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. 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 hymenopterans possess a conserved and ancient 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 (Hermann & Landis, 2017), and will even engage in behaviors that protect their close relatives (e.g. offspring) from predation. 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 is 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 will 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; Muijres et al., 2014; 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)

78   Female oviposition choices are particularly interesting because they can represent trans-
79   generational anti-predation behaviors (a type of parental care) (Refsnider & Janzen, 2010). The
80   decision about where, when, and how to lay an egg is complicated and relies on multiple kinds
81   of sensory information (Cury et al., 2019; Richmond & Gerking, 1979; Rockwell & Grossfield,
82   1978; Wang et al., 2020; Zhang et al., 2020). This information includes the mating and
83   reproductive status of the female, aspects of the abiotic environment like the weather, the time
84   of day and season, aspects of the food source like water content, stiffness, color, odor, and
85   taste, and aspects of the biotic environment like presence of conspecifics and potentially the
86   presence of biotic threats. Might flies alter their oviposition choices (i.e. trigger an innate
87   induced behavior) in the presence of predatory threats to their offspring?

88   Ants are ubiquitous and occupy diverse niches in nearly all natural ecosystems
89   (Hölldobler & Wilson, 1990). Many are facultative or obligate predators of other organisms,
90   including insects of all life stages (Fernandes et al., 2012), which provide necessary proteins and
91   fats for colony growth (Hölldobler & Wilson, 1990). A number of studies have shown that
92   presence of ants, and specifically visual or olfactory ant signals, cause diverse insect species to
93   avoid oviposition in the ant-infested area (Freitas & Oliveira, 1996; Sendoya et al., 2009; Taylor
94   et al., 1998; Van Mele et al., 2009). Although published data on interactions between *D.*
95   *melanogaster* and ants are scarce (Soto-Yéber et al., 2018), ants have been shown to be
96   important predators of the eggs and larvae of other *Drosophila* species (Escalante & Benado,
97   1990; Lewis & Worthen, 1992; Worthen et al., 1993). Anecdotally, we have noticed that rotting
98   fruit traps meant to attract *D. melanogaster* attract far fewer flies when ants are present, and
99   we have seen ants carrying off what appear to be *D. melanogaster* eggs and larvae.

100   Here we tested whether naïve flies alter their oviposition behavior in the presence of
101   predatory ants. We discovered that flies push their eggs deeply into the food substrate when
102   exposed to ants, which protects eggs from ant predation. This innate, induced oviposition
103   depth behavior is a conserved trait across the genus Drosophila. Unlike previous work with
104   other fly predators (de la Flor et al., 2017), olfaction, but not vision, is required for the
105   oviposition depth behavior. Furthermore, we show that flies deploy this behavior in response to
106   diverse ant species, as well as other hymenopterans. This system can serve as a model for how
107   innate threat recognition and downstream defensive behaviors are encoded in the germline
108   and hardwired into the brain.

109

110   **Materials and Methods:**

111   **Species used**:

112   *Flies*: All flies were raised on cornmeal molasses food (10L water, 75g agar, 275g yeast, 520g
113   cornmeal, 110g sugar, 1046g molasses, 45mL propionic acid, and 100mL 20% (w/v) Tegosept)
114   and maintained at 24°C in ~60% humidity with a 16:8 light:dark cycle. We used a *D.*
115   *melanogaster* Oregon R (OreR) strain as our wild-type strain. The following *D. melanogaster*
116   mutants were obtained from the Bloomington Drosophila Stock Center (with stock number
117   given): vision mutants GMR-hid (5771) and ninaB[1] (24776), olfaction mutant orco[2] (23130), the
118   driver strain Or49a-GAL4 (9985), and the responder strains UAS-Kir2.1 (6595) and UAS-hid

119   (65403). The mutant chromosomes from these strains were crossed into the OreR background
120   to reduce genetic variability. White-eyed w[1118] flies were provided by Daniela Zarnescu and
121   used as hosts for growing parasitoid wasps. Of the other *Drosophila* species used, wild *D.*
122   *simulans* were caught in Tucson, AZ in 2018 and maintained in the lab as a single strain. *D.*
123   *yakuba* and *D. virilis* were acquired from the Drosophila Species Stock Center (stock numbers
124   14021-0261.01 and 15010-1051.87, respectively).

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

145   Ant species were identified morphologically to genus using a dichotomous key (Fisher &
146   Cover, 2007), and identified to species by regional species level keys (see Supplementary Table
147   1 for all keys and sources). No species used in this study are protected or endangered. To
148   confirm morphological identifications, DNA was extracted from the ant samples for Sanger
149   sequencing. First, 10-50 ants were homogenized in 20-30 μL extraction buffer (0.1M Tris-HCL,
150   pH 9.0, 0.1M EDTA, 1% SDS) with a Kontes pellet pestle in a 1.5 mL Eppendorf tube. Additional
151   extraction buffer was added to bring the volume up to 500 μL, then the samples were
152   incubated for 30 min in a 70°C water bath. Cellular material was precipitated and pelleted by
153   adding 70 μL of 8M potassium acetate to each sample, incubating on ice for 30 min, and
154   centrifuging at 13000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh
155   Eppendorf tube, then the DNA was precipitated and pelleted by adding 300 μL isopropanol and
156   centrifuging at 13000 rpm for 5 min at room temperature. After a second clean-up spin, the
157   resulting DNA pellet was washed with 1 mL 70% ethanol and centrifuged at 13000 rpm for 5
158   min at room temperature to re-pellet. The pellet was allowed to air dry for 1-2 minutes, then
159   resuspended in 75 μL nuclease-free water and stored at -20°C. The mitochondrial *cytochrome*
160   *oxidase I* (*COI*) gene was sequenced to identify the different ant species. Following the GoTaq
161   Green Master Mix (Promega, WI, USA) protocol, we generated 25μL reactions containing 12.5

162 μL GoTaq, 7.5 μL nuclease-free water, 2 μL of both LCO1490 and HC02198 primers (Folmer et
163 al., 1994), and 1 μL extracted DNA. Polymerase chain reaction (PCR) conditions were as follows:
164 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
165 2 min and a hold at 4°C. The PCR products were visualized on a 1% agarose gel with SYBR safe
166 DNA gel stain (Thermo Fisher Scientific) to confirm single DNA bands were present. PCR
167 products were purified using the QIAquick PCR Purification Kit (Qiagen) and then sequenced at
168 the University of Arizona Genetics Core using the PCR primers. All sequences can be found in
169 Supplementary File 1.

