From plants to ants: Fungal modification of leaf lipids for nutrition and communication in the leaf-cutter ant fungal garden ecosystem

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ABSTRACT

Lipids are essential to all living organisms, as an energy source, as an important cellular structural component, and as a communication tool. In this study, we used global lipidomic methods to evaluate the lipids in leaf-cutter ant fungal gardens. Leaf-cutter ants and their coevolved fungal cultivar, *Leucoagaricus gongylophorus*, are a model mutualistic system. The fungus enzymatically digests fresh plant material that the ants cut and deliver, converting energy and nutrients from plants, and providing them to the ants through specialized hyphal swellings called gongylidia. Using combined liquid chromatography, ion mobility spectrometry, and tandem mass spectrometry we evaluated differences between the molecular speciation of lipids in the leaf-cutter ant fungal garden ecosystem. This lipidomic study characterized leaves that are fed to the gardens, gongylidia that are produced by the fungus to feed the ants, and spatially resolved regions of the fungal garden through stages of leaf degradation. Lipids containing alpha-linolenic acid (18:3) were enriched in leaves and the top of the gardens, but not dominant in the middle or bottom regions. Gongylidia were dominated by lipids containing linoleic acid (18:2). To evaluate the communicative potential of the lipids in fungal gardens we conducted a behavioral experiment that showed *Atta* leaf-cutter ants responded differently to 18:3 and 18:2 fatty acids, with aggression towards 18:3 and attraction for 18:2. This work demonstrates the role of lipids in both the transfer of energy and as an inter-kingdom communication tool in leaf-cutter ant fungal gardens.

Importance

In this work we examined the role of lipids in the mutualism between leaf-cutter ants and fungus. These ants cut fresh leaf material, which they provide to their fungal cultivar, that converts energy and nutrients from the plants and provides it to the ants in specialized hyphal swellings called gongylidia. This work constitutes the first example of a global lipidomics study of a symbiotic system and provides insights as to how the fungus modifies plant lipids into a usable

source for the ants. Through a behavioral experiment, this work also demonstrates how lipids can be used as an inter-kingdom communication tool, in this case an attractant, rather than as a repellant, which is more often seen.

INTRODUCTION

Lipids are one of four fundamental components of organisms, the other three being proteins, carbohydrates, and nucleic acids. Lipids serve many roles, and their characterization is critical to understanding how organisms function individually, and in communities. Two roles that we are particularly interested in are as a source of energy and their use in interspecies communication. Lipids are widely used as a form of stored energy in organisms, and in diets represent a dense source of energy, approximately double that of either proteins or carbohydrates (1).

The role of lipids in communication is vital across biological scales. Lipids found in cell membranes are important in cell-to-cell communication, either as a means to identify and differentiate between cells of the same multicellular organism (2), conspecifics in unicellular organisms (3), or cells that do not belong – pathogens (4), predators (5), or food (6). Lipids even direct communication between host cells and virus, thereby impacting the favorability of membrane fusion events (7). At a larger scale, lipids are used as signaling molecules between cells in the form of hormones (between cells of the same organism) or pheromones (between organisms).

The literature on pheromones as a communication tool between conspecifics is extensive (8-10). There is also some work exploring the role of pheromones between taxa, largely in the realm of plant defense against herbivory. When consumed by insect herbivores, some plants emit pheromones that attract the herbivore's natural enemy, parasitic wasps (11). This system is a fascinating example of communication in a multipartite symbiosis between a host, herbivore, and

81 indirect defensive symbiont. We expect that this type of communication would exist in beneficial
82 symbioses as well. Our work here explores the role of lipids, in both nutrition and
83 communication, in a model mutualistic system where fungi serve as an interface between ants
84 and plants.

85 Leaf-cutter ants are dominant herbivores that can consume as much as 17% of the leaf
86 biomass produced in Neotropical ecosystems (12). *Atta* leaf-cutter ant colonies contain millions
87 of workers that fall into specialized casts (13), contributing to the colony's success as ecosystem
88 engineers (14). Foraging leaf-cutter ants are often seen marching in conspicuous trails and
89 bringing pieces of leaves back to their underground nests that can be as large as 30 m^2 to 35
90 m^2 in area and several meters deep. Once to the nest, the ants use the leaves to feed their fungal
91 cultivar, *Leucoagaricus gongylophorus* (15), which is grown in their specialized fungal gardens.
92 By cultivating and feeding these fungal gardens, the ants are able to access nutrients in plant
93 biomass that would otherwise be unavailable (16-20).

94 Leaf-cutter ants and their microbial symbionts are paradigmatic in the field of insect
95 microbial symbiosis. Unlike other herbivores, the microbes in symbiosis with leaf-cutter ants can
96 be easily sampled because they exist as an external digestive system in the form of fungal
97 gardens. These fungal gardens exhibit vertical stratification, characterized by top, middle and
98 bottom regions. From top to bottom, there is a visual gradient of leaf degradation with leaves
99 starting as fresh, undigested leaves at the top, then processing through the initial stages of
100 degradation in the middle, and finally to more complete stages of degradation by the bottom. To
101 promote growth of the fungal garden, leaf-cutter ants perform different activities in the various
102 locations of the garden. Specifically, the ants triturate fresh plant material, incorporate it into the
103 top of the fungal garden, deposit tufts of fungal mycelium, and defecate with excrement
104 containing fungus-derived plant biomass-degrading enzymes to initiate lignocellulose
105 degradation (21, 22). As the plant material placed on the top of the fungal garden is digested, it

106 moves toward the bottom of the garden where it is removed from the fungal garden chamber and
107 deposited in a refuse dump by the ants (23), and by which time it has been depleted of most
108 nutrients. The gradual degradation of the plant material from top to bottom of the garden,
109 combined with specific ant activities, gives each region of the fungal garden a different visual
110 appearance and varied molecular properties (**Figure 1**). The middle of the fungal garden, where
111 the density of fungal hyphal growth is most pronounced, is also where the *L. gongylophorus*
112 produces gongylidia, or specialized hyphal swellings, that worker ants consume and feed to
113 larvae in the colony. While foraging workers obtain some nutrition from the plant sap they drink
114 when they cut the fresh leaf material, garden workers and larvae exclusively consume
115 gongylidia, where they obtain carbohydrates, polysaccharides, and energy-rich lipids (24, 25).
116 To elucidate the relationship between leaf-cutter ants and their fungal cultivar, earlier
117 studies have focused on understanding how the fungus breaks down plant biomass (16, 17). In
118 our previous metaproteomic analyses of the top, middle and bottom regions of the fungal garden,
119 we discovered that *L. gongylophorus* produced a majority of the lignocellulases found (16). The
120 studies also showed the resident bacterial community likely aids in this process by producing
121 amino acids and vitamins, thereby enabling the fungus to thrive (16, 17, 26). These results shed
122 great light into the symbiosis between the ant and the fungal cultivar, but to understand a more
123 complete picture other proteins were evaluated. Interestingly, the gongylidia (16, 22) showed an
124 enrichment in unique lipid-associated proteins when compared to different regions of the garden
125 (Data file S1), ultimately suggesting the gongylidia lipids would differ from those throughout the
126 fungal garden. A detailed analysis of the molecular speciation of lipids is needed to provide
127 biological insight into subpopulations and differential activities of complex samples, such as the
128 fungal garden ecosystem.
129 Here, we conduct the first global lipidomic study of the leaf-cutter ant-microbe symbiosis
130 using comprehensive liquid chromatography–tandem mass spectrometry and liquid

131 chromatography–ion mobility spectrometry-mass spectrometry. We examined spatiotemporal
132 changes in the molecular speciation of lipids across six heterogenous *Atta* leaf-cutter fungal
133 gardens through different stages of leaf degradation. First we evaluated the lipid content of
134 leaves the ants use to feed their cultivar to understand which ones existed initially. Then we
135 assessed the gongylidia, and the top, middle, and bottom regions of their fungal gardens at initial,
136 intermediate, and advanced stages of leaf degradation, respectively (**Figure 1**). The lipid content
137 of the leaf material was compared to the different regions of the fungal garden to understand
138 consumption of the leaf lipids and synthesis of the fungal garden consortium lipids through the
139 various regions. Additionally, the lipid content of the gongylidia was compared to the middle
140 region of the fungal garden to evaluate its specific properties versus the area where it was
141 harvested. Upon finding an enrichment of particular lipids in the leaves and fungal garden
142 components, we conducted a behavior experiment using seven *Atta cephalotes* colonies to
143 observe whether worker ants detected and responded to the lipids differently.
144

145 **RESULTS**

146 We observed that the predominant lipids in the gongylidia and top and bottom of the
147 garden varied greatly. The top of the garden was most similar to the leaves and had predominant
148 lipid subclasses including monogalactosyldiacylglycerol (MGDG),
149 sulfoquinovosyldiacylglycerol (SQDG), and diacylglycerophosphoglycerol (PG), while the lipids
150 in the bottom of the gardens included diacylglycerophosphoethanolamines (PE) and
151 diacylglycerophosphoserine (PS). The gongylidia had dominant lipids including ceramides (Cer),
152 diacylglycerophosphoethanolamines (PE), and triacylglycerols (TG). Across all lipid subclasses
153 interesting trends were observed based on individual fatty acid composition; an evaluation of the
154 lipid subclasses and fatty acid composition differences is given below.
155

