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1 **Developmental timing of *Drosophila pachea* pupae is robust to temperature**  
2 **changes**

3  
4 running title: Temperature and development in *D. pachea*

5  
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16 **Author contributions**

17 BL and ML designed the experiments. BL and SM generated the data. BL analyzed the data,  
18 BL and ML wrote the manuscript. All authors read and approved the final manuscript.

19  
20 **Conflict of interest**

21 The authors have no conflict of interest to declare.

22 **Abstract**

23 Rearing temperature is correlated with the timing and speed of development in a  
24 wide range of poikilotherm animals that do not regulate their body temperature. However,  
25 exceptions exist, especially in species that live in environments with high temperature  
26 extremes or oscillations. *Drosophila pachea* is endemic to the Sonoran desert in Mexico, in  
27 which temperatures and temperature variations are extreme. We wondered if the  
28 developmental timing in *D. pachea* may be sensitive to differing rearing temperatures or if it  
29 remains constant. We determined the overall timing of the *Drosophila pachea* life-cycle at  
30 different temperatures. The duration of pupal development was similar at 25°C, 29°C and  
31 32°C, although the relative progress differed at particular stages. Thus, *D. pachea* may have  
32 evolved mechanisms to buffer temperature effects on developmental speed, potentially to  
33 ensure proper development and individual's fitness in desert climate conditions.

34

35 **Keywords**

36 *Drosophila pachea*, pupal development, temperature, heterochrony

## 37 1. Introduction

38 Poikilotherms animals do not regulate their body temperature contrary to  
39 homeotherms (Precht et al., 1973) and are sensitive to environmental temperature.  
40 Environmental temperature in turn affects their metabolism (Hazel and Prosser, 1974). In  
41 particular, it seems widespread that developmental speed increases with rearing  
42 temperature in poikilothermic species (Abril et al., 2010; Asano and Cassill, 2012; Hrs-  
43 Brenko et al., 1977; Ikemoto, 2005; Manoj Nair and Appukuttan, 2003; Nishizaki et al., 2015;  
44 Pechenik et al., 1990; Porter, 1988; Sharpe and DeMichele, 1977; Vélez and Epifanio,  
45 1981), including various *Drosophila* species (David and Clavel, 1966; James and Partridge,  
46 1995; Kuntz and Eisen, 2014; Powsner, 1935). This phenomenon is proposed to be due to  
47 thermodynamics of enzymes responsible for biochemical reactions underlying  
48 developmental processes (Crapse et al., 2021; Ikemoto, 2005; Schoolfield et al., 1981;  
49 Sharpe and DeMichele, 1977). Thermal-stress can accelerate development and has been  
50 shown to result in an increase of developmental instability (Kristensen et al., 2003; Nishizaki  
51 et al., 2015; Polak and Tomkins, 2013), measured as deviations of an individual's character  
52 from the average phenotype in the population under the same conditions (Palmer, 1994;  
53 Zakharov, 1992). This may result in a decreased individual's survival and reproductive  
54 fitness. In contrast, a slow development may potentially lead to an increased risk of  
55 predation at vulnerable stages, such as immobile pupae in holometabolous insects (Ballman  
56 et al., 2017; Borne et al., 2021; Hennessey, 1997; Thomas, 1993; Urbaneja et al., 2006).  
57 Furthermore, a variable timing of development among individuals of a same species might  
58 induce intraspecific competition (Amarasekare and Coutinho, 2014; Frogner, 1980) as  
59 individuals developing faster may reproduce sooner and for a longer period compared to  
60 those developing more slowly. Different mechanisms have been found to regulate  
61 developmental timing. The so-called heterochronic miRNAs, such as *let-7* and *miR-125*,  
62 were originally discovered in *Caenorhabditis elegans* (Rhabditida: Rhabditidae)(Ambros,  
63 2011; Ambros and Horvitz, 1984). These miRNAs are conserved in a wide range of species,  
64 such as *Drosophila melanogaster* (Diptera : Drosophilidae)(Caygill and Johnston, 2008) or  
65 *Danio rerio* (Cypriniformes: Cyprinidae)(Ouchi et al., 2014), as well as in mammals and  
66 plants (Ambros, 2011). They act at post-transcriptional level to regulate cellular mRNA  
67 levels, and have been found to control the developmental timing, cell fate and cell  
68 differentiation. Hormones are also known to be important regulators of developmental timing.  
69 In *D. melanogaster*, each of the developmental transitions are regulated by ecdysone  
70 pulses, and premature transition from larva to pupa with respect to food conditions or  
71 starvation is prevented by juvenile hormone (Riddiford, 1994; Riddiford and Ashburner,  
72 1991). Thus, developmental timing might be regulated to reach an optimal duration with

73 respect to outer environmental factors.

