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1 Drosophila glue protects from predation

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16

17 Abstract

18 Animals can be permanently attached to a substrate in aerial environments at certain

19 stages of their development. Pupa adhesion has evolved multiple times in insects and is

20 thought to maintain the animal in a place where it is not detectable by predators. Here,

21 we investigate whether pupa adhesion in Drosophila can also protect the animal by

22 preventing potential predators from detaching the pupa. We measured the adhesion of

23 Drosophila species sampled from the same area and found that pupa adhesion varies 24 among species, which can be explained by different glue production strategies. Then, we

25 compared attached and manually detached pupae in both field and laboratory assays to

26 investigate the role of pupa adhesion to prevent predation. First, we found that attached

27 pupae remain on site 30 % more than detached pupae in the field after three days,

28 probably because they are less predated. Second, we observed that attached pupae are

29 less efficiently predated by ants in the laboratory: they are not carried back to the ant

30 nest and more ants are needed to consume them onsite. Our results show that pupa

31 adhesion can prevent the animal from being taken away by predators and is crucial for

32 Drosophila fly survival.33 (198 words)

34

- 05 IZ
- 35 Keywords
- 36 bioadhesive, predation, insect, Drosophila, ant, pupa

37 Background

38 Multiple animals are immobile and permanently attached to a substrate. Most of them are 39 found in aquatic environments (1) and within living hosts for parasites. In aerial 40 environments, animals can also be attached to a substrate during certain stages of 41 development where no feeding from outside nutrients is required. Eggs from multiple 42 invertebrate species are fixed on leaves, wood or on the host tissue in some parasitic species
43 (2-4). This is also the case of pupae, a non-feeding life stage of holometabolous insects
44 between the larval and adult stages (5).

Modes of attachment and the ability to stick to the substrate appear to change rapidly during animal evolution. In stick and leaf insects gluing eggs to a substrate has evolved independently seven times (6). Wasp parasitoids have evolved different pupation strategies consisting in hanging their cocoon to a leaf (*Meteorus pulchricomis*) or attaching their cocoon on a leaf (*Microplitis sp*) (7). In flies, pupae of certain species such as *Drosophila melanogaster* and *Phormia regina* are glued to a substrate whereas others such as *Musca domestica*, *Calliphora erythrocephala* or *Sarcophaga falculata* are not (8).

52 Permanent attachment of eggs and pupae has been associated with several functions. 53 First, attachment can allow the organisms to remain in a favorable environment. Females of many butterfly species choose to lay and attach their eggs directly on the host plant on which 54 55 their larvae will start feeding (3,9). Butterflies laying their eggs during winter have evolved different strategies to avoid their eggs to be blown away if the dead host leaf falls far away 56 from the plant. They lay their eggs on herbal or wooden substrates near the host plant or they 57 58 glue their eggs less strongly to the host leaf so that the eggs would detach from the dead leaf 59 and fall close to the host plant in case of strong wind (2,9,10). Second, hanging chrysalis may facilitate adult emergence (11). Third, attachment may protect immobile animals from 60 predation in various ways. Permanent attachment, when associated with clumping behaviors, 61 62 in which individuals of a particular species group closely to one another, can confer a better 63 protection from predators. For example, in a freshwater caddisfly, pupal grouping behavior 64 with conspecifics confers protection against a planarian flatworm predator (12). Attachment 65 can also prevent predators from accessing the immobile animal. Cocoons of the parasitoid Meteorus pulchricornis are less predated when they are hanging than when they are 66 67 artificially attached to leaves (14). To our knowledge, nothing is known about the function of 68 pupal attachment in Diptera. In this study, we investigated whether the glue attaching Drosophila pupae can protect them from predation. 69

Drosophila larvae produce a glue right before pupariation which allows the pupa to stay attached to a substrate during metamorphosis. After expectoration, the glue spreads between the body and the substrate and dries within a few minutes (15). This glue is made of a few proteins called salivary gland secreted proteins (Sgs) which have evolved rapidly across and within species (16–19).

75 In Drosophila, pupae are found on rotten fruits (20), on or below the soil surface (21– 23) and even on beer glass bottles (24). Aerial pupation sites are usually close to the ground, 76 77 thus accessible to ground dwelling species. The most common predators of fruit fly pupae are 78 ants, rove beetles and spiders (25-27). Birds and small mammals were also found to prey on fruit fly pupae (28,29). To our knowledge, all studies on pupa predation in Drosophila were 79 performed on D. suzukii (21,23,30). In these analyses, ants and spiders were the most 80 81 common predators of pupae and ground beetles, earwigs and crickets were identified as potential natural predators. As ants were previously observed to dig up and carry pupae out of 82 the soil (23), we hypothesized that pupa adhesion to a substrate might have another, yet 83 84 unexplored, effect against predation: preventing potential predators from taking the animal away. Here, we first compared the adhesion strength of pupae from different drosophila 85

species from the same ecological community to explore whether different species have evolved different attachment strategies. Then, we compared the ability of attached and detached pupae of one of these species to remain on site in a natural environment. Finally, we compared the ability of attached and detached pupae of two of these species to resist predation in the laboratory using the most common natural predator found in the field, an ant species.

92

93 Materials and Methods

94 Fly culture

95 Flies were cultured at 25 °C in plastic vials on standard medium [4 liters: 83.5 g yeast, 335.0
96 g cornmeal, 40.0 g agar, 233.5 g saccharose, 67.0 mL Moldex, 6.0 ml propionic acid]. For *D.*97 *suzukii*, this medium was supplemented with 200 g of D-glucose.

