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

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

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

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

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

RESULTS

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

Lipid comparisons of leaves and fungal garden regions

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

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

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

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

composition. All significantly changing 18:3 containing PC, PE, PA, and DG species in the middle garden were depleted in the gongylidia (signified with an * in **Figure 3**). Conversely, all significantly changing 18:2 containing PC, PE, PA, DG, and PI species were enriched in the gongylidia (signified with a ‡ in **Figure 3**). Many TG species contained both 18:3 and 18:2 as one of their three fatty acyl chains, yet ninety percent of all TG species depleted in the gongylidia compared to the surrounding fungal garden contain at least one 18:3 acyl chain and >84% of all TG species enriched in the gongylidia contain at least one 18:2 acyl chain. Additionally, lipid subclasses with high abundance in the leaves (MDGD, SQDG, PG, and DGDG) showed relatively low concentrations in the gongylidia samples.

Behavior experiment

Our lipidomic analyses characterized a prevalence of lipid species containing linoleic acid (18:2) in the gongylidia, the leaf-cutter ant's food source, and lipid species containing alpha-linolenic acid (18:3) in leaf material. We performed a behavior experiment to test if the *Atta cephalotes* workers had different responses to untreated paper discs and paper discs treated with oleic acid (18:1), 18:2, and 18:3 (**Figure 4**; Figure S1, S2, S3, & S4; Movie S1 & S2). Overall, the ants spent significantly more time inspecting 18:2 ($p=0.0012$) and 18:1 ($p=0.015$) treated paper discs than the control paper discs. Similarly, the ants picked up the discs infused with 18:2 ($p=0.039$) more frequently than the control discs, but during the experiment never picked up a disc containing 18:3. However, the ants displayed aggressive behavior, including lunging and biting the discs more frequently toward 18:3 compared to the control ($p=0.014$). The ants did not exhibit aggressive behavior towards the discs with 18:1 or 18:2.

DISCUSSION

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

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

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

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

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

MATERIALS AND METHODS

Material collection

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

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

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

Total lipid extraction

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

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

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

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

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

Lipid targeted database and alignment

We used LIQUID software (39) for lipid targeted database alignment. This is achieved by 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,

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

Statistical Analysis

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

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

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

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

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

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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).

Competing interests: There are no competing interests.

Data and materials availability: All data are available in the Supplementary Materials. Raw lipidomic datasets are deposited on Mass Spectrometry Interactive Virtual Environment (MassIVE). After the manuscript is published and has a PubMed ID, the data will be made public and available on MassIVE; [https://massive.ucsd.edu] accession number MSV000084520.

FIGURE LEGENDS

Figure 1: A schematic diagram of the process by which lipids are processed through the fungal garden. Fresh leaf material is first deposited on the top of the fungal garden and passes through the middle and bottom of the garden. As the leaf material proceeds, lipids are extracted by the fungus and modified. The final lipid profile in the gongylidia (the specialized fungal structure that ants consume) differs from that which is in the leaf material and top of the fungal garden. The gongylidia are characterized by lipids containing linoleic acid (18:2), whereas the leaves are characterized by lipids containing alpha-linolenic acid (18:3). Lipid structures here are only representative and other isomers are possible. Photo credits: Lily Khadempour, except gongylidia by Don Parsons.

Figure 2: Heatmaps representing the relative log2 expression level for lipids in the leaf food source and the top, middle, and bottom strata of six leaf-cutter ant fungal gardens. Lipids significantly increased in the top are denoted with green font; lipids significantly increasing in the bottom are denoted with orange font (p-values <0.05 were deemed significant); lipids not changing across the top to the bottom of the garden are denoted with grey font; _A and _B denote structural isomers. Diacylglycerophosphocholines (PC), monoacylglycerophosphocholines (LPC), sulfoquinovosyldiacylglycerol (SQDG), Monogalactosyldiacylglycerol (MGDG), Cardiolipin (CL), phosphatidic acid (PA), Hexosyl-Ceramide (HexCer), Ceramide (Cer), Diacylglycerols (DG), diacylglycerophosphoglycerol (PG), monoacylglycerophosphoglycerol (LPG), diacylglycerophosphoserine (PS), diacylglycerophosphoethanolamines (PE), monoacylglycerophosphoethanolamines (LPE), diacylglycerophosphoinositols (PI). Lipid abbreviations show the total number of acyl chain carbons: total number of double bonds. ‡, 18:2 containing lipids significantly increased in the bottom of the garden; *, 18:3 containing lipids significantly increased in the top of the garden.