74 More than 1500 described species of the genus *Drosophila* (Bächli et al., 2021;  
75 O'Grady and DeSalle, 2018) occupy a wide range of habitats with various climatic conditions  
76 (Markow and O'Grady, 2008). A dozen of species have been reported to be cosmopolitan  
77 species (Markow and O'Grady, 2008, 2005), such as *Drosophila melanogaster* (David and  
78 Cappy, 1988; Li and Stephan, 2006) that potentially dispersed with humans from Africa  
79 around the globe (Mansourian et al., 2018). These species may be generalists but were also  
80 found to be locally adapted to diverse environments (Kapun et al., 2020; Markow and  
81 O'Grady, 2008). In contrast, the vast majority of species are restricted to certain continental  
82 ranges or are endemic to a specific geographic region that encompasses a unique habitat  
83 with specific food and climate conditions (Markow and O'Grady, 2008, 2005). Because of  
84 their inability to disperse outside their habitat, these endemic species may have evolved  
85 temperature-buffering mechanisms to ensure a constant developmental timing under  
86 variable temperature conditions.

87 *Drosophila pachea* (Diptera : Drosophilidae) is endemic to the Sonoran desert in  
88 Mexico and is an obligate specialist on decayed parts, or rot-pockets, of its single host plant,  
89 the Senita cactus (*Lophocereus schottii*) (Gibbs et al., 2003; Heed and Kircher, 1965; Lang  
90 et al., 2012; Markow and O'Grady, 2005). The micro-climate of the rot-pockets encompasses  
91 important changes of temperature all along the year, with a recorded maximum variation  
92 from 5°C to 42°C within 24 h (Gibbs et al., 2003). Living in an environment with large daily  
93 and annual temperature changes may require a certain temperature robustness with respect  
94 to developmental processes in poikilotherm species. We wondered if the developmental  
95 timing in *D. pachea* may be sensitive to differing rearing temperatures. To test this, we first  
96 determined the overall timing of the *Drosophila pachea* life-cycle. Then, we focussed on  
97 pupal development at four different rearing temperatures to investigate differences in the  
98 pupal timing. Finally, we compared these durations across closely related sister species  
99 *Drosophila acanthoptera* (Diptera : Drosophilidae) and *Drosophila nannopectera* (Diptera :  
100 Drosophilidae) to investigate potential species-specific developmental timing differences.

101

## 102 **2. Materials and methods**

### 103 **2.1. *Drosophila* stock maintenance**

104 *Drosophila* stocks were retrieved from the San Diego *Drosophila* Species Stock  
105 Center (now The National *Drosophila* Species Stock Center, College of Agriculture and Life  
106 Science, Cornell University, USA). The *D. pachea* stock 15090-1698.01 was established in

107 1997 from individuals caught in Arizona, USA. The *D. nannoptera* stocks 15090-1692.00 and  
108 15090-1693.12 were established in 1992 from individuals caught in Oaxaca, Mexico. The *D.*  
109 *acanthoptera* stock 15090-1693.00 was established in 1976 from individuals caught in  
110 Oaxaca, Mexico (UCSC Drosophila species stock center San Diego, now The National  
111 Drosophila Species stock center, Cornell University). These stocks have been kept in good  
112 conditions at 25°C in our laboratory since 2012.

113 Flies were maintained in transparent plastic vials (25 x 95 mm, Dutscher) containing  
114 about 10 mL of standard Drosophila medium. This medium was composed of 66.6 g/L of  
115 cornmeal, 60 g/L of brewer's yeast, 8.6 g/L of agar, 5 g/L of methyl-4-hydroxybenzoate and  
116 2.5% v/v ethanol (standard food). We added 40 µL of 5 mg/mL of 7-dehydrocholesterol  
117 (7DHC) (Sigma, reference 30800-5G-F) dissolved in ethanol into the food for *D. pachea*, as  
118 this species need this sterol for proper development (Heed and Kircher, 1965; Lang et al.,  
119 2012; Warren et al., 2001) (standard *D. pachea* food). As a pupariation support, a piece of  
120 paper sheet (1 cm x 4 cm, BenchGuard) was added to each vial. Stocks were kept at 25°C  
121 or 29°C at a 12 h light:12 h dark photoperiodic cycle with a 30 min transition between light  
122 (1080 lm) and dark (0 lm).