98

99 Fly collection

100 Drosophila flies were collected at the Bois de Vincennes in Paris, France. On July 3 2020, 101 five traps made from 0.5-L plastic bottles were settled in the ornithological reserve 102 (48°50'05.5"N; 2°26'11.4"E). Small holes were made in the sides of the bottles that allowed 103 drosophilid flies to enter but prevented entry by larger insects. Traps were baited with pieces 104 of banana and hung to tree branches or within the understorey vegetation. They were 105 distantly placed, three of them in the forest, one at the margin of a meadow and another close 106 to a pond (Table S1). Flies in the bottles were collected two days and five days later. On July 107 16 2020, drosophilid flies were also collected with a sweeping net over a compost at the 108 forest services facilities. Collected flies were transferred with an aspirator into plastic vials 109 containing a piece of humid tissue paper for the time of transportation. In the laboratory, flies 110 were checked under a binocular stereomicroscope and isolated by species in culture vials 111 with standard commeal. When species could not be precisely determined, females were 112 isolated in small culture vials with instant Drosophila medium (Formula 4-24, Carolina 113 Biological Supply Company, Burlington, NC, USA) and species were then identified based on 114 key morphological characters in the male progeny. Results of the fly collections are presented 115 in Table S1. Combining isofemale lines of the same species when necessary, we managed to 116 obtain mass culture for most species, including D. hydei, D. simulans and D. suzukii. These 117 three stocks were raised in the laboratory for 3-4 months at 25°C before being used in the 118 experiments described below.

119

120 Adhesion assays

121 Third instar wandering larvae were transferred on glass slides (Menzel Superfrost microscope glass slide, ThermoScientific[™] #AGAB000080) with soft forceps and kept in a box with wet 122 123 cotton. 15 to 21 h after transfer, pupae naturally attached to the glass slides with their ventral 124 part adhering to the glass slide were used for the adhesion tests. The pull-off force necessary to detach the pupa from the glass slide was measured using a universal test machine 125 126 (LS1S/H/230V Lloyd Instruments) with a 5N force sensor (YLC-0005-A1 Lloyd Instruments), in a set up similar to the one published earlier (31). Double-sided adhesive tape 127 (tesa, extra strong, #05681-00018) was attached to a cylindrical metal part in contact with the 128

129 force sensor. The force was set to 0 before each run. The force sensor was moved down with a constant speed of 1 mm/min until it pressed the pupa with a force of 0.07 N (0.25 N for D. 130 131 hydei) then let still at a force of 0.03 N (0.21 N for D. hydei) for 10s and finally moved up 132 with a constant speed of 0.2 mm/s until the pupa was detached. Force-by-time curves were 133 recorded using NEXYGENPlus software (Lloyd Instruments). We used the maximal force 134 reached during the experiment, corresponding to the force at which the pupa was detached, as 135 the adhesion force of the individual. Pupae whose pupal case broke during the assay (D. suzukii: 1/27, D. simulans: 2/37; D. hydei: 7/50) or pupae which were not detached (D. 136 suzukii: 0/27, D. simulans: 5/37, D. hydei: 12/50) were excluded from the analysis. After 137 138 pupa detachment, images of glue prints remaining on glass substrates were taken with a Keyence VHX-2000 Z20 x20 or x100. Contours of prints areas were digitized manually by 139 the same person using imageJ (1.50d, java 1.8.0 212, 64-bit) (32). Pictures were anonymized 140 141 for manual contour acquisition so that the digitizer did not know the genotype. We measured 142 the surface of the print corresponding to the pupa-substrate interface as defined previously 143 (31). Three prints for D. suzukii and one print for D. simulans were not detectable on the 144 slides and were not used in the analysis.

To assess pupal size, we used pupae that were raised in the same condition as for the adhesion tests but that were not used for the tests. We imaged attached pupae from the dorsal view and measured the area of the pupal case as described to measure the glue area.

148

149 Predation assay in the field

In the laboratory, D. simulans third instar wandering larvae were collected with soft forceps 150 151 and let to pupate in Petri dishes (55-mm diameter) in a closed plastic box containing wet paper for 17 to 24 hr at room temperature. 15 larvae were put per dish. All Petri dishes were 152 153 then brought to the field. For the condition 'attached pupae', a few pupae were removed in 154 order to have exactly 10 pupae glued in the lid of the Petri dish. For the condition 'detached 155 pupae', pupae were all detached from the dish with featherweight entomology forceps and exactly 10 detached pupae were kept in the lid. To distinguish between the two conditions 156 ("non collées" versus "collées" in French), the letters 'NC' or 'C' were written on pieces of 157 158 paper that were taped on the external surface of the lids to facilitate visualisation and counting of the pupae. The lids of the Petri dishes were put in the center of buckets 159 previously installed in the ornithological reserve of Bois de Vincennes. These 40×35 cm 160 161 buckets contain local soil, leaf litter and vegetation; they are pierced at the bottom for water draining and are semi-buried (10 cm deep) (33). In total, 28 buckets were used and contained 162 163 both one dish with 'attached pupae' and one dish with 'detached pupae' (56 Petri dishes in 164 total). We randomly alternated the West / East orientation of the two conditions inside the 165 buckets. Pupae were counted at 0h (September 8 2020, day 1 morning, at 11 am), 6h30 (day 1 166 afternoon), 22h30 (day 2 morning), 31h (day 2 afternoon), 47h (day 3 morning), and 54h (day 3 afternoon) after the start of the experiment without being touched. Animals present in the 167 168 dishes at counting times were photographed and later identified based on the pictures (Table 169 S3). Insects present in the dishes at the end of recording (54h) were collected and kept in 90% 170 ethanol for identification.