156 *Lipid comparisons of leaves and fungal garden regions*

157 To initiate the study, lipids present in the maple leaves fed to the fungal garden were evaluated
158 so they could be compared to the fungal garden regions and gongylidia. The lipids present in the
159 leaves were evaluated and leaf lipid categories included sphingolipids, glycerophospholipids, and
160 glycerolipids. These identifications were dominated by the PG subclass as well as galactolipids
161 (MGDG and SQDG) with only minor amounts of the other phospholipids
162 (diacylglycerophosphocholines (PC), PE, and diacylglycerophosphoinositols (PI)) (see Data file
163 S2 for all characterized lipids). Evaluation of the fungal garden samples showed that 274 lipids
164 were identified from the top, middle, and bottom regions of the six gardens (Data file S2 (a) &
165 (b)). Both the leaf lipids and garden lipids are depicted in **Figure 2** where the relative log2
166 expression level of each lipid is represented by a red-white-blue color scale with red denoting
167 high abundance and blue low abundance. Leaf lipids are included in the top row (highlighted
168 with a pink box), subsequent rows contain garden lipids, for the six top regions (highlighted with
169 a green bar), six middle regions (highlighted with a yellow bar), and six bottom regions
170 (highlighted with an orange bar). In leaf samples, PGs and galactolipids were observed to be the
171 most abundant, which was distinct from the lipids detected in the top, middle, and bottom
172 regions of the garden, where TGs were found to be most abundant.

173 Garden lipids were also from sphingolipids, glycerophospholipids, and glycerolipids
174 categories and 13 subclasses. The glycerophospholipid and glycerolipid categories represented
175 97% of the identified lipids with 89 glycerophospholipids species and 177 glycerolipids species,
176 while the sphingolipid category was only a minor component with 8 species identified in the
177 garden (Data file S2(b)). Of all subclasses, TGs contained the greatest number of identified lipids
178 in the fungal gardens (Data file S2(b)). **Figure 2** and Data file S2(c) depict 110 lipids, including
179 isomeric species (27), with accurate quantitative values and no co-eluting lipid species detected.
180 When the top and bottom fungal garden regions were statistically compared to evaluate lipid

181 changes and leave degradation, 50 lipid species were found to be statistically significant (p-value
182 < 0.05) and a trend was noted pertaining to chain length and degree of unsaturation. PE, PG,
183 diacylglycerol (DG), and TG species, were more unsaturated (i.e., contained more double bonds
184 in the fatty acid chains) in the top region of the garden, and PG and TG species had longer fatty
185 acid chains.

186 An examination of the individual fatty acid composition of the lipids in the fungal garden
187 also illustrated interesting trends. The fatty acyl 16:0, 18:2, and 18:3 groups were found to
188 dominate the fatty acids profiles for the most abundant lipids identified in the phospholipid and
189 glycerolipid subclasses. **Figure 2** details the acyl chains for each lipid, where those highlighted
190 in green text or orange text are significantly increasing in the top or bottom garden layer,
191 respectively. Lipids highlighted with grey text were not significantly different from the top to
192 bottom regions across our six gardens. For example, PC(18:3_18:3), LPC(18:1)_A,
193 PE(18:3_18:3), and PI(18:3_18:3) are highlighted with green text in **Figure 2** since they greatly
194 decreased from the top to bottom region of the fungal garden. Of the 50 lipid species
195 significantly elevated in the top layer, over half of them contained an 18:3 acyl chain. These 18:3
196 containing lipids significantly elevated in the top layer include MGDG, SQDG,
197 monoacylglycerophosphocholine (LPC), PC, PG, DG, Cardiolipin (CL), PI,
198 monoacylglycerophosphoethanolamines (LPE), and PE species (signified with an * in **Figure 2**)
199 and TG(18:3/18:3/18:3) (Data file S2(b)). Many of these lipids also had a relatively high
200 abundance in the leaves, especially those containing two 18:3 fatty acyl groups (e.g. 18:3_18:3),
201 suggesting 18:3 containing lipids come from the leaves. Only nine lipids were found to
202 significantly increase in the bottom layer of the garden compared to the top and, all of these
203 lipids contained at least one 18:2 (signified with a ‡ in **Figure 2**), suggesting 18:3 is degraded
204 from the top to bottom regions of the garden by fungal garden microbial symbionts, while 18:2 is

205 synthesized by the fungal garden. Based on these results we hypothesized that 18:2 could be
206 enriched in the gongylidia.
207
208 *Lipid changes between fungal garden and gongylidia*
209 Next, the gongylidia was compared to the middle fungal garden where it is located to
210 assess similarities and differences. In the gongylidia, 263 lipids species were identified from 18
211 different subclasses (Data file S3 (a) & (b)). **Figure 3** depicts 183 lipids, including isomeric
212 species, with accurate quantitative values and no co-eluting lipid species detected (Data file
213 S3(c)). Both the middle region garden lipids and gongylidia lipids are depicted in **Figure 3**
214 where the relative log2 abundance of each lipid is represented by a red-white-blue color scale,
215 red denotes high abundance and blue denotes low abundance. Leaf lipids are included in the top
216 row (highlighted with a pink box), subsequent rows contain garden lipids, for the six middle
217 regions (highlighted with a yellow bar), and six gongylidia samples (highlighted with a purple
218 bar). The acyl chains for each lipid are also detailed in **Figure 3**, where those highlighted in
219 purple text or yellow text are significantly increasing in the gongylidia or surrounding middle
220 garden region, respectively. Lipids highlighted with grey text were not significantly changing
221 between the gongylidia and surrounding garden regions across our six gardens. In the statistical
222 comparison of the gongylidia and surrounding middle garden region, 116 lipid species were
223 found to be statistically significant (**Figure 3**, Data file S3(c)), indicating the gongylidia lipids
224 are distinct from the surrounding middle region. Lipid categories with high abundance in leaves
225 from the initial garden study, MDGD, SQDG, and PG, in addition to DGDG, showed low
226 enrichment in the gongylidia samples; whereas, both detected mannosylinositol
227 phosphorylceramide (MIPC) lipids, MIPC(t18:0_24:0(2OH)) and MIPC(t18:0_26:0(2OH)), and
228 all five significant Cer species were enriched in the gongylidia. PC, PE, phosphatidic acid (PA),
229 PI, DG, and TG lipids showed differential enrichment dependent on the lipid fatty acyl

230 composition. All significantly changing 18:3 containing PC, PE, PA, and DG species in the
231 middle garden were depleted in the gongylidia (signified with an * in **Figure 3**). Conversely, all
232 significantly changing 18:2 containing PC, PE, PA, DG, and PI species were enriched in the
233 gongylidia (signified with a ‡ in **Figure 3**). Many TG species contained both 18:3 and 18:2 as
234 one of their three fatty acyl chains, yet ninety percent of all TG species depleted in the
235 gongylidia compared to the surrounding fungal garden contain at least one 18:3 acyl chain and
236 >84% of all TG species enriched in the gongylidia contain at least one 18:2 acyl chain.
237 Additionally, lipid subclasses with high abundance in the leaves (MDGD, SQDG, PG, and
238 DGDG) showed relatively low concentrations in the gongylidia samples.
239
240 *Behavior experiment*
241 Our lipidomic analyses characterized a prevalence of lipid species containing linoleic
242 acid (18:2) in the gongylidia, the leaf-cutter ant's food source, and lipid species containing alpha-
243 linolenic acid (18:3) in leaf material. We performed a behavior experiment to test if the *Atta*
244 *cephalotes* workers had different responses to untreated paper discs and paper discs treated with
245 oleic acid (18:1), 18:2, and 18:3 (**Figure 4**; Figure S1, S2, S3, & S4; Movie S1 & S2). Overall,
246 the ants spent significantly more time inspecting 18:2 (p=0.0012) and 18:1 (p=0.015) treated
247 paper discs than the control paper discs. Similarly, the ants picked up the discs infused with 18:2
248 (p=0.039) more frequently than the control discs, but during the experiment never picked up a
249 disc containing 18:3. However, the ants displayed aggressive behavior, including lunging and
250 biting the discs more frequently toward 18:3 compared to the control (p=0.014). The ants did not
251 exhibit aggressive behavior towards the discs with 18:1 or 18:2.
252

253 **DISCUSSION**

254 Leaf-cutter ants, from the genera *Atta* and *Acromyrmex*, cultivate a fungus on fresh foliar
255 material, which converts plant biomass into new forms that are readily usable by the ants and
256 their larvae. This obligate mutualism is derived from within the ant subtribe Attina, which
257 represents a monophylogenetic lineage of ants that have associated with fungal cultivars for
258 approximately 55 million years. The leaf-cutter ants are the most derived group of the Attina and
259 diverged approximately 12 million years ago, coinciding with their association with *L.*
260 *gongylophorus* (28). Both organisms have undergone co-evolution. *Leucoagaricus*
261 *gongylophorus* has diversified in association with the ants and has undergone gene reduction so
262 that it can no longer function as a saprotroph, like its closest free-living relatives (29). On the
263 other hand, the ant's digestive system has adapted to prevent digestion of fungus-produced plant
264 biomass degradation enzymes so that the ants can redistribute them through fecal fluid onto
265 freshly incorporated leaf material (21). In return, the fungus provides all of the nutrients to feed
266 the colony by producing carbohydrate, polysaccharide, and lipid rich gongylidia (24, 25).