123

## 124 **2.2. Cohort synchronisation of *D. pachea* embryos and time-lapse recording of** 125 **embryonic development**

126 For collection of cohorts of synchronised embryos, about 250-500 adult flies were  
127 transferred into a 9 x 6 cm plastic cylinder, closed by a net on the top and by a 5.5 cm  
128 diameter petri-dish lid at the bottom. The petri-dish contained grape juice agar (24.0 g/L  
129 agar, 26.4 g/L saccharose, 20% grape juice, 50% distilled water, 12% Tegosept [1.1 g/mL in  
130 ethanol] (Dutscher), 4% 7-DHC (Sigma)) and 50-200 µL fresh baker's yeast as food source  
131 and egg laying substrate on top. These plates are named hereinafter "food plates". Female  
132 flies were let to lay eggs on the food plates for 1 h - 2 h (1 h to examine embryos and 2 h to  
133 synchronise larvae). Then, eggs were retrieved from food plates by filtering the yeast paste  
134 through a 100 µm nylon mesh (BS, Falcon 352360).

135 For time-lapse imaging the chorion of embryos was removed by a 90 sec incubation  
136 of the embryo-containing filter in 1.3% bleach (BEC Javel) under constant agitation until  
137 about half of the embryos were floating at the surface of the bleach bath. Embryos were  
138 extensively rinsed with tap water for at least 30 sec. Dechorionated embryos were then  
139 gently glued on a cover slip (ThermoFisher) coated with Tesa glue. For coating, 50 cm TESA

140 tape was transferred into 25 mL n-heptane (Merck) and glue was let to dissolve overnight at  
141 room temperature. A total of 15  $\mu$ L of dissolved glues was finally pipetted onto a cover slip to  
142 form a 5 x 20 mm rectangular stripe and n-heptane was let to evaporate. Embryos were  
143 covered with 40  $\mu$ L of Voltalef 10S halocarbon oil (VWR) to avoid desiccation. Live-imaging  
144 was immediately launched inside a temperature and humidity controlled chamber at 25°C  $\pm$   
145 0.1°C and 80%  $\pm$  1% humidity (Lang and Orgogozo, 2012; Lefèvre et al., 2021; Rhebergen  
146 et al., 2016). Time-lapse acquisition was performed at an acquisition rate of 1 picture every  
147 7.5 sec using a digital camera (Conrad 9-Megapixel USB digital microscope camera) and  
148 Cheese software, version 3.18.1, on a computer with an ubuntu 16.04 linux operating  
149 system. Movies were assembled with avconv (libav-tools).

150 In *Drosophila melanogaster* and closely related species, females were reported to  
151 hold fertilized eggs inside the reproductive tract for >12 hours (Markow et al., 2009), which  
152 could explain the variation observed in our experiments with *D. pachea*. Therefore, we  
153 monitored egg retention in this species by examining dechorionated eggs from a 1 h egg-  
154 laying period. We found that all observed embryos (n=52) were early embryos at the  
155 syncytial blastoderm stage (Wieschaus and Nüsslein-Volhard, 1986) and egg retention was  
156 not observed. Out of 28 embryos monitored, 12 (43%) pursued their development until  
157 hatching while the others did not develop at all (Movie S1, Dataset S1). Such mortality has  
158 been reported previously (Jefferson, 1977; Pitnick, 1993) but potentially also dependent on  
159 the above-mentioned bleach treatment. The embryos that died during the experiment were  
160 excluded from analysis. Furthermore, the duration of hatching, which is the last stage of  
161 embryonic development, has been shown to be more variable in comparison to the other  
162 embryonic stages in various *Drosophila* species (Chong et al., 2018; Kuntz and Eisen,  
163 2014). We thus measured both the total embryonic duration, from collection up to larva  
164 hatching and the embryonic duration up to the trachea gas filling stage, which precedes the  
165 hatching stage (Dataset S1).