172 Ant predation assay in the laboratory

Seven colonies of T. nylanderi were collected on September 17 2020 in Bois de Vincennes 173 174 (48°50'20.0"N 2°26'57.2"E), brought to the lab and allowed to move into artificial nests 175 consisting of two microscope glass slides separated by a 1-mm auto-adhesive plastic foam 176 harboring three chambers, covered with a black plastic film to maintain darkness. Each 177 artificial nest was placed in a foraging area consisting of a plastic box $(11.5 \times 11.5 \times 5.5 \text{ cm})$ as described in (33). Water was always available in a tube plugged with cotton. Colonies 178 179 were fed frozen Drosophila and diluted honey and then they were starved for 10 days until the beginning of the experiment, on October 13 2020. Prior and during the experiment, 180 181 colonies were kept at room temperature on the bench and under indirect sunlight. Each day at around 9h30 am, and for 6 days (between October 13 and October 21 2020), one glass slide 182 presenting two pupae was put into the foraging area of each colony, at about 10 cm from the 183 184 entrances of the artificial nest. On each day, half of the colonies were given one slide with 2 185 D. simulans pupae and the other half one slide with 2 D. suzukii pupae, and we alternated species every day. We used the same two Drosophila lines as for our adhesion assays 186 187 described above.

188 The glass slides were prepared as follows. Six third instar wandering larvae were 189 transferred on glass slides (Menzel Superfrost microscope glass slide, ThermoScientific[™] 190 #AGAB000080) placed in a Petri dish kept in a box containing wet cotton and let to pupate for 14 to 19 hours. On each slide, only one pupa was kept attached, the other ones were 191 192 detached slightly with soft forceps and one detached pupa was left on the slide. Pupae were about 1 cm apart from each other on the slide. Left / right positions of the two pupae were 193 194 randomly assigned per colony and changed every day. The initial locations of the pupae were 195 identified by a mark under the slide.

Ant foraging areas were checked by eye every 5 minutes for 3 hours after the glass slide with the 2 pupae was added. The number of ants in contact with the pupae were counted. If the pupa was brought to the nest, we noted the time when the pupa was present inside the nest for the first time. If the pupa was not brought to the nest, we noted the time when the pupa was fully consumed by the ants (no Drosophila body remnants visible by eye). 201

202 Quantification and Statistical Analysis

We used R v3.6.1 (R Core Team 2015) to conduct our statistical analyses. Adhesion forces 203 204 were not normally distributed and statistical differences in forces between species were tested by Kruskal-Wallis tests followed by multiple pairwise Wilcoxon tests. To test whether 205 206 adhesion forces were correlated to pupa - substrate contact areas for each species, we 207 performed standardized major axis regressions using *sma* function from the rsmatr-package 208 in R (34). Slopes were compared among species. For predation assays in Bois de Vincennes 209 and in the laboratory, means were compared using Wilcoxon tests because data were not normally distributed. We did not correct P-values for multiple testing, as suggested by 210 211 Nakagawa (2004).

213 **Results**

214 Glue adhesion strength varies between species from a same location

We collected drosophilid flies in the forest near Paris in June 2020 and noted the presence of 215 216 9 Drosophila species (Table S1). We established fly stocks of the most common species. 217 Three of them, Drosophila simulans, D. hydei and D. suzukii, were assayed for pupal 218 adhesion. We found that D. simulans detached at a median strength of 234.2 mN (Fig. 1A), 219 similar to what has been found previously for its sister species D. melanogaster (31). We 220 measured a lower adhesion for *D. suzukii* pupae with a median strength of 78.7 mN and higher adhesion for D. hydei with a median strength of 482.6 mN. Adhesion strength was 221 222 significatively different between the three species (Kruskal-Wallis chi-squared = 63.77, df =223 2, p = 1.4e-14, followed by all pairwise comparison Wilcoxon test, p < 0.001).

224 By examining the glue prints left by the pupae on glass slides after detachment, we 225 found that adhesion forces correlated with the surface of the glue print delimiting the contact between the pupa and the glass slide for each species (Fig. 1B, D. suzukii: $R^2 = 0.41$, p =226 0.0009, D. simulans: $R^2 = 0.55$, p = 5e-06, D. hydei, $R^2 = 0.17$, p = 0.02). There was no 227 228 difference in slope among the three species (p = 0.2) and the common slope was about 491, 229 meaning that adhesion force increases by 491 mN for 1 mm² increase of the pupa - substrate 230 contact area for each species. After dividing the adhesion force by the surface of contact, we found a difference in adhesion between D. simulans and D. hydei (Fig. 1C, Kruskal-Wallis 231 chi-squared = 9.61, df = 2, p = 0.008, followed by all pairwise comparison Wilcoxon test, p =232 0.0008) but not between D. simulans and D. suzukii (p = 0.1) and D. suzukii and D. hydei (p = 0.1)233 0.6). 234

235 To test whether the production of the glue was related to the size of the pupa in each 236 species, we imaged attached pupae raised in the same condition as the pupae used for the adhesion tests and measured the area of the pupal case. We found that the three species had 237 different pupal size (Kruskal-Wallis *chi-squared* = 73.876, dt = 2, p < 2e-16, followed by all 238 pairwise comparison Wilcoxon test, p < 0.001) with D. hydei pupae presenting the biggest 239 240 size (4.63 mm²), then D. suzukii (2.78 mm²) and D. simulans (2.12 mm²). The ratio of glue print area over pupal case area was 0.21 for D. simulans, 0.30 for D. hydei and 0.06 for D. 241 242 suzukii, suggesting that D. suzukii pupae produce less glue relative to their size than D. 243 simulans and D. hydei.



246 Fig 1. Pupa adhesion varies between species originating from the same location. (A) 247 Adhesion strength of three Drosophila lines collected in Vincennes. Force indicates the force 248 required to detach a pupa naturally attached to a glass slide. Each dot corresponds to a single pupa and n indicates the total number of pupae tested for each species. Ends of the boxes 249 250 define the first and third quartiles. The black horizontal line represents the median. The vertical line on the top of the box extends to the largest value no further than 1.5 * IQR from 251 252 the upper hinge of the box. The vertical line on the bottom of the box extends to the smallest value at most 1.5 * IQR of the hinge. (IQR: inter-quartile range is the distance between the 253 first and the third quartiles). Data beyond the end of these lines are "outlying" points. * 254 255 indicates significant differences between D. suzukii and D. hydei, D. suzukii and D. simulans 256 and D. simulans and D. hydei (p < 0.05). (B) Relation between pupa adhesion force and pupa-substrate contact area. Each dot corresponds to a single pupa. D. suzukii pupae are 257 258 represented as circles, D. simulans as triangles and D. hydei as stars. (C) Adhesion strength corrected by the pupa-substrate contact area. Boxplots and * as in 1A. n.s. indicates not 259 260 significant (p > 0.05).

