

Leaf-cutter ant species (Hymenoptera: *Atta*) differ in the types of cues used to differentiate between self and others

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Hernández *et al.* Worker recognition in *Atta*.

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Nestmate recognition among most social insects is thought to be mediated by cuticular hydrocarbons. Here we show that among two closely related ant species, the chemical cues used for recognition vary between them and that they are not cuticular hydrocarbons. Experiments with free living colonies of *Atta laevigata* and *Atta cephalotes* using live and dead dummies, some impregnated with glandular extracts, allowed us to reconstruct the cues by which *A. laevigata* differentiates itself from *A. cephalotes* workers. The results suggest that the same odor cues (chemicals from the alarm pheromones and from abdominal exocrine secretions) are used for both inter- and intra-specific recognition systems, achieving discrimination of self, i.e. a nestmate, from others, and that both species differ in the chemical nature of the odor cues used for recognition. Our results suggest that recognition mechanisms vary among ants, and may thus vary among other social insects, and are shaped by various evolutionary forces in addition to kin selection.

42
43 The working of kin selection is often thought to depend on efficient mechanisms of kin
44 recognition. Yet we ignore how the recognition mechanisms work for most animals and if they
45 allow for kin recognition. Among social insects, which show the most cohesive societies
46 among animals, we should expect evolution to have shaped sophisticated kin recognition
47 mechanisms, but recognition mechanisms might also fulfill other socially relevant functions,
48 such as detecting potentially dangerous intruders. In ants, recognition between (intra-specific)
49 and within species (inter-specific) is important for the cohesiveness of a colony and the
50 effective exclusion of foreigners. They are mostly based on chemical cues, but the
51 mechanisms underlying these abilities are poorly known. For leaf cutter ants, contradictory
52 reports exist: Spencer (1894), describing the work of J. H. Hart, reported the acceptance of
53 leaf-cutting ants by colonies of the same species; however, aggression between ants of
54 different species of leaf-cutting ants is known to occur (Autori 1941; Mariconi 1970; Rockwood
55 1973; Fowler 1977, Whitehouse & Jaffe 1996). One of the functions of intra-specific and inter-
56 specific aggression may be defense and enlargement of the territory to secure food for the
57 colony, such as reported for wood ants (Mabelis 1984). Other functions might be to detect
58 predators and parasites. Whatever the function, con-specific and inter-specific aggression has
59 to be based on an ability to discriminate self from others, yet little is known about how ants
60 achieve inter-specific discrimination.

61
62 Intercolony discrimination (intra-specific recognition systems) has been studied more
63 extensively than inter-specific recognition. For intra-specific recognition, different mechanisms
64 based on cephalic odor cues (Jaffe 1983; 1987; Jaffe & Sanchez 1984; Whitehouse & Jaffe
65 1995; Hernández *et al.* 2002) have been postulated for ants. Most of the reports by other
66 authors, however, suggest a role for cuticular hydrocarbons as nestmate recognition cues
67 (Vander Meer *et al.* 1997, Lahav *et al.* 1999, Katzav *et al.* 2004, Howard & Blomquist 2005),
68 including for species recognition among *Cataglyphis* ants (Oldham *et al.* 1999), the group for
69 which recognition systems have been most extensively studied. For some ants, however,
70 especially for leaf-cutters, the chemicals in the alarm pheromone seem to work as nestmate
71 recognition cues (see below). A switch in the nestmate recognition system of ants based on
72 cuticular hydrocarbons or other odor cues widely distributed on the body, to the use of volatile

73 cues in the alarm pheromone, seems to be related to an evolutionary of agonistic behaviors
74 that include territorial marking behavior (Jaffe1987). That is, in “primitive ants” (ants with
75 simple chemical communication systems) odors produced by several glands are used as cues
76 for nestmate recognition (Jaffe & Marcuse (1983); whereas among species with complex
77 societies, the chemicals of the colony-specific alarm pheromones are used as recognition
78 cues; e.g., *Atta cephalotes* (Jaffe *et al.* 1979), *Camponotus rufipes* (Jaffe & Sánchez 1984),
79 *Solenopsis geminata* (Jaffe & Puche 1984) and *Atta laevigata* (Salzemann & Jaffe 1990;
80 Salzemann *et al.* 1992, Hughes *et al.* 2001; Hernández *et al.* 2002).

