1 **"The glue produced by *Drosophila melanogaster* for pupa adhesion is universal"**

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12 **ABSTRACT**

13 Insects produce a variety of adhesives for diverse functions such as locomotion, mating, egg or

14 pupal anchorage to substrates. Although they are important for the biology of organisms and

15 potentially represent a great resource for developing new materials, insect adhesives have been little

16 studied so far. Here, we examined the adhesive properties of the larval glue of *D. melanogaster*.

17 This glue is made of glycosylated proteins and allows the animal to adhere to a substrate during

18 metamorphosis. We designed an adhesion test to measure the pull-off force required to detach a

19 pupa from a substrate and to evaluate the contact area covered by the glue. We found that the pupa

20 adheres with similar forces to a variety of substrates (with distinct roughness, hydrophilic and

21 charge properties). We obtained an average pull-off force of 217 mN, corresponding to 15 500 times

22 the weight of a pupa and adhesion strength of 137-244 kPa. Surprisingly, the pull-off forces did not

23 depend on the contact area. Our study paves the way for a genetic dissection of the components of

24 *Drosophila melanogaster* glue that confer its particular adhesive properties.

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26 (184 words, max 250 words)

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28 **Abbreviations**. PLL: Poly-L-Lysine, PLL-PEG: Poly-L-Lysine-Polyethyl glycol, SEM: Scanning

29 Electron Microscopy

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31 **Keywords**. Bioadhesion, Drosophila, insect, attachment, adhesion assay

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INTRODUCTION

Natural adhesives have a very important significance for the biology of organisms and are a great material for innovation of biologically-inspired technical adhesives. The most studied bioadhesives are from marine organisms, mussels and barnacles (Power et al., 2010), and they are now used in a variety of biomimetic applications such as surgical sealants to repair tissues or synthetic polymer coatings (Lee et al., 2011). Bioadhesives in insects are less studied although they are extremely diverse (Gorb, 2001; Graham, 2008; Li et al., 2008; Betz, 2010). Some insects produce glue to secure their eggs or cocoon (Betz, 2010). A few studies have been carried out on egg glue composition and particularly on the glue adhesive strength in different insect species. For example, in *Opodiphthera* moths, females secrete a viscous fluid from their accessory reproductive gland that sticks their eggs to each other and to the substratum. This hydrogel-type glue is highly elastic and mainly made of proteins. The shear strength reaches 1-2 MPa on wood, which could potentially be enough for some industrial applications (Li et al., 2008). Furthermore, these eggs and eggs from other species such as *Crioceris asparagi* have the ability to attach to plants covered with crystalline epicuticular waxes known to be non-adhesive (Voigt and Gorb, 2010). Similar properties have been revealed in the egg glue of codling moth (Al Bitar et al., 2012; 2014). Thus, investigating the glue of various insect species appears to be a great strategy to find novel universal and substrate-specific adhesives. Surprisingly, although *Drosophila melanogaster* is a model organism that is extensively used in laboratories, little is known about pupal adhesion in this species.

Drosophila melanogaster larvae secrete a glue from their salivary glands right before pupariation (Fraenkel and Brookes, 1953). After expectoration, following larval peristaltic movements, the secreted liquid spreads between the body and the substrate and dries within a few minutes (Beňová-Liszeková et al., 2019). This glue allows the animal to stay firmly attached to an external surface for several days, until the adult fly emerges from the pupal case, while the pupal case remains attached to the substrate. The pupa of other Brachycera fly species is also often attached to a substrate during metamorphosis (Fraenkel and Brookes, 1953).

In the wild, *Drosophila* pupae have been reported to adhere to a great variety of substrates from fruits to beer bottles (Vouidibio et al., 1989) and in diverse environments. Particularly, pupae have been observed on dry parts of various fruits, some species have also been found fixed to wood, wet rotten parts of fruits, deep in the soil and to one another (Grossfield, 1978; Sokolowski, 1985; Vandal et al., 2008; Beltramí et al., 2010; Castillo et al., 2014). Pupal attachment might be important for not being taken away by predators, to resist environmental conditions (wind or rain) or to help adult flies emerge from the pupal case after metamorphosis (Da Lage et al., 2019).

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66 In *D. melanogaster* (Korge, 1975; 1977) and other *Drosophila* species (*D. virilis* (Kress,
67 1982), *D. natusa* (Ramesh and Kalisch, 1988), *D. gibberosa* (Shirk et al., 1988)), the glue is
68 composed of a small number of proteins called salivary gland secreted (Sgs) proteins. These
69 proteins present repeated motifs and glycosylations, which are commonly found in adhesive
70 proteins (Graham, 2008; Betz, 2010). Some of the *Drosophila* glue proteins are rich in cysteine, like
71 many marine adhesive proteins and this property could be important to maintain a secondary
72 structure by forming disulfide bonds (Graham, 2008). Other *Drosophila* glue proteins are rich in
73 serine and threonine and highly O-glycosylated, suggesting that they may interact with water to
74 hydrate or dehydrate the glue (Farkaš, 2016). The *Sgs* genes have evolved rapidly between species
75 and within species (Korge, 1975; 1977; Beckendorf and Kafatos, 1976; Da Lage et al., 2019).

76 Here, we investigated the adhesive properties of the glue of *D. melanogaster* on different
77 substrates. First, we analyzed the contact region between the pupa and the surface to which it is
78 attached. Then, we designed a pull-off force measurement set up. We assessed the force required to
79 detach the pupa from different substrates, with different hydrophilic and charge properties or with
80 different roughnesses, and analyzed the way rupture occurs.

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83 **METHODS**

84 **Flies**

85 *D. melanogaster* Canton S flies (gift from Roger Karess) were cultured in plastic vials on standard
86 medium (1L: 62.5 g yeast, 62.5 g cornmeal, 10.0 g agar-agar, 20.0 g glucose monohydrate, 30.0 g
87 molasse, 30.0 g sugar beet syrup, 10.0 ml propionic acid (10%), nipagin (10%)) at room
88 temperature.

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90 **Larva preparation**

91 Third instar wandering larvae were washed in PBS to remove traces of food and microorganisms
92 from their surface, put in empty Petri dishes with a paintbrush and kept in high humidity
93 atmosphere at room temperature in a closed plastic box (15x10x5 cm) containing wet cotton. When
94 larvae stopped moving, they were transferred on the substrate of interest with soft forceps, kept at
95 high humidity as mentioned above and let to pupariate. Five to 24 h after transfer to the substrate of
96 interest, the substrate with pupae attached to it was put for 1 h at room humidity to allow the glue to
97 dry completely and then the pupae were used for adhesion assays. Pupae not used for assays were

98   weighed individually using Mettler Toledo AG203 (DeltaRange®, Gießen, Germany). Temperature,

99   humidity and atmospheric pressure were monitored daily.

