A hymenopteran odorant alerts flies to bury eggs

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Abstract: Ants are ubiquitous and consume insects at all life stages, presumably creating a strong selective pressure for ant avoidance behaviors across insects. The insect egg stage can be especially defenseless against predation given that eggs are usually immobile and unguarded, suggesting insect mothers may have evolved oviposition strategies to minimize the ant predation risk to their offspring. Given the lack of parental care in most insects, these oviposition strategies would likely be innate rather than learned, since insect mothers are not usually present to assess predation of their eggs. Here, we use the vinegar fly *Drosophila melanogaster* as a model system for examining parental defensive responses to ant presence. Flies usually lay eggs partially inserted into the food substrate, although some are laid on top of the food and a few are inserted deeply into the food. We found that exposure to ants significantly alters fly oviposition depth: the proportion of eggs on the food surface decreased while the proportion of buried eggs increased. Buried eggs survive ant foraging bouts better than surface eggs, showing that this oviposition depth behavior is adaptive. This induced behavior is conserved across the genus Drosophila and is dependent on the fly olfactory system: anosmic mutant flies fail to bury their eggs in the presence of ants, and ant odor extracts are sufficient to induce egg burying. By fractionating ant body washes and using GC-MS to identify fraction constituents, we identified the saturated, long-chain alcohol 1-octadecanol as the odorant flies use to sense ant presence. To further delineate the ant lineages to which flies respond, we exposed flies to the odors from numerous species of ants and other insects. Surprisingly, flies buried their eggs in response to the odors of nearly all hymenopterans tested, including hymenopteran groups that flies rarely interact with in nature like bees and paper wasps. Our data suggest that 1-octadecanol is a conserved and ancient hymenopteran odorant, and that drosophilids evolved a mechanism for sensing this odorant early in their evolution as a means of protecting their offspring from ant predation. This study sheds light on the ecology and mechanisms underlying a common biotic interaction in nature, that between insect parents and the ants that would consume their offspring.

Introduction:

One of the greatest threats that animals face in nature is predation, being killed and eaten by another organism (Bowman & Hacker, 2020). A common type of defense against predation are defensive behaviors, e.g. where animals hide or escape from nearby predators. Organisms have also evolved to assess and behaviorally respond to the mere risk of predation, not just direct predator exposure (Hermann & Landis, 2017). Because predation results in death, anti-predation behaviors, especially in non-social organisms, are usually innate rather than learned, encoded in an organism's germ line and brain (Baker et al., 2001; Ren & Tao, 2020). The neurogenetic basis of how naïve prey organisms perceive and respond to predators to which they have never been exposed remains poorly understood. Therefore, it would be useful to develop a model system for studying the mechanistic basis of innate anti-predation behaviors.

The vinegar fly *Drosophila melanogaster* has been a genetic 'model organism' for more than a century, and has already proven useful for understanding interactions with a different kind of biotic threat: parasitism (Lemaitre & Hoffmann, 2007). Furthermore, recent technological innovations have made it possible to genetically manipulate the activity of relatively small groups of neurons in the fly nervous system, and thus define the neurological circuitry underlying defensive behaviors (Venken et al., 2011). These tools have been used to uncover the neurological basis of fly responses to simulated predation scenarios, such as a visual 'looming' stimulus (Ache et al., 2019; Morimoto et al., 2020; Muijres et al., 2014). Nevertheless, experiments with natural predators may yield a more nuanced and complete picture of fly defense behaviors. Because flies and their potential predators are relatively small, these natural predator-prey interactions can be studied in controlled lab settings. However, one drawback of developing flies as a model for anti-predation behaviors is that natural history data describing the relative importance of different predator types is limited for natural populations of *Drosophila* (Markow, 2015; Reaume & Sokolowski, 2006; Soto-Yéber et al., 2018).

A large diversity of generalist predators likely consume *D. melanogaster* in nature. We have observed adults being caught in the air or picked off surfaces by predatory flies, dragonflies, spiders, lizards, and hummingbirds. Flies have evolved constitutively erratic flight patterns, as well as induced behaviors like evasive flight maneuvers, avoidance, freezing, jumping, and posturing to escape capture in these contexts (Combes et al., 2012; de la Flor et al., 2017; Parigi et al., 2019). Less is known about predation of *D. melanogaster* eggs, larvae, and pupae in nature, although we have observed rove beetles, predatory beetle larvae, predatory fly larvae, and ants consuming these juvenile stages. Given that fly eggs and pupae are immobile and that larvae are relatively slow crawlers, options for behavioral defenses against predators in these life stages appear limited. However, some constitutive behaviors have presumably evolved to limit predation of juvenile flies, such as adult females preferring to oviposit in food crevices and chemically masking their eggs with pheromones, larvae preferring darker (more hidden) parts of the food and often burrowing as they are eating, and pupating larvae dispersing away from the food and gluing themselves to a substrate (Borne et al., 2021; Narasimha et al., 2019; Rockwell & Grossfield, 1978; Sawin-McCormack et al., 1995; Soto-Yéber et al., 2018; Vijendravarma et al., 2013)

Female oviposition choices are particularly interesting because they can represent trans-generational anti-predation behaviors (a type of parental care) (Refsnider & Janzen, 2010). The decision about where, when, and how to lay an egg is complicated and relies on multiple kinds of sensory information (Cury et al., 2019; Richmond & Gerking, 1979; Rockwell & Grossfield, 1978; Wang et al., 2020; Zhang et al., 2020). This information includes the mating and reproductive status of the female, aspects of the abiotic environment like the weather, the time of day and season, aspects of the food source like water content, stiffness, color, odor, and taste, and aspects of the biotic environment like presence of conspecifics and potentially the presence of biotic threats. Might flies alter their oviposition choices (i.e. trigger an innate induced behavior) in the presence of predatory threats to their offspring?

Ants are ubiquitous and occupy diverse niches in nearly all natural ecosystems (Hölldobler & Wilson, 1990). Many are facultative or obligate predators of other organisms, including insects of all life stages (Fernandes et al., 2012), which provide necessary proteins and fats for colony growth (Hölldobler & Wilson, 1990). A number of studies have shown that presence of ants, and specifically visual or olfactory ant signals, cause diverse insect species to avoid oviposition in the ant-infested area (Freitas & Oliveira, 1996; Sendoya et al., 2009; Taylor et al., 1998; Van Mele et al., 2009). Although published data on interactions between *D. melanogaster* and ants are scarce (Soto-Yéber et al., 2018), ants have been shown to be important predators of the eggs and larvae of other *Drosophila* species (Escalante & Benado, 1990; Lewis & Worthen, 1992; Worthen et al., 1993). Anecdotally, we have noticed that rotting fruit traps meant to attract *D. melanogaster* attract far fewer flies when ants are present, and we have seen ants carrying off what appear to be *D. melanogaster* eggs and larvae.

Here we tested whether naïve flies alter their oviposition behavior in the presence of predatory ants. We discovered that flies push their eggs deeply into the food substrate when exposed to ants, which protects eggs from ant predation. This innate, induced oviposition depth behavior is a conserved trait across the genus Drosophila and is exhibited in response to diverse ant species, as well as other hymenopterans. Unlike previous work with other fly predators (de la Flor et al., 2017), olfaction, but not vision, is required for the oviposition depth behavior. We identified the saturated long chain alcohol 1-octadecanol as a conserved hymenopteran odorant that alerts flies to ant presence. This system can serve as a model for how innate threat recognition and downstream defensive behaviors are encoded in the germline and hardwired into the brain.

Materials and Methods:

Species used:

Flies: All flies were raised on cornmeal molasses food (10L water, 75g agar, 275g yeast, 520g cornmeal, 110g sugar, 1046g molasses, 45mL propionic acid, and 100mL 20% (w/v) Tegosept) and maintained at 24°C in ~60% humidity with a 16:8 light:dark cycle. We used a *D. melanogaster* Oregon R (OreR) strain as our wild-type strain. The following *D. melanogaster* mutants were obtained from the Bloomington Drosophila Stock Center (with stock number given): vision mutants GMR-hid (5771) and ninaB^{1} (24776), olfaction mutant orco^{2} (23130), the

121 driver strain Or49a-GAL4 (9985), and the responder strains UAS-Kir2.1 (6595) and UAS-hid
122 (65403). The mutant chromosomes from these strains were crossed into the OreR background
123 to reduce genetic variability. White-eyed w^{1118} flies were provided by Daniela Zarnescu and
124 used as hosts for growing parasitoid wasps. Of the other *Drosophila* species used, wild *D.*
125 *simulans* were caught in Tucson, AZ in 2018 and maintained in the lab as a single strain. *D.*
126 *yakuba* and *D. virilis* were acquired from the Drosophila Species Stock Center (stock numbers
127 14021-0261.01 and 15010-1051.87, respectively).

128 *Ants:* A laboratory culture of *Pheidole hyatti* was originally started in June 2018 from a single
129 queen collected post-nuptial flight and kept in a small 5ml cotton-stopped tube. Once the
130 queen's first brood emerged the housing tube was placed into 17.5cm x 12.5cm x 6cm plastic
131 container with inside walls coated in 'insect-a-slip' (BioQuip product #2871A) to prevent
132 escape. The colony was given cotton ball stopped, water-filled 5 ml plastic tubes, and were fed
133 ad libitum weekly with both a 2 ml microcentrifuge tubes of honey water (1/4 teaspoon per 50
134 mL water), and 1/8 of a fresh-frozen cockroach (approximately 0.075g) (*Nauphoeta cinerea*).
135 During acclimation, the colony was kept in a laboratory at 20°C with a 12:12 h light cycle and
136 20-25% relative humidity. Once the colony outgrew the initial nest chamber, larger 3.5 cm
137 diameter water-filled glass tubes, stopped with cotton, and a housing container measuring
138 31.5cm x 23.5cm x 10.5cm was provided. Other species of wild ants were trapped around
139 Tucson, AZ using small amounts of protein (tuna) and sugar (honey) arranged on pieces of
140 cardboard placed near active ant trails. After roughly 1 hour, ant-covered cardboards were
141 transferred to gallon-sized zip lock bags and brought back to the lab, where most were frozen
142 at -20°C for a later experiment (Fig. 5). Because the laboratory *P. hyatti* colony collapsed during
143 the course of these experiments, we turned to readily-available *Forelius mccooki* wild ants for
144 many live exposure experiments. Live *F. mccooki* workers were collected and placed in a
145 31.5cm x 23.5cm x 10.5cm bin (walls coated with insect-a-slip) and provided with 2 cm and 3.5
146 cm diameter cotton-stopped glass tubes of water. Live ants were never reused across
147 experiments.