81
82 The nestmate recognition mechanisms proposed vary with respect to the importance
83 given to individual odor markers, i.e. those produced and carried by each individual, relative to
84 communal odor marks, i.e. those shared by the whole colony. Some authors have suggested
85 that environmental odors produced by food and nest materials are adsorbed on the cuticle of
86 the insects and are used for nestmate recognition (Jutsum *et al.* 1979). Other authors have
87 proposed that each individual possesses genetically predetermined odors that serve as
88 discrimination cues (Crozier & Dix 1979). Ants might not use these individual odors directly for
89 nestmate recognition, but rather a blend of odors produced by each individual of the society
90 could serve as recognition cue (Jaffe 1983, Jaffe & Marcuse 1983, Stuart 1987, Whitehouse &
91 Jaffe 1995). Such a blend or “Gestalt” odor could be produced through the interchange of
92 odors between individuals by trophalaxis and allogrooming (Crozier & Dix 1979, Soroker *et al.*
93 1994), or by an exchange of odors between individuals exposed in the nest to volatiles derive
94 from mandibular glands secretions of nestmates (Hernández *et al.* 2002). Other authors have
95 proposed the existence of a queen discriminator that is carried by all members of the society
96 (Carlin & Hölldobler 1986; 1987; however, see Peeters 1988; Tsuji & Ito 1986; Crosland
97 1990a; b).

98
99 Whatever the behavioral mechanisms of nestmate recognition, most evidence hints
100 that the recognition cues are either cuticular hydrocarbons or compounds in the alarm
101 pheromone. Soroker *et al.* (1994), Soroker *et al.* (1995), Lahav *et al.* (1999), and others,
102 provided experimental evidence that cuticular hydrocarbons accumulated in the post-
103 pharyngeal gland are responsible for nestmate recognition in the ant *Cataglyphis niger*. These

104 odors are produced in the epidermal cells, transported through the hemolymph, stored in the
105 post-pharyngeal glands, and are transferred between colony members by allogrooming and
106 trophallaxis. Lahav *et al.* (1999) proposed that the mechanism involving the post-pharyngeal
107 glands in nestmate recognition could be the system used for most ant species. However, Jaffe
108 (1983), Salzeman and Jaffe (1991) and Hernández *et al.* (2002) showed that alarm
109 pheromones derived from mandibular glands are involved, at least in part, in nestmate
110 recognition in *Atta laevigata*. Whitehouse & Jaffe (1995) reported that odors responsible for
111 nestmate recognition in *A. laevigata* are concentrated in the head and can also be found on
112 other parts of the ant. Experimental evidence for this was provided by Hernández *et al.* (2002)
113 who reported the presence of volatiles compounds from the mandibular gland on the cuticle of
114 the thorax and gaster.

115
116 Discrimination between a nestmate and a worker from a different species (inter-specific
117 recognition) seems to be achieved much faster and with less error, than discrimination
118 between a nestmate and a con-specific alien worker. For example, in the case of leaf-cutting
119 ants, Jutsum (1979) observed that discrimination of individuals from a different species
120 occurred at greater distances (0.5-1.5 cm) than discrimination of ants from a different colony
121 of the same species (0-0.8 cm). By exploiting the odor cues at work in inter-specific
122 discrimination, we continue to gain valuable insights into the mechanisms responsible for
123 discriminating “self” from “other” in ants. The present study examines the cues that enable
124 workers of *A. cephalotes* and *A. laevigata* to discriminate between a nestmate and an alien
125 ant from a different, but sympatric, species of the same genus.

126 127 **Methods**

128
129 For this study, we used an adult colony of *Atta cephalotes* L., located on the Campus of
130 the Universidad Simón Bolívar (USB), Caracas-Venezuela, and an adult colony of *Atta*
131 *laevigata* Fr. Smith, also located on the USB Campus. Three more adult *A. laevigata* colonies
132 located in pine tree plantations of CVG-PROFORCA at El Merey, Estado Monagas,
133 Venezuela (8⁰29' to 9⁰25'N and 62⁰ 30'to 64⁰ 40' W) were also used. At each colony, at least
134 fifteen different nest entrances were used for the experiments. Tests were carried out during

135 the first hours of the morning and in the late evening, when both species showed strong
136 activity on the foraging trails.