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Preparation of glass and Teflon substrates

102   Two types of non-coated microscopic glass slides were used to measure adhesion on glass: Menzel

103   Superfrost microscope glass slide from ThermoScientific™ (#AGAB000080) and microscopic

104   glass slide from Roth (#0656.1). Atmospheric plasma-treated glass slides were prepared using a

105   PlasmaBeam (Diener electronic GmbH, Ebhausen, Germany) on Roth glass slides for 1 min. To

106   prepare Poly-L-Lysine-Polyethyl glycol-coated (PLL-PEG-coated) glass slides, Roth glass slides

107   were cleaned with a plasma cleaner then coated with 0.1 mg·ml^{-1} non-biofunctionalized PEG side-

108   chains (methoxy-terminated) (SuSos). Poly-L-Lysine-coated (PLL-coated) glass slides from

109   Thermo Scientific™ (J2800AMNZ) and pieces of Teflon (Polytetrafluorethylen, Kelux, Geldern,

110   Germany) were also tested.

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**Preparation of Spurr epoxy resin substrates**

113   First, a silicone cast was made by pouring polyvinylsiloxane (light body Affinis, Coltène/Whaledent

114   GmbH + Co. KG, Langenau, Germany) over a cleaned Roth glass slide (to create the smooth resin,

115   Ra=80 nm (Salerno et al., 2014)) or over glass slides covered by polished papers with different

116   grain sizes: 1 μm and 9 μm (Ra=0.54 and 2.47 μm measured over 1400x1050 μm area using

117   NewView 6k (Zygo, Middlefield, CT, USA) white light interferometer; both with FiberMet

118   Abrasive disks, Buehler) (Salerno et al., 2014), P1000 and P80 (Ra=3.94 and 40.40 μm measured

119   over the same area using VR 3100 (Keyence, Neu-Isenburg, Germany) 3D profilometer; polishing

120   papers, Bahaus). After a couple of minutes, the slide and the polished paper were removed and the

121   edges of the polyvinylsiloxane cast were made higher. Then, Spurr epoxy resin (Spurr, 1969) was

122   poured into the polyvinylsiloxane cast and polymerized at 65 °C overnight in Memmert U 15 oven

123   (Schwabach, Germany). Resin was then allowed to cool down for a couple of hours before

124   unmolding. Each resin was used for several assays.

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Contact angle measurements

127   Water contact angles on different substrate surfaces were measured using a contact angle

128   measurement device OCA20 (DataPhysics Instruments, Filderstadt, Germany). A 2-μl water droplet

129   was deposited on a substrate, then an image of the droplet was taken after five seconds, and contact

130   angles were determined from the fit of the droplets shape with a sphere using SCA 202 software

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131 (DataPhysics Instruments, Filderstadt, Germany). All the contact angle measurements were done

132 before the pull-off force assays except for PLL-PEG-coated glass.

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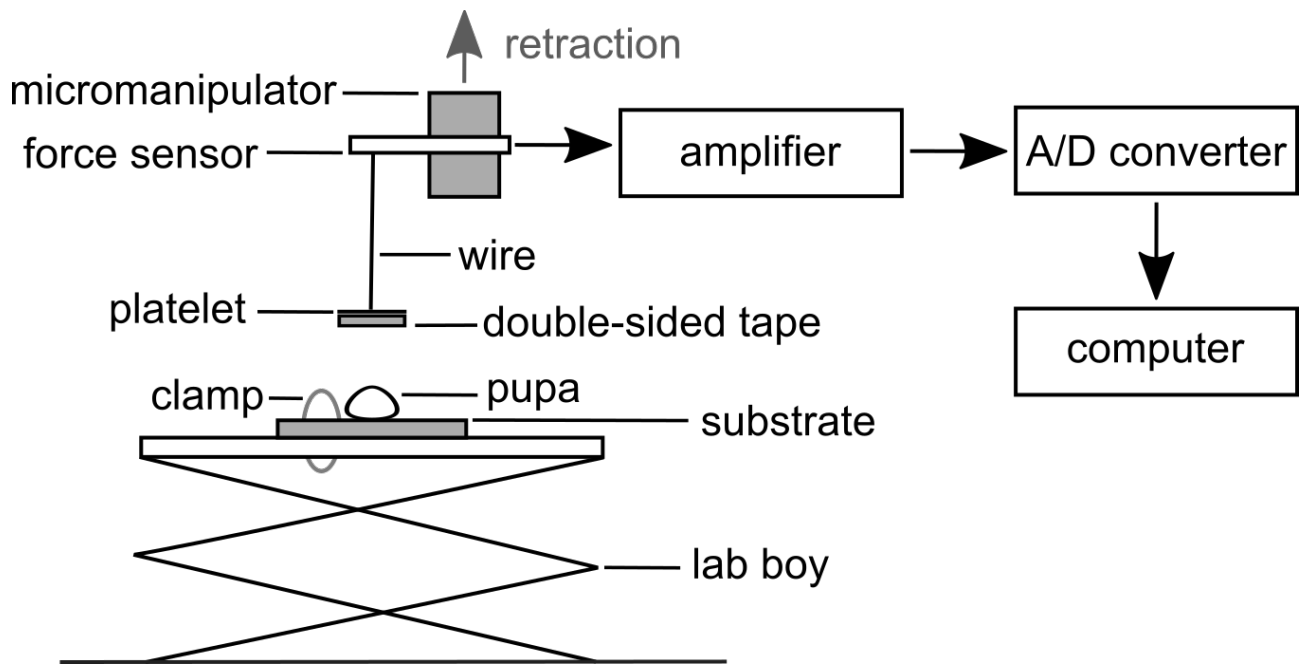
Figure 1. Experimental set-up for measuring adhesion force of individual *Drosophila melanogaster* pupae. The substrate on which the pupa was naturally sticking was fixed on a lab boy using a clamp and brought into contact with a piece of double-sided sticky tape attached to a metal platelet. To detach the pupa from the substrate, a force sensor linked to the platelet by a metal wire was moved up using a motorized micromanipulator. The time-force sensor signal was amplified and converted before being processed in a computer.