148 Ant species were identified morphologically to genus using a dichotomous key (Fisher &
149 Cover, 2007), and identified to species by regional species level keys (see Supplementary Table
150 1 for all keys and sources). No species used in this study are protected or endangered. To
151 confirm morphological identifications, DNA was extracted from the ant samples for Sanger
152 sequencing. First, 10-50 ants were homogenized in 20-30 μL extraction buffer (0.1M Tris-HCL,
153 pH 9.0, 0.1M EDTA, 1% SDS) with a Kontes pellet pestle in a 1.5 mL Eppendorf tube. Additional
154 extraction buffer was added to bring the volume up to 500 μL, then the samples were
155 incubated for 30 min in a 70°C water bath. Cellular material was precipitated and pelleted by
156 adding 70 μL of 8M potassium acetate to each sample, incubating on ice for 30 min, and
157 centrifuging at 13000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh
158 Eppendorf tube, then the DNA was precipitated and pelleted by adding 300 μL isopropanol and
159 centrifuging at 13000 rpm for 5 min at room temperature. After a second clean-up spin, the
160 resulting DNA pellet was washed with 1 mL 70% ethanol and centrifuged at 13000 rpm for 5
161 min at room temperature to re-pellet. The pellet was allowed to air dry for 1-2 minutes, then
162 resuspended in 75 μL nuclease-free water and stored at -20°C. The mitochondrial *cytochrome*
163 *oxidase I* (*COI*) gene was sequenced to identify the different ant species. Following the GoTaq

164 Green Master Mix (Promega, WI, USA) protocol, we generated 25μL reactions containing 12.5
165 μL GoTaq, 7.5 μL nuclease-free water, 2 μL of both LCO1490 and HC02198 primers (Folmer et
166 al., 1994), and 1 μL extracted DNA. Polymerase chain reaction (PCR) conditions were as follows:
167 94°C for 5 min, 37 cycles of 94°C for 1 min, 51°C for 1 min, 72°C for 1 min, followed by 72°C for
168 2 min and a hold at 4°C. The PCR products were visualized on a 1% agarose gel with SYBR safe
169 DNA gel stain (Thermo Fisher Scientific) to confirm single DNA bands were present. PCR
170 products were purified using the QIAquick PCR Purification Kit (Qiagen) and then sequenced at
171 the University of Arizona Genetics Core using the PCR primers. All sequences can be found in
172 Supplementary File 1.

173 *Wasps*: The *Drosophila* parasitoid wasp *Leptopilina heterotoma* (strain Lh14) and *L. boulardi*
174 (strain Lb17) (Schlenke et al., 2007) were used in various wasp exposure assays, and a number
175 of other live parasitoid species maintained in the Schlenke lab were used in a later experiment
176 (Fig. 5). To culture most of these wasps, adult *D. melanogaster* w^{1118} flies were allowed to lay
177 eggs in vials containing *Drosophila* media for 3-4 days, after which the flies were replaced with
178 5-10 female and 2-3 male wasps. The wasps *L. clavipes*, *Ganaspis brasiliensis*, and *Asobara*
179 *tabida* were reared the same way, except that they were grown on the host species *D. virilis*
180 and in bottles rather than vials. The adult wasps were aged for at least 2 days before being used
181 in exposure experiments. Live wasps were never reused across experiments.

182 *Other species*: A variety of other arthropod species were used in an experiment to determine
183 the range of organisms to which *D. melanogaster* responds (Fig. 5). Many of these species were
184 collected around Tucson, AZ, and identified by morphology to the most specific taxonomic
185 group possible. Several other species were provided by research labs at the University of
186 Arizona. See Supplementary Table 2 for more information about all the species used. The NCBI
187 taxonomy ID was used to create a phylogeny of the arthropods used using phyloT and iTOL
188 software (Letunic & Bork, 2021).

189 **Exposure experiments**: Flies were grown in 6oz square bottom culture bottles (Genessee
190 Scientific). On day 0, all adults were cleared from the bottles, the flies were allowed to eclose
191 for three days, then collected and kept in standard molasses food vials for roughly 24 hours
192 until day 4. New vials were prepared with 0.5g ± 1mg Instant Drosophila Medium (Carolina
193 Biological Supply Company) and hydrated with 1.6 mL water with 1% red food coloring
194 (McCormick) (to enhance contrast between the food and eggs). The vials were supplemented
195 with 3-4 drops of hydrated yeast to promote egg development in female flies. The flies were
196 sorted into groups of ten females (of the appropriate genotype) plus two OreR males per vial
197 and kept overnight on this food until day 5 to allow them to recover from CO_2 exposure before
198 the experiment. The experimental vials were also prepared using the same red instant food but
199 without the added yeast. For live insect exposure trials, on the day prior to the start of the
200 experiment eight live insects, either *F. mccooki* or *P. hyatti* ants, or Lh14 or Lb17 wasps, were
201 added to the experimental vials to allow the odorous compounds to accumulate (day 4).
202 Control, unexposed vials contained no insects. The following day (day 5), the 2-5 day old flies
203 were flipped to the experimental vials, with or without live insects or insect odors. For the
204 whole-body wash odor exposure trials (see below), 50 μL of the solvent (control) or odor
205 extract was added to each experimental vial. Once 1-octadecanol was identified as the odorant

206 of interest, we similarly used 50 μL of 1-octadecanol standard (MilliporeSigma, 258768
207 ReagentPlus® 99%, Burlington, MA, USA) in hexane at indicated concentrations in each
208 experimental vial. The vials were kept in a fume hood to allow the solvent to evaporate for
209 fifteen minutes before the flies were added to start the experiment.

210 For all exposure experiments, we ran five replicates per treatment. The flies were kept
211 in the experimental vials for 24 hours at 24°C in ~60% humidity with a 16:8 light:dark cycle,
212 then all insects were removed and the vials were moved to -20°C to stop egg development. The
213 following parameters were used to categorize each egg position: 'surface' eggs were those for
214 which their entire circumference, from anterior to posterior ends, was entirely visible; 'buried'
215 eggs were those where the position at which the dorsal appendages emerges from the eggshell
216 was located below the surface of the substrate; 'partial' eggs were those that were positioned
217 in the food substrate, but not to the extent of the buried eggs. Unlike *D. melanogaster* eggs, *D.*
218 *virilis* eggs have two pairs of dorsal appendages, so the more posterior pair was used to
219 determine the 'buried' position. To calculate the egg position index (EPI), we used the following
220 equation: EPI = (S – B)/T where S is the number of eggs on the surface, B is the number of eggs
221 that are buried, and T is the total number of eggs across all three categories.

222 **Odor extracts**: All insects were frozen at -20°C for at least 24 hours prior to odor extraction. A
223 freeze-thaw cycle was shown to improve detection of ant volatile compounds (Chen, 2017). We
224 used 1.5 mL Eppendorf tubes, washed two times with 500 μL solvent, for the odor extraction.
225 Initial experiments with ants or parasitoid wasps used 70 insects washed with 350 μL solvent.
226 Tubes were vortexed for 1-2 minutes, centrifuged briefly, then 50 μL aliquots (representing the
227 odor equivalent of ten insects) were pipetted out of the tube and added to each of the five
228 replicate experimental vials. For control vials, the pure solvent underwent the same procedure
229 but without insects. For the ant and wasp fractionation experiments, we increased the odorant
230 concentration five-fold by reducing the solvent-to-insect ratio to 1 μL solvent per insect. For
231 odor extractions from especially small or large arthropod species, we used body mass instead of
232 number of individuals to determine the solvent-to-insect ratio. A minimum of 30 mg was
233 washed with 350 μL solvent. See Supplementary Table 1 for more information about how body
234 washes were generated.

235 **Ant predation experiment**: OreR flies laid eggs on standard molasses food in 5.5cm diameter
236 petri dishes for up to two hours. Eggs were gently removed and manually positioned on 5.5cm
237 diameter petri dish filled with 0.5% agarose gel with red food coloring. Surface eggs were set on
238 top of the agarose gel, partially buried eggs were inserted lengthwise roughly 50% into the gel,
239 and the buried eggs were inserted until the gel covered the point at which the dorsal
240 appendages connect to the eggshell (twenty eggs per category for a total of sixty eggs). The *P.*
241 *hyatti* laboratory colony was used under the assumption that it displays normal foraging
242 activity. A secondary 17.5cm x 12.5cm x 6cm plastic container, with walls coated in 'insect-a-
243 slip,' was connected to the main colony chamber by a 2cm (length) x 1.5cm (diameter) plastic
244 tube plugged with cotton. The cotton separator was then removed, and ants were allowed to
245 freely explore the smaller chamber for thirty minutes before the dish with fly eggs was added.
246 The ants were allowed to forage for thirty minutes before removal of the egg dish, at which
247 point the remaining eggs were counted. The connection between the colony chamber and the

248 foraging chamber was then blocked, the remaining ants were transferred back to the colony
249 chamber, and the bottom of the foraging chamber was washed with ethanol to remove any ant
250 pheromone trails. After one hour, the cotton separator was removed and the next replicate
251 trial began. Two replicates were run per day over the course of ten days, for a total of twenty
252 replicates. After counting the remaining fly eggs, we scored egg hatching rate 48 hours after the
253 ant exposure to confirm that the remaining eggs were viable.

254 **Testing tradeoffs to deep oviposition**: To test whether buried fly eggs show lower survival or
255 slower development to the adult stage, we had OreR flies lay eggs on standard molasses food in
256 5.5cm diameter petri dishes for two hours. Groups of thirty eggs were each transferred to a vial
257 with molasses food and manually positioned such that all eggs were either on the surface or
258 buried deeply in the food. Starting eleven days post egg lay, the number of flies that eclosed
259 per vial was counted daily. To test whether buried fly eggs are more susceptible to a biotic risk
260 (being further buried by foraging fly larvae), twenty white-eyed (w^{1118}) third instar larvae were
261 added to the vials of fly eggs, and eclosion was assayed. To test for cold temperature risk, vials
262 with fly eggs were placed overnight (~15 hours) at 4°C, then transferred to 24°C to allow the
263 flies to continue development, before assaying eclosion. To test for risk of drowning, vials with
264 fly eggs were sprayed with water using a 4oz fine mist sprayer bottle such that the bottom of
265 the vial had a thin layer of water, before assaying eclosion.

266 To test whether there is a tradeoff to adult female flies when ovipositing deeply into the
267 food, we measured the duration of the oviposition phase and the clean-and-rest phase post-
268 oviposition by taking videos of groups of ovipositing flies. Batches of five OreR females plus one
269 male were collected from bottles three days after being cleared and kept on molasses food
270 overnight in rectangular 3 cm (length) x 3 cm (width) x 6.3 cm (height) chambers. The following
271 day, five female wasps (strain Lh14) were added and kept co-housed with the flies overnight.
272 The next day, all insects were transferred to a new rectangular chamber with molasses food.
273 Two strips of parafilm were placed in parallel on top of the food, leaving a 2-3 mm wide area
274 available for oviposition at a depth of field amenable to video capture. Multiple 1-hour videos
275 were recorded per day using a Basler 2.3-megapixel acA1920-155µm camera (Graftek Imaging).
276 Frames were captured using Pylon Viewer (version 6.2.4.9387 64-bit) at 15 Hz, and converted
277 to .avi video format using MATLAB code (Chowdhury et al., 2021). Egg position was scored,
278 then the oviposition and clean-and-rest phase durations were determined by examination of
279 the video files.