137
138 Bioassays tested if “test” ants (free walking foraging workers) were able to discriminate
139 between two “subject” ants (restrained on a pin and placed on the foraging trail). Subjects ants
140 represented always “self” (nestmates) and “other” (alien ants from another species of the
141 same genus). That is, in all bioassays the two subjects consisted of a nestmate representing
142 self (**S**) and an alien or other (**O**). Test ants were free walking workers of the field colonies of
143 *A. cephalotes* and *A. laevigata*. The methodology is described in detail in Hernández *et al.*
144 (2002). All bioassays were carried out blind so that the observer did not know the type of
145 treatment each subject received. All bioassays were performed on colonies of both species.
146 When the colony of *A. cephalotes* was used as test colony, the workers of *A. laevigata* or the
147 gland extracts of this species were defined as “other” and vice versa. Three different
148 bioassays were designed:

- 149
150 1) Bioassays using whole bodies. These were tested 0, 12 and 24 hours after being collected
151 from the nest.
- 152 2) Bioassays using body parts (head, thorax and gaster), tested 0 and 12 hours after being
153 collected from the nest,
- 154 3) Bioassays using glandular extracts placed on clean odorless plastic ants. The extracts
155 tested were mandibular, intra-mandibular, postpharyngeal glands and gaster extracts.

156
157 In each bioassay the two subjects were either whole ants, heads, thoraces and heads,
158 thoraces, gasters or plastic ants odorized with an extract. Subjects were restrained by placing
159 clean entomological pins through their thoraces and into the substrate, as described in
160 Whitehouse & Jaffe (1995) and Hernández *et al.* (2002). Each pair of restrained subjects was
161 placed near the nest entrance on both sides of the central axis of the trail, separated by
162 approximately 2-3 cm. After each replicate, both subjects were removed from the trial. None of
163 the nest entrances was used again for the next 30 minutes.

164
165

166 **Bioassays using whole bodies and body parts:**

167 For experiments with whole bodies, the ants were collected with clean forceps and
168 immediately placed into a clean 5 ml glass vial. For the experiments involving body parts only,
169 ants were collected as above, immediately dissected and each body part to be evaluated was
170 placed into a clean vial. The whole ants and the body parts were used both immediately (t=0)
171 or twelve hours (t=12) after collecting the worker from the nest. In the case of the treatment
172 with whole bodies, the evaluation was also repeated after a 24 hour-period.

173

174 **Bioassays using glandular extracts:**

175 Foraging workers from the selected field colonies were collected using clean forceps.
176 Each ant was placed into a 4 ml glass vial and its activity reduced by placing it in a cool
177 thermal container. Ants were transported to the laboratory and kept at -5°C while their
178 cephalic glands were dissected under a stereoscopic microscopic: Mandibular glands,
179 intramandibular glands and postpharyngeal glands. Gland extracts were prepared using 2 ml
180 ethanol, dichloromethane (CH_2Cl_2) or *n*-hexane (pesticide grade solvent, Fisher Scientific). The
181 solvents were chosen so as to cover a broad spectrum of extractable compounds, from very
182 polar (ethanol), slightly polar (dichloromethane), to not-polar (hexane). In the case of
183 mandibular and intra-mandibular gland extracts, 15 pairs of glands were dissected. For
184 postpharyngeal glands 20 ants were dissected. In all cases the glands were left in the solvent.
185 For the bioassay, 2 μl of the extract, which contained the equivalent of 0.15 % and 1 % of the
186 content of one gland respectively, were placed over the plastic ants using a 10 μl Hamilton
187 syringe. Preliminary assays had shown that these concentrations were the lowest ones to
188 elicit aggressive behavior. Plastic ants were cleaned, prior to the application of the extract,
189 with solvent in a Soxhlet for a 48 hour-period. The ants were then dried in an oven, at 40°C
190 and were used only once. Each extract was evaluated separately. Controls were clean plastic
191 ants and plastic ants with 2 μl of pure solvent.

192

193 **Treatments:**

194 The bioassays used whole ants, body parts and plastic ants as subjects. By using
195 different body parts we tried to locate or exclude the source of recognition odors. By isolating
196 whole ants and the body parts, we evaluated if the odor cues were produced by specific

197 glands of the ant, or were volatiles adsorbed on the cuticle. Seventeen different treatments
198 using different odor cues, including controls, were undertaken. We measured the difference in
199 levels of aggression received by two subjects: self (**S**) and other (**O**). **S**: ants or odors derived
200 from the test colony, and **O**: ants or odors derived from a colony of the other species. The
201 subjects used in each test are indicated in Tables 1 and 2.