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Adhesion force measurement

143 To measure pupal adhesion, a force transducer (100 g capacity, FORT100, World Precision

144 Instruments, Sarasota, FL, USA) mounted on a motorized 3D micromanipulator (DC3001R, World

145 Precision Instruments, Sarasota, FL, USA) was used (Fig. 1). The substrate was horizontally

146 clamped to a lab boy. A piece of aluminum platelet of 1.0 x 2.0 x 0.2 mm attached to the force

147 transducer by a metal wire (0.1 mm) was covered by a piece of double-sided adhesive tape (tesa,

148 extra strong, #05681-00018). A new piece of tape was glued for each measurement. Only pupae

149 attached on their ventral side and which did not contact other pupae were used for measurements.

150 The tape on aluminum platelet was brought into contact with the dorsal part of the pupa by

151 adjusting manually the height of the lab boy. The force transducer was then moved upwards with a

152 velocity of 200 μm/s until detachment of the pupa. The signal from the force transducer was

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153   amplified using Transbridge TBM4M and digitized using Lab-Trax-4 data acquisition hardware
154   (WPI, Sarasota, FL, USA). Three phases could be distinguished on force curves recorded using
155   LabScribe v3 (iWorks, Dover, NH, USA) (Fig. 2A). During the initial phase, the pupa was not
156   attached to the tape and force with no pupa weight was measured. The pulling phase started when
157   the pupa came into contact with the tape. During this phase, the pupa was stretched until it detaches
158   from the substrate (maximal force). Finally, during the resting phase, the force went down to a basal
159   value including pupal weight. The set up was calibrated before the first assay with a standard
160   weight. Pull-off force corresponding to the force at pupa detachment was calculated as the
161   difference between the maximal pulling force and the mean force at the initial phase (offset). The
162   force corresponding to the adjustment of the lab boy until pupa glued to the tape between the initial
163   phase and the pulling phase was not recorded.

164   328 pupae were measured using our pull-off force measurement set up. In 59 (17%) cases,
165   the pupal case cracked on the dorsal side between the head and the thorax during the pulling
166   process. For 36 (11%) of them, the pupal case covering the ventral part of the head detached from
167   the rest of the pupal case and remained glued to the substrate at the end of the trial, while the
168   complete pupa body was detached together with the posterior part of the pupal case. For the 23
169   (7%) other cases, the whole animal together with the broken pupal case were detached from the
170   substrate. These 59 (18%) cases were excluded from further analysis. Furthermore, two or more
171   trials were sometimes necessary to detach a pupa, when the connection between the double-sided
172   tape and the pupa failed. If the maximal force on the last trial was higher than the previous ones
173   (Fig. 2E), the last trial was used for analysis unless the pupal case was damaged. If the maximal
174   force was higher in one of the first trials, the measurement was excluded, suggesting that the pupa
175   has been partly detached during the first trials (n=23 (7%), Fig. 2F). 11 other cases (3%) were
176   excluded from the analysis: imperfections in the substrates (n=3), pupa not attached ventrally (n=6),
177   pupa not attached, with no glue (n=1) and tape glued to the substrate (n=1). In total, 93 cases (28%)
178   were excluded.

179   We then organized the remaining 235 cases (100%) into four groups. The first group gathers
180   the trials for which the whole pupa detached at once and the tape stayed well fixed to the pupa
181   (n=56 (24%), Fig. 2A). The second group gathers the trials for which the piece of tape started to
182   peel from the pupal case surface during the pulling process and pupa detached at once. For this
183   group, the force-time curve had a negative derivative at some point during the pulling phase (n=
184   106 (45%), Fig. 2B). The third group gathers the trials for which the whole pupa did not detach at
185   once but the head part stayed attached longer than the rest of the body. In such cases, two peaks

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186 were observed on the force-time curve (n=29 (12%), Fig. 2C): the first one corresponds to the main
187 body part detachment and the second one to the head part detachment. The first peak always
188 displayed the maximal force. Finally, the fourth group gathers the trials for which tape started to
189 peel before pupa detachment and for which the whole pupae did not detach at once. For this group,
190 the force-time curve had a negative derivative at some point during the pulling phase and a second
191 peak was observed after the peak corresponding to the maximal force (n=44 (19%), Fig. 2D).

192 We tested if these four groups having different detachment behavior have an effect on the
193 resulting adhesion force. We performed a two-way ANOVA considering force as the dependent
194 variable. We found that substrates but not groups had a significant impact on pupa adhesion force
195 and that there was no significant interaction between groups and substrates (substrate: F=12.86,
196 P<2e-16, group and interaction group-substrate: P<0.05).