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263 Attached pupae are taken away less frequently than detached pupae in a semi-natural264 environment

265 To test whether glue attachment may protect pupae from predation in a semi-natural 266 environment, we chose to use *D. simulans*, as we could obtain a large number of pupae from 267 our fly strain. We compared the disappearance of pupae naturally attached to the plastic lid of 268 Petri dishes with pupae mechanically detached from the lid. We placed two dishes containing 269 respectively 10 attached and 10 detached pupae in 28 open buckets in Bois de Vincennes 270 (two Petri dishes in each bucket) for 54 h and monitored the number of remaining pupae 271 twice a day (Fig. 2A,B). At the end of the experiment, 10 pupae (median = 10) remained in 272 the dish with attached pupae (all still attached) compared to 6-7 pupae (median = 6.5) in the 273 dish with detached pupae. We found that attached pupae stayed significantly more in the Petri 274 dishes than detached ones, with differences becoming significant from day 2 AM until the 275 end of the experiment (Fig. 2F; paired Wilcoxon rank tests with continuity correction: day 1 PM: V = 9, p = 0.2; day 2 AM: V = 89.5, p = 0.02; day 2 PM: V = 92.5, p = 0.01; day 3 AM: 276

V = 91, p = 0.01; day 3 PM: V = 110, p = 0.005). After three days, attached pupae remained 277 on site 30 % more than detached pupae. During the countings, a few species were observed in 278 279 the Petri dishes: Temnothorax nylanderi ants, red spider mites, cockroaches and woodlice 280 (Table S5). Red spider mites were seen to fix themselves to both attached and detached pupae 281 (Fig. 2C). Temnothorax nylanderi was the only species that was clearly seen consuming both 282 attached and detached pupae in the dishes (Fig. 2D). Cockroaches were also observed, but only on the first day (Fig. 2E). Woodlice were also found in the dishes but never in contact 283 284 with the pupae.







287 Fig 2. Attached pupae are taken away less frequently than detached pupae in nature.

(A) Picture of the half-buried buckets installed in Bois de Vincennes. (B) Picture of two 288 289 dishes placed in the center of a bucket and containing attached ("C") or detached ("NC") 290 pupae. (C-E) Predators observed in the dishes during the experiments: a red mite spider (C), 291 two Temnothorax nylanderi ants (D) and a cockroach (E). (F) Boxplot represents the number 292 of pupae present in one dish at the counting time. Pupae were counted twice a day in the 293 morning (AM) and in the afternoon (PM). Each dot represents the count for one dish. White 294 boxes represent dishes with detached pupae and grey boxes dishes with attached pupae. Boxplots are defined as previously (Fig. 1A). * represents a significant difference between 295 the number of remaining pupae between the attached and detached conditions (p < 0.05, 296 Wilcoxon test). Scale bars: (B) 1 cm, (C-E) 1 mm. 297



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Fig 3. Attached pupae require more time and more ants to go away. (A) Number of ants 301 in direct contact with the pupa over the duration of the experiment. Each cell represents one 302 303 experiment with X Y corresponding to the experiment with the colony X during the trial Y. Grey cells represent experiments using D. suzukii and white cells using D. simulans. Red 304 lines represent the number of ants over time on attached pupa and blue lines on detached 305 306 pupa. Vertical lines represent the time when the attached pupa (in red) or the detached pupa (in blue) arrives in the nest (dashed line) or is fully consumed outside the nest (full line) (B) 307 Time during which ants are in contact with attached pupa (grey box) and detached pupa 308 309 (white box) outside the nest (until the pupa is completely consumed outside the nest or enters into the nest). Each dot represents one experiment. (C) Maximum number of ants observed in 310

311 contact with detached pupa (white box) and attached pupa (grey box) during the duration of 312 the experiment. Each dot represents one experiment. Boxplots are defined as previously (Fig. 313 1A). * represents significant differences (p < 0.05, Wilcoxon tests).

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315

316 Attached pupae are predated less efficiently by ants

To understand further how predators may act when they encounter strongly attached, loosely 317 318 attached or detached pupae, we decided to monitor in the laboratory pupae predation by the ant Temnothorax nylanderi, which was the most commonly found predator of D. simulans 319 320 pupae in our field assay. Seven ant colonies were collected in Bois de Vincennes. After 10-321 day starvation, each ant colony was given on each day one glass slide with two pupae, an 322 attached and a detached one (either two pupae of D. simulans, or two pupae of D. suzukii, we 323 alternated colonies each day). We examined the ant-Drosophila interactions every 5 minutes 324 for 3 hours after adding the glass slide with pupae.

325 We found that in all replicates all pupae were consumed by the ants. For both fly 326 species, we observed a difference between detached and attached pupae: detached pupae 327 were mostly taken to the nest and eaten there while most of the attached pupae were eaten on 328 site (Fig. 3A, number of pupae taken to the nest in D. simulans: detached 21/21, attached 3/21, *chi2* = 28.10, *df* = 1, *p* < 10⁻⁶; in *D. suzukii*: detached 19/21, attached 7/21, *chi2* = 12.22, 329 df = 1, p = 0.0005). In 9 cases (6 for D. suzukii and 3 for D. simulans), both the attached and 330 331 the detached pupa were taken to the nest; in all those cases the detached pupa was always 332 taken to the nest first (about 37 min earlier for D. suzukii and 50 min earlier for D. simulans 333 in median, Fig. 3A).