280 **GC-MS:** N-hexane (Optima grade), acetonitrile (Optima grade), and dichloromethane (Optima
281 grade) were purchased from Fisher Scientific (Pittsburg, PA, USA). 1-octadecanol (99%), 1,3-di-
282 *tert*-butylbenzene (97%), and C7 - C30 Saturated Alkanes (certified reference material) were
283 purchased from Sigma-Aldrich (St. Louis, MO, USA).

284 Ant and wasp body washes were fractionated using a Thermo Vanquish LC system
285 (Waltham, MA USA) with an Agilent Technologies AdvanceBio RP-mAb C4, 4.6 × 50 mm, 3.5 µm
286 column (Santa Clara, CA, USA). Mobile phase A was acetonitrile and mobile phase B was 50/50
287 dichloromethane/hexane. The flow rate was 0.830 mL/min. 50 µL of sample was injected onto
288 the column and fractions were collected using an Ultimate 3000 Automated Fraction Collector
289 (Thermo Scientific, Waltham, MA, USA). The gradient was started with 100% phase A and was

290 held for 1 minute. The solvent composition was changed linearly to 100% phase B at 9 minutes
291 and was held for 2 minutes. The solvent was immediately returned to 100% phase A and
292 allowed to re-equilibrate for 5 minutes. The fractions were collected every 1 minute until a total
293 of 11 fractions had been collected. Fractions were dried down under a gentle stream of
294 nitrogen and reconstituted in hexane.

295 GC-MS analysis was performed using an Agilent Technologies 7890B GC with 7000D
296 Triple Quadrupole MS (Santa Clara, CA, USA) with MassHunter Acquisition software (v.
297 B.07.06.2704, Agilent Technologies, Santa Clara, CA, USA). Helium was used as the carrier gas at
298 a flow rate of 2 mL/min. Inlet temperature was 250 °C. 1 µL of sample was injected onto a DB-5
299 30m × 0.25 mm × 0.25 µm column (Agilent Technologies, Santa Clara, CA, USA). Initial oven
300 temperature was set to 40 °C and held for 2 minutes, then increased at 5 °C/min to 300 °C and
301 held for 2 minutes. The transfer line was set to 230 °C. The electron impact ionization source
302 was set at 70 eV and the source temperature was set to 230 °C. The quench flow was set to 2.5
303 mL/min. The mass range was scanned from 35-500 *m/z* at a scan time of 200 ms.

304 Data analysis was performed using MassHunter Qualitative Analysis Workflows software
305 (v. B.08.00, Agilent Technologies, Santa Clara, CA, USA). Peaks were identified using the Agile
306 integrator and compounds were identified using the NIST 17 database with a reverse match
307 score of at least 50.00 (National Institute of Standards and Technology 2017). Compounds
308 identified as siloxanes or other silicone containing molecules were ignored as these compounds
309 are from the sample vial septa or the inlet septum. Compounds that were identified in both the
310 ant and wasp fractions constituting a significant portion of the peak area were confirmed by
311 matching retention times to known standards.

312 **Statistical analysis**: All graphs and statistical tests were performed using GraphPad Prism 9 (v
313 9.2.0) software. All data were tested for normality using the Shapiro-Wilk test. Oviposition
314 rates, egg position index, and eclosion rates were tested using the unpaired, two-tailed t-test
315 with Welch's correction or two-way ANOVA followed by Dunnett's multiple comparison test
316 against the control condition. The Brown-Forsythe and Welch's ANOVA with Dunnett's multiple
317 comparison test was used on egg position data with multiple, independent samples. Fly egg
318 survival after ant predation, oviposition timing, and clean-and-rest phase timing were
319 compared using the nonparametric Kruskal-Wallis test with Dunn's multiple comparison test.
320 Throughout the paper, statistical notation are as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$,
321 **** $p<0.0001$, ns: not significant.

322

323 **Results**:

324 **Flies bury their eggs when exposed to ants**

325 To test whether adult female flies change their oviposition behavior in the presence of
326 predatory ants, we co-housed flies with either wild-caught *Forelius mccooki* or laboratory raised
327 *Pheidole hyatti* ants for 24 hours. Unlike with exposure to parasitoid wasps (Supp. Fig. 1A and C)
328 (Kacsoh et al., 2015; Lefèvre et al., 2012; Lynch et al., 2016), female flies did not reduce
329 oviposition in the presence of these ants (Fig. 1A and B). The non-significant trend towards
330 reduced egg numbers from the *P. hyatti* exposure is likely due to the ants actively capturing the

331 adult flies during the experiment, as exemplified by flies that had sections missing from their
332 wings or legs at the end of the experimental period (Fig. 1C). Ants of the genus *Pheidole* have
333 been described as highly aggressive in terms of prey capture (Traniello, 2010).

334 Although there was no reduced oviposition, we noticed while counting the fly eggs that
335 the eggs of ant-exposed flies tended to be less visible than those of control unexposed flies.
336 Flies oviposit their eggs either fully on the surface of the food substrate, partially inserted into
337 the food, or completely submerged beneath the surface (Fig. 1D-E). Further examination
338 revealed that the majority of the eggs, regardless of ant exposure condition, were laid partially
339 submerged into the food substrate (Fig. 1F), but that the second most common category
340 switched depending upon ant exposure. Unexposed flies lay more of their eggs on the surface
341 while those exposed to ants push more of their eggs deeply into the food substrate (Fig 1F). To
342 better describe this alteration in oviposition depth, we calculated the egg position index, which
343 is the proportional difference between the surface vs buried eggs (see methods). A positive
344 value indicates a stronger preference to lay eggs on the surface of the food while a negative
345 value indicates a stronger preference for burying eggs under the food. Exposure to both *F.*
346 *mccooki* and *P. hyatti* ants significantly altered the flies' oviposition preference towards the
347 buried egg category (Fig. 1G-H). Interestingly, this behavioral modification also occurs when
348 flies were exposed to parasitoid wasps (Supp. Fig. 1B and D).

349 **Buried eggs survive ant predation**

350 We hypothesized that eggs laid deeper into the food substrate would be protected
351 against ant consumption. We provided the laboratory culture of *P. hyatti* ants with fly eggs
352 manually positioned at three different depths, similar to the three natural depth categories (Fig.
353 1D-E, 2A). After a 30 minute ant foraging bout, the remaining fly eggs were counted. Eggs
354 positioned on the surface of the media were the most susceptible to predation, while eggs
355 positioned beneath the surface were strongly protected (Fig. 2B). Indeed, ants readily removed
356 the eggs on the surface but struggled (attempted and often failed) to remove the deeply
357 positioned eggs (Supp. videos 1 and 2). Of the eggs remaining after the ant foraging bout,
358 hatching rates were high and similar across the depth categories (Fig. 2C), showing that the ants
359 were not consuming or otherwise harming the buried eggs on-site. These results suggest that
360 the fly oviposition depth behavior is an adaptation to protect fly offspring from ant predation.

361 Given that laying eggs deeply into the substrate protects them from predation, why
362 don't fly mothers perform this behavior constitutively? We tested whether buried eggs suffer
363 some fitness cost compared with eggs laid on the substrate surface. First, we tested whether
364 there was any reduction in offspring developmental time or survival for flies that were buried in
365 the egg stage, but did not find any difference in eclosion time or eclosion success between flies
366 manually positioned on the food surface versus buried at the egg stage (Supp. Fig. 2A). We also
367 tested whether the presence of older fly larvae churning the food, reduced temperature, or
368 simulated rain (water misting) harmed the buried eggs, but once again found no difference in
369 survival to eclosion (Supp. Fig. 2B-D). These results suggest that any cost of deep oviposition is
370 not incurred by the offspring, but instead may be borne by fly mothers. The *D. melanogaster*
371 oviposition program has been described as a progression of stereotyped phases starting with a
372 searching phase, then the oviposition phase, and finally the clean-and-rest phase (C.-H. Yang et

al., 2008). We hypothesized that female flies might require more time to lay buried eggs or to rest after laying buried eggs. However, video analysis of the fly oviposition program during parasitoid wasp exposure showed no difference in the average length of either of these phases when the flies laid a surface egg versus laid a buried egg (Supp. Fig. 2E-F). Curiously, female flies spent significantly longer periods laying partially buried eggs as compared to surface eggs or fully buried eggs (Supp. Fig. 2E), suggesting that partially buried eggs need more care to position. In summary, we were unable to detect any negative consequences of the induced egg burying behavior. Either there is no fitness cost associated with this behavior, or there is a cost that we have not yet identified.

Egg burying is a conserved trait across *Drosophila*

To test whether ant-induced egg burying is a conserved defensive behavior in flies, we assayed the behavior in three other *Drosophila* species of varying evolutionary distances from *D. melanogaster: D. simulans* (~3MY since the most recent common ancestor), *D. yakuba* (~5MY), and *D. virilis* (~30MY) (Powell, 1997). Like *D. melanogaster*, the other *Drosophila* species maintained their egg production level when exposed to *F. mccooki* ants (Fig. 3A), and also like *D. melanogaster*, all three *Drosophila* species consistently laid their eggs deeper in the substrate in the presence of ants (Fig. 3B). This result holds even though *D. virilis*, and to some extent *D. yakuba*, tend to lay more deeply buried eggs than *D. melanogaster* in the unexposed state (Fig. 3B). These data suggest that although normal egg depth behavior varies across species, the switch to laying more buried eggs to prevent egg predation is a conserved trait.

Olfaction mediates ant detection

Fly mothers sense and respond to the presence of ants by burying their eggs, but how do they know that ants are present? Flies have been shown to detect parasitoid wasps through the combined effects of the visual and olfactory systems (Ebrahim et al., 2015; Kacsoh et al., 2015, 2013; Lynch et al., 2016), and given that flies also bury their eggs in the presence of wasps (Supp. Fig. 1) we tested both of these sensory modalities. *GMR-hid* flies express a pro-apoptotic factor in the eye, making their eyes significantly reduced in size (Grether et al., 1995). These sight-deficient flies maintained their ability to respond to the presence of *F. mccooki* ants by burying their eggs (Fig. 4A). *GMR-hid* as well as *ninaB*^{1} mutant flies (which are blind due to loss of photoreceptors) also maintained their ability to sense and respond to *L. heterotoma* parasitoid wasps (Supp. Fig. 3A-B). These data suggest that vision is dispensable for the altered egg depth behavior. We next tested the necessity of the olfactory system: *orco*^{2} mutant flies lack the odorant receptor co-receptor and therefore lack most olfactory ability (Larsson et al., 2004). Heterozygous *orco*^{2} mutant flies showed a strong induced egg burying response in the presence of *F. mccooki* ants, but the anosmic homozygous *orco2* mutant flies showed a much weaker (albeit still significant) response (Fig. 4B). Exposing the anosmic flies to either *P. hyatti* ants or *L. heterotoma* parasitoid wasps resulted in a complete failure to alter egg depth (Supp. Fig. 3C-D). To further test the necessity of olfaction for the egg burying behavior, we manually ablated the two main olfactory sensing organs from experimental flies either individually or in tandem. While ablating the maxillary palps had no effect on fly ability to bury eggs in the presence of *F. mccooki* ants, ablating the fly antennae abolished their ability to respond to the ants (Supp. Fig. 3E). Altogether, these data demonstrate that flies require olfactory input

415 through their antennae to sense ants in their environment and alter oviposition depth behavior
416 accordingly.