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

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

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

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

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

227   **Ant predation experiment**: OreR flies laid eggs on standard molasses food in 5.5cm diameter
228   petri dishes for up to two hours. Eggs were gently removed and manually positioned on 5.5cm
229   diameter petri dish filled with 0.5% agarose gel with red food coloring. Surface eggs were set on
230   top of the agarose gel, partially buried eggs were inserted lengthwise roughly 50% into the gel,
231   and the buried eggs were inserted until the gel covered the point at which the dorsal
232   appendages connect to the eggshell (twenty eggs per category for a total of sixty eggs). The *P.*
233   *hyatti* laboratory colony was used under the assumption that it displays normal foraging
234   activity. A secondary 17.5cm x 12.5cm x 6cm plastic container, with walls coated in 'insect-a-
235   slip,' was connected to the main colony chamber by a 2cm (length) x 1.5cm (diameter) plastic
236   tube plugged with cotton. The cotton separator was then removed, and ants were allowed to
237   freely explore the smaller chamber for thirty minutes before the dish with fly eggs was added.
238   The ants were allowed to forage for thirty minutes before removal of the egg dish, at which
239   point the remaining eggs were counted. The connection between the colony chamber and the
240   foraging chamber was then blocked, the remaining ants were transferred back to the colony
241   chamber, and the bottom of the foraging chamber was washed with ethanol to remove any ant
242   pheromone trails. After one hour, the cotton separator was removed and the next replicate
243   trial began. Two replicates were run per day over the course of ten days, for a total of twenty
244   replicates. After counting the remaining fly eggs, we scored egg hatching rate 48 hours after the
245   ant exposure to confirm that the remaining eggs were viable.

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

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

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

Results:

Flies bury their eggs when exposed to ants

To test whether adult female flies change their oviposition behavior in the presence of predatory ants, we co-housed flies with either wild-caught *Forelius mccooki* or laboratory raised

287   *Pheidole hyatti* ants for 24 hours. Unlike with exposure to parasitoid wasps (Supp. Fig. 1A and C)
288   (Kacsoh et al., 2015; Lefèvre et al., 2012; Lynch et al., 2016), female flies did not reduce
289   oviposition in the presence of these ants (Fig. 1A and B). The non-significant trend towards
290   reduced egg numbers from the *P. hyatti* exposure is likely due to the ants actively capturing the
291   adult flies during the experiment, as exemplified by flies that had sections missing from their
292   wings or legs at the end of the experimental period (Fig. 1C). Ants of the genus *Pheidole* have
293   been described as highly aggressive in terms of prey capture (Traniello, 2010).

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

309   **Buried eggs survive ant predation**

310   We hypothesized that eggs laid deeper into the food substrate would be protected
311   against ant consumption. We provided the laboratory culture of *P. hyatti* ants with fly eggs
312   manually positioned at three different depths, similar to the three natural depth categories (Fig.
313   1D-E, 2A). After a 30 minute ant foraging bout, the remaining fly eggs were counted. Eggs
314   positioned on the surface of the media were the most susceptible to predation, while eggs
315   positioned beneath the surface were strongly protected (Fig. 2B). Indeed, ants readily removed
316   the eggs on the surface but struggled (attempted and often failed) to remove the deeply
317   positioned eggs (Supp. videos 1 and 2). Of the eggs remaining after the ant foraging bout,
318   hatching rates were high and similar across the depth categories (Fig. 2C), showing that the ants
319   were not consuming or otherwise harming the buried eggs on-site. These results suggest that
320   the fly oviposition depth behavior is an adaptation to protect fly offspring from ant predation.

321   Given that laying eggs deeply into the substrate protects them from predation, why
322   don't fly mothers perform this behavior constitutively? We tested whether buried eggs suffer
323   some fitness cost compared with eggs laid on the substrate surface. First, we tested whether
324   there was any reduction in offspring developmental time or survival for flies that were buried in
325   the egg stage, but did not find any difference in eclosion time or eclosion success between flies
326   manually positioned on the food surface versus buried at the egg stage (Supp. Fig. 2A). We also
327   tested whether the presence of older fly larvae churning the food, reduced temperature, or
328   simulated rain (water misting) harmed the buried eggs, but once again found no difference in

329   survival to eclosion (Supp. Fig. 2B-D). These results suggest that any cost of deep oviposition is
330   not incurred by the offspring, but instead may be borne by fly mothers. The *D. melanogaster*
331   oviposition program has been described as a progression of stereotyped phases starting with a
332   searching phase, then the oviposition phase, and finally the clean-and-rest phase (Yang et al.,
333   2008). We hypothesized that female flies might require more time to lay buried eggs or to rest
334   after laying buried eggs. However, video analysis of the fly oviposition program during
335   parasitoid wasp exposure showed no difference in the average length of either of these phases
336   when the flies laid a surface egg versus laid a buried egg (Supp. Fig. 2E-F). Curiously, female flies
337   spent significantly longer periods laying partially buried eggs as compared to surface eggs or
338   fully buried eggs (Supp. Fig. 2E), suggesting that partially buried eggs need more care to
339   position. In summary, we were unable to detect any negative consequences of the induced egg
340   burying behavior. Either there is no fitness cost associated with this behavior, or there is a cost
341   that we have not yet identified.

342   **Egg burying is a conserved trait across *Drosophila***

343   To test whether ant-induced egg burying is a conserved defensive behavior in flies, we
344   assayed the behavior in three other *Drosophila* species of varying evolutionary distances from
345   *D. melanogaster: D. simulans* (~3MY since the most recent common ancestor), *D. yakuba*
346   (~5MY), and *D. virilis* (~30MY) (Powell, 1997). Like *D. melanogaster*, the other *Drosophila*
347   species maintained their egg production level when exposed to *F. mccooki* ants (Fig. 3A), and
348   also like *D. melanogaster*, all three *Drosophila* species consistently laid their eggs deeper in the
349   substrate in the presence of ants (Fig. 3B). This result holds even though *D. virilis*, and to some
350   extent *D. yakuba*, tend to lay more deeply buried eggs than *D. melanogaster* in the unexposed
351   state (Fig. 3B). These data suggest that although normal egg depth behavior varies across
352   species, the switch to laying more buried eggs to prevent egg predation is a conserved trait.

353   **Olfaction mediates ant detection**

354   Fly mothers sense and respond to the presence of ants by burying their eggs, but how
355   do they know that ants are present? Flies have been shown to detect parasitoid wasps through
356   the combined effects of the visual and olfactory systems (Ebrahim et al., 2015; Kacsoh et al.,
357   2015, 2013; Lynch et al., 2016), and given that flies also bury their eggs in the presence of
358   wasps (Supp. Fig. 1) we tested both of these sensory modalities. *GMR-hid* flies express a pro-
359   apoptotic factor in the eye, making their eyes significantly reduced in size (Grether et al., 1995).
360   These sight-deficient flies maintained their ability to respond to the presence of *F. mccooki* ants
361   by burying their eggs (Fig. 4A). *GMR-hid* as well as *ninaB[1]* mutant flies (which are blind due to
362   loss of photoreceptors) also maintained their ability to sense and respond to *L. heterotoma*
363   parasitoid wasps (Supp. Fig. 3A-B). These data suggest that vision is dispensable for the altered
364   egg depth behavior. We next tested the necessity of the olfactory system: *orco[2]* mutant flies
365   lack the odorant receptor co-receptor and therefore lack most olfactory ability (Larsson et al.,
366   2004). Heterozygous *orco[2]* mutant flies showed a strong induced egg burying response in the
367   presence of *F. mccooki* ants, but the anosmic homozygous *orco[2]* mutant flies showed a much
368   weaker (albeit still significant) response (Fig. 4B). Exposing the anosmic flies to either *P. hyatti*
369   ants or *L. heterotoma* parasitoid wasps resulted in a complete failure to alter egg depth (Supp.
370   Fig. 3C-D). To further test the necessity of olfaction for the egg burying behavior, we manually