267 In this study, characterization of both the lipid content of leaf material and lipids
268 synthesized by the fungus, allowed us to gain novel insight into the conversion of lipid nutrients
269 from the leaf material deposited on the top of the fungal garden to the enrichment of lipid
270 nutrients in the gongylidia for the ant to consume. We observed significant subclass trends to
271 each region and a correlation of lipids containing 18:3 from the leaves and the top of the garden
272 and lipids containing 18:2 at the bottom of the garden and enriched in the gongylidia. Lipid
273 subclasses that were significantly increased in the top of the garden and leaf material included
274 PG, MGDG, SQDG, and DGDG species, all of which are known to be significant contributors to
275 photosynthetic organelles (30). In addition, 18:3 has notable signaling properties in plant tissues.
276 When leaves are wounded, the polypeptide systemin is emitted from the damaged cells into the
277 apoplast, signaling the liberation of 18:3 from plant membrane lipids into the cells. 18:3 begins

278 the defense pathway by being converted to 12-oxophytodienoic acid and then through beta-
279 oxidation is converted into jasmonic acid (31). The main defense mechanism in these leaves is
280 jasmonic acid, and 18:3 is crucial to its synthesis.

281 The abundant lipids in the fungal garden top to gongylidia lipids sections differ greatly.
282 This is an important result because while the ants ingest biomass-degrading enzymes from the
283 gongylidia and deposit them on top, the lipids are not being recycled in this manner. It is clear
284 then that the fungal garden metabolizes the lipids from the leaves and synthesizes new lipids
285 which are provided to the ants and larvae through the gongylidia, where they are ingested and
286 absorbed into their bodies. While this was assumed previously (24, 25), our study is the first to
287 actually track the molecular speciation and dynamic changes of these lipids through the fungal
288 garden.

289 We conducted the behavior experiment because we wanted to evaluate whether the ants
290 were able to detect the various lipids in the fungal gardens, and to observe any respective
291 differences in their behavior. We expected *Atta cephalotes* workers to be attracted to all three
292 tested lipids. Previous studies had shown that ants from the genera *Pogonomyrmex, Solenopsis,*
293 and *Atta* are attracted to 18:1, which is a lipid that dead ants emit from their bodies as a cue for
294 workers to move them out of the colony or toward the dump (32-34). We observed similar
295 attractive results with our ants, validating our methods. We expected that the workers would also
296 be attracted to 18:3, which is abundant in leaves they routinely cut and incorporate into their
297 gardens. However, the workers instead behaved aggressively toward 18:3 by lunging and biting
298 at the infused discs indicating that it is not this component of leaves that leaf-cutter ants are
299 attracted to. This ant response to the 18:3 lipids might be related to the lipid's role as a
300 constituent of the plant defense response lipid, jasmonic acid, which may be playing a role in
301 deterring herbivory by leaf-cutter ants. Finally, we also expected that the *Atta* workers would be
302 attracted to 18:2 since it has been shown attractive to another ant species (*Cataglyphis fortis*)

303 (35) and is abundant in the gongylidia they consume. The ants' attractive responses to 18:2 (both
304 disc pickup and time spent inspecting) were the most different from the controls, compared to the
305 two other lipids tested, which suggests that there is a strong attraction to this lipid. These
306 responses all relate back to the fungal garden metabolizing 18:3 containing lipids from leaves to
307 synthesize 18:2. 18:3 is repulsive to the ants, while 18:2 is highly attractive to them. One very
308 clear example of coevolution between the leaf-cutter ants and *L. gongylophrous* is the cultivar's
309 morphological and physiological adaptation in the production of gongylidia for ant
310 consumption(36). Our result, indicating that the gongylidia contain lipids that the leaf-cutter ants
311 are attracted to, provides a further example of the intricacies of coevolution through lipid
312 communication in this system. By restricting the enrichment of 18:2 lipids to the gongylidia, the
313 fungus can focus the ants' consumption to these specialized structures, thereby preventing
314 damage to its hyphae. Such communication from the fungus to the ants is vital for maintaining
315 fungal fitness, and for maintaining this mutualism.
316 As an important constitute of food, lipids act as a valuable source of energy, cellular
317 structural component, and as a communication tool. Thus, understanding what lipids are
318 consumed by leaf-cutter ants and how their cultivar functions to obtain, modify and deliver plant
319 lipids, further sheds light on this complex mutualistic relationship between these organisms. In a
320 broader context, it provides an important example of how microbes mediate the relationship
321 between herbivores and the plants that they consume, either directly, with microbes interfacing
322 with their hosts in the gut, or less directly, with the fungal garden serving as the ants' external
323 gut. Future lipidomic studies may help us to further understand how these lipids are used by the
324 ants in communication and symbiont maintenance, and if specific lipids play a role in
325 communication throughout the Attina, and other fungus farming insects, or if this phenomenon is
326 limited to the leaf-cutter ants and their cultivar *L. gongylophorus*.
327

328 **MATERIALS AND METHODS**

329 *Material collection*

330 Six lab-maintained leaf-cutter ant colonies (five *Atta cephalotes* and one *Atta sexdens*)
331 were provided exclusively maple leaves for the two weeks before sample collection. We
332 collected leaf material as well as fungal garden from the top, middle, and bottom of leaf-cutter
333 ant fungal garden ecosystems. These layers are differentiated based on color, texture and location
334 in the garden (**Figure 1**).

335 Gongylidia are difficult to collect because of their small size and their sparseness in the
336 garden. To collect gongylidia, we first collected fungal garden in deep petri dishes and reduced
337 the number of workers. With fewer workers in the fungal garden, the gongylidia proliferate.
338 After several days, the gongylidia were picked off by needlepoint under a dissecting microscope
339 and they were placed in a microcentrifuge tube with water.

340 The leaf material used in this experiment was collected in the summer and kept frozen at
341 -20°C in vacuum sealed bags so that the ant colonies can be fed during the winter when fresh
342 leaves are not available.

343 *Total lipid extraction*

344 The collected material was lyophilized for untargeted lipidomics analysis. Lipids were
345 extracted using a modified Folch extraction (37). To break up the biological material,
346 approximately 25 mg of sample was bead beaten using a 3 mm tungsten carbide bead in 750 µl
347 of methanol for 2 min at a frequency of 30 Hz. The sample was then removed and transferred
348 into a 20 mL clean EPS glass vial with a Teflon lined cap. Another 750 µl of methanol was
349 added for a final volume of 1.5 mL methanol. Next, 3 mL of chloroform and 200 µl of water
350 were added to each sample. The samples were vortexed for 30 s, sonicated for 30 min, vortexed
351 again for 30 s, and then 0.925 µl of water was added to induce a phase separation. The samples

352 were incubated at 4ºC overnight and then the lower lipid layer was removed, dried down, and
353 stored at -20ºC at a concentration of 2 µg/µl in 2:1 chloroform/methanol until Mass
354 Spectrometry (MS) analysis.

355 *Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) and LC-ion mobility*
356 *spectrometry-mass spectrometry (LC-IMS-MS) analyses*

357 All extracted lipids in this manuscript were analyzed by LC-MS/MS using a Waters
358 NanoAquity UPLC system interfaced with a Velos Orbitrap mass spectrometer (Thermo
359 Scientific, San Jose, CA) and an Agilent 6560 Ion Mobility QTOF MS system (Agilent, Santa
360 Clara, CA). The total lipid extracts (TLE) were reconstituted in methanol for a final abundance
361 of 0.4 µg TLE/µl. Seven µl of the TLE were injected onto Waters column (HSS T3 1.0 mm x
362 150 mm x 1.8 µm particle size). Lipids were separated over a 90 min gradient elution (mobile
363 phase A: ACN/H_2O (40:60) containing 10 mM ammonium acetate; mobile phase B: ACN/IPA
364 (10:90) containing 10 mM ammonium acetate) at a flow rate of 30 µl/min. Samples were
365 analyzed in both positive and negative ionization using HCD (higher-energy collision
366 dissociation) and CID (collision-induced dissociation) to obtain high coverage of the lipidome.
367 Velos Orbitrap MS abundance data is depicted in **Figure 2**, **Figure 3**, Data file S2, and Data file
368 S3.

369 The LC-IMS-MS analyses were also performed in both positive and negative ion mode
370 and collected from 100-3200 m/z at a MS resolution of 40,000. The LC-IMS-MS data were
371 analyzed using in-house PNNL software for deisotoping and feature finding the
372 multidimensional LC, IMS and MS data (38).

