumina
444 NovaSeq 6000 S4 flow cell platform using a paired-end 150 bp. Approximately 43 million
445 raw reads were generated from each library (Dataset S3).

446

447 **Gene expression analyses**

448 Raw data obtained from Novogene were checked using FastQC v.0.11.9 [124], and
449 Illumina adapters were removed using Trimmomatic v.0.36 [125]. The protein-coding
450 genes of *T. longispinosus* (GCA_004794745.1; [126] and the congener *T. curvispinosus*
451 (GCA_003070985.1) were retrieved from the NCBI database and transferred to the
452 recently published *T. longispinosus* genome [71] using the Liftoff v.1.6.1 tool [127]. In total,
453 10,029 of 13,061 (~77%) annotated protein-coding genes were transferred from the
454 original *T. longispinosus* assembly (genes identified as "DBV15") and 4,808 were
455 transferred from *T. curvispinosus* (genes identified as "LOC"), for a total of 14,837. For
456 gene expression analysis, reads were mapped to our *T. longispinosus* genome assembly,
457 and the read counts table was generated using STAR 2.7.0 [128] with default settings.
458 Detailed mapping statistics for each sample is available in Dataset S3. We used the
459 deseq2 v1.16.1 package to identify differentially expressed genes [129]. To avoid biased
460 results due to low read counts, we removed from the counting matrix those genes for
461 which less than 10 of the reads mapped to at least 6 of our 14 samples (n - 1 of the
462 smallest sample size). Then, we conducted a differential gene expression analysis with
463 DESeq2 [130]. We began with comparisons between nurses and foragers using the
464 ~Colony+Task model, followed by a likelihood ratio test (LRT) approach, with colony ID
465 as a fixed factor. Genes were considered differentially expressed if the false discovery
466 rate (FDR), using Benjamini-Hochberg procedure, had an adjusted p-value of ≤ 0.05. The
467 resulting lists of DEG refer to genes that are overexpressed and underexpressed in
468 foragers compared to nurses. We used the online tool Venny v.2.1
469 (https://bioinfogp.cnb.csic.es/tools/venny) to generate a Venn diagram containing the
470 DEG associated with task and tissues. Separation of differentially expressed genes by

task was visualized by performing principal component analysis (PCA) on transformed reads of filtered transcriptomes from all contigs using the plotPCA function provided by DESeq2. Finally, ORs genes that were upregulated in each behavioral phenotype was visualized in a volcano plot using the EnhancedVolcano package in R.

Identification of behavior candidate genes and odorant receptors

We used gene annotations based on a BlastX search of the *T. longispinosus* transcriptome compared to a list of different invertebrate proteomes (i.e., *Acromyrmex echinatior, Apis mellifera, Camponotus floridanus, Drosophila melanogaster, Harpegnathos saltator, Odontomachus brunneus, Temnothorax curvispinosus*) 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 [131], including amino acid sequences from the *T. longispinosus* proteome [126], 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 [38,41,87,93,120,132–135]. 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 [136]. 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 [137].

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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Data. RNAseq data that support the findings of this study have been deposited in GenBank with the BioProject accession codes PRJNA926589 (http://www.ncbi.nlm.nih.gov/bioproject/926589).