202 203 **Measuring aggression:**

204 Following procedures outlined in Hernández *et al.* (2002), the reaction of “test”
205 workers from the resident colony towards “subjects” placed on the trail was evaluated for 20 s
206 every minute, for five minutes. The number of aggressive interactions received by each of two
207 subjects was recorded. For each consecutive replicate, the position of the subjects was
208 interchanged to avoid site habituation. The agonistic interactions we recorded were the
209 number of times each subject was threatened (attempts to bite) and bitten, following the
210 criteria established in Whitehouse & Jaffe (1995). These numbers were used to compute the
211 aggression index which quantified the difference in the number of threat and bites the free
212 walking workers on the trail directed towards each of the two subjects, **O** and **S**, where **S**
213 served as control. It was estimated by adding threats and bites received by **O** subject in each
214 replicate of each treatment and dividing by the same number computed for **S** subjects using
215 the equation:

$$216 \text{Aggression index} = (\text{bites and treats to } \mathbf{O} + 1) / (\text{bites and treats to } \mathbf{S} + 1)$$

217
218
219 Statistical analyses were undertaken by comparing the number of aggressive behaviors
220 received by each subject in each treatment, using the Wilcoxon test (Siegel & Castellan,
221 1988). A Mann Whitney test was used to compare the aggression indices of *A. laevigata* and
222 *A. cephalotes*.

223 224 **Chemical analyses of mandibular gland extract:**

225 Mandibular gland chemicals were analyzed as previously reported for *A. laevigata*
226 (Hernandez *et al.* 1999). Because mandibular glands are much more difficult to dissect than
227 postpharyngeal glands, mandibular gland extracts were analyzed by gas chromatography

(GC) in order to confirm the presence of mandibular gland compounds in the extracts. GC analyses were undertaken on a Hp 5890 GC, equipped with a FID detector, a fused silica DB-5 capillary column (25m x 0.18 ID). The carrier gas was helium (1 ml/min) and the oven was programmed at an initial temperature of 40 °C for 4 minutes and then heated at 6°C/min to 280°C and held at this temperature for 30 min. The main components of the mandibular glands were identified by comparing the retention times with the corresponding synthetic standards (Aldrich 99% pure). In order to identify the components of the mandibular glands of *A. cephalotes*, as previously done for *A. laevigata* (Hernández *et al.*1999), solid phase microextraction (SPME) technique was used for analyses (Borg-Karlson & Mozuraitis, 1996) with modifications. Workers were collected individually with forceps from field colonies and were immediately placed in a vial and cooled (aprox. 5°C) to reduce the ant activity. They were then transported to the laboratory and the head was dissected while remaining cold. Ten heads were placed into a silane treated glass 2 ml vial and then were crushed using a clean glass rod, immediately sealed with an aluminum seal and a Teflon rubber septum. The vial was then placed in a water bath and kept at 33°C for 10 min. A SPME fiber coated with 100 µm poly-dimethyl-siloxane was introduced for 10 min for adsorption of headspace volatiles, then desorbed in the GC injection port at 250°C for 10 sec. GC and GC-MS analyses were carried out on GC Hewlett Packard 5890A equipped with DB-5 column (30 m x 0.25 mm ID, Quadrex, New Haven, Connecticut) and GC-MS Perkin Elmer Qmass-910 linked to a GC-Autosystem 2000, equipped with DB-5 column (25 m x 0.18 mm ID, Quadrex, New Haven, Connecticut). The oven was programmed at an initial temperature of 50°C for 5 minutes and then heated at 6°C/min to 150°C and held at this temperature for 0 minutes, then heated at 20°C/min to 225°C and held at this temperature for 20 minutes. In both cases the carrier gas was helium (1 ml/min). The methods reported by Schäfer *et al.* (1997) were used to obtain the approximate quantities of compounds.

Chemical analyses of post-pharyngeal glands

Post-pharyngeal gland analyses were identical to those reported for *A. laevigata* (Hernández *et al.* 2002). A *n*-hexane extract of 20 dissected post-pharyngeal glands was prepared. The extract was concentrated by gently blowing a nitrogen current. One microliter of this solution was analyzed in a GC-MS as described above. The oven was programmed at an

259 initial temperature of 40°C for 4 minutes and then heated at 3°C/min to 280°C for 20 minutes.
260 Straight-chained and branched hydrocarbons were identified using the presence of
261 characteristic ions in the GC-MS spectra, produced by the α cleavage from the branching
262 point. We also used *n*-alkanes standard solutions to compare their mass spectra and retention
263 times.