417 It was previously shown that the fly odorant receptors Or49a and Or85f, which are
418 expressed in the same olfactory receptor neurons in adult flies, detect specific odorants from
419 parasitoid wasps in the genus *Leptopilina*, in turn driving avoidance behaviors during fly
420 oviposition (Ebrahim et al., 2015). One of these compounds, iridomyrmecin, was first isolated
421 from the Argentine Ant, *Linepithema humile* (formerly *Iridomyrmex humilis*) (Pavan, 1948)
422 suggesting flies may also detect ants via this mechanism. Using an *Or49a-Gal4* driver, we
423 inhibited activity of Or49a/Or85a-expressing olfactory receptor neurons by driving expression
424 of *Kir2.1* (which causes neuronal membrane hyperpolarization) in these neurons, or we
425 completely ablated these neurons by expressing in them the proapoptotic gene *hid*. Neither
426 modification blocked the fly egg burying behavior in the presence of *L. heterotoma* parasitoid
427 wasps (Supp. Fig. 3F-G). These data suggest that flies are using a different type of olfactory
428 sensory neuron and olfactory receptor to detect a novel odorant associated with ants.

429 If an ant olfactory stimulus is sufficient to induce the fly oviposition depth switch, we
430 hypothesized that ant body-wash extracts could replace live ants as the egg burying stimulus.
431 We used the solvent hexane to extract odorants from the bodies of *P. hyatti* ants and added the
432 odors directly onto the food substrate. While flies that were exposed to live *P. hyatti* ants as a
433 positive control showed a strong induced egg burying behavior, flies exposed to ant odors alone
434 showed a weaker, though still significant, response (Fig. 4C). There are at least three reasons
435 why odor extracts do not fully recapitulate the direct ant exposure condition. First, it is possible
436 that the body wash extracts only possess a subset of multiple distinct odorants required for the
437 full behavioral response. To test this, we used different solvents that varied in their polarity to
438 target different molecules. Interestingly, extracts collected in non-polar hexane (Fig 4C, Supp.
439 Fig. 4A) and weakly polar dichloromethane (Supp. Fig. 4B) were both sufficient to induce the
440 partial egg burying behavior, while use of more strongly polar solvents failed to induce any fly
441 response (Supp. Fig. 4C-E). However, combining body wash odorants extracted using hexane
442 and dichloromethane did not induce the full egg burying behavior (data not shown), showing
443 that no additive effect is achieved by combining the odorant subsets extracted by each solvent.
444 Second, it is possible that exposure to insect body wash extracts fails to fully recapitulate the
445 live insect exposure results because the body wash odorants dissipate over the course of the
446 experiment without being replaced. To test this idea, we exposed flies to both dead *L.*
447 *heterotoma* parasitoid wasps, and to vials in which the wasps had been housed, both
448 treatments of which should have the full wasp odorant profile but no new odorants being
449 produced. In both cases, flies showed a partial egg burying response similar to their response to
450 insect body wash extracts, supporting the odorant dissipation hypothesis (data not shown).
451 Third, it is possible that flies are using some other sensory modality besides olfaction or vision
452 to detect ant/wasp presence, such as gustation, a hypothesis that remains untested.
453 Regardless, we were able to extract an odorant from insect bodies that flies detected, causing
454 them to induce at least a partial egg burying response.

455 **Flies sense the odorant 1-octadecanol**

456 To identify the odorant (or odorants) in insect body washes that induces flies to bury
457 their eggs, we used regular-phase column chromatography to generate fractions from the
458 whole-body washes. These fractions are expected to pull out different subsets of the molecules
459 from the whole-body washes, allowing us to narrow down and identify the causal odorant(s)
460 using gas-chromatography mass-spectrometry (GC-MS). Eleven fractions were generated from
461 *F. mccooki* ant whole-body washes and assayed for phenotypic effects on flies. As expected,
462 none of the fractions altered fly oviposition rates (Fig. 5A), but fraction 9 did induce the flies to
463 lay eggs significantly deeper in the food than control hexane treatments (Fig. 5B). Fraction 9
464 from a *L. heterotoma* parasitoid wasp body wash showed a similar result (data not shown). GC-
465 MS of the *F. mccooki* whole-body wash compared to the fraction 9 sample revealed that the
466 fraction contained a small subset of the molecules found in the whole-body wash, and in
467 particular contained one shared high intensity peak (Fig. 5C-D). This peak was identified as the
468 saturated, linear eighteen-carbon alcohol, 1-octadecanol (Fig. 5C-D, peak 2). While the *F.*
469 *mccooki* whole-body wash contained iridomyrmecin (Fig. 5C, peak 1), an odorant found in
470 parasitoid wasps and previously shown to affect fly oviposition (Ebrahim et al., 2015), fraction 9
471 did not possess this odorant, consistent with the idea that the fly egg burying behavior is
472 triggered by a novel odorant. To confirm the role of 1-octadecanol in the egg burying behavior,
473 we purchased pure 1-octadecanol and exposed flies to various concentrations. At
474 concentrations as low as 10^{-8} M (similar to 1-octadecanol concentration in the whole-body
475 wash sample as measured by a standard), flies responded to 1-octadecanol as strongly as they
476 responded to whole ant body washes (Fig. 5E). Thus, we concluded that 1-octadecanol is the
477 ant odorant (shared with *L. heterotoma* parasitoid wasps) that alerts flies to the presence of
478 dangerous predators, inducing flies to bury their eggs in order to protect their offspring.

479 **Flies respond to most hymenopterans**

480 We have shown that flies lay eggs more deeply into the food substrate when exposed to
481 ants (Fig. 1), and that this behavior protects the eggs from ant predation (Fig. 2). Flies also
482 induce this behavior when exposed to a parasitoid wasp, although it is unclear what benefit
483 there is to burying eggs in this context given that *L. heterotoma* does not attack fly offspring
484 until they reach the larval stage, when they are crawling on top of the food. To determine the
485 breadth of ants and potentially other insects that induce flies to bury their eggs, we exposed
486 flies to body washes or live samples of numerous arthropods. Flies responded to the vast
487 majority of ant species tested representing the three ant subfamilies Dolichoderinae,
488 Formicinae, and Myrmicinae (Fig. 6). Exceptions included the Myrmicinae species *Monomorium*
489 *cyaneum*, as well as some trials from several other ant species (Fig. 6). Parasitoid wasps
490 represent another large branch of hymenopterans that impose strong selective pressures on fly
491 offspring survival. Flies that were exposed directly to parasitoid wasps that infect fly larvae
492 (genus *Leptopilina*, *Ganaspis*, and *Asobara*) or fly pupae (*Pachycrepoideus* and *Trichopria*)
493 significantly altered their oviposition depth (Fig. 6). However, flies also responded to the body
494 wash extract of the whitefly parasitoid, *Encarsia inaron*, as well as extracts from every other
495 hymenopteran tested (bees and paper wasps), even though *D. melanogaster* has no known
496 ecological interactions with these insects (Fig. 6).

497 The odor extracts from non-hymenopteran insects generally did not induce flies to alter
498 their oviposition behavior, but there were two exceptions. First, odor extract from the leaf-

499 footed bug, *Leptoglossus zonatus*, did induce the fly egg burying behavior in two separate trials,
500 despite body washes from other hemipterans showing no effect on the flies (Fig. 6). Second,
501 odor extract from the nymph stage of the Madagascar hissing cockroach, *Gromphadorhina*
502 *portentosa*, also affected fly oviposition behavior. In sum, these data suggest that a common
503 odorant, 1-octadecanol, evolved early in the hymenopterans and has been maintained over
504 time, with sporadic gains and losses in the broader insect group. Further, our data suggest that
505 flies evolved to sense this odorant as a means of protecting their offspring from predatory ants.

506

507 **Discussion**:

508 Recognition of environmental threats, and execution of an appropriate response, is
509 critical to organismal survival. Here, we have shown that flies detect ant presence through
510 olfactory sensing of the hymenopteran odorant 1-octadecanol, and switch their oviposition
511 behavior to more deeply position their eggs in the food substrate. This protects fly eggs from
512 ant predation.

513 Egg laying behavior follows a similar stereotyped pattern across different fly species. It is
514 first characterized by a search-like, informational gathering processes about the local nutrient
515 availability and substrate stiffness through labellum and leg contact (Bräcker et al., 2019; C.-H.
516 Yang et al., 2008; Zhang et al., 2020). Once a suitable site is found, flies switch to a more refined
517 search behavior, including ovipositor contact with the substrate (Bräcker et al., 2019), before an
518 egg is finally laid. These discrete micro-behaviors are conserved across *Drosophila*, with minor
519 variations depending on the ecology of each species. For example, the agricultural pest *D.*
520 *suzukii* spends significantly more time than *D. melanogaster* contacting the food substrate with
521 its ovipositor and expelling its eggs (Bräcker et al., 2019), presumably because it tends to lay
522 eggs more deeply into less ripened fruits (Karageorgi et al., 2017). Furthermore, *Drosophila*
523 species that oviposit on mushrooms tend to deeply bury their eggs (Rouquette & Davis, 2003),
524 perhaps because mushroom flesh is soft, or because ants commonly visit mushrooms (Lewis &
525 Worthen, 1992). While fly oviposition behaviors are well-studied (Bräcker et al., 2019; Cury et
526 al., 2019; Karageorgi et al., 2017; Van Mele et al., 2009; Wang et al., 2020; C.-H. Yang et al.,
527 2008; Zhang et al., 2020), this is the first report describing an induced change in oviposition
528 depth.

529 Our measure of oviposition depth, the egg position index, is a blunt tool. While egg
530 depth is a continuous trait, we bin the eggs into one of three depth categories, and most of the
531 eggs are classified as 'partially buried'. A lot of the behavior may be missed when an egg that is
532 only 20% inserted into the substrate is scored the same as an egg that is 80% inserted,
533 especially if these eggs have different levels of protection against foraging ants. Ideally a more
534 sensitive measure of egg depth could be devised. Nevertheless, the fact that we see significant
535 differences in the egg position index across treatments indicates that egg burying is a robust
536 and important fly behavior.