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Figures and Tables

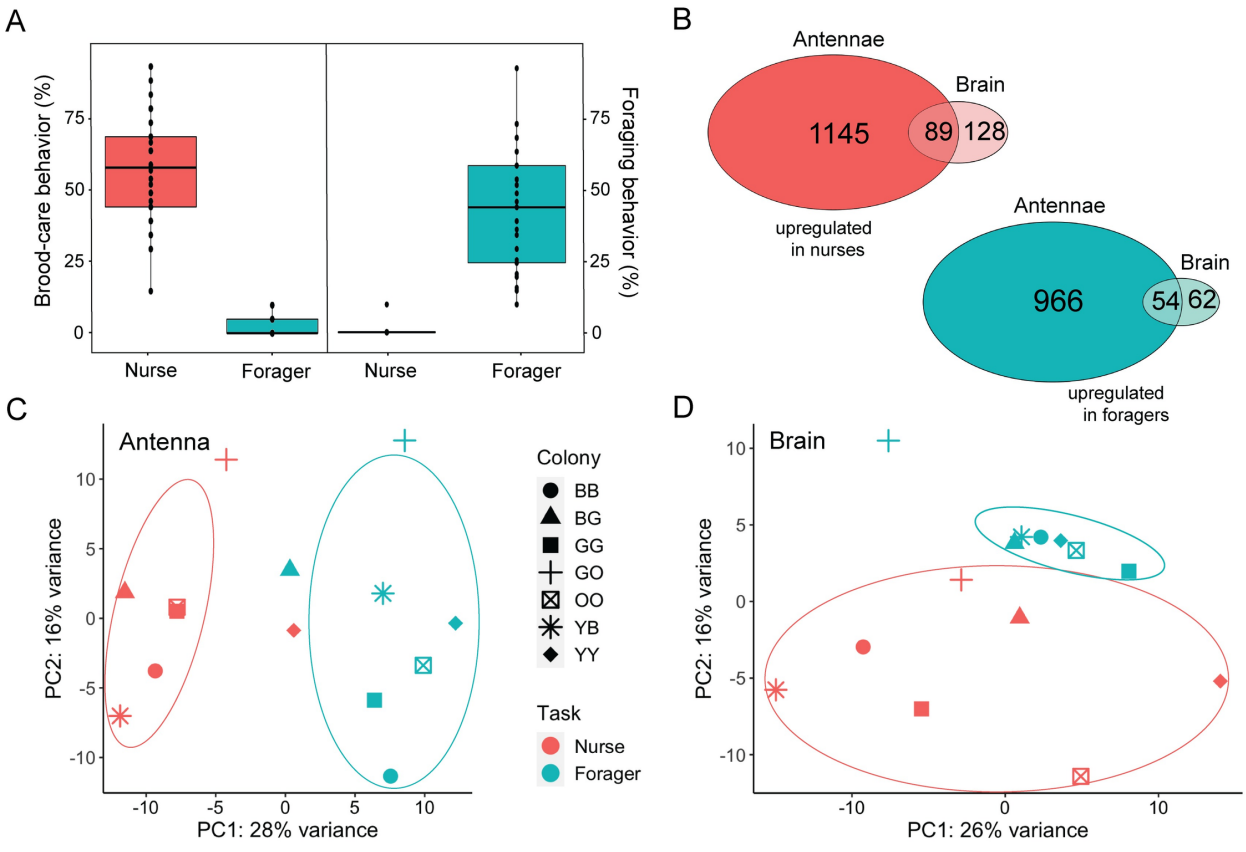


Fig 1. Variation in behavior, brain and antenna gene expression between nurses and foragers. (*A*) Boxplot showing behavioral differences between ants selected for transcriptomic analysis. Each black dots represent individual ants. For differential expression analysis, each sample contains the pooled RNA of seven ants of the respective behavioral phenotype. (*B*) Venn diagrams showing the number of DEG that were overexpressed in nurses (red) and foragers (turquoise) for both antenna and brain tissues. Principal component analysis (PCA) plots based on all expressed genes for (*C*) antenna and (*D*) brain samples. The color of each sample represents task (red = nurse, turquoise = forager), and the shape the colony of origin. The ellipses were defined by a

95% confidence interval and show how similar/different the samples are within task groups.