Figure 3: Heatmaps representing the relative log2 expression level for lipids in the leaf food source and the gongylidia and surrounding fungal garden of six leaf-cutter ant fungal garden ecosystems. Lipids significantly enriched in the gongylidia compared to the surrounding fungal garden are denoted with purple font; lipids significantly enriched in the fungal garden are denoted with yellow font (p-values <0.05 were deemed significant); lipids not significantly changing between the gongylidia and the surrounding fungal garden are denoted with grey font; _A and _B denote structural isomers. Digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), Monogalactosyldiacylglycerol (MGDG), diacylglycerophosphoglycerol (PG), monoacylglycerophosphoglycerol (LPG), Diacylglycerophosphocholines (PC), monoacylglycerophosphocholines (LPC), mannosylinositol phosphorylceramide (MIPC), Ceramide phosphoinositol (PI-Cer), diacylglycerophosphoinositols (PI), phosphatidic acid (PA), lysophosphatidic acid (LPA), Hexosyl-Ceramide (HexCer), Ceramide (Cer), diacylglycerophosphoethanolamines (PE), monoacylglycerophosphoethanolamines (LPE), Triacylglycerols (TG), Diacylglycerols (DG). Lipid abbreviations show the total number of acyl chain carbons: total number of double bonds. *, 18:3 containing lipids significantly decreased in the gongylidia; ‡, 18:2 containing lipids significantly increased in the gongylidia.

Figure 4: **Results of the behavior experiment**. *Atta cephalotes* ants are attracted to linoleic acid (18:2) and oleic acid (18:1) but show aggression toward alpha-linolenic acid (18:3). Each point represents one experimental replicate, where the values for the control count or duration are 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.

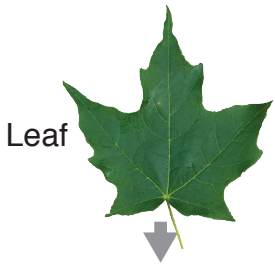
Data file S1: Proteomic identification of lipid associated proteins. Quantities of protein spectra related to lipid metabolism identified throughout the fungus garden, from the dataset produced for Aylward et al. 2015.

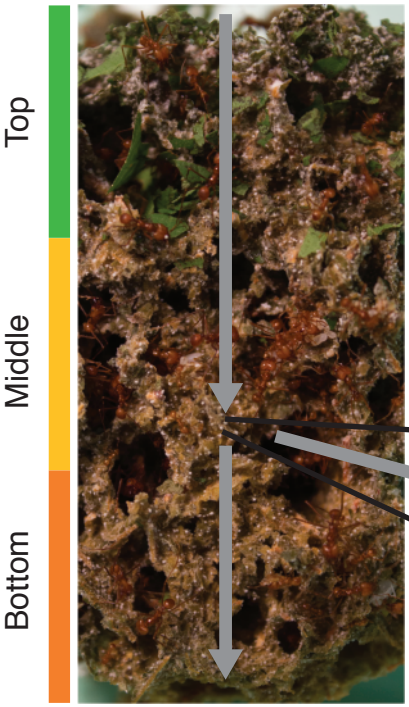
Data file S2: Lipid comparisons of top, middle, and bottom fungal garden regions. Lipidomic intensity data and statistical analysis results for comparisons across the top, middle, and bottom fungal garden regions.

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.

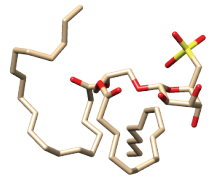
Movie S1: Aggressive behaviors. Discs labeled D are the negative control infused with only ethanol. Discs labeled A are infused with ethanol and alpha-linolenic acid. In the first 34 s, ants can be seen near or on both types of discs with their mandibles open, moving very slowly or standing still. In 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.