166

### 167 **2.3. Cohort synchronisation of larvae, dissection and imaging of larval mouth hooks**

168 In order to collect cohorts of larvae at a synchronous developmental stage, we first  
169 collected embryos from a 2 h egg laying interval (see above) that were placed on a food  
170 plate together with fresh yeast. Freshly hatched larvae were retrieved from the yeast paste  
171 with fine forceps (Dumont #5, Fine Science Tool) or by filtering the yeast through a nylon  
172 mesh (see above). Larvae were transferred into vials containing standard *Drosophila*  
173 *pachea* food and were examined once a day until all larvae had turned into pupae (Dataset

174 S2).

175 For imaging of the larval teeth, entire larvae were mounted in 20  $\mu$ L dimethyl-  
176 hydantoin formaldehyde (DMHF) medium (Entomopraxis) beneath a cover slip (0.17 mm  $\pm$   
177 0.01 mm thick, ThermoScientific), which was gently pressed against the microscope slide  
178 (ThermoScientific) to orient larval teeth in a flat, lateral orientation to the microscope  
179 objective. Larval teeth were imaged at 100 or 400 fold magnification in bright field  
180 illumination (Strasburger, 1935) using the microscope IX83 (Olympus). The instar stage of  
181 each dissected individual was determined based on tooth morphology (Strasburger, 1935)  
182 (Figure S1).

183

#### 184 **2.4. Measurement of the duration of puparium formation in *D. pachea***

185 The precise duration of puparium formation was characterized by monitoring nine *D.*  
186 *pachea* pupariating larvae by time-lapse imaging. Larvae at the third instar stage and third  
187 instar wandering stage were collected from the *D. pachea* stock and were transferred into  
188 fresh *D. pachea* standard medium, inside a 5 cm diameter petri-dish and a piece of 1 cm x 4  
189 cm paper sheet (BenchGuard). The dish was then placed into the temperature and humidity  
190 controlled chamber at 25°C  $\pm$  0.1°C and 80%  $\pm$  1% humidity, as previously described. Time-  
191 lapse acquisition was performed for about 72 h as previously described for embryonic timing  
192 characterization. The duration of the white puparium stage was measured from the moment  
193 when the larva had everted the anterior spiracles and had stopped moving until the moment  
194 when the pupal case had turned brown.

195

#### 196 **2.5. Characterization of developmental timing in pupae**

197 The developmental duration of *D. pachea*, *D. nanoptera* and *D. acanthoptera* was  
198 examined by observation of pupae at different time points after puparium formation (APF).  
199 Synchronised pupae were obtained from each species by collecting so-called “white pupae”  
200 that had just formed the puparium (Dataset S3). Specimens were collected with a wet brush  
201 directly from stock vials. Individuals of the same cohort were placed onto moist Kimtech  
202 tissue (Kimberly-Clark) inside a 5 cm diameter petri dish. Petri dishes with pupae were kept  
203 at 22°C, 25°C, 29°C, or 32°C inside plastic boxes, which also contained wet tissue paper.  
204 Specimens analyzed at 22°C and 32°C were taken from a stock at 25°C at the stage of  
205 puparium formation and subsequently incubated at the desired temperature. A group of



206 pupae resulting from a single collection event was considered as a synchronised cohort.  
207 Developmental progress of synchronised cohorts (Table 1, Figure S2) was examined at  
208 various time points by visual examination of the pupae using a stereomicroscope VisiScope  
209 SZB 200 (VWR) (Dataset S4). Developmental stages were assigned according to  
210 morphological markers defined for *D. melanogaster* by Bainbridge and Bownes (1981)  
211 (Table 2). The markers used to characterize stages 8 to 12 (eye, wing or body pigmentation,  
212 Table 2) were not convenient for the characterization by direct observation of *D.*  
213 *acanthoptera* pupae as these flies develop black eyes, as opposed to most other Drosophila  
214 species that have red eyes. In addition, *D. acanthoptera* is generally less pigmented  
215 compared to *D. pachea* and *D. nanoptera* (Pitnick and Heed, 1994) and pigmentation  
216 changes were not easily detectable through the pupal case. Therefore, we additionally  
217 carried out time-lapse imaging of one cohort with five *D. acanthoptera* pupae to investigate  
218 the developmental durations of stages 8-12. The anterior part of the pupal case was  
219 removed, letting the head and the anterior part of the thorax visible. Image acquisition was  
220 done at  $25^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$  and  $80\% \pm 1\%$  humidity, as previously described. Time-lapse  
221 acquisition was performed as previously described and recorded with the VLC media player,  
222 version 3.0 at an acquisition rate of 1 picture every 13:02 min. Two pupae died during  
223 acquisition and were excluded from the analysis (Movie S2).