264 265 **Results**

266
267 In general, workers of *A. cephalotes* and *A. laevigata* showed aggressive behaviors
268 towards **O** workers, although *A. laevigata* had higher aggression scores towards
269 heterospecifics. Table 1 shows the results from the different treatments using whole ants as
270 subjects. Clearly both species can discriminate odor from **S** and **O** worker as is shown by the
271 higher aggression directed towards alien ants compared to nestmates. This is expressed by a
272 significantly higher aggression towards O relative to S (aggression index O/S \gg 1) in
273 treatment 1 ($p < 0.05$; Wilcoxon's test). This higher aggression towards O subjects was
274 maintained even after 24 h of isolating the subjects (Treatments 2 and 3, $p < 0.05$; Wilcoxon's
275 test).

276
277 In the case of body parts, both species were able to recognize head, thorax and gaster
278 odors of their nestmates (Treatments 4 to 6) when collected and tested immediately. When
279 the thoraces were isolated for 12 hours the recognition ability was lost (Treatment 7).
280 Thoraces plus gasters of both species were discriminated when freshly collected (Treatment
281 8). In the case of *A. cephalotes*, recognition ability was lost when the thoraces plus gasters
282 were isolated from the colony 12 hours prior to the test (Treatment 9), but *A. laevigata*
283 discriminated between thoraxes and gasters isolated from the colony 12 hours prior to the
284 test.

285
286 Table 2 shows the results from the different treatments using plastic ants as subjects
287 and glandular solvent extracts as odor cues. The test with glandular extracts showed the
288 presence of a recognition cue usable for *A. cephalotes* in the hexane extracts of the
289 mandibular glands and the ethanol extracts of the gaster. *A. laevigata* ants, by contrast,
290 responded to recognition cues only in the ethanol and CH₂Cl₂ extracts of mandibular glands

291 and in the CH₂Cl₂ extracts of gasters. The chemicals to which *A. laevigata* responded were
292 those extracted by the more polar solvent, which also took longer to evaporate as suggested
293 by results of Treatment 9 (Table 1). *A. cephalotes* responded to extracts from less polar
294 solvents. No recognition cues could be extracted from intra-mandibular or post-pharyngeal
295 glands.

296
297 Both species produce 4-methyl-3-heptanone as the main component of mandibular
298 gland secretions (Table 3). However, differences in the chemical composition and relative
299 concentrations of the various compounds of mandibular glands are evident between both
300 species. In both species, the postpharyngeal gland secretions are characterized by *n*-alkanes
301 and methyl branched alkanes (Table 4). In the case of *A. cephalotes*, the mix was dominated
302 by 11-methyl,+13-methyl,+15-methyl tritriacontane, 11-methyl,+16-methyl-hexatriacontane and
303 10-methyl,+12-methyl,+13-methyl,+14-methyl pentatriacontane. In the case of *A. laevigata*,
304 the mix was dominated by nonacosane, heptacosane and hentriacontane. Both species
305 produce hydrocarbons but show clear differences in this secretion, where *A. cephalotes*
306 produces hydrocarbons with higher molecular weight than *A. laevigata*.

307

308

309

Discussion

310

311 Previous studies have described aggression between *Atta* species (Autori 1941;
312 Mariconi 1970; Rockwood 1973; Fowler 1977, Whitehouse & Jaffe 1996). The present study is
313 to our knowledge the first to assess the recognition cues mediating this inter-specific
314 aggression. Our results with glandular extracts show that *A. cephalotes* and *A. laevigata* have
315 the capacity to discriminate between a nestmate and an ant from a different *Atta* species, and
316 that this capacity is based on odor cues that are part of the exocrine secretions of these ants.

317

318 The ant species studied differed in the chemical nature (chemical polarity of
319 compounds) of the cues used to discriminate between nestmates and heterospecific workers.
320 The recognition cue used were, at least in part, volatile cephalic odors from the mandibular
321 glands, which have been reported as part of the mechanisms for intra-specific nestmate

322 recognition in *Atta* spp. (Jaffe 1983; Jaffe 1987; Salzemann & Jaffe 1990; Whitehouse & Jaffe
323 1995; Hernández *et al.* 2002).

324
325 We showed that both species also use abdominal odors as discrimination cue, as had
326 been reported previously for *A. cephalotes* (Jaffe 1983). Whitehouse & Jaffe (1995) reported
327 that the odors responsible for nestmate recognition in *A. laevigata* are concentrated in the
328 head, although they can be found in other body parts. For example, Hernández *et al.* (2002)
329 showed that compounds from the mandibular gland, which are involved in recognition, are
330 dispersed on the entire body surface of ants, where they serve as a cue for nestmate
331 recognition. It is possible that the ant is constantly releasing the mandibular glands secretions
332 and these compounds are adsorbed in the cuticular hydrocarbons layer (Jaffe & Marcuse
333 1983; Jaffe & Márquez 1987; Hernández *et al.* 2002). Once absorbed on the cuticular waxes,
334 the odors may be slowly released. The experimental results presented here are consistent
335 with this mechanism. A role for cuticular hydrocarbons, or for queen pheromones, as the
336 chemicals responsible for providing the recognition cues, is not supported by our results using
337 postpharyngeal gland extracts.