371   ablated the two main olfactory sensing organs from experimental flies either individually or in
372   tandem. While ablating the maxillary palps had no effect on fly ability to bury eggs in the
373   presence of *F. mccooki* ants, ablating the fly antennae abolished their ability to respond to the
374   ants (Supp. Fig. 3E). Altogether, these data demonstrate that flies require olfactory input
375   through their antennae to sense ants in their environment and alter oviposition depth behavior
376   accordingly.

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

389   If an ant olfactory stimulus is sufficient to induce the fly oviposition depth switch, we
390   hypothesized that ant body-wash extracts could replace live ants as the egg burying stimulus.
391   We used the solvent hexane to extract odorants from the bodies of *P. hyatti* ants and added the
392   odors directly onto the food substrate. While flies that were exposed to live *P. hyatti* ants as a
393   positive control showed a strong induced egg burying behavior, flies exposed to ant odors alone
394   showed a weaker, though still significant, response (Fig. 4C). There are at least three reasons
395   why odor extracts do not fully recapitulate the direct ant exposure condition. First, it is possible
396   that the body wash extracts only possess a subset of multiple distinct odorants required for the
397   full behavioral response. To test this, we used different solvents that varied in their polarity to
398   target different molecules. Interestingly, extracts collected in non-polar hexane (Fig 4C, Supp.
399   Fig. 4A) and dichloromethane (Supp. Fig. 4B) were both sufficient to induce the partial egg
400   burying behavior, while use of more strongly polar solvents failed to induce any fly response
401   (Supp. Fig. 4C-E). However, combining body wash odorants extracted using hexane and
402   dichloromethane did not induce the full egg burying behavior (data not shown), showing that
403   no additive effect is achieved by combining the odorant subsets extracted by each solvent.
404   Second, it is possible that exposure to insect body wash extracts fails to fully recapitulate the
405   live insect exposure results because the body wash odorants dissipate over the course of the
406   experiment without being replaced. To test this idea, we exposed flies to both dead *L.
407   heterotoma* parasitoid wasps, and to vials in which the wasps had been housed, both
408   treatments of which should have the full wasp odorant profile but no new odorants being
409   produced. In both cases, flies showed a partial egg burying response similar to their response to
410   insect body wash extracts, supporting the odorant dissipation hypothesis (data not shown).
411   Third, it is possible that flies are using some other sensory modality besides olfaction or vision
412   to detect ant/wasp presence, such as gustation, a hypothesis that remains untested.

413   Regardless, we were able to extract an odorant from insect bodies that flies detected, causing
414   them to induce at least a partial egg burying response.

415   **Flies respond to most hymenopterans**

416   We have shown that flies lay eggs more deeply into the food substrate when exposed to
417   ants (Fig. 1), and that this behavior protects the eggs from ant predation (Fig. 2). Flies also
418   induce this behavior when exposed to a parasitoid wasp, although it is unclear what benefit
419   there is to burying eggs in this context given that *L. heterotoma* does not attack fly offspring
420   until they reach the larval stage, when they are crawling on top of the food. To determine the
421   breadth of ants and potentially other insects that induce flies to bury their eggs, we exposed
422   flies to body washes or live samples of numerous arthropods. Flies responded to the vast
423   majority of ant species tested representing the three ant subfamilies Dolichoderinae,
424   Formicinae, and Myrmicinae (Fig. 5). Exceptions included the Myrmicinae species *Monomorium*
425   *cyaneum*, as well as some trials from several other ant species (Fig. 5). Parasitoid wasps
426   represent another large branch of hymenopterans that impose strong selective pressures on fly
427   offspring survival. Flies that were exposed directly to parasitoid wasps that infect fly larvae
428   (genus *Leptopilina*, *Ganaspis*, and *Asobara*) or fly pupae (*Pachycrepoideus* and *Trichopria*)
429   significantly altered their oviposition depth (Fig. 5). However, flies also responded to the body
430   wash extract of the whitefly parasitoid, *Encarsia inaron*, as well as extracts from every other
431   hymenopteran tested (bees and paper wasps), even though *D. melanogaster* has no known
432   ecological interactions with these insects (Fig. 5).

433   The odor extracts from non-hymenopteran insects generally did not induce flies to alter
434   their oviposition behavior, but there were three exceptions. First, odor extract from the leaf-
435   footed bug, *Leptoglossus zonatus*, did induce the fly egg burying behavior in two separate trials,
436   despite body washes from other hemipterans showing no effect on the flies (Fig. 5). Second,
437   odor extract from the nymph stage of the Madagascar hissing cockroach, *Gromphadorhina*
438   *portentosa*, also affected fly oviposition behavior. Third, odors from the adult stage of the moth
439   *Hyles lineata* altered fly oviposition behavior, but odors from the larval stage had no such
440   effect. In sum, flies responded to odors from 27 of 28 hymenopteran insects, but only 3 of 19
441   non-hymenopteran insects. Our data suggest that an odorant evolved early in the
442   hymenopterans and has been maintained over time, with sporadic gains and losses in the
443   broader insect group, and that flies evolved to sense this odorant as a means of protecting their
444   offspring from predatory ants.

445

446   **Discussion**:

447   Recognition of environmental threats, and execution of an appropriate response, is
448   critical to organismal survival. Here, we have shown that flies detect ant presence through
449   olfactory sensing of hymenopteran odors, and switch their oviposition behavior to more deeply
450   position their eggs in the food substrate. This protects fly eggs from ant predation.

451   Egg laying behavior follows a similar stereotyped pattern across different fly species. It is
452   first characterized by a search-like, informational gathering processes about the local nutrient
453   availability and substrate stiffness through labellum and leg contact (Bräcker et al., 2019; Yang

454 et al., 2008; Zhang et al., 2020). Once a suitable site is found, flies switch to a more
455 refined search behavior, including ovipositor contact with the substrate (Bräcker et al.,
456 2019), before an egg is finally laid. These discrete micro-behaviors are conserved across
457 *Drosophila*, with minor variations depending on the ecology of each species. For
458 example, the agricultural pest *D. suzukii* spends significantly more time than *D.*
459 *melanogaster* contacting the food substrate with its ovipositor and expelling its eggs
460 (Bräcker et al., 2019), presumably because it tends to lay eggs more deeply into less
461 ripened fruits (Karageorgi et al., 2017). Furthermore, *Drosophila* species that oviposit on
462 mushrooms tend to deeply bury their eggs (Rouquette & Davis, 2003), perhaps because
463 mushroom flesh is soft, or because ants commonly visit mushrooms (Lewis & Worthen,
464 1992). While fly oviposition behaviors are well-studied (Bräcker et al., 2019; Cury et al.,
465 2019; Karageorgi et al., 2017; Van Mele et al., 2009; Wang et al., 2020; Yang et al., 2008;
466 Zhang et al., 2020), this is the first report describing an induced change in oviposition
467 depth.