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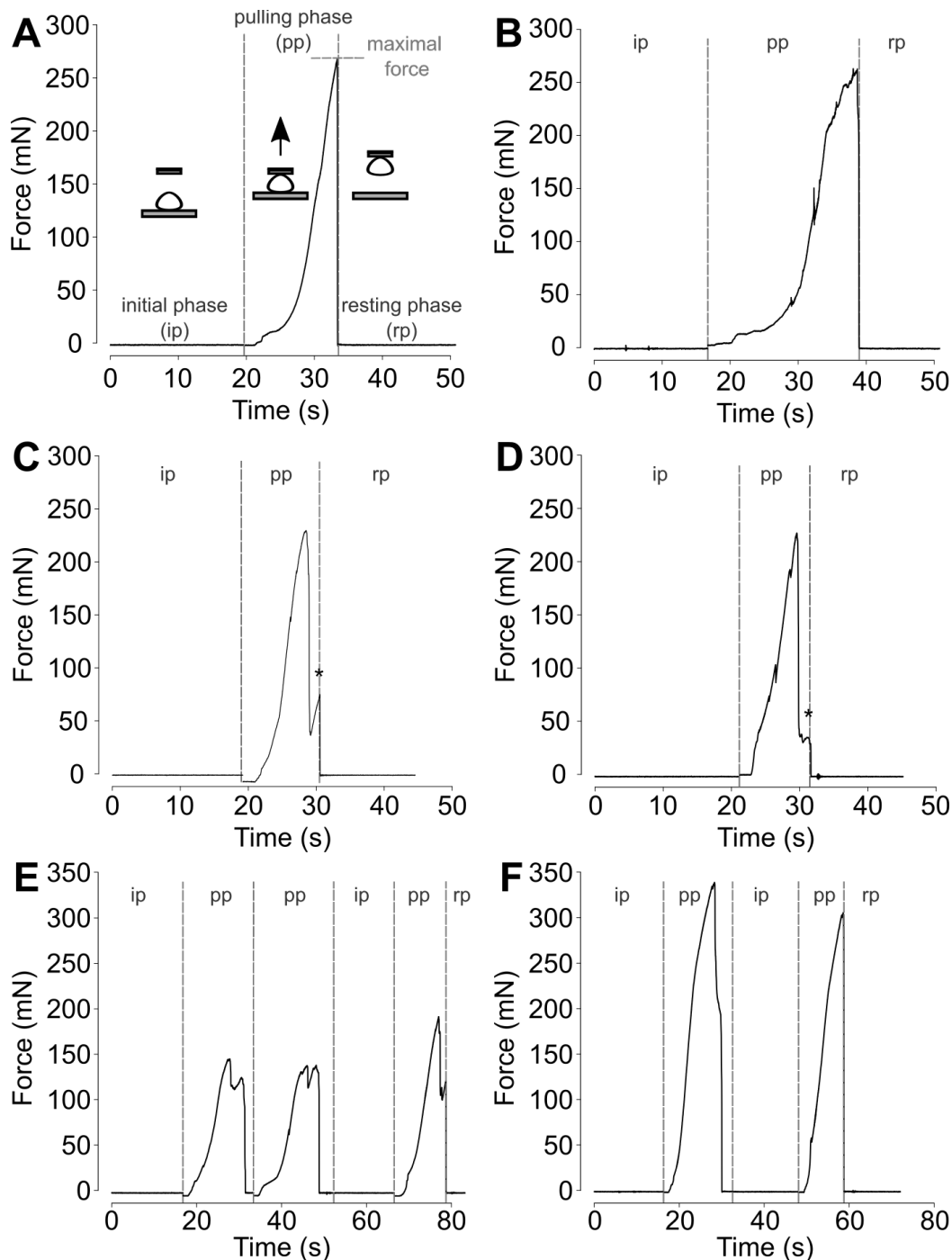215
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Figure 2. Representative force-time curves obtained in pull-off force measurements. (A) Force-time curve consists of three phases: the initial phase (ip) corresponding to the force before pupa is attached to the tape, the pulling phase (pp) from the time the pupa is attached to the tape to the maximal force, and the resting phase (rp) after detachment. The maximal force corresponds to the force at pupa detachment. Force-time curve corresponding to the attachment of the pupa to the tape (between initial phase and pulling phase) is not shown. Every phase is separated with a vertical dot line. Representative curves of the following cases are shown: **(A)** the tape does not peel from the

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227   pupa during the pulling phase and the pupa is detached at once, **(B)** the tape peels from the pupa

228   during the pulling phase and at the end the pupa is detached at once, **(C)** first the posterior part and

229   then the head of the pupa are detaching. Head detachment produces a small peak on the curve

230   (marked with a star), **(D)** the tape peels from the pupa, then the posterior part of the pupa first

231   detaches and then the head of the pupa detaches (marked with a star), **(E)** the tape detaches from the

232   pupa twice and then the pupa detaches from the substrate at the third trial, maximal force is

233   observed during pupa detachment **(F)** tape detaches from pupa once and pupa detaches from the

234   substrate at the second trial, maximal force is observed during tape detachment from the pupa.

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236   **Microscopy**

237   After pupal detachment, images of glue prints remaining on glass substrates (100 cases) were taken

238   with a Keyence VR 3100 microscope at x40 or x80. Prints areas were measured manually using

239   imageJ (1.50d, java 1.8.0_212, 64-bit). For 1 case, prints were altered before the picture and areas

240   could not be measured. For 11 cases, the red area could not be visualized and was not measured.

241   One pupa detached from Menzel non-coated glass slide was mounted on aluminum stubs by

242   using double-sided carbon conductive tape (Plano, Wetzlar, Germany). The sample was frozen in

243   liquid nitrogen, sputter-coated with gold–palladium (8 nm thickness) at -140°C, and examined in a

244   cryo-SEM (Hitachi S-4800; Hitachi Ltd., Tokyo, Japan) at -120°C and an accelerating voltage of 3

245   kV.

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247   **Statistical analysis**

248   Statistical differences in pull-off forces between substrates, groups and interactions were tested by

249   two-way ANOVA using R *aov* function (R version 3.4.4, Team, 2013). Statistical differences in

250   pull-force between substrates were tested by one-way ANOVA with the same R *aov* function

251   followed by multiple pairwise comparison tests using Tukey test with R *TukeyHSD* function.

252   Pearson correlation between pull-off force and contact areas were tested using R *lm* function. Effect

253   of humidity, temperature, pressure and age were also tested using R *lm* function.
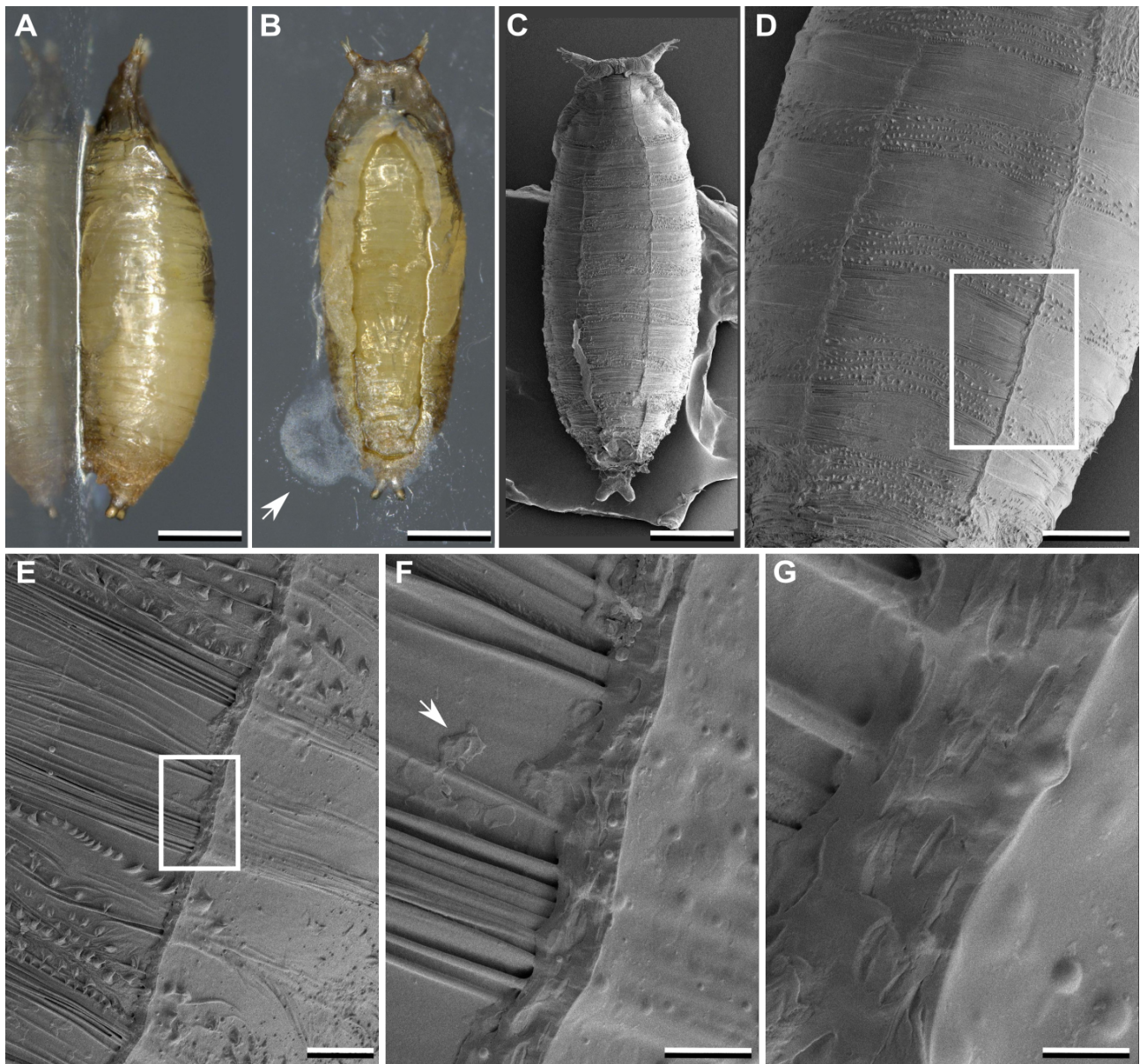
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RESULTS