537 Several examples exist of organisms altering oviposition behavior in response to biotic
538 threats. For example, water striders (*Aquarius paludum insularis*) oviposit their eggs deeper in
539 the water column after exposure to parasitoid wasps, which limits egg parasitism (Hirayama &

Kasuya, 2009). Newts (*Taricha granulosa*) lay their eggs attached to plants higher in the water column to avoid egg predator caddisfly larvae (*Limnephilus flavastellus*) (Gall et al., 2012). There are even examples in fruit flies: *Drosophila* species avoid ovipositing at sites infested by toxic microbes, which they detect via the odorant geosmin (Stensmyr et al., 2012). They also alter oviposition in the presence of parasitoid wasps (Carton et al., 1986) using both visual and olfactory cues: female flies lay fewer eggs during forced exposures, they preferentially choose non-infested sites when given a choice by sensing the wasp odorant iridomyrmecin, and they preferentially lay their eggs in more toxic (alcoholic) environments when exposed (Kacsoh et al., 2013; Lefèvre et al., 2012; Lynch et al., 2016; Ebrahim et al., 2015). All of these are examples of threat-induced behavioral changes that organisms make about 'when' or 'where' to oviposit, whereas the new behavior described here is about 'how' flies choose to oviposit. Furthermore, we know that the fly responses to geosmin and iridomyrmecin, and likely 1-octadecanol as well, are hardwired into the fly, using specific olfactory receptors and neuronal circuits in the brain. It will be interesting to determine how these different environmental cues are signaled through the brain to regulate different types of egg laying behaviors, and whether there is any overlap in each circuit.

Ants are a ubiquitous presence across diverse habitats, and many insects have evolved ant avoidance behaviors triggered by distinct sensory modalities. For example, honeybees avoid flowers in the field that have live ants or their odors nearby (Li et al., 2014; Sidhu & Rankin, 2016), and tephritid fruit flies avoid fruits perfumed with ant pheromones (Van Mele et al., 2009). Furthermore, butterflies (*Eunica bechina*) were reported to avoid oviposition sites due to visual detection of ant presence, although the fact that ants were pinned to plant leaves suggests that ant odors may also have contributed to the butterfly avoidance (Sendoya et al., 2009). Surprisingly, termites (*Coptotermes acinaciformis*) were shown to detect and avoid ants based on the vibrational pattern of ants walking through their woody substrates (Oberst et al., 2017). This may reflect a keen sense of vibration detection in termites, which have been shown to communicate alarm signals via similar vibrational cues (Inta et al., 2009). These examples show that detection of ant threats is common in the insect world, and that insects have evolved diverse sensory mechanisms for identifying ant presence. Given that *D. melanogaster* invests more resources into its olfactory system than other sensory systems (Keesey et al., 2019), it may not be surprising that they detect ant predators through olfactory signals. In the future, it will be interesting to identify the odorant receptor(s) responsible for fly sensing of ant odors and the neural circuitry that responds to odor sensing by altering oviposition choices (Wang et al., 2020).

Although olfaction is necessary for fly detection of ants, we could not prove that ant odors are sufficient to induce the full fly egg burying phenotype (Fig. 4). We believe there are two likely reasons why providing flies with ant body wash odorant extracts does not fully recapitulate the direct insect-exposure results. First, the ant odor extract experiments likely started with a high concentration of ant odors (like 1-octadecanol), but over the course of the 24 hour experiment these volatile odorant concentrations may have declined to a point where fly behavior was no longer altered. In contrast, the live-ant exposures may have maintained a high concentration of odorants throughout the experiments due to constant release from the insects. It would be interesting to conduct an experiment in which ant odors are constantly

passed over the fly habitat to test whether exposure to a constant concentration of ant odors induces a more robust egg burying phenotype. Second, it is possible that fly oviposition behaviors like egg burying require multimodal sensory integration such that ant odors suppress the preference for 'surface' eggs, but a second stimulus enhances the magnitude of preference for 'buried' eggs. This second stimulus might include gustatory or auditory cues from the ants. This kind of gating mechanism has been observed for fly larval rolling behavior during parasitoid attack, where vibrational cues enhance the fly rolling response to mechanical poking by the the wasp ovipositor (Ohyama et al., 2015). Further testing of fly sensory systems will help identify additional inputs into the oviposition depth behavioral response.

There are sporadic reports in the literature about insects producing or behaviorally responding to 1-octadecanol. For example, 1-octadecanol is a component in the pheromone blend secreted from the male hairpencil glands of various male lepidopterans to attract females (Bober & Rafaeli, 2010; Teal & Tumlinson, 1989). However, 1-octadecanol has been reported to have repulsive effects on other insects: aphids (*Rhopalosiphum padi*) avoid leaves coated with *Trichoderma* fungi, which produce 1-octadecanol (Ganassi et al., 2016), and the beet armyworm, *Spodoptera exigua,* avoids ovipositing on roses infected with the powdery mildew *Podosphaera pannosa* (Fa-zhong Yang et al., 2013), due to increased plant production of volatiles like 1-octadecanol (Fazhong Yang et al., 2019). In our system, flies do not seem deterred from ovipositing on food substrates with ant odors or 1-octadecanol, they just lay their eggs deeper into the substrate. Still, allowing flies a choice between oviposition sites with and without 1-octadecanol would offer interesting insight into potential tradeoffs – do flies prefer to spend time searching for non-contaminated oviposition sites or potentially spend more energy laying eggs on a contaminated oviposition site?

We showed that the ability of flies to sense 1-octadecanol from ants, and induce the egg burying behavior, is beneficial to the flies due to the increased survival of their eggs during ant predation events (Fig. 2). However, flies also induce the egg burying behavior in response to the odors of other hymenopterans like wasps and bees, even though wasps and bees are not known to harm fly eggs. This suggests that 1-octadecanol is a conserved odorant dating back to the common ancestor of all hymenopterans. Likewise, the ant odor-induced egg burying behavior is conserved across the genus Drosophila (Fig. 3). Given that the expansion of ant, wasp, and bee lineages seems to coincide with the expansion of dipterans in the Jurassic period over 150 million years ago (Misof et al., 2014; Peters et al., 2017), it is possible that fly recognition of hymenopteran threats via 1-octadecanol sensing is ancient. In the future, it will be interesting to determine if other dipterans mount behavioral defenses in response to 1-octadecanol, and if the neurogenetic basis of this sensing is conserved across dipterans.

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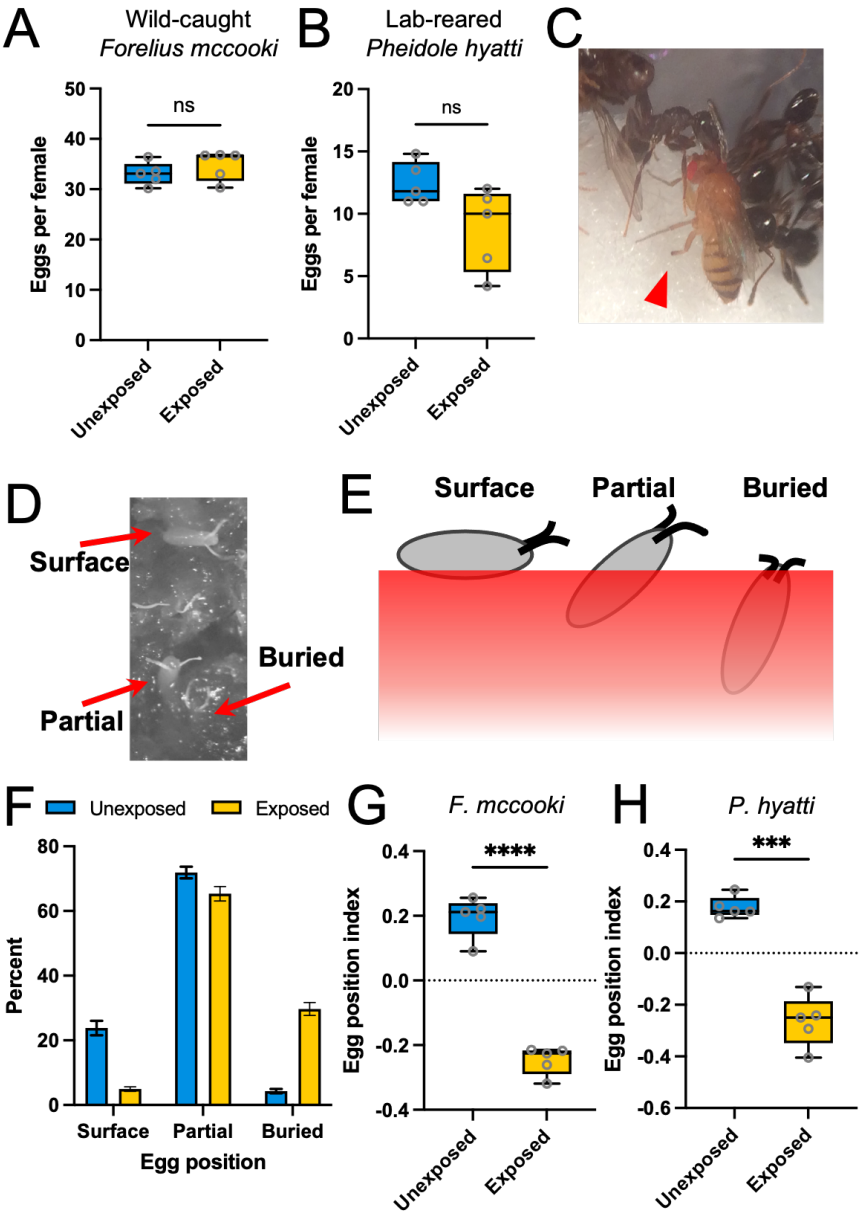


Fig. 1: Flies alter egg depth when exposed to ants. *D. melanogaster* flies exposed to either wild-caught *Forelius mccooki* (**A**) or lab-reared *Pheidole hyatti* (**B**) ants did not reduce oviposition rates (unpaired, Welch's t-test; *F. mccooki*: *P*=0.3551; *P. hyatti*: *P*=0.0713). The lower, but non-significant, exposed egg numbers in (B) are likely due to some flies being caught by the ants (**C**). The arrowhead indicates a leg that was severed. We did not observe any flies being caught by *F. mccooki* ants. (**D**) Photo of eggs in an exposure experiment, indicating the three different egg depth categories. (**E**) Schematic of oviposition depth categories. (**F**) Ant exposure significantly alters the distribution of eggs in each category (χ^2=28.93, *P*<0.0001, bars represent mean ± S.E.M). (**G** and **H**) Both *F. mccooki* and *P. hyatti* ants significantly altered the egg position index towards a preference for buried eggs rather than surface eggs (unpaired, Welch's t-test: *F. mccooki*: *P*<0.0001; *P. hyatti*: *P*=0.0002).