373 *Lipid targeted database and alignment*

374 We used LIQUID software (39) for lipid targeted database alignment. This is achieved by
375 aligning all datasets (grouped by sample type and ionization mode) and matching unidentified

376 features to their identified counterparts using MZmine2 (40). Aligned features are manually
377 verified and peak apex intensity values are exported for statistical analysis.
378 *Behavior experiment*
379 An ant behavior experiment was conducted in order to determine if *A. cephalotes* ants
380 could detect various lipids found in the fungal garden, and to observe their corresponding
381 behavioral response to them. We tested the ants' response to linoleic acid (abundant in the
382 gongylidia, 18:2 fatty acids), alpha-linolenic acid (abundant in the leaves and top of garden, 18:3
383 fatty acids), and oleic acid (excreted by dead ants, 18:1 fatty acids). To test the response of the *A.*
384 *cephalotes* to these lipids, we used a method similar to those from López-Riquelme *et al.*(34).
385 All three of the lipids were dissolved in ethanol at 1 mg/mL concentration. We pipetted 5 µL of
386 the mixture onto 6 mm paper discs (Whatman 2017-006), that had been pre-labeled with pencil,
387 then waited several seconds for the ethanol to evaporate before introducing the discs to the *A.*
388 *cephalotes* colonies. Discs with only ethanol were used as a negative control. Each treatment
389 replicate was set up as a binary choice between the treatment oil, and the negative control. We
390 used seven *A. cephalotes* queenright colonies, which were all collected from La Selva Biological
391 Research Station in Costa Rica in April 2018 and were maintained at the University of
392 Wisconsin-Madison. The colonies were housed in 42.5 cm x 30.2 cm x 17.8 cm plastic
393 containers. Three treatment discs and three control discs were used for each assay and were
394 placed on a flat surface in the colony box. The ants were observed for five minutes from the time
395 the discs were placed in the colony. Behavior was coded using the BORIS program (41). We
396 recorded the number of times discs were picked up, characterized by ants using their mandibles
397 to lift the disc in the same manner that they lift leaf pieces when foraging. We recorded the
398 number and duration of ant inspections, characterized by ants approaching the discs while
399 waving antennae toward the discs, touching the disks with their antennae, or walking over the
400 discs. Finally, we recorded the number and duration of aggressive behaviors toward the discs,

401 characterized by lunging and biting, or moving slowly or stopping nearby with open mandibles
402 (42) (example behaviors can be seen in Movie S1 and Movie S2). It should be noted that if
403 several ants were simultaneously exhibiting the same behavior, this was not considered a
404 separate event. The event would be considered active as long as at least one ant was exhibiting
405 the behavior. We tested all three treatments with each colony.

406 *Statistical Analysis*

407 To determine if individual lipids varied in their abundance across the samples, statistics
408 were performed on the aligned lipid peak apex intensity values from each of the gardens and
409 their respective sections from the bottom, middle and top of the gardens. Initial analysis
410 evaluated if the samples from within a garden are more highly correlated than across gardens.
411 Gardens were compared using a standard Pearson correlation values for all samples to one and
412 gardens were compared to one another using a simple two-sample t-test. There is no obvious
413 correlation structure by garden in **Figure 2** and **Figure 3** and the t-test confirms this with p-
414 values of 0.933 and 0.894, respectively, for the negative and positive datasets. This validated our
415 below statistical approach of grouping sample types (e.g., bottom, middle, top) across gardens.

416 Outlier identification was conducted using the algorithm RMD-PAV(43). There were no
417 outliers found and additionally, the data had very few missing values and thus no samples or
418 lipid identifications were removed as part of the quality control processing. A standard Analysis
419 of Variance (ANOVA) was used to evaluate each lipid for a statistical difference between the
420 top, middle and bottom for the first comparison (**Figure 2**) and between the middle and
421 gongylidia for the second comparison (**Figure 3**) with a Tukey's post-hoc test to compare the
422 individual levels to one another.

423 We evaluated if there was a multivariate difference in the lipid profiles between garden
424 components by Principal Components Analysis (PCA). We conduced one PCA to compare the

425 layers of the garden and another for the comparison between gongylidia and the middle of the
426 garden. In both cases, we used the function prcomp in the stats package of R with the scale
427 option. To determine if the distance between groups was significantly different than random, we
428 used ANOSIM and PERMANOVA, both in R (44).

429 For the behavior experiment, we did not compare the various treatments to each other,
430 but only to the control. Because the colonies differed in terms of their activity, for the duration of
431 time we evaluated the difference from control and for the number pickups and aggression events
432 we used the proportion of times the event happened to compensate for different activity levels or
433 number of ants between colonies. For the duration of time we conducted a one sample two tailed
434 t-test, where a mean different from zero indicated a significant difference between the treatment
435 and the control, and for the pickup and aggression events we performed a test of proportions,
436 where a proportion different from 0.5 indicated a significant difference (44).

437

438 **REFERENCES**

439 1. Merrill AL, Watt BK. 1973. Energy Value Of Foods - Basis and Derivation.
440 2. Yeagle PL. 1989. Lipid regulation of cell membrane structure and function. FASEB J
441 3:1833-42.
442 3. Waters CM, Bassler BL. 2005. Quorum sensing: Cell-to-cell communication in bacteria.
443 Annual Review of Cell and Developmental Biology 21:319-346.
444 4. Lebeer S, Vanderleyden J, De Keersmaecker SCJ. 2010. Host interactions of probiotic
445 bacterial surface molecules: comparison with commensals and pathogens. Nature
446 Reviews Microbiology 8:171-184.
447 5. Selander E, Kubanek J, Hamberg M, Andersson MX, Cervin G, Pavia H. 2015. Predator
448 lipids induce paralytic shellfish toxins in bloom-forming algae. Proceedings of the
449 National Academy of Sciences of the United States of America 112:6395-6400.
450 6. Kearns DB, Shimkets LJ. 2001. Lipid chemotaxis and signal transduction in Myxococcus
451 xanthus. Trends in Microbiology 9:126-129.
452 7. Ketter E, Randall G. 2019. Virus Impact on Lipids and Membranes. Annual Review of
453 Virology 6:319-340.
454 8. Brand JM, Young JC, Silverstein RM. 1979. Insect Pheromones: A Critical Review of
455 Recent Advances in Their Chemistry, Biology, and Application, p 1-190. *In* Herz W,
456 Grisebach H, Kirby GW (ed), Fortschritte der Chemie organischer Naturstoffe / Progress
457 in the Chemistry of Organic Natural Products doi:10.1007/978-3-7091-8545-2_1.
458 Springer Vienna, Vienna.

459 9. Belanger RM, Corkum LD. 2009. Review of Aquatic Sex Pheromones and Chemical
460 Communication in Anurans. Journal of Herpetology 43:184-191.
461 10. Liberles SD. 2014. Mammalian Pheromones. Annual Review of Physiology, Vol 76
462 76:151-175.
463 11. Magalhaes DM, Da Silva ITFA, Borges M, Laumann RA, Blassioli-Moraes MC. 2019.
464 Anthonomus grandis aggregation pheromone induces cotton indirect defence and attracts
465 the parasitic wasp Bracon vulgaris. Journal of Experimental Botany 70:1891-1901.
466 12. Costa AN, Vasconcelos HL, Vieira-Neto EHM, Bruna EM. 2008. Do herbivores exert
467 top-down effects in Neotropical savannas? Estimates of biomass consumption by leaf-
468 cutter ants. Journal of Vegetation Science 19:849-854.
469 13. Hölldobler B, Wilson EO. 2009. The superorganism: the beauty, elegance, and
470 strangeness of insect societies. WW Norton & Company, New York, NY.
471 14. Meyer ST, Leal IR, Tabarelli M, Wirth R. 2011. Ecosystem engineering by leaf-cutting
472 ants: nests of *Atta cephalotes* drastically alter forest structure and microclimate.
473 Ecological Entomology 36:14-24.
474 15. Holldobler B, Wilson EO. 2010. The Leafcutter Ants: Civilization by Instinct. W.W.
475 Norton and Company.
476 16. Aylward FO, Burnum-Johnson KE, Tringe SG, Teiling C, Tremmel DM, Moeller JA,
477 Scott JJ, Barry KW, Piehowski PD, Nicora CD, Malfatti SA, Monroe ME, Purvine SO,
478 Goodwin LA, Smith RD, Weinstock GM, Gerardo NM, Suen G, Lipton MS, Currie CR.
479 2013. *Leucoagaricus gongylophorus* produces diverse enzymes for the degradation of
480 recalcitrant plant polymers in leaf-cutter ant fungus gardens. Appl Environ Microbiol
481 79:3770-8.
482 17. Khadempour L, Burnum-Johnson KE, Baker ES, Nicora CD, Webb-Robertson BM,
483 White RA, 3rd, Monroe ME, Huang EL, Smith RD, Currie CR. 2016. The fungal cultivar
484 of leaf-cutter ants produces specific enzymes in response to different plant substrates.
485 Mol Ecol 25:5795-5805.
486 18. Suen G, Teiling C, Li L, Holt C, Abouheif E, Bornberg-Bauer E, Bouffard P, Caldera EJ,
487 Cash E, Cavanaugh A, Denas O, Elhaik E, Fave MJ, Gadau J, Gibson JD, Graur D,
488 Grubbs KJ, Hagen DE, Harkins TT, Helmkampf M, Hu H, Johnson BR, Kim J, Marsh
489 SE, Moeller JA, Munoz-Torres MC, Murphy MC, Naughton MC, Nigam S, Overson R,
490 Rajakumar R, Reese JT, Scott JJ, Smith CR, Tao S, Tsutsui ND, Viljakainen L, Wissler
491 L, Yandell MD, Zimmer F, Taylor J, Slater SC, Clifton SW, Warren WC, Elsik CG,
492 Smith CD, Weinstock GM, Gerardo NM, Currie CR. 2011. The genome sequence of the
493 leaf-cutter ant Atta cephalotes reveals insights into its obligate symbiotic lifestyle. PLoS
494 Genet 7:e1002007.
495 19. Nagamoto NS, Garcia MG, Forti LC, Verza SS, Noronha NC, Rodella RA. 2011.
496 Microscopic evidence supports the hypothesis of high cellulose degradation capacity by
497 the symbiotic fungus of leaf-cutting ants. Journal of Biological Research-Thessaloniki
498 16:308-312.
499 20. Suen G, Scott JJ, Aylward FO, Currie CR. 2011. The Microbiome of Leaf-Cutter Ant
500 Fungus Gardens, p 367-379. *In* de Bruijn FJ (ed), Handbook of Molecular Microbial
501 Ecology II doi:10.1002/9781118010549.ch36. John Wiley & Sons, Inc., Hoboken, NJ,
502 USA.
503 21. De Fine Licht HH, Schiott M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P,
504 Boomsma JJ. 2013. Laccase detoxification mediates the nutritional alliance between leaf-
505 cutting ants and fungus-garden symbionts. Proc Natl Acad Sci U S A 110:583-7.
506 22. Aylward FO, Khadempour L, Tremmel DM, McDonald BR, Nicora CD, Wu S, Moore
507 RJ, Orton DJ, Monroe ME, Piehowski PD, Purvine SO, Smith RD, Lipton MS, Burnum-