Morphology of the glue

Figure 3. Morphology of *Drosophila melanogaster* glue. (A,B) Pupa naturally attached to a glass slide viewed from the side **(A)** and ventrally **(B).** Glue at the posterior part of the animal sometimes forms a plug on the substrate (**B**, arrow). **(C-G)** Cryo-SEM micrographs of a pupa after detachment from a glass slide. The white squares in **(D)** and **(E)** indicate respectively the location of the images **(E)** and **(F)**. After detachment, glue is not present on the former contact area on the ventral side except thin traces (**F**, arrow) and remains on the sides of the ventral part. Scale bars: **(A-C)** 500 μm, **(D)** 250 μm, **(E)** 50 μm, **(F)** 20 μm. **(G)** 5 μm.

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270   Pupae of *D. melanogaster* attach naturally to substrates on their ventral side via a layer of glue
271   which forms an oval-shaped patch visible on glass slides of approximately 2 mm length and 0.5 mm
272   width (Fig. 3A-B). Glue near the posterior part of the animal usually forms a bigger plug that can
273   spread on the substrate (arrow, Fig. 3B). SEM observations of pupae detached from non-coated
274   glass slides show that sometimes only thin traces of glue remain on the former contact area (Fig.
275   3C-G arrow in Fig. 3F). The glue layer covering the pupal case appears to be organized in thin
276   layers (Fig. 3F-G). Air bubbles are observed between the layers and the glue does not fill all the
277   asperities of the cuticle surface (Fig. 3I-J).

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279   **Pupal adhesion on different substrates**
280   We performed pull-off force measurements using pupae naturally attached to 11 different substrates:
281   non-coated glass (from Roth and from Menzel), PLL-coated glass, PLL-PEG-coated glass, oxygen-
282   activated glass, Teflon, and Spurr epoxy resin with 5 different roughnesses. We measured the
283   contact angle of these 11 substrates (Table S2). Contact angle values ranged from 11° (highly
284   hydrophilic substrate) to 112° (highly hydrophobic substrate). In total, 328 pupae were measured.
285   We excluded 93 cases for which adhesion measures were not reliable, for instance when the pupal
286   case was damaged during the assay or when several trials with the same pupa disrupted its adhesion
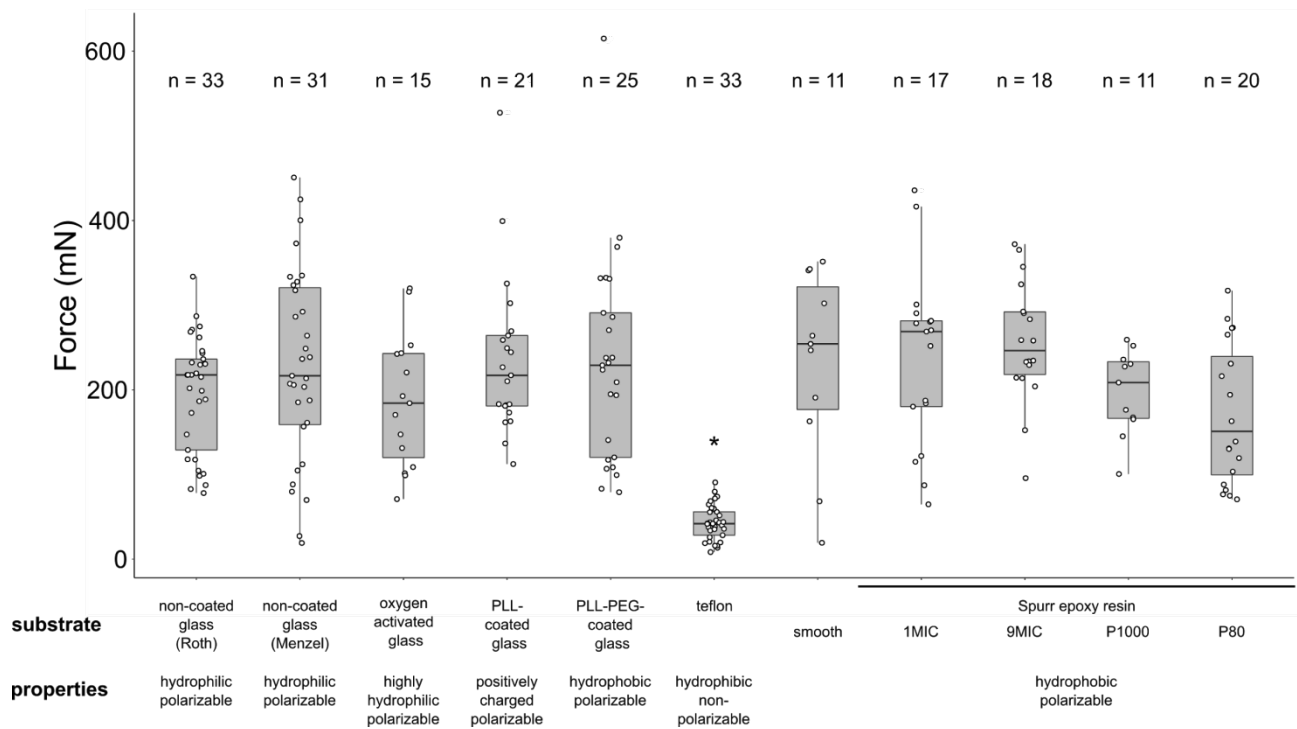287   (see Methods).