224

## 225 **2.6. Data analysis**

226 Data was manually entered into spreadsheets (Datasets S1, S2, S3 and S4) and  
227 analysis was performed in R version 3.6 (R Core Team, 2014). Ages expressed in hours  
228 after pupa formation were automatically calculated with respect to the time point of white  
229 pupa collection.

230

## 231 **3. Results**

### 232 **3.1. *D. pachea* embryonic and larval development at 25°C last for about 33 h and 216** 233 **h, respectively**

234 We roughly examined the duration of embryonic and larval development in *D.*  
235 *pachea* at 25°C. The average duration of the total embryonic development in *D. pachea* at  
236 25°C, until hatching of the larva was 32 h 48 min  $\pm$  1 h 13 min (mean  $\pm$  standard deviation ; n  
237 = 12) (Figure 1, Movie S1). Embryonic development up to the trachea gas filling stage (see  
238 Material and Methods for details) was estimated to be 26 h 48 min  $\pm$  1 h 13 min (mean  $\pm$

239 standard deviation ; n = 12) (Movie S1). These durations appeared to be longer in *D. pachea*  
240 compared to those reported for various other *Drosophila* species, such as *Drosophila*  
241 *melanogaster*, *Drosophila simulans*, *Drosophila sechellia*, *Drosophila yakuba*, *Drosophila*  
242 *pseudoobscura*, *Drosophila mojavensis* (Figure 2) (David and Clavel, 1966; Kuntz and  
243 Eisen, 2014; Powsner, 1935).

244 The total duration of *D. pachea* larval development on standard *D. pachea* food at  
245 25°C was approximately 9 days (~216 h). The duration of the first and second instar larva  
246 were about 2 days each and the third instar stage lasted for about 5 days (Figure 1). In *D.*  
247 *melanogaster*, the total duration of the larval stage was about 5 days for larvae reared on  
248 optimal food at 25°C, the first and second instars lasting for 1 day each, and the third instar  
249 for three days, according to Strasburger, (1935). The larval development of *D. pachea*  
250 appeared thus to be longer compared to those of *D. melanogaster* at 25°C.

251

### 252 **3.2. Similar durations of pupal development in *D. pachea* at 25°C, 29°C and 32°C**

253 The duration of larval development appears to be sensitive to various environmental  
254 factors, such as diet (Matzkin et al., 2011), crowding, or access to food (Vijendravarma et al.,  
255 2013). Since pupal development is apparently less affected by such factors, we focussed on  
256 the pupal stage to investigate the effect of the rearing temperature on timing of development  
257 in *D. pachea*. We evaluated pupal developmental progress at four temperatures: 22°C, 25,  
258 29°C, and 32°C. Preliminary tests revealed that rearing of *D. pachea* at temperatures lower  
259 than 25°C is prolonged which favors the accumulation of bacterial infections in the food and  
260 decreased survival of the flies. At 34°C, *D. pachea* individuals died within a few days and at  
261 32°C flies survived but did not reproduce. Since we could not cultivate *D. pachea* at the  
262 extreme temperatures of 22°C and 32°C, individuals were selected at the stage of puparium  
263 formation in a stock at 25°C and incubated at either temperature.

264 At 25°C - 32°C, *D. pachea* pupae reached the pharate adult stage in less than 55 h  
265 but timing was prolonged at 22°C (Figure 3A-E). However, pupal development was  
266 accelerated at 29°C and 32°C between stages 8 and 13 (beginning of eye pigmentation until  
267 the end of body and wing pigmentation) compared to development at 25°C (Figure 3A-E). In  
268 addition, development was consistently slower at 22°C compared to 25°C. However, stages  
269 14 and 15 required more time at 29°C and 32°C with respect to developmental progress at  
270 25°C and resulted in a similar overall duration of about 100 - 145 h. Only at 22°C,  
271 development was globally slower and adults emerged later, between 150 - 190 h. Thus, in

272 *D. pachea* the rearing temperature influences the relative progress of pupal development at  
273 particular stages. While pupal development is slowed-down at temperatures below 25°C, the  
274 overall duration appears to be similar at higher temperatures.