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

476 Several examples exist of organisms altering oviposition behavior in response to biotic
477 threats. For example, water striders (*Aquarius paludum insularis*) oviposit their eggs deeper in
478 the water column after exposure to parasitoid wasps, which limits egg parasitism (Hirayama &
479 Kasuya, 2009). Newts (*Taricha granulosa*) lay their eggs attached to plants higher in the water
480 column to avoid egg predator caddisfly larvae (*Limnephilus flavastellus*) (Gall et al., 2012). There
481 are even examples in fruit flies: *Drosophila* species avoid ovipositing at sites infested by toxic
482 microbes, which they detect via the odorant geosmin (Stensmyr et al., 2012). They also alter
483 oviposition in the presence of parasitoid wasps (Carton et al., 1986) using both visual and
484 olfactory cues: female flies lay fewer eggs during forced exposures, they preferentially choose
485 non-infested sites when given a choice by sensing the wasp odorant iridomyrmecin, and they
486 preferentially lay their eggs in more toxic (alcoholic) environments when exposed (Kacsoh et al.,
487 2013; Lefèvre et al., 2012; Lynch et al., 2016; Ebrahim et al., 2015). All of these are examples of
488 threat-induced behavioral changes that organisms make about 'when' or 'where' to oviposit,
489 whereas the new behavior described here is about 'how' flies choose to oviposit. Furthermore,
490 we know that the fly responses to geosmin and iridomyrmecin are hardwired into the fly, using
491 specific olfactory receptors and neuronal circuits in the brain. It will be interesting to determine
492 how these different environmental cues are signaled through the brain to regulate different
493 types of egg laying behaviors, and whether there is any overlap in each circuit.

494 Ants are a ubiquitous presence across diverse habitats, and many insects have evolved
495 ant avoidance behaviors triggered by distinct sensory modalities. For example, honeybees avoid
496 flowers in the field that have live ants or their odors nearby (Li et al., 2014; Sidhu & Rankin,

497   2016), and tephritid fruit flies avoid fruits perfumed with ant pheromones (Van Mele et al.,
498   2009). Furthermore, butterflies (*Eunica bechina*) were reported to avoid oviposition sites due to
499   visual detection of ant presence, although the fact that ants were pinned to plant leaves
500   suggests that ant odors may also have contributed to the butterfly avoidance (Sendoya et al.,
501   2009). Surprisingly, termites (*Coptotermes acinaciformis*) were shown to detect and avoid ants
502   based on the vibrational pattern of ants walking through their woody substrates (Oberst et al.,
503   2017). This may reflect a keen sense of vibration detection in termites, which have been shown
504   to communicate alarm signals via similar vibrational cues (Inta et al., 2009). These examples
505   show that detection of ant threats is common in the insect world, and that insects have evolved
506   diverse sensory mechanisms for identifying ant presence. Given that *D. melanogaster* invests
507   more resources into its olfactory system than other sensory systems (Keesey et al., 2019), it
508   may not be surprising that they detect ant predators through olfactory signals. In the future, it
509   will be interesting to identify the specific hymenopertan odorant and the fly odorant
510   receptor(s) responsible for sensing of ant odors and the neural circuitry that responds to odor
511   sensing by altering oviposition choices (Wang et al., 2020).

512   Although olfaction is necessary for fly detection of ants, we could not prove that ant
513   odors are sufficient to induce the full fly egg burying phenotype (Fig. 4). We believe there are
514   two likely reasons why providing flies with ant body wash odorant extracts does not fully
515   recapitulate the direct insect-exposure results. First, the ant odor extract experiments likely
516   started with a high concentration of ant odors, but over the course of the 24 hour experiment
517   these volatile odorant concentrations may have declined to a point where fly behavior was no
518   longer altered. In contrast, the live-ant exposures may have maintained a high concentration of
519   odorants throughout the experiments due to constant release from the insects. Second, it is
520   possible that fly oviposition behaviors like egg burying require multimodal sensory integration
521   such that ant odors suppress the preference for 'surface' eggs, but a second stimulus enhances
522   the magnitude of preference for 'buried' eggs. This second stimulus might include gustatory or
523   auditory cues from the ants. This kind of gating mechanism has been observed for fly larval
524   rolling behavior during parasitoid attack, where vibrational cues enhance the fly rolling
525   response to mechanical poking by the wasp ovipositor (Ohyama et al., 2015). Further testing of
526   fly sensory systems will help identify additional inputs into the oviposition depth behavioral
527   response.