334 Ants spent 15 min and 45 min (median) outside the nest in contact with D. simulans 335 and D. suzukii detached pupae, respectively, while they spent respectively six and two times 336 longer in contact with attached pupae (Fig. 3B, respectively 100 min and 110 min in D. simulans and D. suzukii, paired Wilcoxon rank tests with continuity correction D. simulans: V 337 338 = 4.5, p = 0.0001; D. suzukii: V = 18, p = 0.0007). The maximum number of ants observed in 339 direct contact with the fly pupa over the duration of the experiment was significantly higher 340 for attached pupae than for detached pupae (Fig. 3A,C, median of 9 for D. simulans and 8 for D. suzukii for attached pupae compared to median of 2 for detached pupae in both species, 341 paired Wilcoxon rank tests with continuity correction D. simulans: V = 2.5, p = 0.0002; D. 342 suzukii: V = 10.5, p = 0.0007). The difference was still significant after correcting for the 343 344 amount of time, by comparing the maximum number of ants in contact with the pupa until the 345 first pupa is brought to the nest or fully consumed (paired Wilcoxon rank tests with continuity correction, D. simulans: 3 vs 2, V = 24, p = 0.02; D. suzukii 6 vs 2, V = 7, p =346 347 0.004).

The time for the first ant to touch a pupa was slightly significantly different between attached and detached pupae only in *D. suzukii* (*D. suzukii*: 15 min vs 20 min, V = 31, p = 0.03; *D. simulans*: 15 min vs 20 min, V = 62.5, p = 0.3) but not different between *D. simulans* and *D. suzukii* (Wilcoxon rank tests with continuity correction, attached pupae: W = 195.5, p = 0.5; detached pupae: W = 200, p = 0.6). No differences were found between *D. simulans* and *D. suzukii* regarding the time until the detached pupae is brought to the nest from the start of the experiment (45 min vs. 65 min, W = 160.5, p = 0.296), the time that ants spent in 355 contact with attached pupa (W = 194, p = 0.5) and detached pupa (W = 161, p = 0.1), the 356 maximum number of ants over the duration of the experiment on detached pupae (W = 196.5, 357 p = 0.5) or on attached pupae (W = 190.5, p = 0.5), the maximum number of ants until the 358 first pupa disappeared on detached pupae (W = 231.5, p = 0.8) and the maximum number of 359 ants on attached pupae (W = 265, p = 0.3).

360

361 Discussion

362 In the same environment, pupa adhesion strength varies among species

Using our previously published pull-force measurement assay (31), we provide here the first evidence that pupa adhesion varies between Drosophila species. Our result is in agreement with the rapid evolution of glue genes (17). Our analysis unravels at least two mechanisms leading to changes in the quantity of glue produced and resulting in changes in adhesion among species: (1) a change in body size (probably linked with a change in salivary gland size), as observed between *D. hydei* and *D. simulans*, (2) a change in the amount of glue production independently of body size, as observed between *D. suzukii* and *D. simulans*.

370 The variation that we observed in adhesion force among individuals within a given 371 species is much higher than measurement error (our universal test machine has an accuracy of 372 $\pm 0.5\%$) and could be due to individual variation in size, shape, weight, glue production, or 373 position of the pupa relative to the substrate. We note that our experiment might not reflect 374 natural conditions as we have not tested adhesion on natural substrates and in natural 375 conditions. Drosophila pupation behavior and pupation sites have been thoroughly investigated in the lab (36-38) and more rarely in nature (39). Pupation behavior depends on 376 377 abiotic factors such as temperature (37), darkness (40) or the nature of the substrates (41). In 378 particular, D. simulans prefers to pupate on rough and humid surfaces while D. hydei prefers 379 dry and smooth surfaces. D. simulans was often reported as pupating in fruits in the lab (36,38) and from field sampling (39) but other natural sites have not been investigated. In D. 380 381 suzukii, recent studies in the field have found that pupae are present in the soil rather than in 382 fruits (21,23). Additionally, pupation behavior depends on biotic factors and particularly on 383 the presence of conspecifics and alien species. In D. simulans and D. hydei and in other Drosophila species, pupae are aggregated with conspecifics (39,42). In D. simulans and D. 384 385 *buzzatii*, larvae change their site choice in presence of heterospecific larval cues (20). As 386 many parameters seem to influence pupation behavior, it is hard to know how differences in pupa ecology lead to differences in pupa adhesion between species. 387

388

389 Fixation of the pupa prevents predation

390 Comparing the disappearance of attached and detached D. simulans pupae in the field, 391 we found that being attached allows pupae to stay on site more efficiently. Because pupae 392 lied within the lid of petri dishes, we can infer that pupae were not blown away by small wind 393 but we cannot know whether pupae that disappeared were indeed predated or not. The ant Temnothorax nylanderi was the main predator that we observed in the field consuming 394 395 pupae. This observation is in agreement with previous studies which found that ants prev on 396 fruit fly pupae (21,23,30). During our laboratory assay, T. nylandei ants predated attached and detached pupae with distinct behaviours: they brought most of the detached pupae to the 397

398 nest while they ate the attached ones directly on site. The latter strategy requires ants to spend 399 more time outside the nest and to recruit more foragers. The presence of parasites, predators 400 and competitors in the wild would certainly make this strategy costly. Additionally, *T*. 401 *nylanderi* is a solitary foraging species that recruits nestmates one by one (43). Under natural 402 conditions, it would take a relatively long time to gather many foragers around the pupae. In 403 our field assay, no more than two ants were observed together in a lid (Table S5). We found 404 that ants act similarly on *D. suzukii* and *D. simulans* pupae, suggesting that there is no 405 difference in strategy to predate loosely attached pupae such as *D. suzukii* or more strongly 406 attached ones such as *D. simulans*, and that both species are equally attractive as preys.