Johnson KE, Currie CR. 2015. Enrichment and Broad Representation of Plant Biomass-Degrading Enzymes in the Specialized Hyphal Swellings of Leucoagaricus gongylophorus, the Fungal Symbiont of Leaf-Cutter Ants. PLoS One 10:e0134752.

23. Farji-Brener AG, Medina CA. 2000. The Importance of Where to Dump the Refuse: Seed Banks and Fine Roots in Nests of the Leaf-Cutting Ants Atta cephalotes and A. colombica1. Biotropica 32:120-126.

24. Bass M, Cherrett JM. 1995. Fungal Hyphae as a Source of Nutrients for the Leaf-Cutting Ant Atta Sexdens. Physiological Entomology 20:1-6.

25. Shik JZ, Rytter W, Arnan X, Michelsen A. 2018. Disentangling nutritional pathways linking leafcutter ants and their co-evolved fungal symbionts using stable isotopes. Ecology 99:1999-2009.

26. Aylward FO, Burnum KE, Scott JJ, Suen G, Tringe SG, Adams SM, Barry KW, Nicora CD, Piehowski PD, Purvine SO, Starrett GJ, Goodwin LA, Smith RD, Lipton MS, Currie CR. 2012. Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. Isme Journal 6:1688-1701.

27. Kyle JE, Zhang X, Weitz KK, Monroe ME, Ibrahim YM, Moore RJ, Cha J, Sun X, Lovelace ES, Wagoner J, Polyak SJ, Metz TO, Dey SK, Smith RD, Burnum-Johnson KE, Baker ES. 2016. Uncovering biologically significant lipid isomers with liquid chromatography, ion mobility spectrometry and mass spectrometry. Analyst 141:1649-59.

28. Schultz TR, Brady SG. 2008. Major evolutionary transitions in ant agriculture. Proc Natl Acad Sci U S A 105:5435-40.

29. Nygaard S, Hu H, Li C, Schiøtt M, Chen Z, Yang Z, Xie Q, Ma C, Deng Y, Dikow RB, Rabeling C, Nash DR, Wcislo WT, Brady SG, Schultz TR, Zhang G, Boomsma JJ. 2016. Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. Nature Communications 7:12233.

30. Kobayashi K. 2016. Role of membrane glycerolipids in photosynthesis, thylakoid biogenesis and chloroplast development. J Plant Res 129:565-580.

31. Conconi A, Miquel M, Browse JA, Ryan CA. 1996. Intracellular Levels of Free Linolenic and Linoleic Acids Increase in Tomato Leaves in Response to Wounding. Plant Physiol 111:797-803.

32. Gordon DM. 1983. Dependence of necrophoric response to oleic acid on social context in the ant,Pogonomyrmex badius. J Chem Ecol 9:105-11.

33. Wilson EO, Durlach NI, Roth LM. 1958. Chemical Releaser of Necrophoric Behavior in Ants. Psyche: A Journal of Entomology 65:108-114.

34. Lopez-Riquelme GO, Malo EA, Cruz-Lopez L, Fanjul-Moles ML. 2006. Antennal olfactory sensitivity in response to task-related odours of three castes of the ant Atta mexicana (Hymenoptera : Formicidae). Physiological Entomology 31:353-360.

35. Buehlmann C, Graham P, Hansson BS, Knaden M. 2014. Desert ants locate food by combining high sensitivity to food odors with extensive crosswind runs. Curr Biol 24:960-4.

36. De Fine Licht HH, Boomsma JJ, Tunlid A. 2014. Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nat Commun 5:5675.

37. Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509.

38. Crowell KL, Slysz GW, Baker ES, LaMarche BL, Monroe ME, Ibrahim YM, Payne SH, Anderson GA, Smith RD. 2013. LC-IMS-MS Feature Finder: detecting multidimensional liquid chromatography, ion mobility and mass spectrometry features in complex datasets. Bioinformatics 29:2804-5.

39. Kyle JE, Crowell KL, Casey CP, Fujimoto GM, Kim S, Dautel SE, Smith RD, Payne SH, Metz TO. 2017. LIQUID: an-open source software for identifying lipids in LC-MS/MS-based lipidomics data. Bioinformatics 33:1744-1746.
40. Pluskal T, Castillo S, Villar-Briones A, Oresic M. 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics 11:395.
41. Friard O, Gamba M. 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods in Ecology and Evolution 7:1325-1330.
42. Hernandez JV, Lopez H, Jaffe K. 2002. Nestmate recognition signals of the leaf-cutting ant Atta laevigata. Journal of Insect Physiology 48:287-295.
43. Matzke MM, Waters KM, Metz TO, Jacobs JM, Sims AC, Baric RS, Pounds JG, Webb-Robertson BJ. 2011. Improved quality control processing of peptide-centric LC-MS proteomics data. Bioinformatics 27:2866-72.
44. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available at: http://wwwR-projectorg/ (Accessed: 23rd December 2013).

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Author contributions: Conceived of the project (KEBJ, ESB, CC, LK), conducted experiments (LK, CN, KEBJ, ESB) performed statistical analysis (BJMWR, LK), analyzed data (LK, KEBJ, ESB, JEK, FBS) designed sampling methodology (LK, KEBJ, CC), provided resources and facilities (KEBJ, ESB, CC, MSL, RDS) and wrote the manuscript (LK, KEBJ, ESB).