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Figure 4. Force required to pull-off pupae from different substrates. Each dot corresponds to a single pupa and n indicates the total number of pupae tested for each surface. Ends of the boxes 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 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 first and the third quartiles). Data beyond the end of these lines are "outlying" points. Asterisks indicate significant difference (P<0.001) between Teflon and all the other substrates.

Medians of the pull-off forces on glass-substrates (non-coated, PLL-coated, PLL-PEG-coated and oxygen-activated glass) ranged from 184 mN (oxygen-activated glass, SD= 78) to 229 mN (PLL-PEG-coated glass, SD= 122) while for Teflon, force was 42 mN (SD= 20) (Fig. 4). Only pull-off force on Teflon was significantly different from forces obtained on other substrates (one-way ANOVA F=12.92, P<2e-16, followed by all pairwise comparison Tukey-Test, P<0.001 for all comparisons with Teflon). Similarly, pull-off force medians on resin with different roughnesses ranged from 151 mN (P80, SD= 82) to 269 mN (1MIC, SD= 105) and no statistical differences were found (from same ANOVA and Tukey tests). During our assays, the ranges for humidity, temperature, pressure and age of pupa were the following: 34.4 - 58.8 %, 23.5 - 27.9 °C, 1005 - 1026 mb, 3.5 - 23 h after deposition of the wandering L3 larva on the substrate. No effect of

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323   humidity, temperature, pressure and age of pupa was found (multiple linear regression with force as

324   dependent variable and substrate, humidity, pressure, age as independent variables).

325   In conclusion, we found that the pull-off force is independent of substrate surface chemistry

326   (except Teflon) and of roughness in wide range (80 nm – 40.40 µm).

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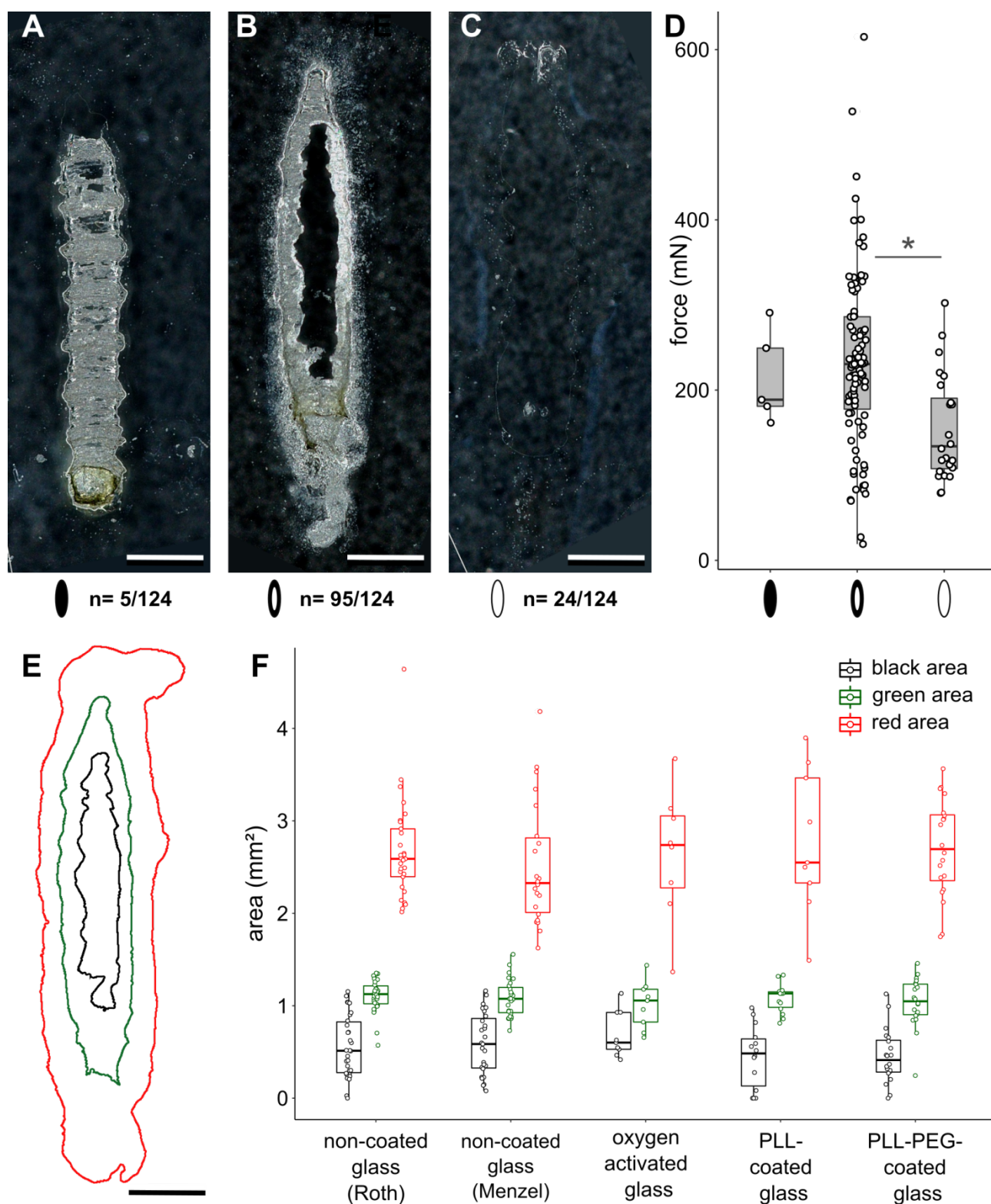344

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The glue-substrate interface