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

540

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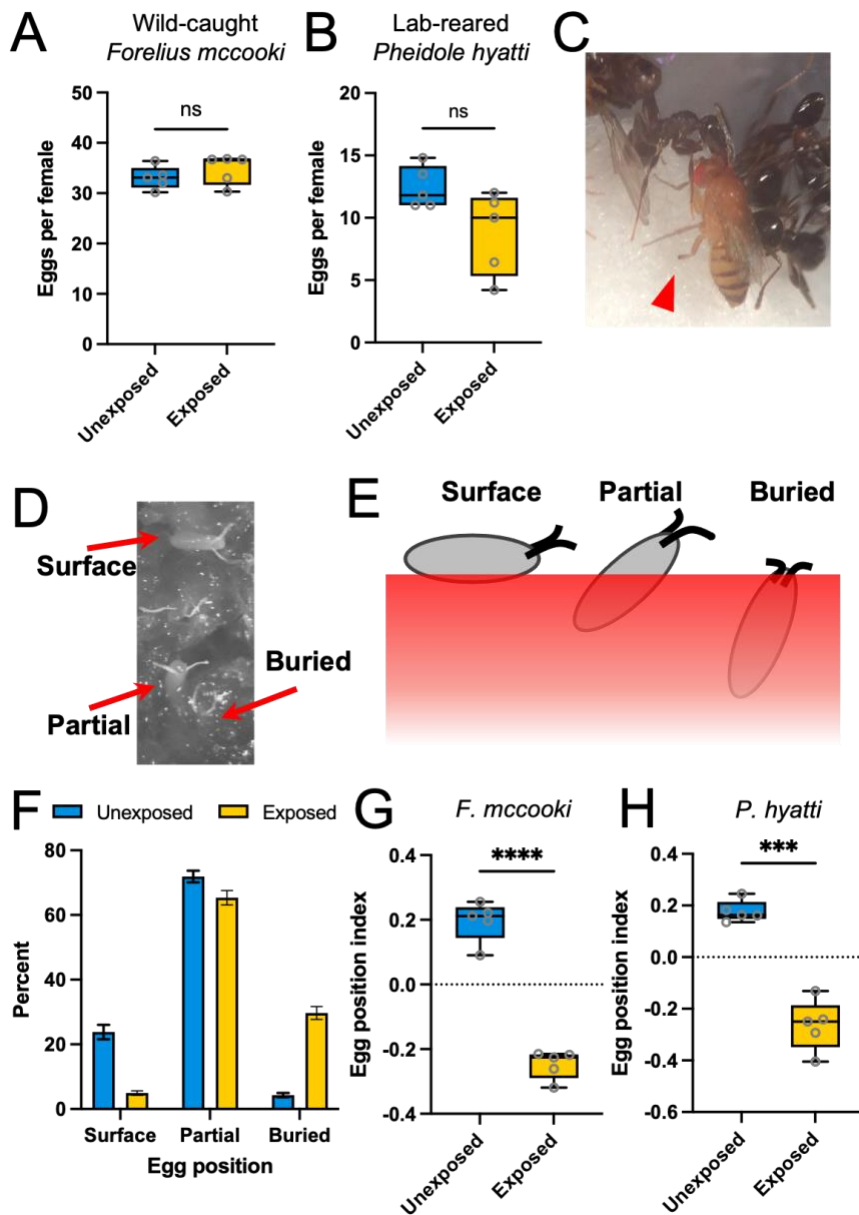
Fig. 1: Flies alter egg depth when exposed to ants. *D. melanogaster* flies exposed to either wild-caught *Forelius mccooki* (**A**) or lab-reared *Pheidole hyatti* (**B**) ants did not reduce oviposition rates (unpaired, Welch's t-test; *F. mccooki*: P=0.3551; *P. hyatti*: P=0.0713). The lower, but 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).
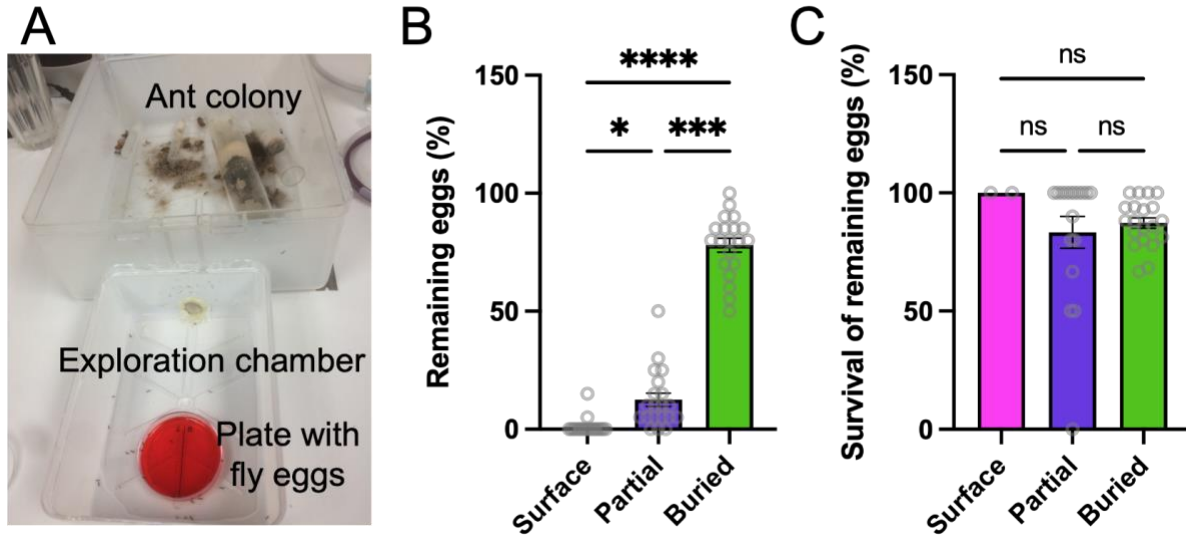
Fig. 2: Fly eggs positioned deeply are protected from foraging ants. (**A**) Photo of ant foraging experiment with *P. hyatti* ants. (**B**) Buried fly eggs were significantly more likely to remain after ant foraging, while surface and partially buried eggs were removed by the ants (Surface vs. Partial: *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.
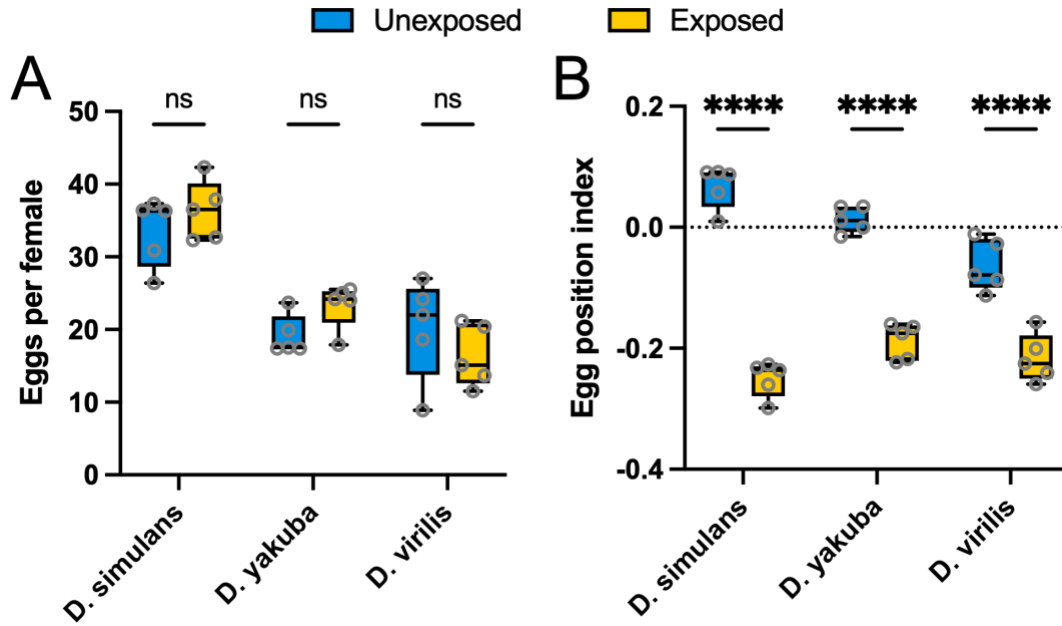
Fig. 3: The induced egg burying behavior is conserved across Drosophilids. (A)Three other fly species did not reduce oviposition rates when exposed to *F. mccooki* ants (*D. simulans*: *P*=0.9729; *D. yakuba*: *P*=0.4829; *D. virilis*: *P*=0.6040). (B) All three species altered their oviposition depth when exposed to ants (*P*<0.0001 for all comparisons). (Two-way ANOVA with Bonferroni multiple comparison test).
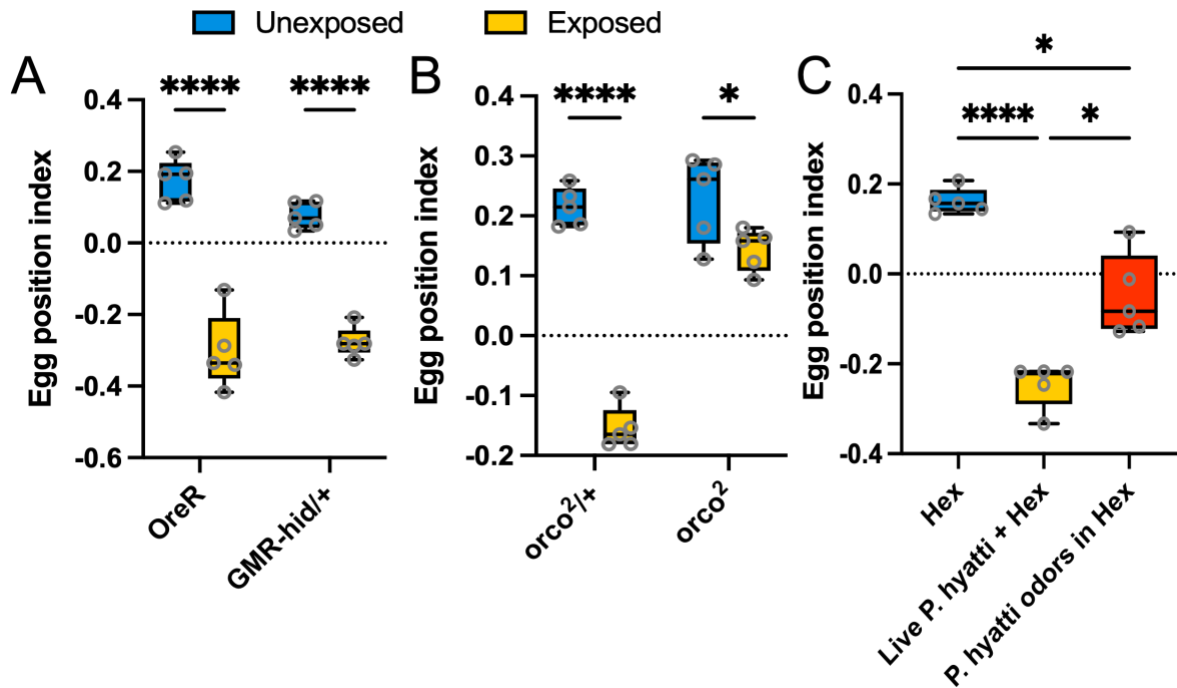
Fig. 4: Flies detect ants through olfactory cues. (**A**) Blind flies continued to bury their eggs when exposed to *F. mccooki* ants, similar to control OreR flies ($P<0.0001$ for both comparisons). (**B**) Both control heterozygous *orco* mutant flies and homozygous *orco* mutants buried their eggs 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).
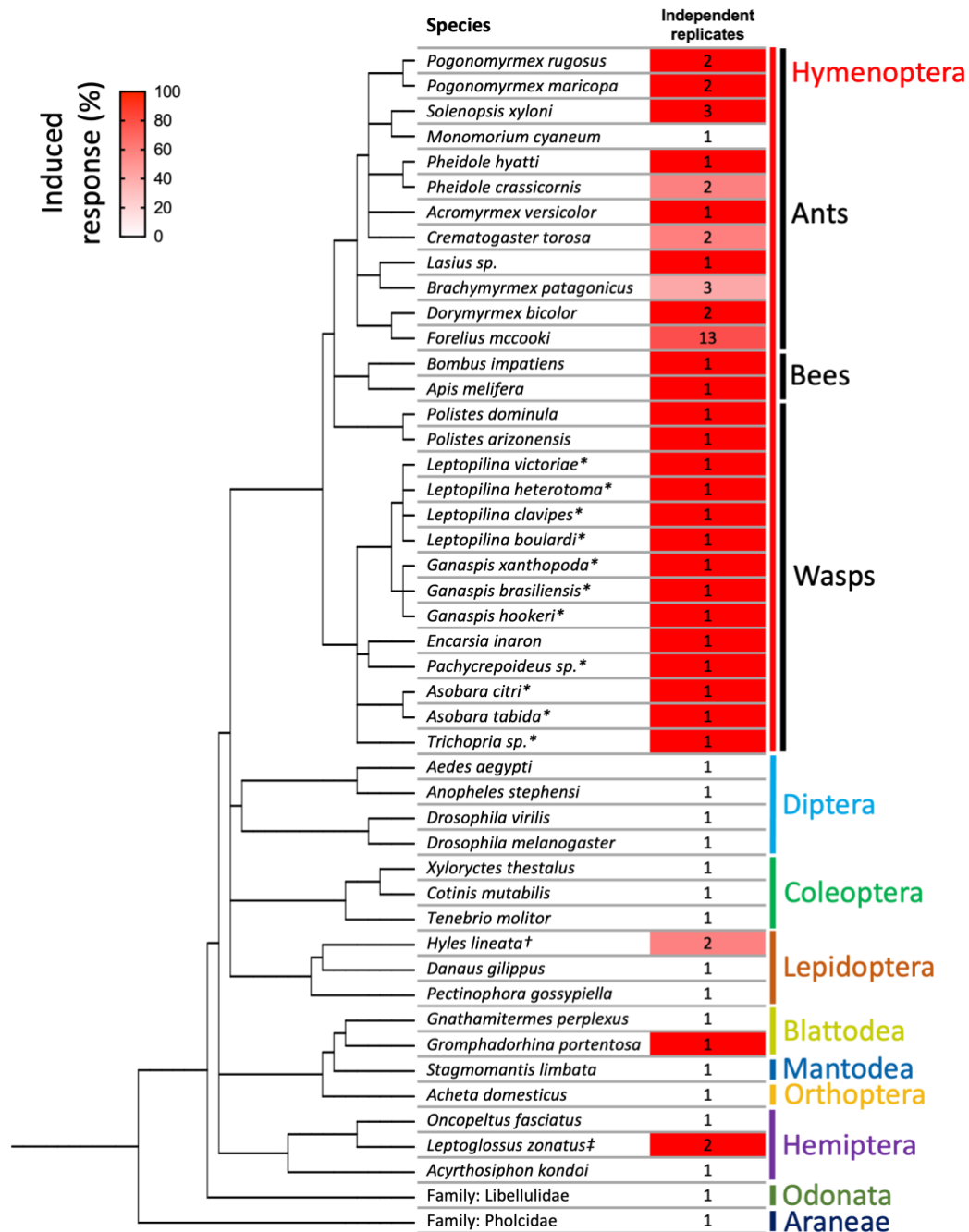
Fig. 5: Hymenopteran odors trigger the fly egg burying behavior. A phylogeny is shown of the different insect species whose odors were tested for the ability to induce the fly egg burying behavior. The number of independent replicates run for each insect species is shown, while the percentage of replicates 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 or adult 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.