Lipids prevalent in leaf and top of fungus garden



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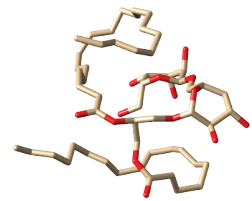

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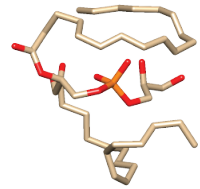
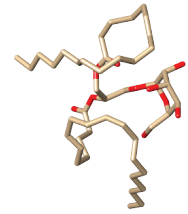
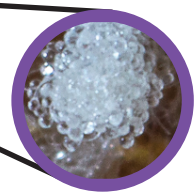


PG(16:1_18:3)



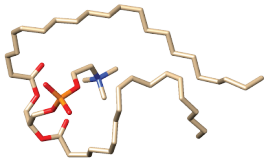
MGDG(18:3_18:3)



Gongylidia

Ant

Lipids prevalent gongylidia



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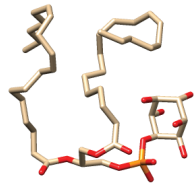

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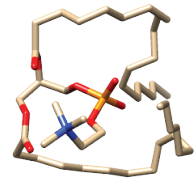

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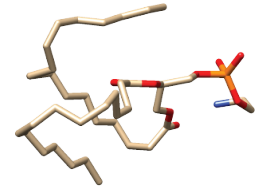

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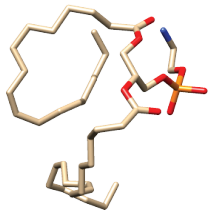

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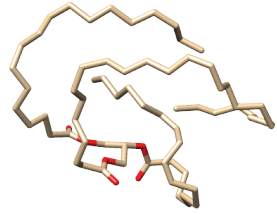

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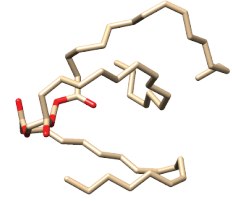
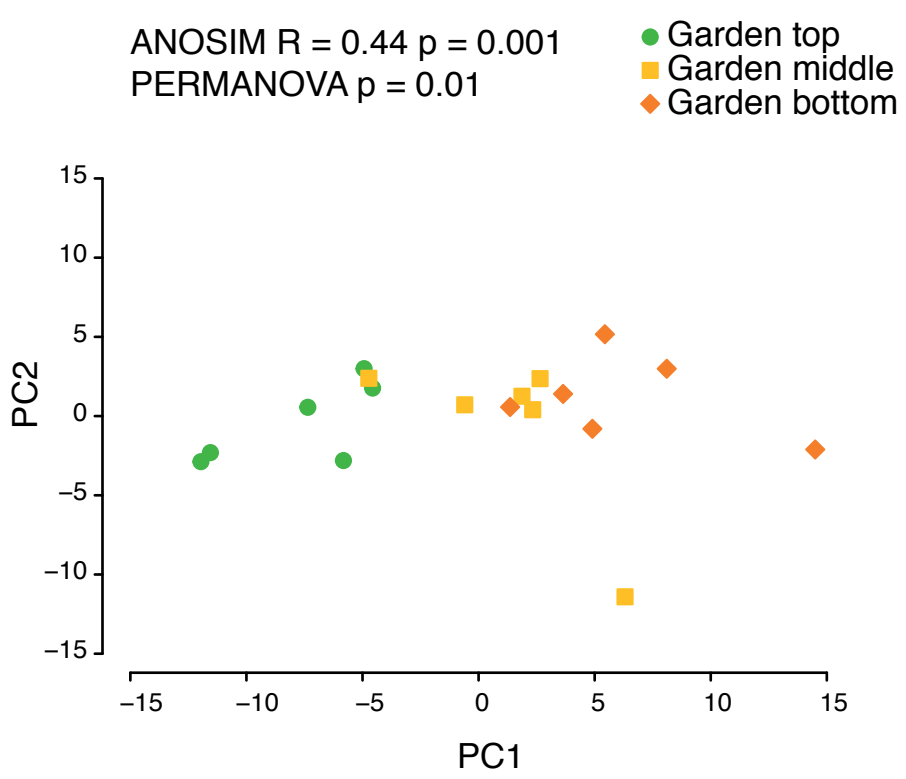


TG(16:0_16:1_18:2)