407 To our knowledge, this study is the first to show that fly pupa adhesion can protect 408 from predation. Our experiments are simple and can be easily applicable to other species, and 409 not only for pupae but also for eggs, to check if this phenomenon is general in flies and 410 insects.

411

412 Other strategies can protect pupae from predation

413 Pupal adhesion is only one of several strategies for pupae to escape predators. A 414 common strategy is cryptic coloration to hide from visual predators (44,45). The brownish 415 color of Drosophila pupae could contribute to hiding the animal when pupating in the leaf 416 litter or in the soil. In some cases, pupae mimic non-living things such as leaves or sticks like 417 the Common Maplet butterfly chrysalis (45). To avoid non visual predators, pupa has also 418 evolved chemical defences either to chemically hide from predators (46) or to make the pupa 419 toxic (47). Pupae have also evolved different types of physical defence (spin, hard pupal case, 420 urticating hairs...). In Drosophila, the pupa is covered by a relatively thick (about 20 μ m, 421 (48)) and hard cuticle which has been hypothesized to protect the animal from predator 422 attacks. Additionally, pupae have evolved different behaviors such as interacting with 423 conspecifics during larval stage particularly to form aggregation. Aggregated pupae may be 424 more visible to visual predators but predation risk is diluted in group living (12). This last 425 strategy is found in Drosophila species (39,42) but its contribution to protection from 426 predators has not been tested.

427

For the first time, we report that pupa adhesion varies among Drosophila species and that pupa attachment can protect from predation. Our results unravel a previously unknown important trait for Drosophila survival in the wild, the ability of pupae to firmly adhere to a substrate. Further studies of Drosophila glue combining genetic and phenotypic approaches should provide insight on the molecular basis for diverse bioadhesive properties, adapted to various habitats and climates.

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451

452 Author Contributions

FB, MM and VCO designed the experiments, VCO supervised the project, SRP collected,
identified and raised wild-caught flies, FB performed all the other experiments, FB, MM and
VCO analyzed the results, FB wrote the original draft and all authors edited and contributed
to the final version of this paper.

458 Conflict of Interest Statement

459 The authors have no conflict of interest to declare.

460 Data Availability Statement

- 461 Raw data and scripts have been deposited in DRYAD (temporary link for private access:
- 462 https://datadryad.org/stash/dataset/doi:10.5061/dryad.x3ffbg7hg.
- 463 doi:10.5061/dryad.x3ffbg7hg

464 **References**

- 465 1. Wahl M. Living attached: aufwuchs, fouling, epibiosis. Fouling organisms of the Indian
- 466 Ocean: biology and control technology. Oxf IBH Publ Co Put Ltd New Delhi. 1997 Jan
 467 1;31–83.
- Fordyce JA, Nice CC. Variation in butterfly egg adhesion: adaptation to local host plant
 senescence characteristics?: Variation in butterfly egg adhesion. Ecol Lett. 2002 Dec
 13;6(1):23-7.
- 471 3. Hinton HE. Biology of insect eggs. 1st ed. Oxford [Eng.]; New York: Pergamon Press;
 472 1981. 3 p.
- 473 4. Voigt D, Gorb S. Egg attachment of the asparagus beetle Crioceris asparagi to the
 474 crystalline waxy surface of Asparagus officinalis. Proc R Soc B Biol Sci. 2010 Mar
 475 22;277(1683):895–903.
- 476 5. Heming BS. Insect Development and Evolution [Internet]. Cornell University Press;
 477 2003 [cited 2020 Dec 17]. 246–247 p. Available from:
- 478 https://www.jstor.org/stable/10.7591/j.ctv75d5sv
- 479 6. Robertson JA, Bradler S, Whiting MF. Evolution of Oviposition Techniques in Stick
- and Leaf Insects (Phasmatodea). Front Ecol Evol. 2018 Dec 19;6:216.