### 275 **3.3. The timing of the pupal development is conserved up to the pharate adult stage** 276 **between *D. pachea* and various *Drosophila* species at 25°C**

277 The white pupa stage (see Material and Methods for details) in *D. pachea* was  
278 estimated to last for 102 min ± 41 min (mean ± standard deviation) (n=9) at 25°C. This  
279 duration has to be considered as the remaining variation of developmental progress between  
280 examined individual pupae in later timing analyses (see Materials and Methods). This  
281 duration was similar to previously reported durations for *D. melanogaster* white pupae of 80-  
282 120 min, at 25°C (Bainbridge and Bownes, 1981).

283 At 25°C, the pharate adult stage (stage 7, Table 2) was observed about 55 h after  
284 puparium formation and emergence of adults between 115 - 145 h after puparium formation  
285 (Figures 2A). This timing was similar to those of *D. acanthoptera* and *D. nannoptera* (Figure  
286 3B). The developmental duration from puparium formation to pharate adult (stages 1 to 7,  
287 from 0 h APF to about 55 h APF) was also similar to those reported for *D. melanogaster* and  
288 *D. guttifera* (Figure 3B) (Bainbridge and Bownes, 1981; Fukutomi et al., 2017). However, at  
289 later pupal development durations of stages were prolonged in *D. pachea*, *D. nannoptera*  
290 and *D. acanthoptera* compared to *D. melanogaster* and *D. guttifera*.

291 The emergence of the adult fly from the pupal case (stage 15) is highly variable  
292 within *D. pachea*, *D. nannoptera* and *D. acanthoptera*. *D. pachea* adults emerge between  
293 115 - 144 h APF, *D. nannoptera* adults between 112 - 140 h APF and *D. acanthoptera*  
294 adults between 102 h - 142 h APF. The variance of this stage was significantly different  
295 between the three species (Levene's test: F = 3.4414, Df = 2, p = 0.03847), the stage 15  
296 being longer in *D. acanthoptera* compared to *D. pachea* and *D. nannoptera* (Figure 3B).

297

## 298 **4. Discussion**

299

### 300 **4.1. A possible temperature-buffering mechanism during pupal development**

301 The trend of a decrease of developmental duration when rearing temperature  
302 increases was not observed in *D. pachea* at high temperatures. Overall, pupal development  
303 duration were similar at 25°C and 32°C, while it was prolonged at 22°C. On the contrary, the  
304 duration of the overall pupal development decreases with increasing rearing temperature in

305 *D. melanogaster* (Ashburner and Thompson Jr, 1978; Powsner, 1935). In addition,  
306 temperature fluctuations during pupal development of *D. melanogaster* are known to either  
307 increase or decrease developmental speed (Ludwig and Cable, 1933; Petavy et al., 2001).  
308 In this species, the first 24 h of pupal development are more sensitive to temperature  
309 changes compared to the rest of the pupal stage (Ludwig and Cable, 1933; Petavy et al.,  
310 2001). While *D. melanogaster* is a cosmopolitan species that lives in a wide climate range  
311 (David and Capy, 1988), *D. pachea* is a desert species endemic of the Sonora (Heed and  
312 Kircher, 1965; Markow and O'Grady, 2005). The mean daily variations of temperature of this  
313 habitat are 18°C - 42°C in spring/summer and 6°C - 32°C in fall/winter (Gibbs et al., 2003).  
314 *D. pachea* is found in the wild throughout the year but undergoes a strong population decline  
315 during August, when the seasonal temperatures are highest (Breitmeyer and Markow, 1998).  
316 However, adult *D. pachea* are particularly resistant to high-temperatures and survive up to  
317 44°C in the wild, while most other *Drosophila* species revealed a decreasing survival already  
318 at 38°C (Stratman and Markow, 1998). Thus, this species may have developed some heat  
319 resistance mechanisms, physiological and/or behavioral, that results in a certain tolerance to  
320 temperature variations and would buffer temperature changes on the developmental  
321 progress. This buffering effect could potentially be important for proper development since  
322 heat stress has been reported to increase developmental instability in various species  
323 (Kristensen et al., 2003; Nishizaki et al., 2015; Polak and Tomkins, 2013). However, the  
324 specific mechanism by which temperature affects developmental stability is not well  
325 understood (Abrieux et al., 2020; Breuker and Brakefield, 2003; Carvalho et al., 2017;  
326 Enriquez et al., 2018). Rearing at a lower temperature (< 25°C) revealed slower  
327 developmental progress, indicating that a potential buffering for colder temperatures does  
328 not exist in *D. pachea*.