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603 **FIGURE LEGENDS**

604 **Figure 1: A schematic diagram of the process by which lipids are processed through the**
605 **fungal garden**. Fresh leaf material is first deposited on the top of the fungal garden and passes
606 through the middle and bottom of the garden. As the leaf material proceeds, lipids are extracted
607 by the fungus and modified. The final lipid profile in the gongylidia (the specialized fungal
608 structure that ants consume) differs from that which is in the leaf material and top of the fungal
609 garden. The gongylidia are characterized by lipids containing linoleic acid (18:2), whereas the
610 leaves are characterized by lipids containing alpha-linolenic acid (18:3). Lipid structures here are
611 only representative and other isomers are possible. Photo credits: Lily Khadempour, except
612 gongylidia by Don Parsons.
613
614 **Figure 2: Heatmaps representing the relative log2 expression level for lipids in the leaf food**
615 **source and the top, middle, and bottom strata of six leaf-cutter ant fungal gardens**. Lipids
616 significantly increased in the top are denoted with green font; lipids significantly increasing in
617 the bottom are denoted with orange font (p-values <0.05 were deemed significant); lipids not
618 changing across the top to the bottom of the garden are denoted with grey font; _A and _B
619 denote structural isomers. Diacylglycerophosphocholines (PC),
620 monoacylglycerophosphocholines (LPC), sulfoquinovosyldiacylglycerol (SQDG),
621 Monogalactosyldiacylglycerol (MGDG), Cardiolipin (CL), phosphatidic acid (PA), Hexosyl-
622 Ceramide (HexCer), Ceramide (Cer), Diacylglycerols (DG), diacylglycerophosphoglycerol (PG),
623 monoacylglycerophosphoglycerol (LPG), diacylglycerophosphoserine (PS),
624 diacylglycerophosphoethanolamines (PE), monoacylglycerophosphoethanolamines (LPE),
625 diacylglycerophosphoinositols (PI). Lipid abbreviations show the total number of acyl chain
626 carbons: total number of double bonds. ‡, 18:2 containing lipids significantly increased in the
627 bottom of the garden; *, 18:3 containing lipids significantly increased in the top of the garden.
628
629 **Figure 3: Heatmaps representing the relative log2 expression level for lipids in the leaf food**
630 **source and the gongylidia and surrounding fungal garden of six leaf-cutter ant fungal**
631 **garden ecosystems**. Lipids significantly enriched in the gongylidia compared to the surrounding
632 fungal garden are denoted with purple font; lipids significantly enriched in the fungal garden are
633 denoted with yellow font (p-values <0.05 were deemed significant); lipids not significantly
634 changing between the gongylidia and the surrounding fungal garden are denoted with grey font;
635 _A and _B denote structural isomers. Digalactosyldiacylglycerol (DGDG),
636 sulfoquinovosyldiacylglycerol (SQDG), Monogalactosyldiacylglycerol (MGDG),
637 diacylglycerophosphoglycerol (PG), monoacylglycerophosphoglycerol (LPG),
638 Diacylglycerophosphocholines (PC), monoacylglycerophosphocholines (LPC), mannosylinositol
639 phosphorylceramide (MIPC), Ceramide phosphoinositol (PI-Cer), diacylglycerophosphoinositols
640 (PI), phosphatidic acid (PA), lysophosphatidic acid (LPA), Hexosyl-Ceramide (HexCer),
641 Ceramide (Cer), diacylglycerophosphoethanolamines (PE),
642 monoacylglycerophosphoethanolamines (LPE), Triacylglycerols (TG), Diacylglycerols (DG).
643 Lipid abbreviations show the total number of acyl chain carbons: total number of double bonds.
644 *, 18:3 containing lipids significantly decreased in the gongylidia; ‡, 18:2 containing lipids
645 significantly increased in the gongylidia.
646
647 **Figure 4**: **Results of the behavior experiment**. *Atta cephalotes* ants are attracted to linoleic acid
648 (18:2) and oleic acid (18:1) but show aggression toward alpha-linolenic acid (18:3). Each point
649 represents one experimental replicate, where the values for the control count or duration are
650 subtracted from the treatment count or duration. A one sample t-test was conducted for the

difference in duration and a one sample test of proportion was conducted for the number of pickups and aggressions. Those whose mean is significantly different from zero are marked with * $p < 0.05$ and ** $p < 0.01$. Box plot definitions: center line – median, upper and lower box limits – upper and lower quartiles, whiskers – 1.5 x inter-quartile range, outliers – any points outside the 1.5x inter-quartile range.

SUPPLEMENTARY MATERIALS

Figure S1: Results of the behavior experiment. Similar to the boxplots in Figure 4, each point represents one experimental replicate, where the values for the control count or duration are subtracted from the treatment count or duration. Here the difference in the number of inspections as well as the difference in duration of aggressions are displayed. Again, a one sample t-tests was conducted for the time-based comparison and a one sample test of proportions was conducted for the difference in counts. Neither were found to be significantly different between treatments at a p-value threshold of 0.05. Box plot definitions: center line – median, upper and lower box limits – upper and lower quartiles, whiskers – 1.5 x inter-quartile range, outliers – any points outside the 1.5x inter-quartile range.

Figure S2: *Atta* ant behavior in response to alpha-linolenic acid (18:3) in comparison to control. Labels above panels represent the different colonies that were tested. Tracks represent the times of event occurrences and their durations.

Figure S3: *Atta* ant behavior in response to linoleic acid (18:2) in comparison to control. Labels above panels represent the different colonies that were tested. Tracks represent the times of event occurrences and their durations.

Figure S4: *Atta* ant behavior in response to oleic acid (18:1) in comparison to control. Labels above panels represent the different colonies that were tested. Tracks represent the times of event occurrences and their durations.

Data file S1: Proteomic identification of lipid associated proteins. Quantities of protein spectra related to lipid metabolism identified throughout the fungus garden, from the dataset produced for Aylward et al. 2015.

Data file S2: Lipid comparisons of top, middle, and bottom fungal garden regions. Lipidomic intensity data and statistical analysis results for comparisons across the top, middle, and bottom fungal garden regions.

Data file S3: Lipid changes between fungal garden and gongylidia. Lipidomic intensity data and statistical analysis results for comparisons across the middle fungal garden and gongylidia.

Movie S1: Aggressive behaviors. Discs labeled D are the negative control infused with only ethanol. Discs labeled A are infused with ethanol and alpha-linolenic acid. In the first 34 s, ants can be seen near or on both types of discs with their mandibles open, moving very slowly or standing still. In the second part of the movie, an ant can be seen lunging and biting at the alpha-linolenic acid infused discs. Movie can be viewed at https://youtu.be/fq6SVF-8v6M.

Movie S2: Interest/attractive behavior. Discs labeled D are the negative control infused with only ethanol. Discs labeled B are infused with ethanol and linoleic acid. In the first 12 s of the movie, ants can be seen approaching the linoleic acid discs, and waving their antennae. In the second part of the movie, ants are inspecting the linoleic acid discs and one ant picks up a disc at 30 s. Movie can be viewed at https://youtu.be/unUL-ets3Vk.

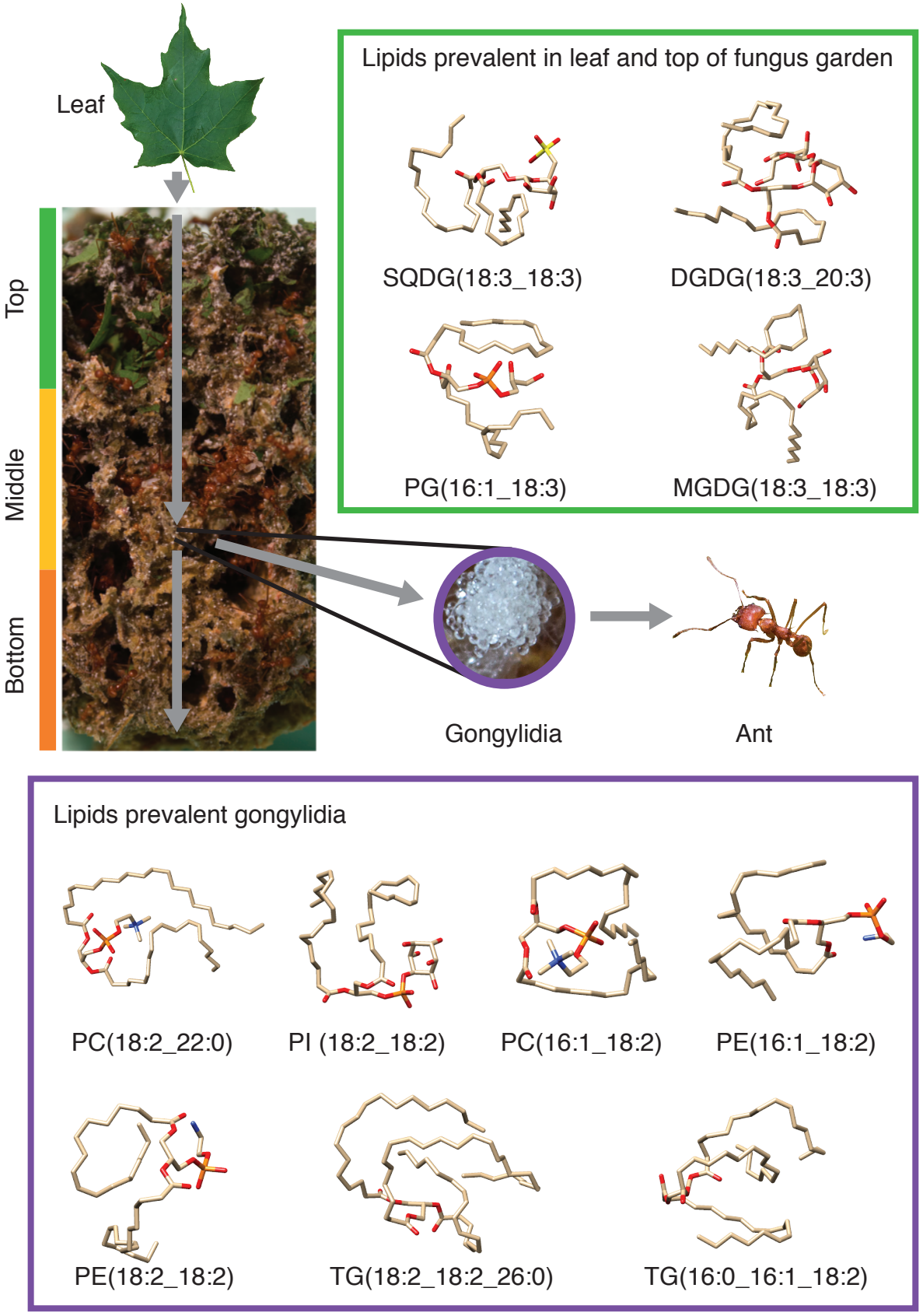

Leaf
Top
Middle
Bottom
Lipids prevalent in leaf and top of fungus garden
SQDG(18:3_18:3)
DGDG(18:3_20:3)
PG(16:1_18:3)
MGDG(18:3_18:3)
Gongylidia
Ant
Lipids prevalent gongylidia
PC(18:2_22:0)
PI (18:2_18:2)
PC(16:1_18:2)
PE(16:1_18:2)
PE(18:2_18:2)
TG(18:2_18:2_26:0)
TG(16:0_16:1_18:2)