- 481 7. Harvey JA, Gols R, Tanaka T. Differing Success of Defense Strategies in Two
 482 Parasitoid Wasps in Protecting Their Pupae Against a Secondary Hyperparasitoid. Ann
 483 Entomol Soc Am. 2011 Sep 1;104(5):1005–11.
- 484 8. Fraenkel G, Brookes VJ. The process by which the puparia of many species of flies
 485 become fixed to a substrate. Biol Bull. 1953 Dec;105(3):442–9.
- 486 9. Wiklund C. Egg-laying patterns in butterflies in relation to their phenology and the
 487 visual apparency and abundance of their host plants. Oecologia. 1984 Jul;63(1):23–9.
- 488 10. Hayes JL. A Study of the Relationships of Diapause Phenomena and Other Life History
 489 Characters in Temperate Butterflies. Am Nat. 1982 Aug;120(2):160–70.
- 490 11. Chapman RF. The Insects: structure and function. Cambridge (Mass.): Harvard University Press; 1982.
- 492 12. Wrona FJ, Dixon RWJ. Group Size and Predation Risk: A Field Analysis of Encounter
 493 and Dilution Effects. Am Nat. 1991;137(2):186–201.
- Hieber CS. Spider cocoons and their suspension systems as barriers to generalist and
 specialist predators. Oecologia. 1992 Oct;91(4):530–5.
- 496 14. Shirai S, Maeto K. Suspending cocoons to evade ant predation in *Meteorus*497 *pulchricornis*, a braconid parasitoid of exposed-living lepidopteran larvae. Entomol Sci.
 498 2009 Mar;12(1):107–9.
- 499 15. Beňová-Liszeková D, Beňo M, Farkaš R. Fine infrastructure of released and solidified
 500 *Drosophila* larval salivary secretory glue using SEM. Bioinspir Biomim. 2019 Jul
 501 11;14(5):055002.
- 502 16. Beckendorf SK, Kafatos FC. Differentiation in the salivary glands of Drosophila
 503 melanogaster: Characterization of the glue proteins and their developmental appearance.
 504 Cell. 1976 Nov 1;9(3):365–73.
- 505 17. Da Lage J-L, Thomas GWC, Bonneau M, Courtier-Orgogozo V. Evolution of salivary
 glue genes in Drosophila species. BMC Evol Biol. 2019 Dec;19(1):36.
- 507 18. Korge G. Chromosome puff activity and protein synthesis in larval salivary glands of 508 Drosophila melanogaster. Proc Natl Acad Sci. 1975 Nov 1;72(11):4550–4.
- 509 19. Korge G. Larval saliva in Drosophila melanogaster: Production, composition, and
 relationship to chromosome puffs. Dev Biol. 1977 Jul;58(2):339–55.
- 511 20. Beltramí M, Medina-Muñoz M, Pino F, Ferveur J-F, Godoy-Herrera R. Chemical Cues
 512 Influence Pupation Behavior of Drosophila simulans and Drosophila buzzatii in Nature
 513 and in the Laboratory. PloS One. 2012 Jun 21;7:e39393.
- 514 21. Ballman ES, Collins JA, Drummond FA. Pupation Behavior and Predation on
 515 Drosophila suzukii (Diptera: Drosophilidae) Pupae in Maine Wild Blueberry Fields. J
 516 Econ Entomol. 2017 Dec 5;110(6):2308–17.
- 517 22. Grossfield. Non-sexual behavior of Drosophila. In: The genetics and biology of
- 518 Drosophila. Ashburner M, Wright TRF. London, New York, San Francisco: Academic
 519 Press; 1978. p. 3–126.
- 520 23. Woltz JM, Lee JC. Pupation behavior and larval and pupal biocontrol of Drosophila
 521 suzukii in the field. Biol Control. 2017 Jul;110:62–9.
- 522 24. Vouidibio J, Capy P, Defaye D, Pla E, Sandrin J, Csink A, et al. Short-range genetic
 523 structure of Drosophila melanogaster populations in an Afrotropical urban area and its
- 524 significance. Proc Natl Acad Sci. 1989 Nov 1;86(21):8442–6.
- 525 25. Hennessey MK. Predation on wandering larvae and pupae of caribbean fruit fly (diptera:
 526 tephritidae) in guava and carambola grove soils. J Agric Urban Entomol.
 527 1997;14(2):129–38.
- 528 26. Thomas DB. Predation on the soil inhabiting stages of the Mexican fruit fly. Southwest
 529 Entomol. 1995;20(1):61–71.
- 530 27. Urbaneja A, Marí FG, Tortosa D, Navarro C, Vanaclocha P, Bargues L, et al. Influence
- of Ground Predators on the Survival of the Mediterranean Fruit Fly Pupae, Ceratitis

- 532 capitata, in Spanish Citrus Orchards. Biocontrol. 2006 Oct 3;51(5):611–26.
- 533 28. Bigler F, Neuenschwander P, Delucchi V, Michelakis S. Natural enemies of preimaginal
 534 stages of Dacus oleae Gmel.(Dipt., Tephritidae) in Western Crete. II. Impact on olive fly
 535 populations. Boll Lab Entomol Agrar Filippo Silvestri Italy. 1986;43:79–96.
- 536 29. Thomas DB. Survivorship of the Pupal Stages of the Mexican Fruit Fly Anastrepha
- 537 ludens (Loew) (Diptera: Tephritidae) in an Agricultural and a Nonagricultural Situation.
 538 J Entomol Sci. 1993 Oct 1;28(4):350–62.
- 539 30. Gabarra R, Riudavets J, Rodríguez GA, Pujade-Villar J, Arnó J. Prospects for the
 biological control of Drosophila suzukii. BioControl. 2015 Jun;60(3):331–9.
- 541 31. Borne F, Kovalev A, Gorb S, Courtier-Orgogozo V. The glue produced by *Drosophila melanogaster* for pupa adhesion is universal. J Exp Biol. 2020 Apr
- 543 15;223(8):jeb220608.
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image
 analysis. Nat Methods. 2012 Jul;9(7):671–5.
- 546 33. Honorio R, Doums C, Molet M. Manipulation of worker size diversity does not affect
 547 colony fitness under natural conditions in the ant Temnothorax nylanderi. Behav Ecol
 548 Sociobiol. 2020 Aug;74(8):104.
- 549 34. Warton DI, Duursma RA, Falster DS, Taskinen S. smatr 3– an R package for estimation and inference about allometric lines. Methods Ecol Evol. 2012;3(2):257–9.
- 551 35. Nakagawa S. A farewell to Bonferroni: the problems of low statistical power and publication bias. Behav Ecol. 2004 Nov 1;15(6):1044–5.
- 553 36. Erezyilmaz DF, Stern DL. Pupariation Site Preference Within and Between Drosophila
 554 Sibling Species. Evolution. 2013;67(9):2714–27.
- 555 37. Schnebel EM, Grossfield J. Temperature effects on pupation-height response in four
 556 Drosophila species group triads. J Insect Physiol. 1992 Oct 1;38(10):727–32.
- 557 38. Vandal NB, Siddalingamurthy GS, Shivanna N. Larval pupation site preference on fruit
 in different species of *Drosophila*. Entomol Res. 2008 Sep;38(3):188–94.
- 559 39. Beltramí M, Muñoz M, Arce D, Godoy-Herrera R. Drosophila pupation behavior in the
 wild. Evol Ecol. 2010 Mar 1;24.
- 40. Rizki MTM, Davis Charles G. Light as an Ecological Determinant of Interspecific
 Competition between Drosophila willistoni and Drosophila melanogaster. Am Nat. 1953
 Nov 1;87(837):389–92.
- 564 41. Godoy-Herrera R, Luis S-C. The behavior of sympatric Chilean populations of
 565 Drosophila larvae during pupation. Genet Mol Biol. 1998 Mar 1;21.
- 566 42. Ringo J, Dowse H. Pupation Site Selection in Four Drosophilid Species: Aggregation
 and Contact. J Insect Behav. 2012 Nov 1;25.
- 568 43. Glaser S, Grueter C. Ants (Temnothorax nylanderi) adjust tandem running when food
 569 source distance exposes them to greater risks. Behav Ecol Sociobiol. 2018 Feb
 570 19:72:40.
- 571 44. Gaitonde N, Joshi J, Kunte K. Evolution of ontogenic change in color defenses of 572 swallowtail butterflies. Ecol Evol. 2018 Oct;8(19):9751–63.
- 573 45. Lindstedt C, Murphy L, Mappes J. Antipredator strategies of pupae: how to avoid
 574 predation in an immobile life stage? Philos Trans R Soc B Biol Sci. 2019 Oct
 575 14;374(1783):20190069.
- 576 46. Mizuno T, Hagiwara Y, Akino T. Chemical tactic of facultative myrmecophilous
 577 lycaenid pupa to suppress ant aggression. Chemoecology. 2018 Dec;28(6):173–82.
- 578 47. Deyrup ST, Eckman LE, Lucadamo EE, McCarthy PH, Knapp JC, Smedley SR.
- Antipredator activity and endogenous biosynthesis of defensive secretion in larval and
 pupal Delphastus catalinae (Horn) (Coleoptera: Coccinellidae). Chemoecology. 2014
 Aug;24(4):145–57.
- 582 48. Mitchell HK, Weber-Tracy UM, Schaar G. Aspects of cuticle formation in Drosophila