Figure 1



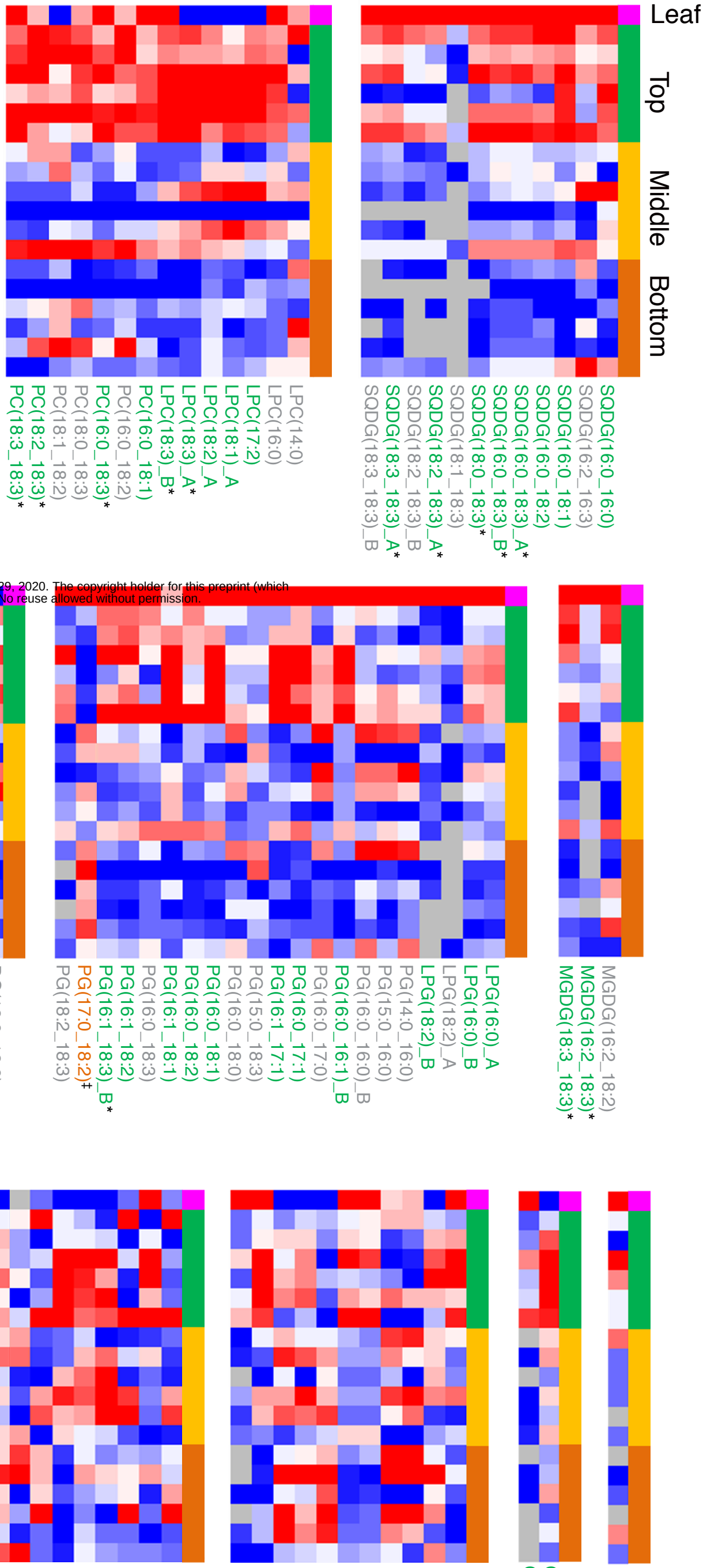