Figure 5. Glue-substrate interface on glass-type substrates. (A-C) Examples of typical glue prints obtained after pull-off force assays on glass-type substrates: **(A,C)** PLL-coated glass, **(B)** non-coated glass (Roth). Three cases are defined: **(A)** when the glue fully remained on the substrate,

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361 represented as a black oval, **(B)** when the glue partly detached from the substrate, represented as a

362 white oval with thick black contour, **(C)** when the glue completely went off from the substrate,

363 represented as a white oval. n indicates the total number of glue prints obtained for each case. **(D)**

364 Box plots representing the force required to detach pupae depending on the print types defined in

365 **(A-C)**. One dot corresponds to a single pupa. Asterisk indicates significant difference (P<0.05)

366 between cases where the glue partly detached from the substrate and cases where the glue

367 completely went off from the substrate. **(E)** Scheme of the various glue prints with the glue-pupa

368 interface delimited in green, the glue-glass interface delimited in red and the border of the glue

369 removed from the glass after pull-off adhesion test in black. **(F)** Box plots showing the size of the

370 three areas defined in **(E)** for the five glass-substrates. Only prints where the glue fully remained

371 and partly detached from the substrates were analyzed (see Methods). Scale bars: **(A-C,E)** 500 μm.

372 Box plots are defined as previously (Fig. 4).

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374 After pull-off force measurements, glue prints were analyzed for the five glass-type substrates. For

375 these substrates, three types of prints can be defined: (1) the glue fully remained on the substrate

376 (Fig. 5A), (2) the glue partly detached from the substrate (Fig. 5B) or (3) the glue went off

377 completely with the pupa (Fig. 5C). Most of the time, the glue was partly detached from the

378 substrate (n= 95/124). For the five glass-type substrates, print types (but not substrate types) have a

379 significant impact on pupa adhesion (two-way ANOVA, print types: F=5.6, P=0.0013). When the

380 glue completely went off with the pupa, corresponding pull-off force was significantly lower than

381 force obtained when glue was partly detached (134 mN, SD= 62 compared to 231 mN, SD= 102 ,

382 Tukey test P<0.05) (Fig. 5D). On Teflon, the glue always went off with the pupa (n=33/33).

383 Furthermore, from the glue print three distinct areas can be defined. The pupa-substrate

384 interface area corresponds to the area where the pupa was in contact with the substrate (green

385 outline in Fig. 5E). The glue-substrate interface area (red outline in Fig. 5E) corresponds to the total

386 surface of the substrate covered by the glue. After pupa detachment, a third area where glue went

387 away with the pupa can be defined (black outline in Fig. 5E). For all cases (as in Fig. 5A,B) where

388 the glue did not go off completely with the pupa, the three areas were measured from the print

389 images of the five glass-type substrates. A relatively high variation in glue-substrate interface area

390 was observed (from 1.3 to 4.6 mm^2) compared to the pupa-substrate area which was relatively

391 invariant, around 0.2 to 1.5 mm^2 (Fig. 5F). No difference was found in areas between substrates

392 (one-way ANOVA for each area, P>0.05) (Fig. 5F). No correlation was found between pull-off

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393   force and any of the three areas when combining the five substrates or when testing each substrate

394   individually (linear regression for each area, P>0.05) (Fig. S1).

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396   **DISCUSSION**

397   *Drosophila* glue spreads over an external substrate and sticks the pupa's ventral part to the

398   substrate, forming an oval-shaped interface (Fig. 3B). From SEM images, we observed flat holes

399   that seem to correspond to air bubbles that were trapped between different layers of glue. Several

400   layers may have been deposited successively on the surface of the pupa by the back and forth

401   movements of the larvae during expectoration (Fraenkel and Brookes, 1953). The presence of

402   bubbles and the fact that the glue does not fill all the asperities of the cuticles suggest that the glue

403   is relatively viscous when it comes out. It is known that the glue is produced by the salivary glands

404   and is secreted by the mouth (Fraenkel and Brookes, 1953). However, on the glue prints we

405   sometimes observed a plug around the posterior tip of the larva (Fig. 3B). Recently, Benova-

406   Liszekova et al. (2019) reported that a few larvae did not empty their guts entirely before

407   pupariation. One possibility is that this plug might be a posterior secretion from the digestive tube,

408   which mixes with the anteriorly secreted glue.

409   During our pull-off force assays, the piece of tape used to stick the pupa in order to pull it

410   peeled out from the pupal case in 153 of the 235 analysed cases, meaning that the adhesion strength

411   between the double-sided tape and the pupal case is close to the adhesion strength between the glue

412   and the pupa. In our experiments, the tape thus adheres to the pupal case just enough for pupa glue

413   measurements. Furthermore, in 76/235 cases, the posterior part of the animal first detached from the

414   substrate before the head detached. In these cases, the pull-off force was probably not applied

415   exactly at the center of the pupa but more posteriorly due to the asymmetrical shape of the pupa.

416   Glue concentration and quantity may also be higher around the mouth, from which it is secreted.

417   Furthermore, the pupal case is more fragile around the puparial opercular seam, a seam which runs

418   across the front of the puparium and extends along the sides, and which will break when the adult

419   will hatch (Tyler, 1994). In addition, when using forceps to extract the animal from its pupal case,

420   the pupal case generally splits into annular strips (Held, 1992). The zone of breakage that we

421   observed on the pupal case in our pull-off assays therefore corresponds to the most fragile region of

422   the pupal case.

423   In any case, no significant difference was found between cases for which pupae detached at

424   once and cases where the posterior part detached before the head part. This suggests that stronger

425   head adhesion does not affect the maximal pull-off force.

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426   Pull-off forces required to detach pupae from substrates other than Teflon ranged from 151
427 mN (P80, SD=20) to 269 mN (1MIC, SD=105). Using pupa-substrate interface (green outline in
428 Fig. 5E) as a measure of contact area between the glue and the substrate, we found a median contact
429 area of 1.10 mm², which leads to an adhesive strength ranging from 137 to 244 kPa. Few similar
430 studies have been carried on insect egg adhesive strength. Two of them reported higher values (1-2
431 MPa for *Opodiphthera* eggs on wood (Li et al., 2008), 4.3-12.2 MPa for whitefly eggs on rose
432 leaves (Voigt et al., 2019), one study found equivalent values (38.8-271.3 kPa for beetle *C.*
433 *asparagi* on plant surface (Voigt and Gorb, 2010)) or another lower adhesive strength (13.9-97.8
434 kPa for the moth *C. pomonella* eggs on fruits (Al Bitar et al., 2014)). To our knowledge, our study is
435 the first one to report adhesion strength measurements for Diptera pupa.