583 melanogaster. J Exp Zool. 1971;176(4):429–43.

585 Supplementary materials

Table S1. List of Drosophila flies collected in Bois de Vincennes. The number corresponds
to the total number of individuals collected at each site. Traps were emptied twice, two days
and five days after traps were set up in the field. GPS coordinates are shown for each trap and
for the compost.

590

584

591 Table S2. Adhesion force and pupa-substrate interface measurements. Results of the 592 adhesion assay performed on pupae from D. suzukii, D. simulans and D. hydei strain 593 originating from Vincennes. Sample ID column corresponds to a unique identification for 594 each pupa, Temperature assay, Humidity, Pressure mba indicate respectively the room temperature (°C), the room humidity (%) and the atmospheric pressure (mba) at the moment 595 of the assay. date measurement and time measurement correspond to the day and hours of 596 the assay, date substrate and time substrate correspond to the day and hours at which larvae 597 598 were put on the substrate. force detachment mN corresponds to the maximum force reached 599 during the experiment in mN. Comment on this sample reports particular observations: the pupa did not detach from the substrate ("not detached"), the pupal case broke during the 600 601 assay ("cuticle broke") or nothing special happened during the assay ("ok"). Area px corresponds to the pupa-substrate contact surface measured in pixel and scale px, scale mm 602 603 correspond respectively to the scale present on the picture in pixel and mm.

604

Table S3. Pupal size measurement. Measurements of the size of pupae of *D. simulans*, *D. suzukii*, *D. hydei*. ID column corresponds to a unique identification for each pupa. area corresponds to the area obtained by measuring the contour of the pupa in pixel. scale_px and scale_mm are defined as in Table S2.

609

Table S4. Pupal count in the field experiment. Results of the experiment performed in the field. bucket_ID column corresponds to a unique identification for each bucket. Orientation_C and Orientation_NC give, respectively, the orientation of attached and detached pupa within the bucket. Count_C and Count_NC indicate, respectively, the number of attached and detached pupa in a dish. Time indicates the time at which pupae were counted: t0 after 0h (8 September 2020, day 1 morning, at 11 am) t1 after 6h30 (day 1 PM), t2 after 22h30 (day 2 AM), t3 after 31h (day 2 PM), t4 after 47h (day 3 AM), and t5 after 54h (day 3 PM).

618

619 Table S5. Animals observed in the dishes during the experiment with half-buried

620 buckets in Bois de Vincennes. The number indicates the number of dishes where the

621 respective animals were observed. In total, 56 dishes were examined at each time point.

622

623 **Table S6. Ant count during predation assay in the laboratory.** The number indicates the 624 number of ants observed in direct contact with the pupa at each time point. The column 625 "X_Y" corresponds to the count over one experiment with the ant colony X at the trial Y.

626

627 **Table S7. Results table of the predation assay in the laboratory.** Each row corresponds to 628 the description of the experiment for one pupa. condition column indicates the initial state of 629 the pupa ("attached" or "detached"), strategies indicates whether the pupa was brought to the 630 nest over the duration of the experiment ("to nest") or was never brought to the nest and 631 consumed outside the nest ("on site"). Time corresponds to the time at which the pupa was 632 brought to the nest or fully consumed outside the nest in min. max ant corresponds to the 633 maximum number of ants observed in contact with the pupa over the duration of the 634 experiment. orientation indicates whether the pupa was initially on the left or on the right side 635 of the glass slide at the beginning of the experiment. first ant corresponds to the time in min 636 at which the first interaction between the pupa and an ant is observed. max ant at first done corresponds to the maximum number of ants observed in direct interaction with the pupa 637 between the beginning and the time when the first pupa is brought to the nest or fully 638 639 consumed outside the nest.

640

641 **R script. Drosophila_glue_predation.R** R script used to prepare the figures and run the 642 statistical tests.

643

644 pupa_prints Folder containing pictures of the prints left by the pupae on glass slides after645 detachment during our adhesion assay force measurements.

646 **pupal_size** Folder containing the pictures of pupae used to measure the size of the pupae of 647 the different species.

648 **Pictures_Vincennes** Folder containing pictures taken during and after the experiments in the 649 field.

650 Pictures_predation_lab Folder containing pictures and video of the predation assays in the651 laboratory.