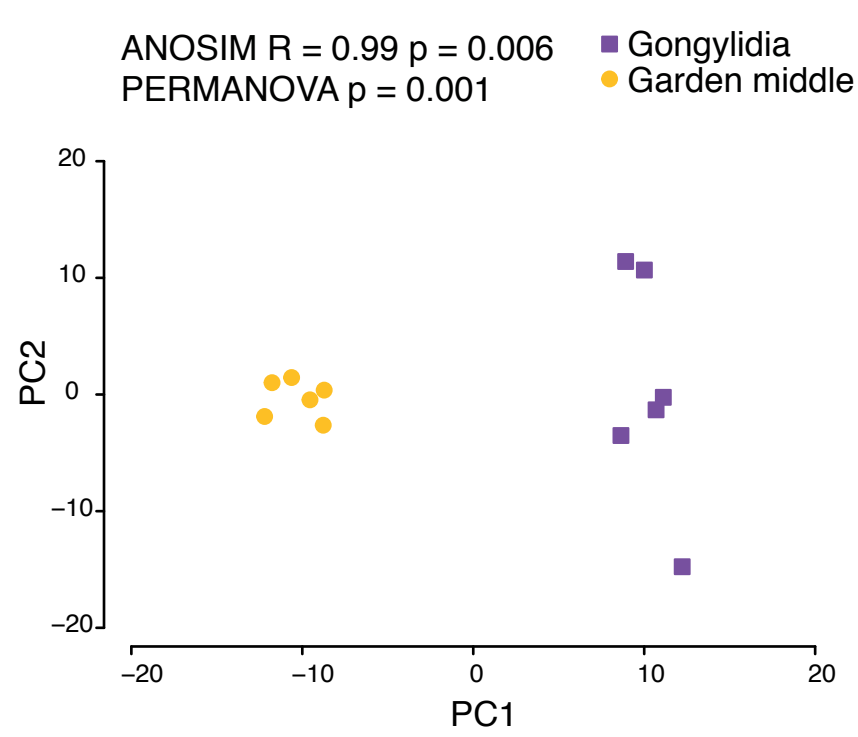
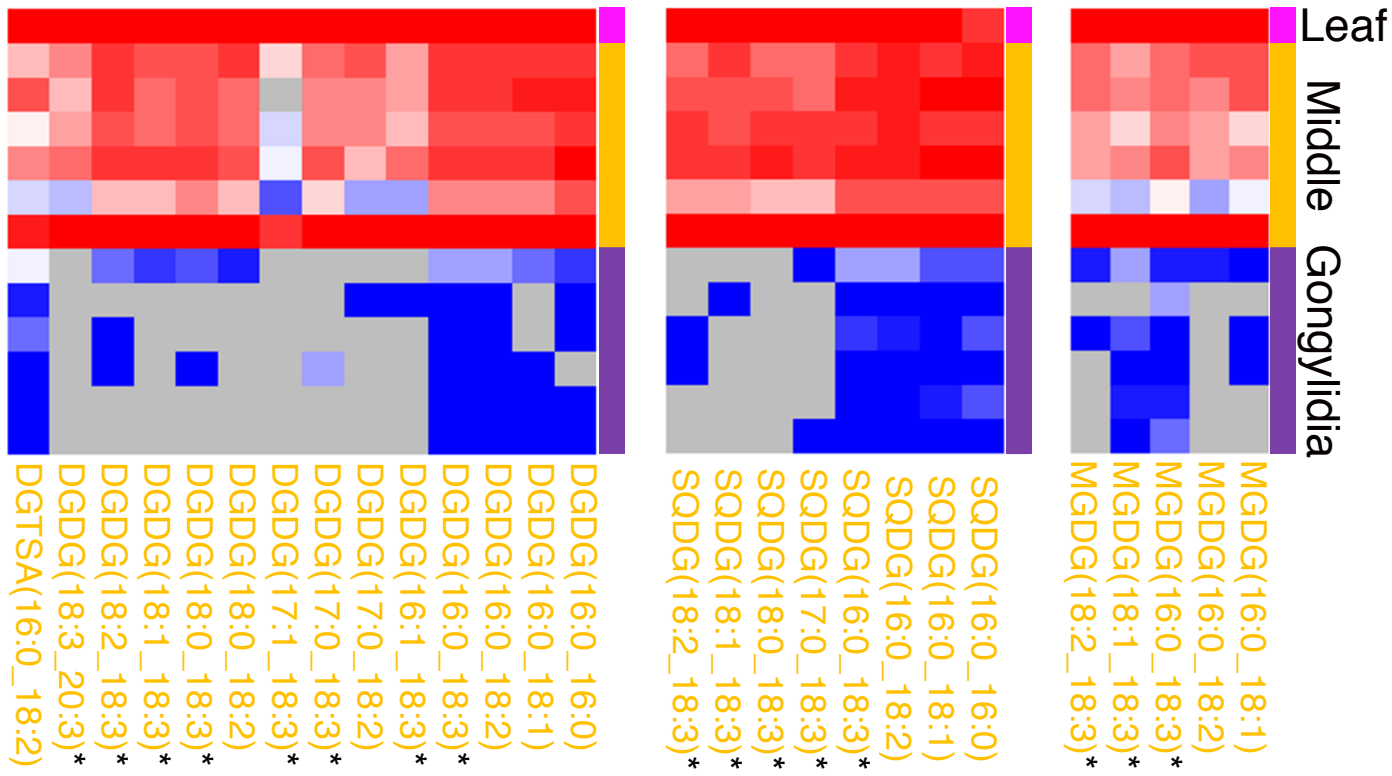