Figure 1

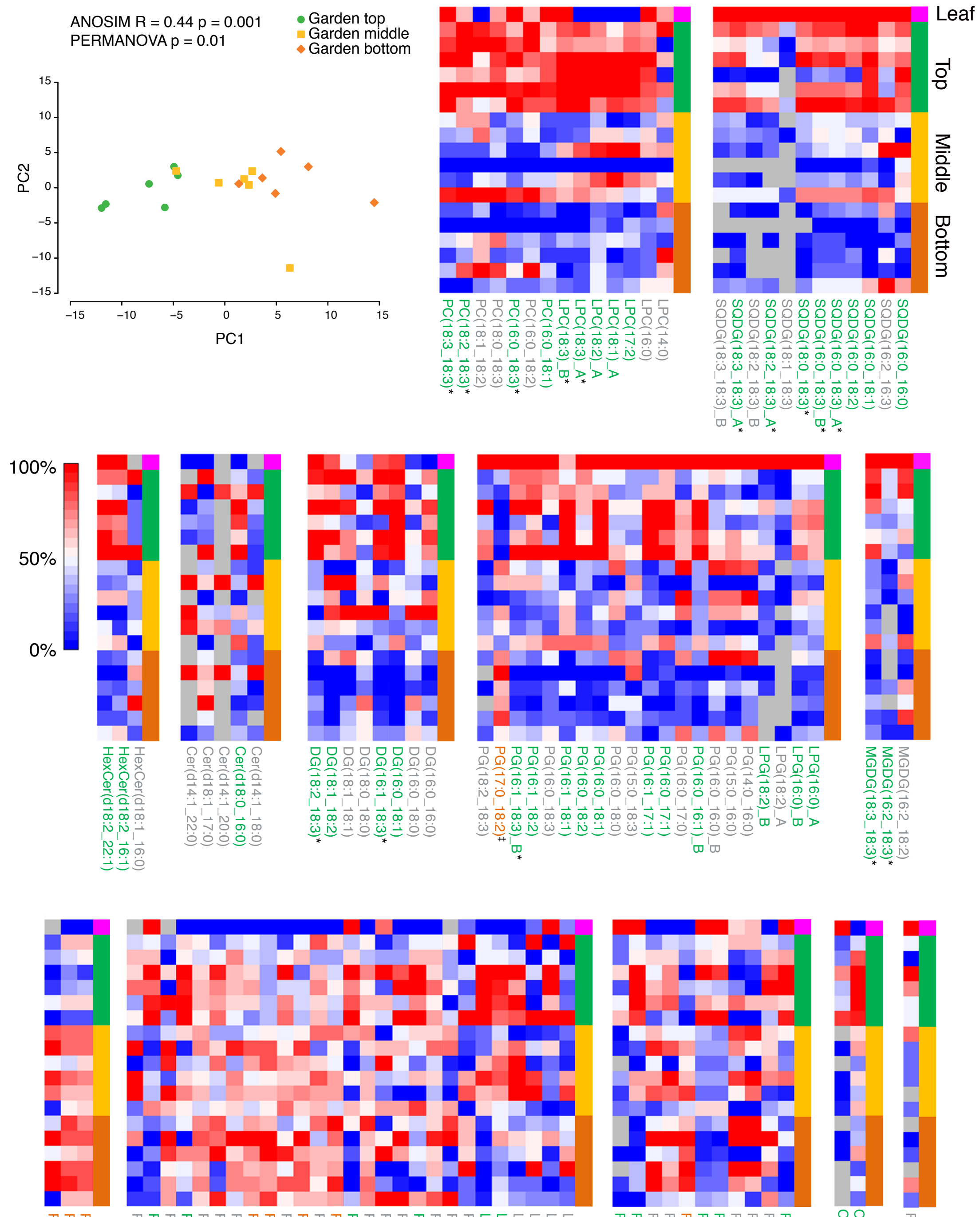
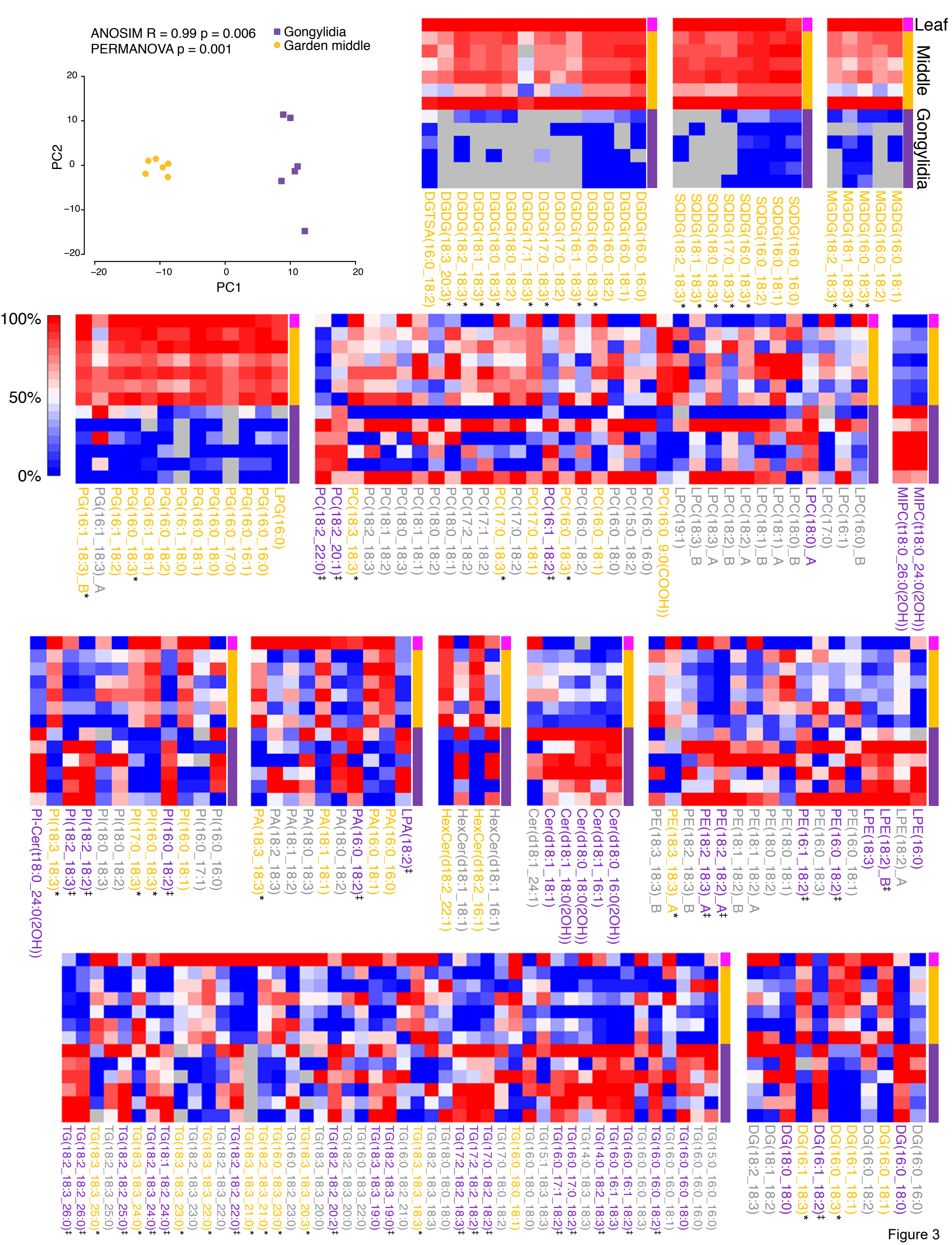
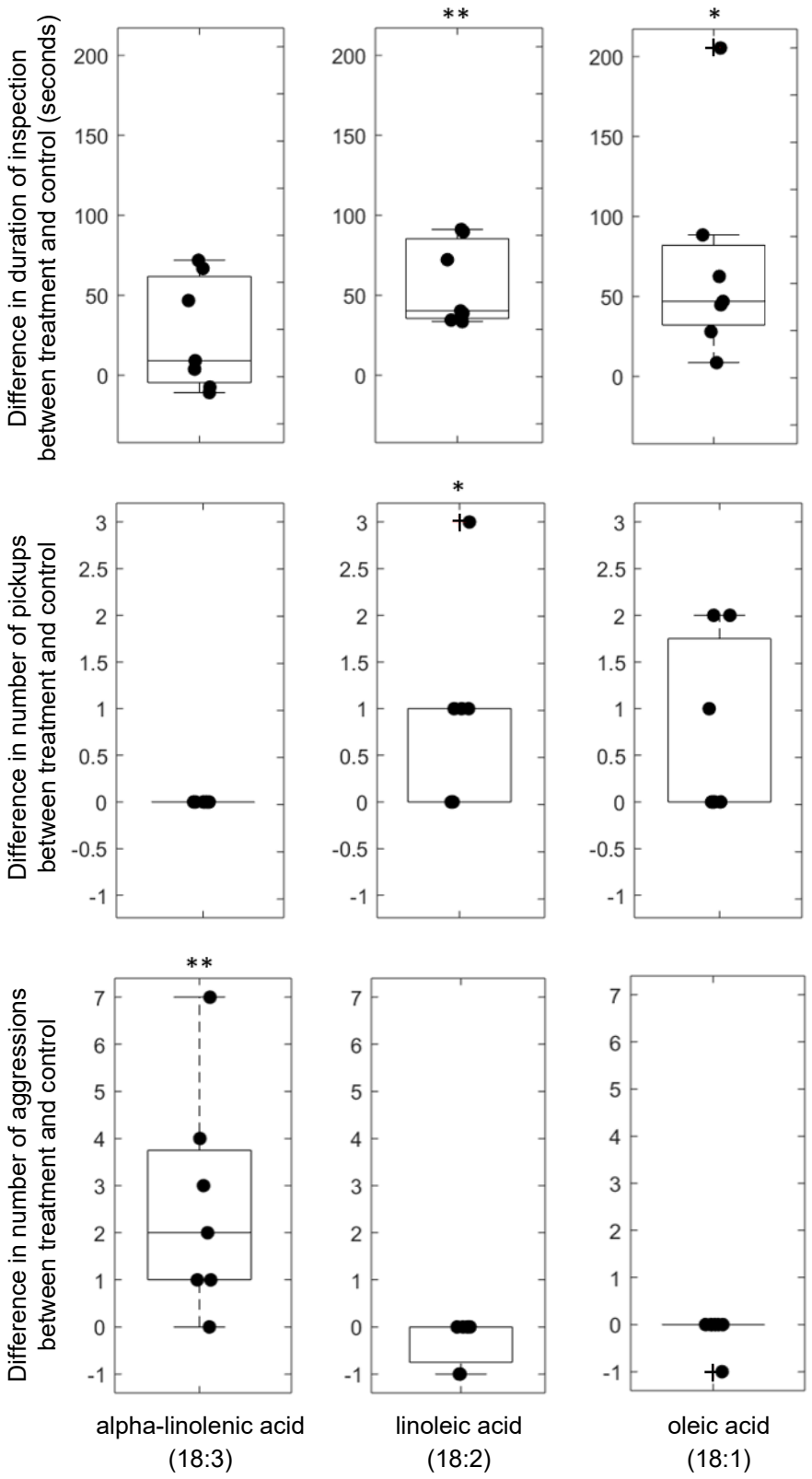