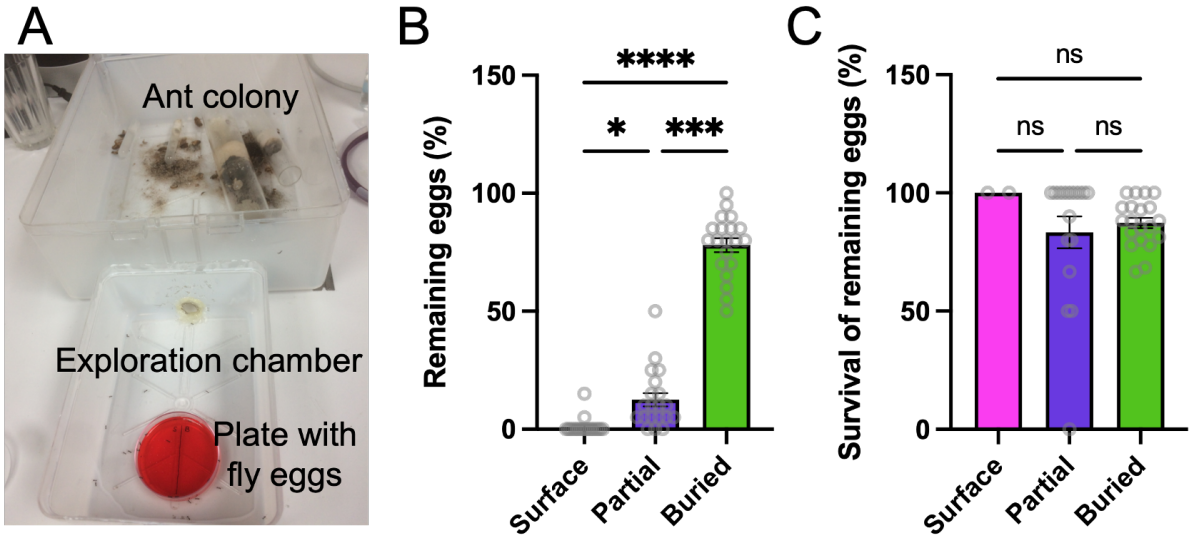


Fig. 2: Fly eggs positioned deeply are protected from foraging ants. (**A**) Photo of ant foraging experiment with *P. hyatti* ants. (**B**) Buried fly eggs were significantly more likely to remain after ant foraging, while surface and partially buried eggs were removed by the ants (Surface vs. Partial: P=0.0135; Surface vs Buried: P<0.0001; Partial vs Buried: P=0.0001). (**C**) There was no significant difference in egg hatching rates across all three categories of the remaining eggs after ant foraging (Surface vs. Partial: P=0.6613; Surface vs Buried: P=0.2665; Partial vs Buried: P=0.8782). (Kruskal-Wallis test with Dunn's multiple comparison test). Bars represent mean ± S.E.M.

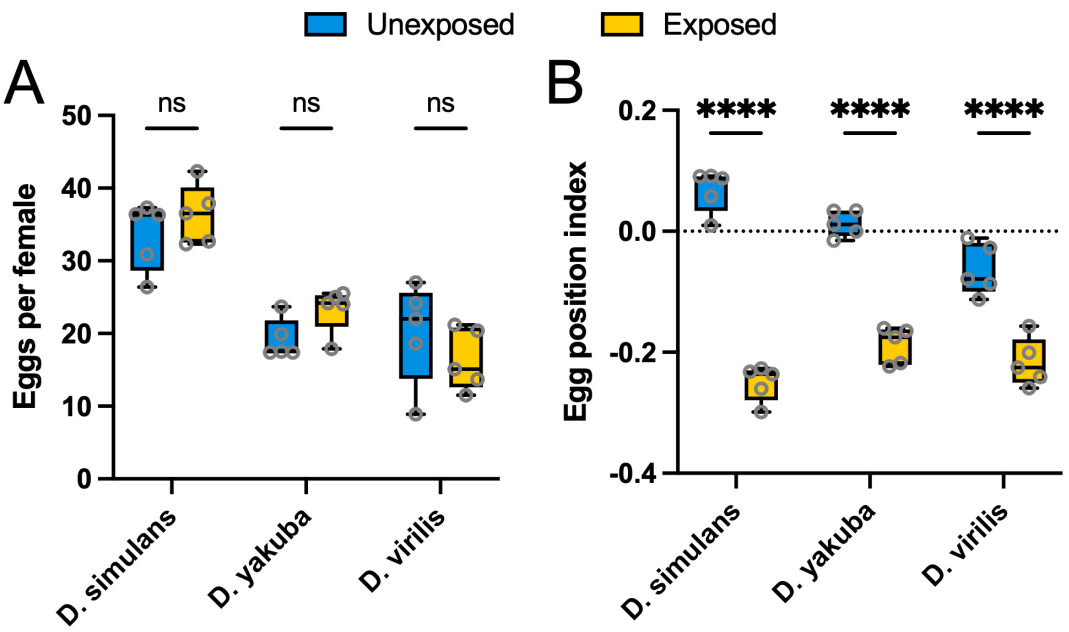


Fig. 3: The induced egg burying behavior is conserved across Drosophilids. (**A**)Three other fly species did not reduce oviposition rates when exposed to *F. mccooki* ants (*D. simulans*: *P*=0.9729; *D. yakuba*: *P*=0.4829; *D. virilis*: *P*=0.6040). (**B**) All three species altered their oviposition depth when exposed to ants (*P*<0.0001 for all comparisons). (Two-way ANOVA with Bonferroni multiple comparison test).

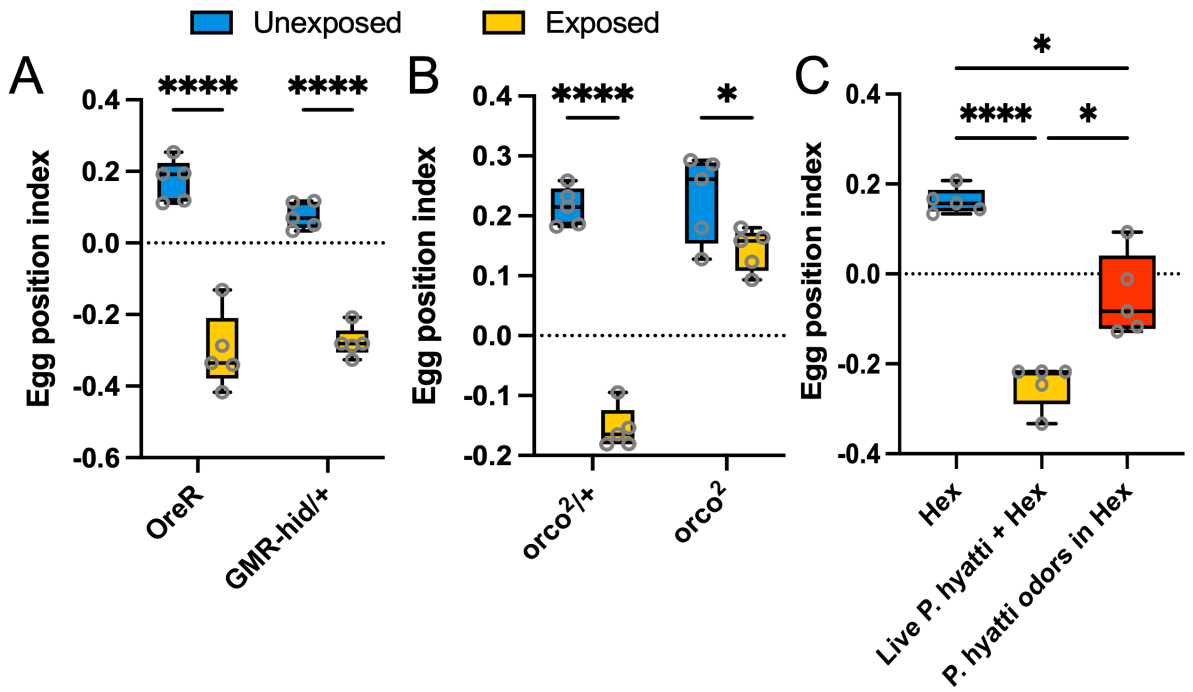
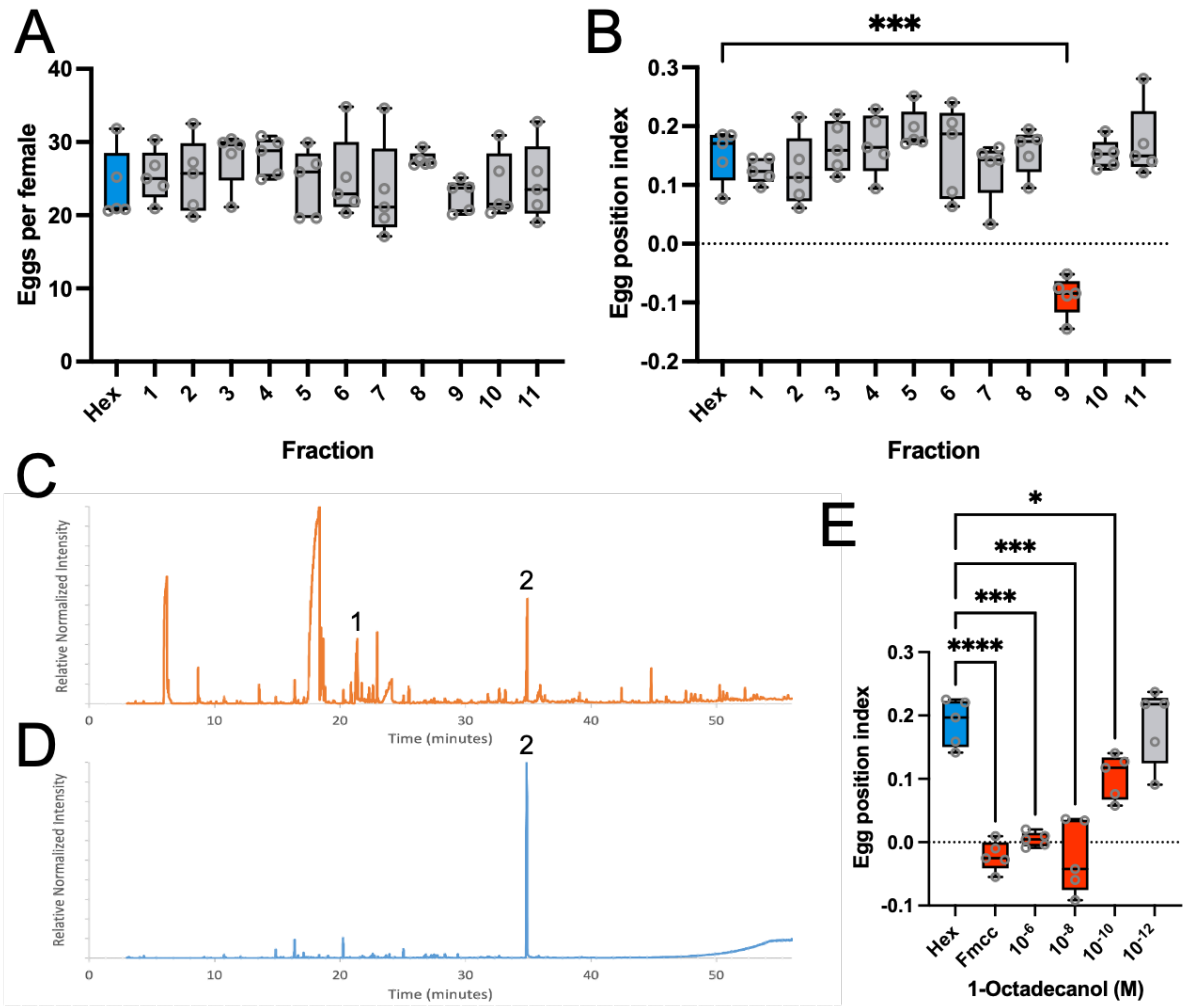