436   No statistical differences in pull-off forces were found between the different substrates
437 except for Teflon (median pull-off force is 42 mN, SD= 20), suggesting that the glue can stick to
438 hydrophobic substrates as well as hydrophilic substrates. We can hypothesize that proteins of the
439 glue are polarizable, which allows the glue to stick strongly to polarizable substrates that are
440 differently charged and not to Teflon, which is not polarizable. Indeed, Sgs proteins are highly
441 charged. They are mostly composed of positively charged amino acids and they are highly
442 glycosylated with negatively charged sugars (Beckendorf and Kafatos, 1976; Korge, 1977).

443   No differences were found on resin substrates with different roughnesses. We expected that
444 pull-off force would increase with roughness as contact area on rough substrate is bigger.
445 Furthermore, we did not find correlation between force and contact area for any of the substrates
446 used. Both observations can be explained by a possible critical crack initiation stress between the
447 pupal case and the glue. Indeed, we observed on the glue prints on glass-type substrates that the
448 glue from the pupa-substrate contact area is partly detached, suggesting that rupture occurs between
449 the glue and the pupal case. A similar phenomenon may occur on resin substrates, whereas on
450 Teflon the glue completely goes off from the substrate, suggesting that the rupture occurs between
451 the glue and the Teflon. Thus, in all the tested substrates except Teflon, the bond between the glue
452 and the different substrates appears to be stronger than the bond between the glue and the pupal
453 case. It is thus possible that our adhesion tests measure the adhesive force of the bond between the
454 glue and the pupal case, which would explain why we observed the same adhesion strength for all
455 substrates except Teflon. As an alternative explanation for the same adhesiveness measured on all
456 tested substrates except Teflon, it is possible that the animal modulates the amount of glue it
457 produces, or the way it spreads the glue over the substrate and its body. It would be interesting to do
458 adhesion measurement on isolated glue. However, it is challenging to collect the glue as it is

459 secreted in very small volume and it polymerizes within 3-5 min. after expectoration (Beňová-
460 Liszeková et al., 2019).

461   Pull-off force values were rather variable between trials for a given substrate (for example
462 from 19 to 451 mN on Menzel non-coated glass). Such a wide range of values could be due to
463 individual variability. Pupae weight could not be deduced accurately by subtracting the final force
464 from the initial force because pupal weight was within the noise of force values. However,
465 individuals from the same population as the pupae used for trials were weighted instead. We found
466 that pupal weight averaged 1.4 ± 0.3 (SE) mg (n=24). The weight variation is negligible compared
467 to the force variation observed within substrates. Surprisingly, considering this average weight and
468 an average force of 217 mN (mean of all trials kept for analysis except trials on Teflon), the glue
469 holds about 15 500 times the weight of a pupa.

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Conclusions

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472   For the first time, we report here measurements of *Drosophila* pupal adhesion strength. We
473 present a pull-off force test to measure pupa adhesion which could be used in the future to explore
474 pupa adhesion in various strains and species of flies. With the powerful genetic tools available in
475 *Drosophila melanogaster*, we now plan to assess the adhesive function of the glue genes. The use of
476 *D. melanogaster* as a model organism for the study of bioadhesives is very promising as it makes it
477 possible and easy to manipulate the composition of the glue.

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492 **Supplementary**

493 **Table S1. raw dataset: Borne_2020_glue_table_S1.csv**

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495 **Table S2. Contact angle measurements (°) for the different substrates.** Contact angles were

496 measured for each substrate. All the measurements were done before the pull-off force

497 measurements except for PLL-PEG-coated glass.

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| substrate	mean	std error
1MIC-resin	112.68	0.47
P1000-resin	109.19	0.89
P80-resin	103.27	1.29
teflon	96.64	1.88
smooth_resin	84.11	1.93
9MIC-resin	78.63	1.89
PLL-coated glass	55.15	1.28
PLL-PEG-coated glass	38.65	1.06
oxygen-activated glass	37.03	1.39
non-coated glass (Menzel)	28.86	0.91
non-coated glass (Roth) unwashed	20.05	0.57
non-coated glass (Roth) washed	11.84	1.40

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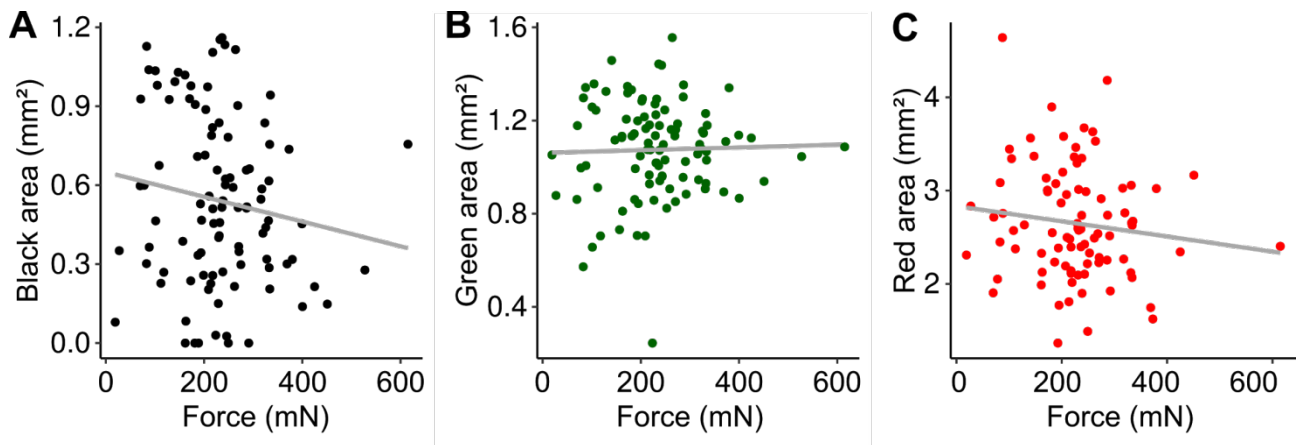
Figure S1. Force in function of (A) black area, (B) green area and (C) red area. Each dot represents one print on glass-type substrates. The grey lines represent the linear regressions: **(A)** r^2= 0.01168, y= 257.69 - 46.03x, p=0.144 ; **(B)** r^2= -0.009391, y= 218.05 + 13.73x, p=0.7793 ; **(C)** r^2= 0.004791, y= 279.36 - 19.97x, p= 0.2369; where r^2 is the adjusted r^2.

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