338
339 Both species studied have 4-methyl-3-heptanone as the main component of mandibular
340 gland secretions, however, they differ in the presence of other chemicals and their relative
341 abundances. Blum *et al.* (1968) reported the mandibular gland composition for six *Atta*
342 species, and 4-methyl-3-heptanone was detected in all ant species studied, including *A.*
343 *laevigata*. Moser *et al.* (1968) also point to 4-methyl-3-heptanone and 2-heptanone as
344 chemicals components of mandibular gland secretions in *A. texana*, where 4-methyl-3-
345 heptanone is 1000 times more active as alarm pheromone than 2-heptanone. Only one chiral
346 form of 4-methyl-3-heptanone has been shown to induce behavioral activity in *Atta* ants
347 (Moser *et al.* 1968, Riley *et al.* 1974 a,b).

348
349 Our experiments did not detect any recognition function for intra-mandibular nor post-
350 pharyngeal gland secretions in the species studied. However, the chemicals in the post-
351 pharyngeal gland secretions are long chain and branched hydrocarbons and show clear
352 differences between the two species (*A. cephalotes* produces heavier molecular weight

353 hydrocarbons than *A. laevigata.*), providing the chemical attributes required for a putative
354 recognition cue. Yet these secretions do not seem to be used for nestmate recognition in *Atta*,
355 although they are used by many other ants (Vander Meer *et al.* 1997, Katzav *et al.* 2004,
356 Howard & Blomquist 2005). Specifically, experiments by Lahav *et al.* (1999) revealed that
357 cuticular hydrocarbons stored in the post-pharyngeal glands are responsible for nestmate
358 recognition in *Cataglyphis niger*. These authors hypothesized that this mechanism is
359 applicable to ants in general. However, based on our experimental results, we have to reject
360 the generality of this hypothesis because it does not apply to *Atta*. Our results suggest that
361 ants (and possibly other social insects) do not share a common nestmate recognition system,
362 as even differences in the chemical nature of nestmate recognition cues can be evidenced
363 among species in a single genus.

364
365 In conclusion, we suggest that the system used by these ants to discriminate between
366 self and other, is the same for intra and inter-specific recognition (Hernandez *et al.* 2002). This
367 implies that speaking of intra- and inter-specific recognition as separate processes may be
368 misdirected. What these ants seem to achieve is recognition of kin (i.e. nestmates), which
369 then allows discrimination of self from others. In the case of *Atta*, this recognition system
370 seems to be based on odors produced by exocrine glands in the head and gaster of the
371 workers. It is very important to note that our results suggest that the chemical nature of the
372 odor cues on which the recognition system is based differs between the two species studied.

373
374 The nestmate recognition systems of *Atta* builds upon a chemical communication
375 system designed to regulate alarm behavior. This shows that our understanding of recognition
376 of individuals among animals is not helped by focusing too narrowly on kin selection alone.
377 Several ecological constraints seem to modulate the evolution of recognition systems in ants
378 in addition to kin selection. The need to recognize and defend a territory, to call nestmates for
379 help, coordinate battles and to recognize aliens, seem to build on the same chemical
380 communication systems in *Atta*, confirming the existence of an evolutionary trend, at least
381 among ants (see Jaffe 1987) whereby individual recognition systems are displaced by a
382 colony segregation mechanisms based on territorial behavior and where chemical
383 communication systems are re-used for different purposes. We suggest that multiple selective

384 forces can be expected to be at work in shaping recognition mechanism of other social
385 animals

386

387 ACKNOWLEDGEMENTS: We thank Angela Shuetrim for help with manuscript translation and
388 critical suggestion, the editors and referees of Animal Behavior for valuable suggestions and
389 much effort, Leonardo Caraballo, Carlos Cárdenas, and Diego Giraldo for their valuable help
390 in the field. This work was partially financed by grant S1-97001402, CONICIT.