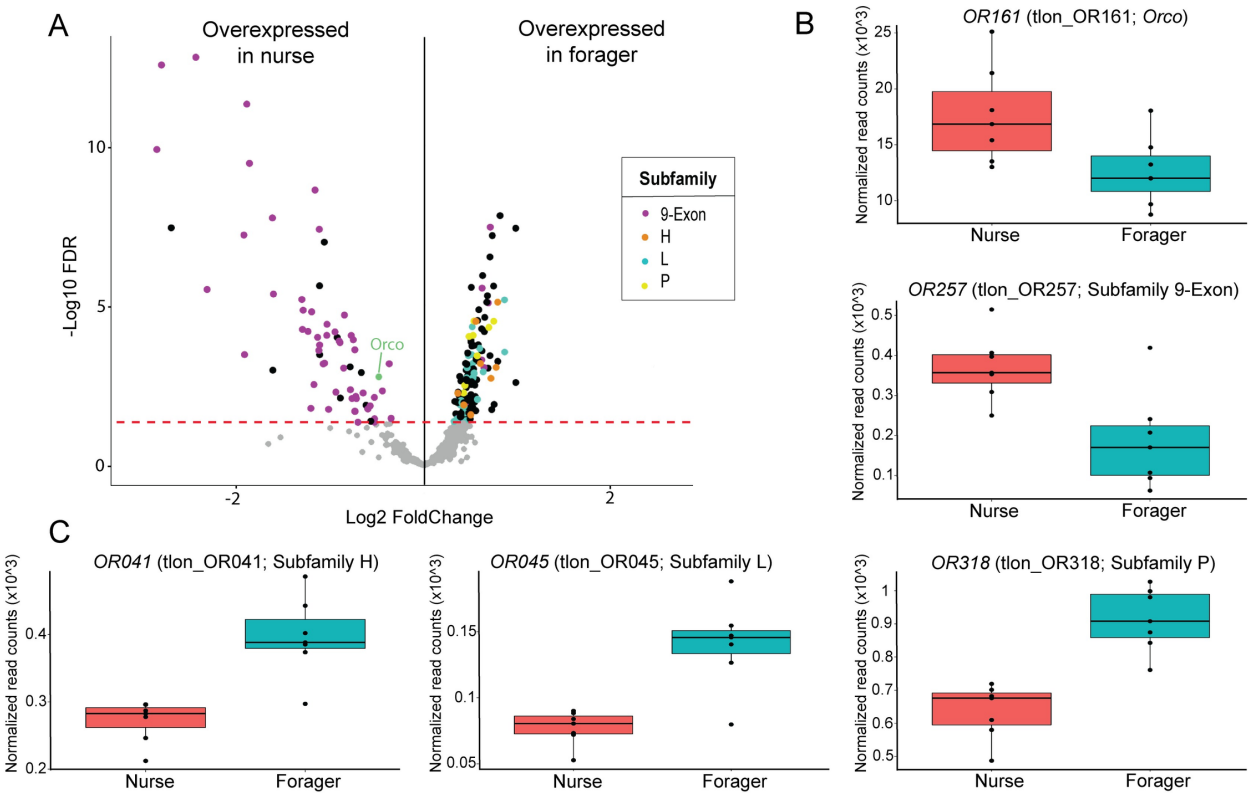
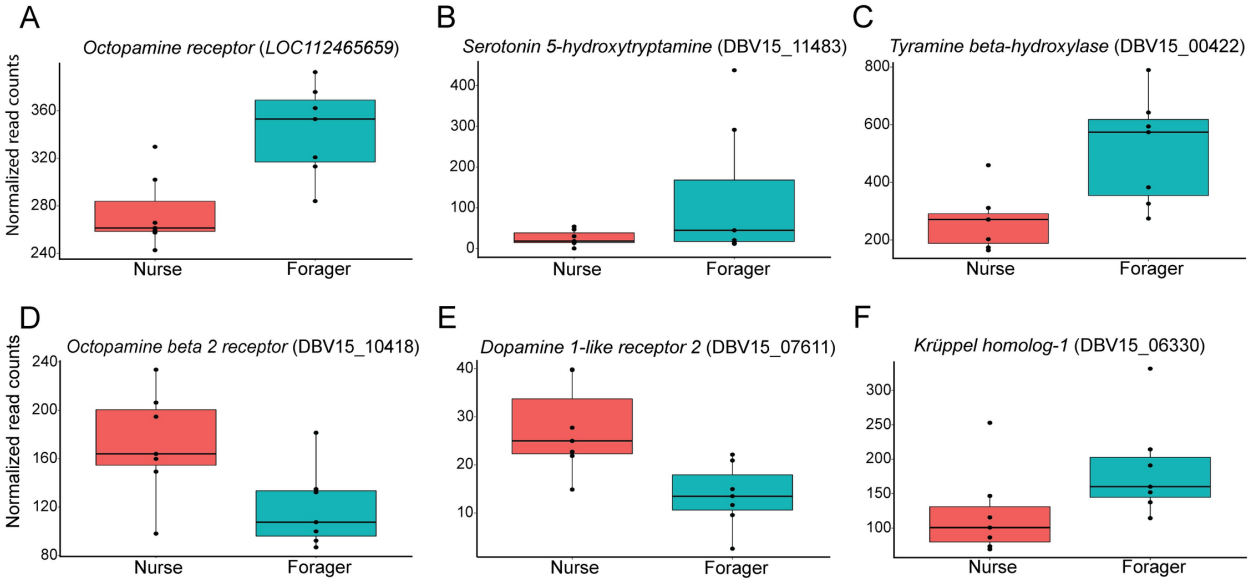


Fig 2. OR expression in the antennae differs between nurses and foragers. (*A*) Among the DEG overexpressed in nurses and foragers, the most represented OR subfamilies are indicated by different colors. The red dotted line represents the significance threshold of our differential expression analysis.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



892

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

897 # Supporting Information

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

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

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

906 **Dataset S1. Gene list with caste-differential expression, NCBI BlastX results, and**
907 **gene read count of 28 RNA-seq samples.**

908 **Dataset S2. OR subfamilies and orthogroups from the DEG lists between nurses**
909 **and foragers in the antenna.**

910 **Dataset S3. Summary of ant brain and antenna transcriptome data sets and**
911 **mapping results.**

912 **Dataset S4. List of orthogroups from the DEG list between nurses and foragers in**
913 ***Temnothorax longispinosus***.