329         Alternatively, the observed buffering phenotype may be temperature independent  
330 and could perhaps ensure the emergence of the adult fly at a particular moment of the day,  
331 such as dawn or dusk, when the environmental temperature might be most suitable for the  
332 freshly emerged individual. In the last pupal stage that corresponds to the adult emergence,  
333 we observed timing variation between individuals in *D. pachea* (up to 75 h between  
334 individuals). This variation could potentially depend on individual differences or on  
335 environmental factors that we could not control, such as the light/dark illumination cycle at  
336 the moment of adult emergence. Such circadian regulation of adult emergence has been  
337 observed in various *Drosophila* species (Ashburner et al., 2004; Mark et al., 2021; Powsner,  
338 1935; Soto et al., 2018). However, the important variation in the last pupal stage is also  
339 found among individuals of the same cohort (Dataset S3 and S4). Future monitoring of the  
340 emergence of adults from various cohorts collected at different moments of the day will be  
341 necessary to test this hypothesis. Future investigations will be needed to further characterize

342 the potential temperature buffering effect during *D. pachea* development and to test the  
343 influence of the circadian rhythm in this species. In addition, we must further assess  
344 temperature dependent pupal development in a wider range of species that live in distinct  
345 climate habitats.

346

#### 347 **4.2. Conservation of the overall developmental progress during early pupal stages**

348 The detailed analysis of the timing of pupal stages revealed that the first stages 1 to  
349 7 appear to be rather synchronous among *D. melanogaster* (Bainbridge and Bownes, 1981),  
350 *D. guttifera* (Fukutomi et al., 2017), and the three closely related species *D. pachea*, *D.*  
351 *acanthoptera* and *D. nannoptera*. Later on, pupal development appears to be more variable  
352 between species. This may indicate the existence of some developmental constraints, which  
353 are limitations of phenotypic variability due to inherent properties of the developmental  
354 system (Smith et al., 1985; Wagner, 2014). Such constraints probably act on outgrowth of  
355 adult organs from primordial structures, so-called imaginal discs, that develop throughout  
356 larval stages but undergo extensive tissue growth during pupal development up to the  
357 pharate adult stage. Thereafter, the timing of development seems to be less constrained and  
358 interspecific variations were observed. At least a part of the variation in the pupal  
359 developmental timing could be attributed to the developmental marker used. As the  
360 coloration markers are qualitative, it is hard to define precise limits of each stage (ie. eyes  
361 turn progressively from yellow to red). A solution might be to identify a combination of  
362 multiple markers for each stage or to establish gene expression markers that are known to  
363 account for specific developmental processes, as it has been recently done for eye  
364 development (Escobedo et al., 2021) or male genitalia development (Vincent et al., 2019).

365

#### 366 **4.3. *D. pachea* embryonic and larval developmental durations appear to be longer** 367 **compared to other *Drosophila* species**

368 The embryonic developmental duration at 25°C has been investigated in 11  
369 drosophila species other than *D. pachea* (Chong et al., 2018; Crapse et al., 2021; David and  
370 Clavel, 1966; Kuntz and Eisen, 2014; Powsner, 1935) (Figure 2) and ranged from 16 h in *D.*  
371 *sechellia* to 25 h in *D. virilis* (Chong et al., 2018; Kuntz and Eisen, 2014) (Figure 2), which  
372 appear to be shorter compared to embryonic development of *D. pachea* at the same  
373 temperature. Interspecific variation in the duration of embryonic development might rely on  
374 genetic factors, as closely related species tend to have similar embryonic developmental  
375 durations compared to those of distantly related ones (Figure 2). Overall, sample size was

376 rather low in our experiments and only present a rough approximation of the time range of  
377 *Drosophila* larval and embryonic development at a single temperature. A detailed examination  
378 would be necessary to adequately refine the duration of each developmental stage.