Figure 2





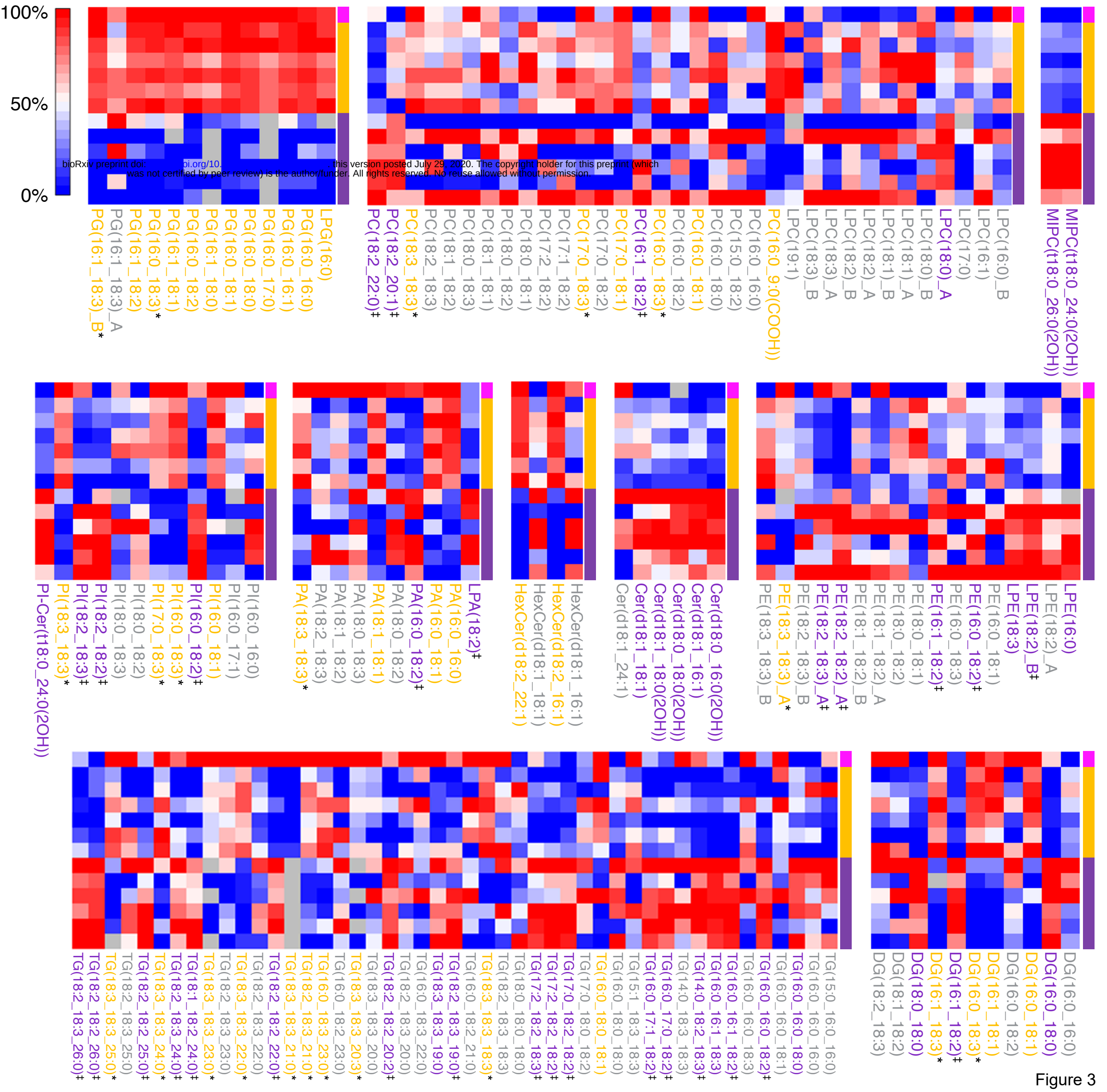