ANOSIM R = 0.44 p = 0.001
PERMANOVA p = 0.01
Garden top
Garden middle
Garden bottom
PC1
PC2
Leaf
Top
Middle
Bottom
100%
50%
0%

Figure 2


ANOSIM R = 0.99 p = 0.006
PERMANOVA p = 0.001
Gongylidia
Garden middle
PC2
PC1
Leaf
Middle
Gongylidia
100%
50%
0%
DGTSA(16:0_18:2)
DGDG(18:3_20:3)*
DGDG(18:2_18:3)*
DGDG(18:1_18:3)*
DGDG(18:0_18:3)*
DGDG(18:0_18:2)
DGDG(17:1_18:3)*
DGDG(17:0_18:3)*
DGDG(17:0_18:2)
DGDG(16:1_18:3)*
DGDG(16:0_18:3)*
DGDG(16:0_18:2)
DGDG(16:0_18:1)
DGDG(16:0_16:0)
SQDG(18:2_18:3)*
SQDG(18:1_18:3)*
SQDG(18:0_18:3)*
SQDG(17:0_18:3)*
SQDG(16:0_18:3)*
SQDG(16:0_18:2)
SQDG(16:0_18:1)
SQDG(16:0_16:0)
MGDG(18:2_18:3)*
MGDG(18:1_18:3)*
MGDG(16:0_18:3)*
MGDG(16:0_18:2)
MGDG(16:0_18:1)
LPG(16:0)
PG(16:0_16:0)
PG(16:0_16:1)
PG(16:0_17:0)
PG(16:0_18:0)
PG(16:0_18:1)
PG(16:0_18:2)
PG(16:1_18:1)
PG(16:0_18:3)*
PG(16:1_18:2)
PG(16:1_18:3)_A
PG(16:1_18:3)_B*
PC(18:2_22:0)‡
PC(18:2_20:1)‡
PC(18:3_18:3)*
PC(18:2_18:3)
PC(18:1_18:2)
PC(18:0_18:3)
PC(18:1_18:1)
PC(18:0_18:2)
PC(18:0_18:1)
PC(17:2_18:2)
PC(17:1_18:2)
PC(17:0_18:2)
PC(17:0_18:3)*
PC(17:0_18:1)
PC(16:1_18:2)‡
PC(16:0_18:3)*
PC(16:0_18:2)
PC(16:0_18:1)
PC(15:0_18:2)
PC(16:0_18:0)
PC(16:0_16:0)
PC(16:0_9:0(COOH))
LPC(19:1)
LPC(18:3)_B
LPC(18:3)_A
LPC(18:2)_B
LPC(18:2)_A
LPC(18:1)_B
LPC(18:1)_A
LPC(18:0)_B
LPC(18:0)_A
LPC(17:0)
LPC(16:1)
LPC(16:0)_B
MIPC(t18:0_24:0(2OH))
MIPC(t18:0_26:0(2OH))
LPE(16:0)
LPE(18:2)_A
LPE(18:2)_B‡
LPE(18:3)
PE(16:0_18:1)
PE(16:0_18:2)‡
PE(16:0_18:3)
PE(16:1_18:2)‡
PE(18:0_18:1)
PE(18:0_18:2)
PE(18:1_18:2)_A
PE(18:1_18:2)_B
PE(18:2_18:2)_A‡
PE(18:2_18:3)_A‡
PE(18:2_18:3)_B
PE(18:3_18:3)_A*
PE(18:3_18:3)_B
Cer(d18:0_16:0(2OH))
Cer(d18:1_16:1)
Cer(d18:0_18:0(2OH))
Cer(d18:1_18:0(2OH))
Cer(d18:1_18:1)
Cer(d18:1_24:1)
HexCer(d18:1_16:1)
HexCer(d18:2_16:1)
HexCer(d18:1_18:1)
HexCer(d18:2_22:1)
LPA(18:2)‡
PA(16:0_16:0)
PA(16:0_18:1)
PA(16:0_18:2)‡
PA(18:0_18:2)
PA(18:1_18:1)
PA(18:0_18:3)
PA(18:1_18:2)
PA(18:2_18:3)
PA(18:3_18:3)*
PI(16:0_16:0)
PI(16:0_17:1)
PI(16:0_18:1)
PI(16:0_18:2)‡
PI(16:0_18:3)*
PI(17:0_18:3)*
PI(18:0_18:2)
PI(18:0_18:3)
PI(18:2_18:2)‡
PI(18:2_18:3)‡
PI(18:3_18:3)*
PI-Cer(t18:0_24:0(2OH))
DG(16:0_16:0)
DG(16:0_18:0)
DG(16:0_18:1)
DG(16:0_18:2)
DG(16:1_18:1)
DG(16:0_18:3)*
DG(16:1_18:2)‡
DG(16:1_18:3)*
DG(18:0_18:0)
DG(18:1_18:2)
DG(18:2_18:3)
TG(15:0_16:0)
TG(16:0_16:0)
TG(16:0_16:0)
TG(16:0_16:0)
TG(16:0_18:1)
TG(16:0_18:2)‡
TG(16:0_16:0)
TG(16:0_16:1)
TG(16:0_16:1)
TG(14:0_18:2)
TG(14:0_18:3)
TG(16:0_17:0)
TG(16:0_17:1)
TG(15:1_18:3)
TG(16:0_18:0)
TG(16:0_18:1)
TG(17:0_18:0)
TG(17:0_18:2)
TG(17:0_18:2)
TG(17:2_18:2)
TG(18:0_18:0)
TG(18:2_18:3)
TG(18:3_18:3)*
TG(16:0_18:2)
TG(16:0_18:3)
TG(18:2_18:3)
TG(18:3_18:3)*
TG(18:2_18:2)
TG(18:2_18:2)
TG(18:3_18:3)
TG(18:3_18:3)*
TG(18:2_18:3)
TG(18:2_18:3)
TG(18:3_18:3)*
TG(18:2_18:2)
TG(18:3_18:3)*
TG(18:3_18:3)*
TG(18:1_18:2)
TG(18:2_18:3)
TG(18:2_18:2)
TG(18:2_18:3)
TG(18:3_18:3)*
TG(18:2_18:2)
TG(18:2_18:3)
TG(18:2_18:3)
TG(18:2_18:3


Difference in duration of inspection between treatment and control (seconds)
Difference in number of pickups between treatment and control
Difference in number of aggressions between treatment and control
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alpha-linolenic acid (18:3)
linoleic acid (18:2)
oleic acid (18:1)

Figure 4