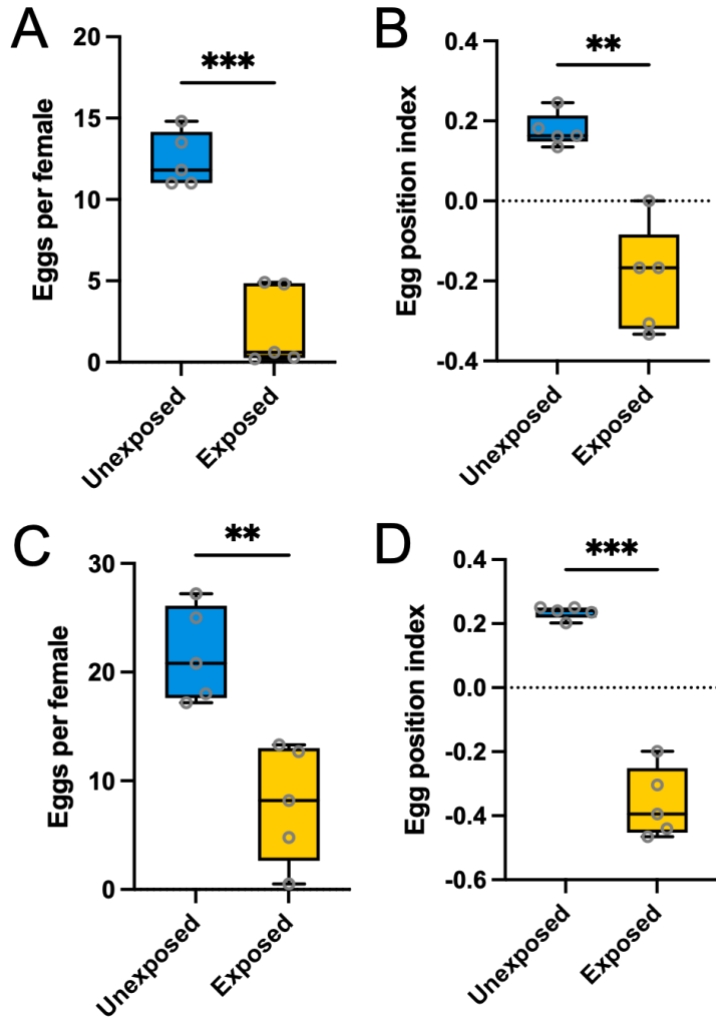
Supp. Fig. 1: Exposure to parasitoid wasps also alters fly oviposition behaviors. Unlike their response to ant exposure, flies reduced oviposition rates when exposed to *L. heterotoma* (**A**, *P*=0.0001) or *L. boulardi* (**C**, *P*=0.0025) parasitoid wasps. However, like their response to ant exposure, 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).
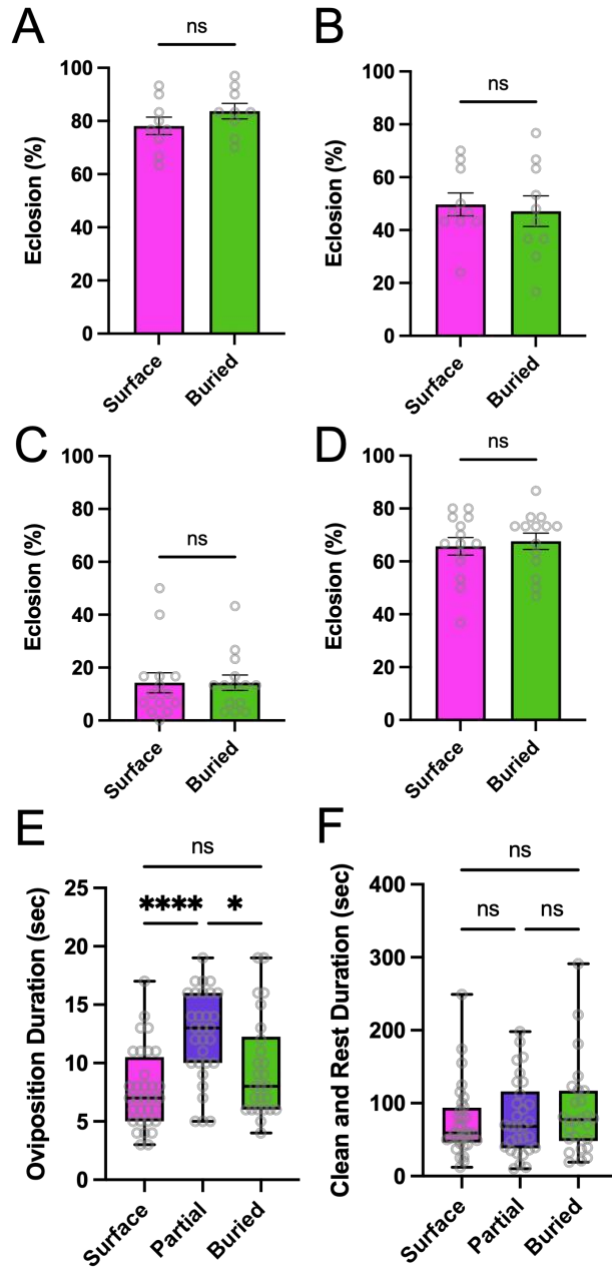
Supp. Fig. 2: Any cost to deeper oviposition remains unknown. Egg depth had no effect on adult eclosion rates under normal conditions (**A**, *P*=0.2230) or when other survival risks were present, such as the presence of older larvae (**B**, *P*=0.7234), cold overnight temperatures (**C**, *P*=0.7577), 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.
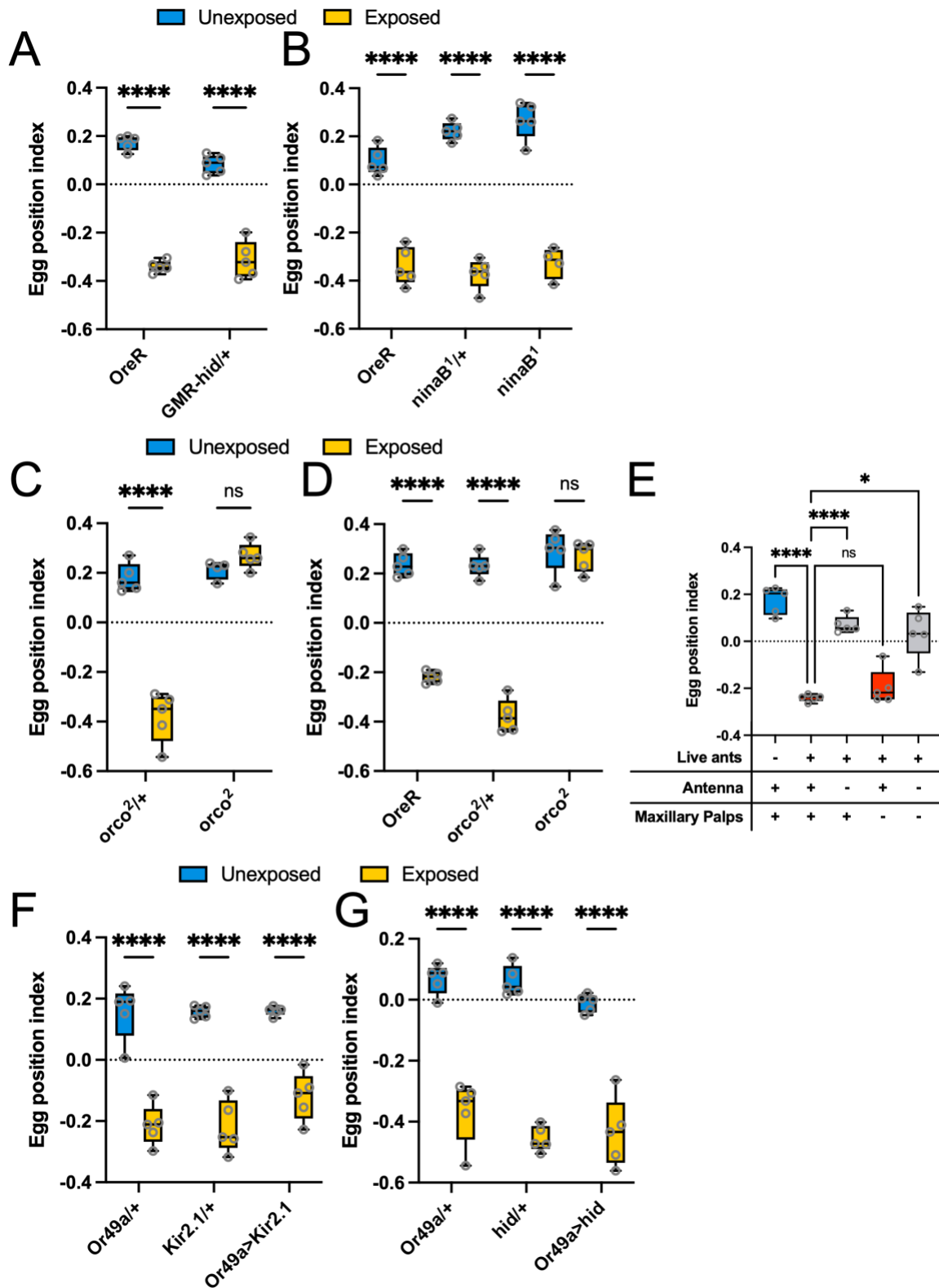
Supp. Fig. 3: Olfaction is required to detect ants and wasps. (**A** and **B**) Blind flies exposed to *L. heterotoma* parasitoid wasps maintained normal oviposition depth change similar to control OreR flies or heterozygous controls (*P*<0.0001 for all comparisons in A and B). (**C**) Anosmic *orco*