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Table 1. Aggressive responses of the leaf-cutting ants *A. cephalotes* and *A. laevigata* using whole bodies and body parts as odor signals. Numbers shown are the average aggression scores (number of aggressive behaviors towards heterospecific stimulus divided by the number of aggressive responses towards conspecific stimulus)

Treatment	Subjects	Aggression of <i>A. cephalotes</i> O/S		Aggression of <i>A. laevigata</i> O/S		Between species comparison
		N	Mean \pm SD	N	Mean \pm SD	p \$
1	Live whole ants t=0	10	5.4 \pm 4 *	10	22 \pm 13 **	0.0008
2	Live whole ants t=12h	10	7.2 \pm 7.5 *	10	5.1 \pm 3.1 **	0.791
3	Live whole ants t=24h	10	7.8 \pm 5.3 **	10	9.7 \pm 5.5 **	0.545
4	Fresh head t=0	10	5.5 \pm 3.4 **	10	8.5 \pm 6.3 **	0.289
5	Fresh gaster t=0	12	2 \pm 1.1	10	3.2 \pm 1.6 **	0.06
6	Fresh thorax t=0	10	3.9 \pm 2.8 **	10	7.9 \pm 5.1 **	0.053
7	Thorax t=12h	10	1 \pm 0.3	10	1.1 \pm 0.8	0.597
8	Thorax+gaster t=0	10	4.7 \pm 3.1 *	10	3.4 \pm 2 **	0.5
9	Thorax+gaster t=12h	10	1.1 \pm 1.2	10	2 \pm 1 *	0.026

* and ** indicate $p < 0.05$ and 0.01 respectively using the Wilcoxon's matched pairs test comparing aggressions towards S and O

\$ Mann Whitney's test comparing aggressions towards O/S of *A. cephalotes* ants with those of *A. laevigata* ants

Table 2. Aggressive responses of the leaf-cutting ants *A. cephalotes* and *A. laevigata* using glandular extracts placed on plastic ants as odor signals. Numbers shown are the average aggression scores (number of aggressive behaviors towards heterospecific stimulus divided by the number of aggressive responses towards conspecific stimulus)

Treatment	Odor signal	Aggression of <i>A. cephalotes</i> O/S		Aggression of <i>A. laevigata</i> O/S		Between species comparison
		N	Mean \pm SD	N	Mean \pm SD	p \$
10 Control 1	None	10	1	10	1	1
11a Control 2	ethanol vs None	10	1.1 \pm 0.4	10	1.5 \pm 1.4	0.879
11b Control 3	CH ₂ Cl ₂ vs None	10	1.3 \pm 0.9	10	1.4 \pm 1.1	0.879
11c Control 4	Hexane vs None	10	1.7 \pm 1.6	10	1.1 \pm 0.3	0.256
12a	PPG ethanol extract	10	1.1 \pm 0.9	10	1.4 \pm 1.2	0.364
12b	PPG CH ₂ Cl ₂ extract	10	1.6 \pm 1.3	10	1.6 \pm 1.3	0.344
12c	PPG hexane extract	10	1.3 \pm 0.7	10	2 \pm 1.6	0.112
13a	MG ethanol extract	10	1.3 \pm 1.4	10	0.5 \pm 0.2 **	0.023
13b	MG CH ₂ Cl ₂ extract	13	0.9 \pm 0.5	13	2.4 \pm 2.5*	0.015
13c	MG hexane extract	10	0.6 \pm 0.4 **	10	1.1 \pm 0.5	0.004
14a	IMG ethanol extract	10	0.9 \pm 0.3	10	1.2 \pm 0.5	0.307
14b	IMG CH ₂ Cl ₂ extract	10	1.2 \pm 0.6	10	1.1 \pm 0.8	0.449
14c	IMG hexane extract	10	1.1 \pm 0.8	10	1.1 \pm 0.4	0.427
15a	Gaster ethanol extract	10	0.7 \pm 0.3 *	10	1.6 \pm 1	0.022
15b	Gaster CH ₂ Cl ₂ extract	10	1.1 \pm 0.7	10	1.4 \pm 0.6 *	0.198
15c	Gaster hexane extract	10	0.9 \pm 0.6	10	1.1 \pm 0.3	0.256

MG= mandibular Gland; **PPG**= Post-pharyngeal Gland **IMG**= intra-mandibular gland.

* and ** indicate $p < 0.05$ and 0.01 respectively using the Wilcoxon's matched pairs test comparing aggressions towards S and O

\$ Mann Whitney's test comparing aggressions towards O/S of *A. cephalotes* ants with those of *A. laevigata* ants

Table 3. Chemical compounds isolated and identified by SPME and solvent micro-extraction from Mandibular glands of *A. cephalotes* and *A. laevigata* respectively.