Fig. 4: Flies detect ants through olfactory cues. (**A**) Blind flies continued to bury their eggs when exposed to *F. mccooki* ants, similar to control OreR flies ($P<0.0001$ for both comparisons). (**B**) Both control heterozygous *orco* mutant flies and homozygous *orco* mutants buried their eggs when exposed to ants, although the magnitude of the effect was much weaker for the homozygous mutants (*orco*^{2}/+: $P<0.0001$; *orco*^{2}: $P=0.0210$; two-way ANOVA with Bonferroni multiple comparison test for A and B). (**C**) Ant (*P. hyatti*) odors extracted in hexane significantly altered fly oviposition depth behavior, although not to the same extent as direct ant exposure (Hex vs live ants: $P<0.0001$; Hex vs ant odors: $P=0.0166$; Live ants vs ant odors: $P=0.0150$; Brown-Forsythe and Welch's ANOVA with Dunnett's multiple comparison test).



877 **Fig. 5: The ant pheromone 1-octadecanol induces fly egg burying.** Whole body-wash extract
878 from *F. mccooki* ants was fractioned, resulting in 11 subfractions. (**A**) Flies did not alter
879 oviposition rate after exposure to any of the odor subfractions, as compared to hexane-only
880 exposed flies. (**B**) However, fraction 9 did significantly alter oviposition depth behavior (Hex vs
881 fraction 9: P=0.0003). (**C**) The chromatogram from GC-MS of the whole-body wash extract
882 revealed many peaks, including iridomyrmecin (peak 1). (**D**) Fraction 9 contained a single large
883 peak that was identified as 1-octadecanol, which was also present in the whole-body wash
884 extract (peak 2). (**E**) Flies exposed to *F. mccooki* (Fmcc) ant body wash, or higher concentrations
885 of 1-octadecanol, significantly altered oviposition depth, as compared to hexane-exposed flies
886 (Hex vs Fmcc: P<0.0001; Hex vs 10^{-6} M: P=0.0005; Hex vs 10^{-8} M: P=0.0010; Hex vs 10^{-10} M:
887 P=0.0271; Hex vs 10^{-12} M: P>0.9999). (A, B, and E: Brown-Forsythe and Welch's ANOVA with
888 Dunnett's multiple comparison test).

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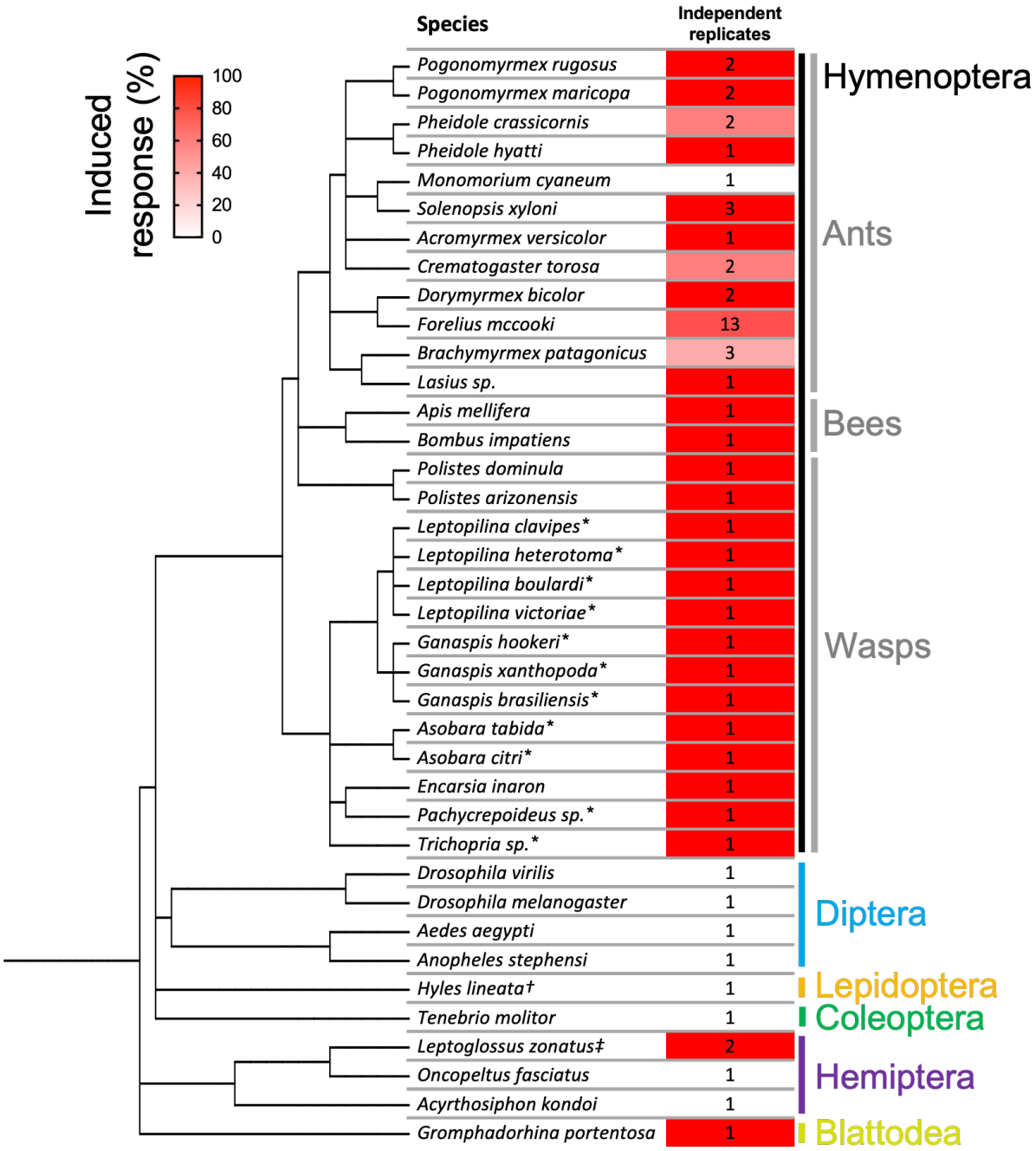
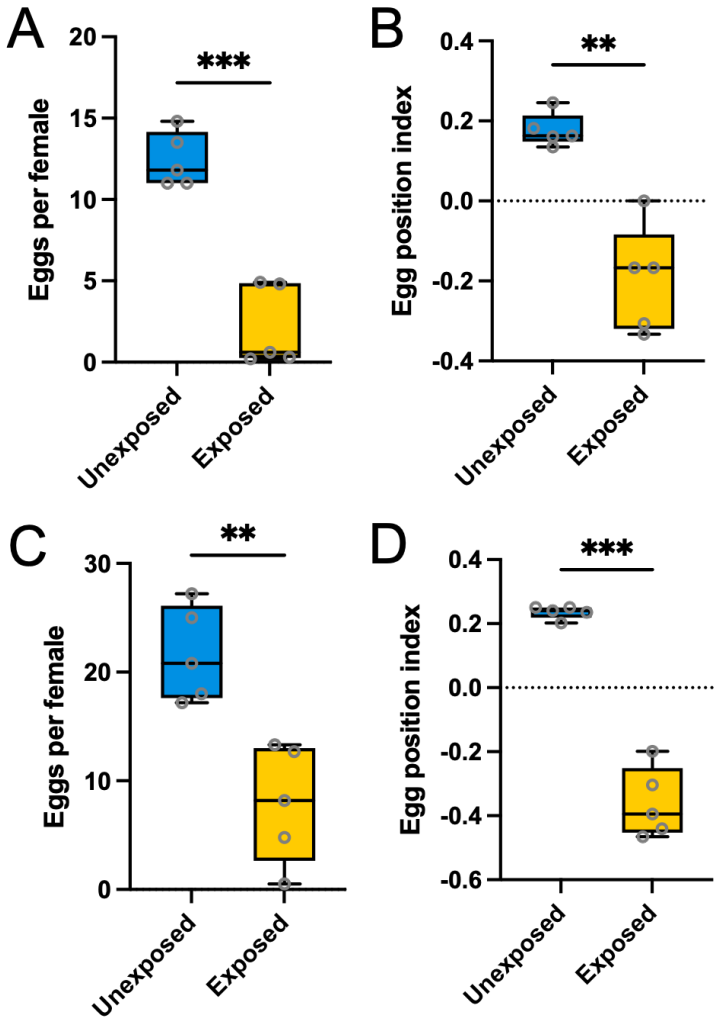
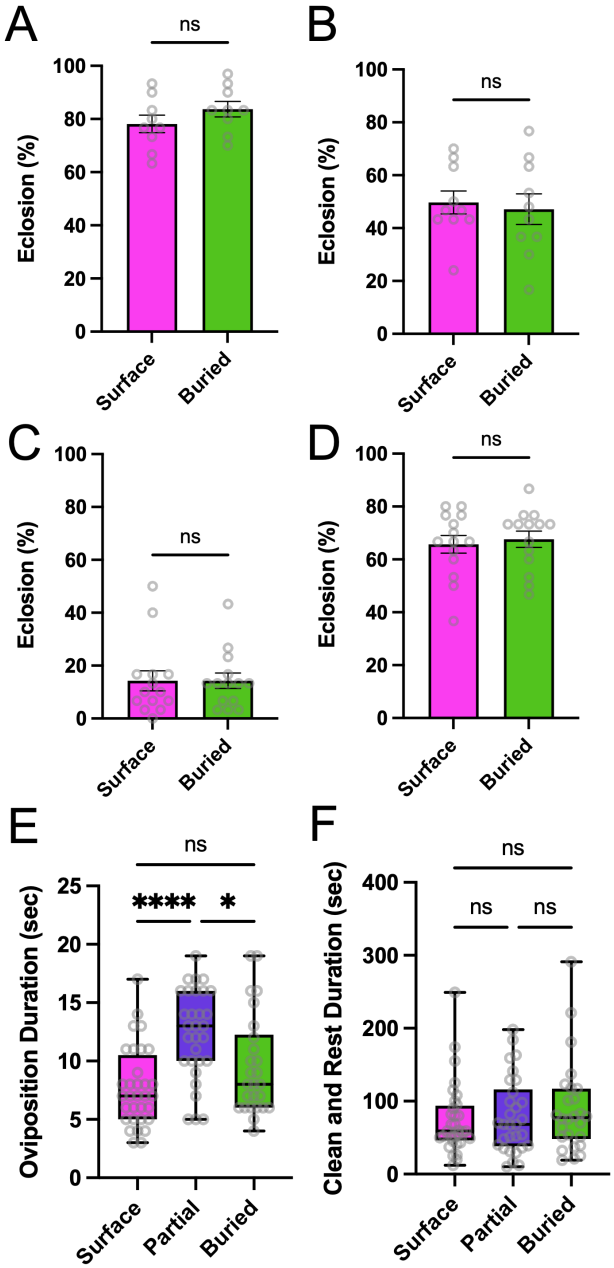