Figure 3

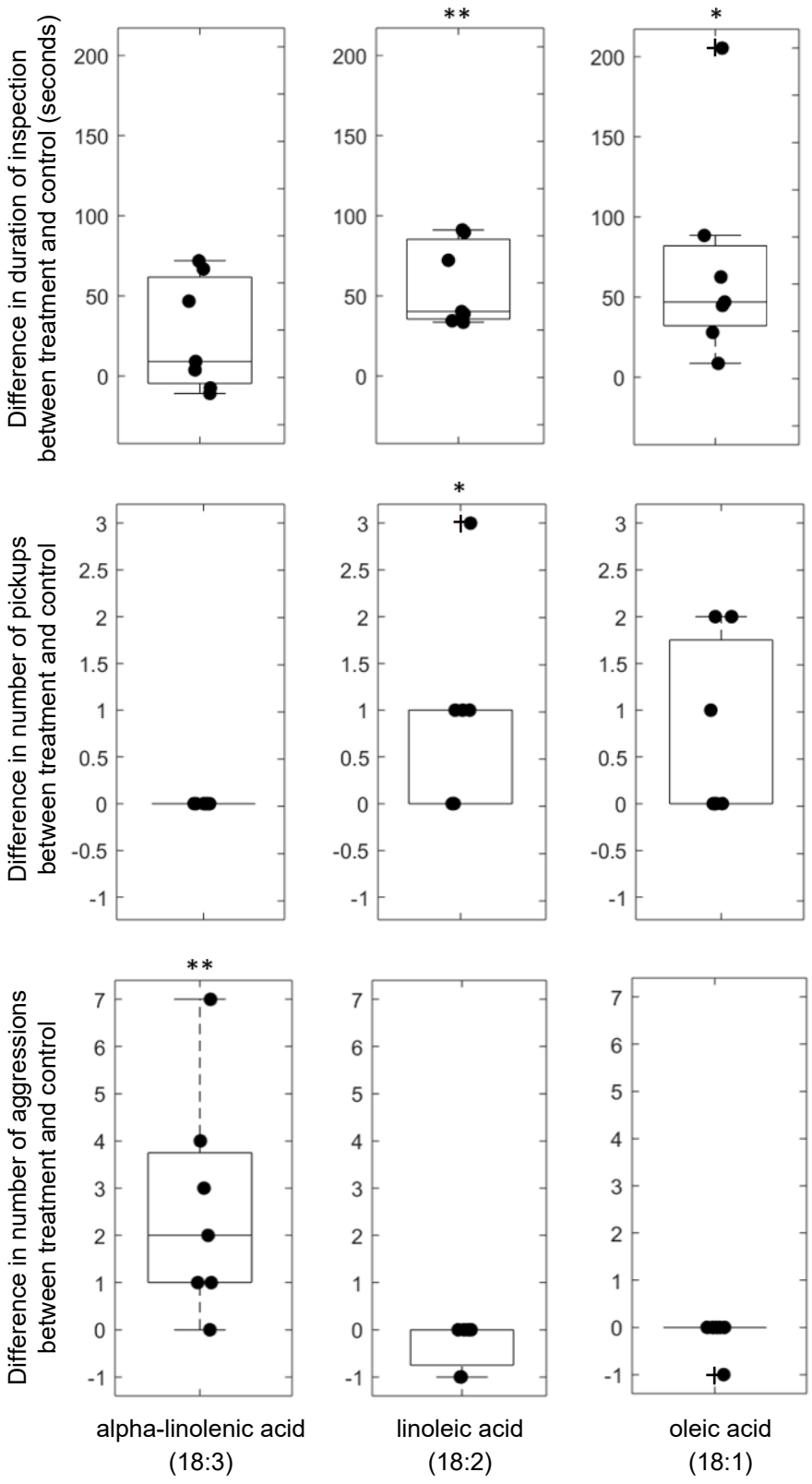


Figure 4