810   mutant flies failed to alter oviposition depth behavior when exposed to *P. hyatti* ants (*orco²*/+:
811   *P*<0.0001; *orco²*: *P*=0.4805). (**D**) Similarly, anosmic flies also failed to respond to *L. heterotoma*
812   wasps (OreR: *P*<0.0001; *orco²*/+: *P*<0.0001; *orco²*: *P*>0.9999). (**E**) Control OreR flies with ablated
813   antenna had a significantly higher egg position index when exposed to *F. mccooki* ants than
814   fully intact flies. Ant-exposed flies with only maxillary palp ablations showed no significant
815   difference in oviposition depth compared to intact flies (intact exposed vs intact unexposed:
816   *P*<0.0001; intact exposed vs antenna-ablated exposed: *P*<0.0001; intact exposed vs maxillary
817   palp-ablated exposed: *P*=0.6090; intact exposed vs both-ablated exposed: *P*=0.0138). (**F** and **G**)
818   Flies responded to *L. heterotoma* wasp presence by burying eggs even with the silencing
819   (*Kir2.1*) or ablation (*hid*) of wasp odor-detecting Or49a sensory neurons (*P*<0.0001 for all
820   comparisons in F and G). (A-D, F, G: Two-way ANOVA with Bonferroni multiple comparison test;
821   E: Brown-Forsythe and Welch's ANOVA with Dunnett's multiple comparison test).
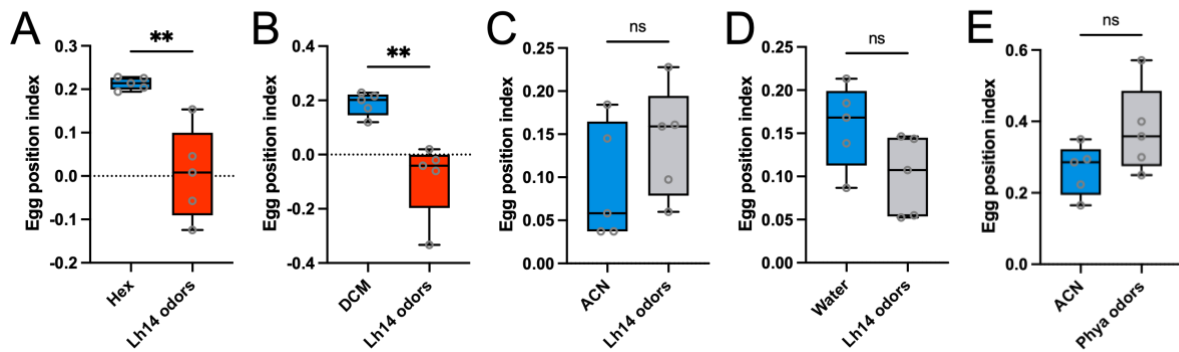
822 **Supp. Fig. 4: Body wash solvent specificity in fly detection of ant and wasp odors.** Different
823 solvents used to extract body odors varied in their success at inducing the fly egg burying
824 behavior. Flies responded to *L. heterotoma* parasitoid wasp body washes when either hexane
825 (Hex) (**A**, *P*=0.0110) or dichloromethane (DCM) (**B**, *P*=0.0099) were used. Body washes using
826 other solvents, such as acetonitrile (ACN) (**C**, *P*=0.2802) and water (**D**, *P*=0.0899), did not induce
827 the fly behavior. *P. hyatti* ant body washes using the ACN solvent also showed no significant
828 change in oviposition behavior (**E**, *P*=0.1249). (Unpaired, Welch's t-test).