Chemical compounds	Identified in <i>A. cephalotes</i>	Workers Head with 2.5-4 mm (ng/head)	Identified in <i>A. laevigata</i> •	Workers Head with 2.5-4 mm (ng/head)
		Mean \pm Std		Mean \pm Std
4-methyl-3-hexanone			X	0.3 \pm 0.4
Unknown	X	0.47 \pm 0.97		
Unknown	X	t		
Unknown	X	t		
Unknown	X	t		
3-heptanone	X	0.12 \pm 0.26		
2-heptanone	X	0.24 \pm 0.48		
Unknown	X	t		
2-heptanol	X	0.22 \pm 0.28		
Unknown	X	t		
4-methyl-6-hepten-3-one				
4-methyl-3-heptanone	X	20.62 \pm 28.25	X	4.5 \pm 2.6
4-methyl-3-heptanol	X	5.80 \pm 6.56	X	t
3-octanone	X	0.56 \pm 0.66	X	t
3-octanol	X	0.27 \pm 0.30		
2-octanol	X	t		
Unknown	X	t		
Unknown	X	0.14 \pm 0.17		
Unknown	X	t		
Unknown	X	0.05 \pm 0.05		
Unknown	X	0.06 \pm 0.08		
Unknown	X	t		
Unknown	X	0.09 \pm 0.07		
Unknown	X	0.03 \pm 0.06		
Unknown	X	t		
Unknown	X	0.07 \pm 0.08		

t: trace compounds

•: Chemical compounds derive from MG glands reported by Hernández *et al.* 1999.

Table 4. Chemical compounds identified from *n*-hexane extracts of postpharyngeal glands of *A. cephalotes* and *A. laevigata* ants.

Peak GC-MS	Identified Compounds in <i>A. cephalotes</i>	Relative proportion (%)	Identified Compounds in <i>A. laevigata</i> •	Relative proportion (%)
1	Tricosene	0.8	Unkonwn	1.69
2	Tetracosane	1.2	Heneicosane	5.93
3	12-methyl tetracosane	0.5	Docosane	2.22
4	Pentacosane	2.8	Tricosane	21.93
5	Hexacosane	1.5	9-methyl tricosane	2.86
6	Hexacosane	2.0	7-methyl tricosane	1.85
7	Heptacosane *	2.7	5-methyl tricosane	0.79
8	Octacosane **	2.9	3-methyl tricosane	1.19
9	Nonacosane ***	13.0	Tetracosane	4.45
10	Triacotane ****	0.7	9-methyl tetracosane	2.76
11	Hentriacontane *****	1.5	7-methyl tetracosane	2.19
12	11-methyl-hentriacontane	6.1	3-methyl tetracosane	2.07
13	13-methyl-hentriacontane	1.4	Pentacosane	19.49
14	6-methyl-hentriacontane	2.2	7,13 dimethyl pentacosane	3.00
15	Dotriacontane	0.9	Unknown	2.34
16	12-methyl dotriacontane	11.1	9+11 methyl hexacosane	13.84
17	8-methyl dotriacontane	5.0	7,11 dimethyl hexacosane	8.63
18	Tritriacontane	1,6	7,X dimethyl hexacosane	1.68
19	11-methyl, + 13-methyl, + 15-methyl- tritriacontane	100	Heptacosane *	92.27
20	9-methyl, + 12-methyl, + 14-methyl-tritriacontane	14.3	9-,11-,13- Methyl heptacosane	63.51
21	13-methyl tetratriacontane	7.4	7,11 dimethyl heptacosane	51.51
22	12-methyl,+14-methyl,+ 16-methyl-tetratriacontane	16.1	Octacosane **	9.29
23	11-methyl pentatriacontane	4.2	11-methyl octacosane	2.40
24	10-methyl,+12-methyl,+ 13-methyl,+14-methyl-pentatriacontane	35.2	9-methyl octacosane	27.01
25	11-methyl, + 16-methyl-hexatriacontane	51.6	Nonacosane ***	100
26			11-methyl nonacosane	2.68
27			6-methyl nonacosane	13.38
28			Triacotane ****	21.29
29			Unknown	1.09
30			13-methyl triacotane	31.63
31			Hentriacontane *****	61.68
32			9-,10-, 13-methylhentriacontane	9.49
33			Unknown	3.41
34			Unknown	2.74

* Chemical compounds shared by *A. cephalotes* y *A. laevigata*

• Chemical compounds derived from PPG reported by Hernández *et al.* 2002.