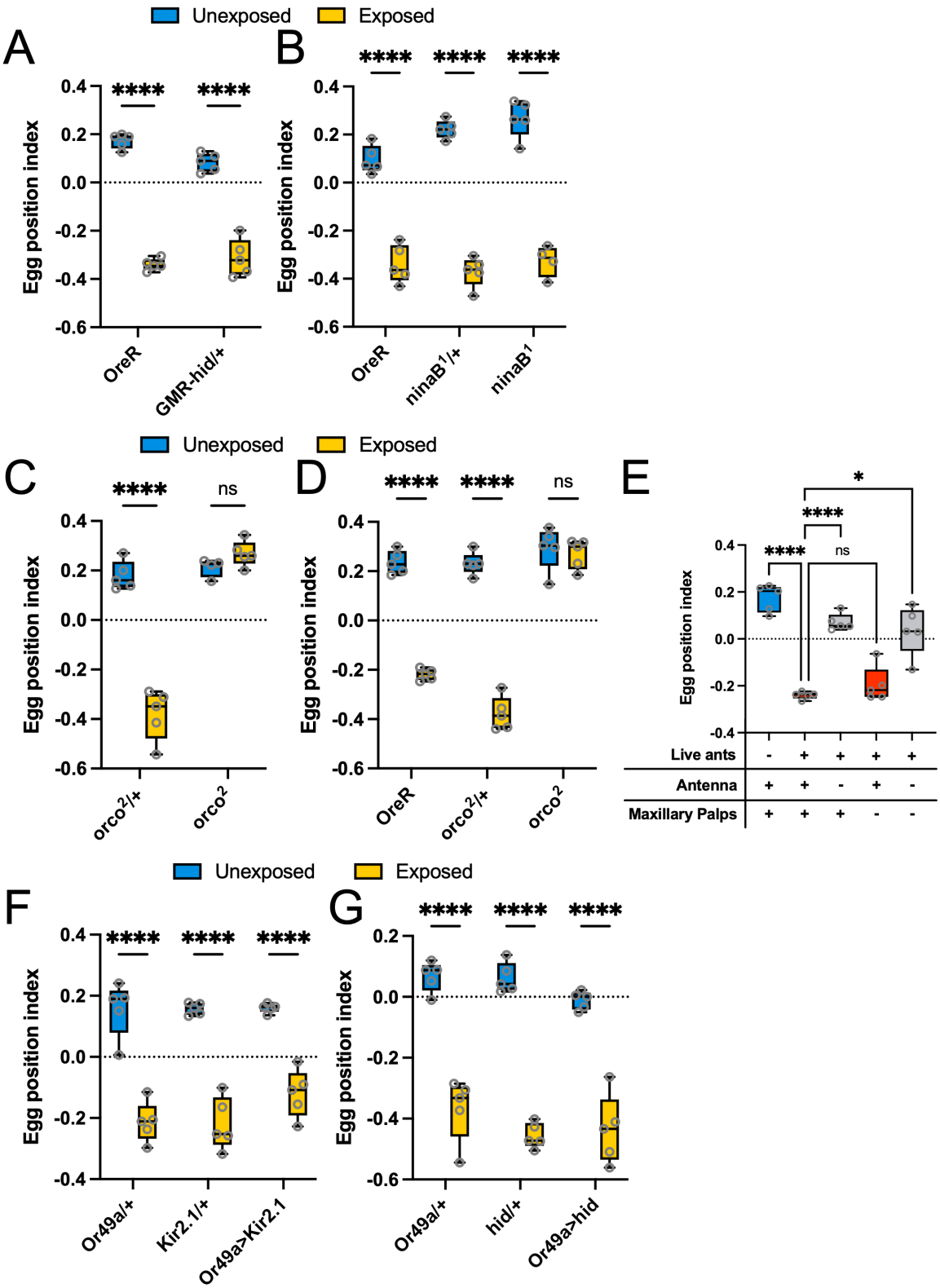
Fig. 6: Hymenopteran odors trigger the fly egg burying behavior. A phylogeny is shown of the different insect species whose odors were tested for the ability to induce the fly egg burying behavior. The number of independent replicates run for each insect species is shown, while the percentage of replicates resulting in significant fly egg burying is colored in red. Flies responded to odors from a diversity of hymenopterans, but rarely to odors from non-hymenopterans. * live insect exposure rather than body wash exposures; † odor extracted from larval stage; ‡ unlike the ant replicates that were derived from independent ant colonies, the *Leptoglossus zonatus* replicates were derived from the same colony, although the odor extractions were independent.



Supp. Fig. 1: Exposure to parasitoid wasps also alters fly oviposition behaviors. Unlike their response to ant exposure, flies reduced oviposition rates when exposed to *L. heterotoma* (**A**, *P*=0.0001) or *L. boulardi* (**C**, *P*=0.0025) parasitoid wasps. However, like their response to ant exposure, flies oviposited eggs more deeply when exposed to *L. heterotoma* (**B**, *P*=0.0022) and *L. boulardi* (**D**, *P*=0.0002) wasps. (Unpaired, Welch's t-test).



Supp. Fig. 2: Any cost to deeper oviposition remains unknown. Egg depth had no effect on adult eclosion rates under normal conditions (**A**, *P*=0.2230) or when other survival risks were present, such as the presence of older larvae (**B**, *P*=0.7234), cold overnight temperatures (**C**, *P*=0.7577), or simulated rain (**D**, *P*=0.6774) (unpaired, Welch's t-test for A, B, and D; Mann-Whitney test for C). (**E**) Maternal oviposition duration was significantly increased for partially inserted eggs, but no timing difference was found between surface and buried eggs (Surface vs. Partial: *P*<0.0001; Surface vs Buried: *P*<0.3475; Partial vs Buried: *P*=0.0386). (**F**) After ovipositing, the female flies showed no difference in the length of their clean-and-rest phase across the three egg position categories (Surface vs. Partial: *P*=0.; Surface vs Buried: *P*<0.; Partial vs Buried: *P*=0.). (Kruskal-Wallis with Dunn's multiple comparison test in E and F). Bars represent mean ± S.E.M.



Supp. Fig. 3: Olfaction is required to detect ants and wasps. (**A** and **B**) Blind flies exposed to *L. heterotoma* parasitoid wasps maintained normal oviposition depth change similar to control OreR flies or heterozygous controls (*P*<0.0001 for all comparisons in A and B). (**C**) Anosmic *orco*

919 mutant flies failed to alter oviposition depth behavior when exposed to *P. hyatti* ants (*orco*^{2}/+:
920 $P<0.0001$; *orco*^{2}: $P=0.4805$). (**D**) Similarly, anosmic flies also failed to respond to *L. heterotoma*
921 wasps (OreR: $P<0.0001$; *orco*^{2}/+: $P<0.0001$; *orco*^{2}: $P>0.9999$). (**E**) Control OreR flies with ablated
922 antenna had a significantly higher egg position index when exposed to *F. mccooki* ants than
923 fully intact flies. Ant-exposed flies with only maxillary palp ablations showed no significant
924 difference in oviposition depth compared to intact flies (intact exposed vs intact unexposed:
925 $P<0.0001$; intact exposed vs antenna-ablated exposed: $P<0.0001$; intact exposed vs maxillary
926 palp-ablated exposed: $P=0.6090$; intact exposed vs both-ablated exposed: $P=0.0138$). (**F** and **G**)
927 Flies responded to *L. heterotoma* wasp presence by burying eggs even with the silencing
928 (*Kir2.1*) or ablation (*hid*) of wasp odor-detecting Or49a sensory neurons ($P<0.0001$ for all
929 comparisons in F and G). (A-D, F, G: Two-way ANOVA with Bonferroni multiple comparison test;
930 E: Brown-Forsythe and Welch's ANOVA with Dunnett's multiple comparison test).

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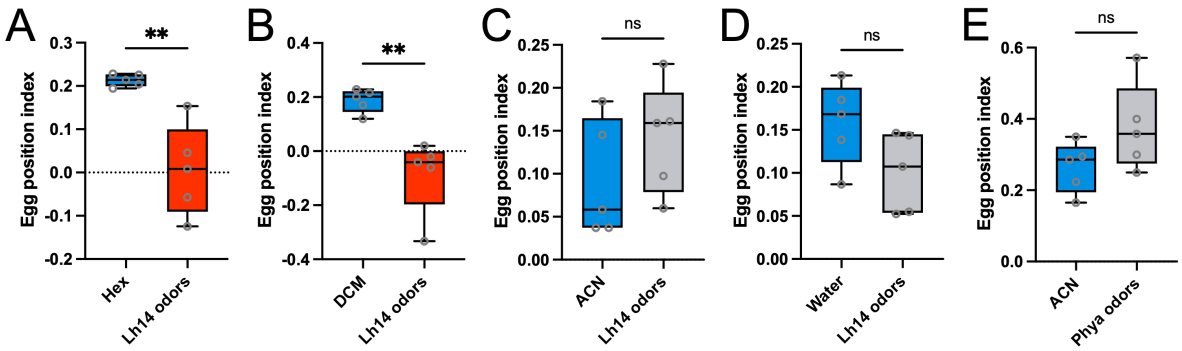

Supp. Fig. 4: Body wash solvent specificity in fly detection of ant and wasp odors. Different solvents used to extract body odors varied in their success at inducing the fly egg burying behavior. Flies responded to *L. heterotoma* parasitoid wasp body washes when either hexane (Hex) (**A**, *P*=0.0110) or dichloromethane (DCM) (**B**, *P*=0.0099) were used. Body washes using other solvents, such as acetonitrile (ACN) (**C**, *P*=0.2802) and water (**D**, *P*=0.0899), did not induce the fly behavior. *P. hyatti* ant body washes using the ACN solvent also showed no significant change in oviposition behavior (**E**, *P*=0.1249). (Unpaired, Welch's t-test).