379 Larval development appeared to be longer in *D. pachea* compared to those in *D.*  
380 *melanogaster* (Bakker, 1959; Strasburger, 1935). However, the duration of this  
381 developmental stage has been shown to be highly variable compared to the other life  
382 stages. In particular it has been shown that larvae are very sensitive to food composition and  
383 to crowding that affect food quality and food access (Matzkin et al., 2011; Vijendravarma et  
384 al., 2013). Food quality and food access in turn prolong the larval developmental duration  
385 (Matzkin et al., 2011). This effect of food on developmental duration might also probably  
386 affect embryonic and pupal stages indirectly due to nutrient contribution from the adult and  
387 larval stages. A slower development observed in *D. pachea* raised in the lab might also be  
388 due to variations in the ecdysone metabolism. In insects, ecdysone is first provided to the  
389 embryo as maternal contribution and then directly produced by the individual (Lafont et al.,  
390 2012). However, in *D. pachea* the first metabolic step of the ecdysone biosynthesis is  
391 different compared to other insect species, the conversion of cholesterol into 7-  
392 dehydrocholesterol being abolished (Lang et al., 2012). Instead, *D. pachea* metabolizes  
393 sterols produced by the *Senita* cactus on which they feed, such as lathosterol, and  
394 potentially campestenol and schottenol (Heed and Kircher, 1965), into steroid hormones  
395 differing in their side residues (Lang et al., 2012). Therefore, in the wild, *D. pachea* may  
396 produce different variants of ecdysone that may also differently affect developmental timing  
397 compared to the lab conditions that only provide the single ecdysone precursor 7-  
398 dehydrocholesterol. Thus, it would be interesting to compare developmental durations of *D.*  
399 *pachea* fed with standard *D. pachea* food used in the lab or with their natural host plant, the  
400 *Senita* cactus. In addition, further investigations would be needed to elucidate how  
401 temperature modulates these mechanisms.

402

#### 403 **4.5. Conclusion**

404 We investigated the effect of temperature on developmental speed in *D. pachea*, a  
405 desert species. We characterized the timing of the life-cycle in this species and observed  
406 prolonged developmental durations compared to other *Drosophila* species. The global  
407 developmental duration during metamorphosis is similar at rearing temperatures between  
408 25°C and 32°C although stage specific timing differences were observed. These  
409 observations indicate that *D. pachea* might potentially have evolved mechanisms to buffer  
410 the effect of temperature on developmental speed. Such mechanisms might be of

411 importance to preserve the fitness of individuals exposed to extreme temperatures and  
412 important temperature variations during their development.

413

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648

649

650 **Tables**

651 **Table 1: Total numbers of pupae and synchronised cohorts used in for pupal timing**  
 652 **characterization**

Species	Temperature (°C)	Total number of pupae	Total number of synchronised cohorts
<i>D. pachea</i>	22	134	21
	25	76	11
	29	42	5
	32	141	21
<i>D. nannoptera</i>	25	40	13
<i>D. acanthoptera</i>	25	61	7

653

654 **Table 2: Summary of morphological markers used to stage pupae, according to**  
 655 **Bainbridges and Bownes (1981)**

Pupal stage	Description
1	Pupariation: extremity of trachea are everted, pupa does not move anymore.
2	Clear, white pupa.
3	Light pigmentation, dorsal trachea still visible.
4	Bubbles appear, dorsal trachea is not visible anymore, light pigmentation of the body.
5	Cranial extremity is retracted, distal extremity of wings appeared.
6	Yellow body is visible.
7	Pharate adult, eyes are not yet pigmented.
8	Eye discs become a bit yellower compared to the rest of the body.
9	Orange eyes, transparent wings are visible.
10	Deep red eyes.
11	Bristles are visible on the thorax.
12	Wings are clear grey, clear pigmentation of the body.
13	Wings are completely black, grey pigmentation appears on the body.

14	Meconium appeared under the form of a dark spot visible through the abdomen.
15	Eclosion of the adult.

656

657 **Figures**

658

659

660 **Figure 1: Timing of the embryonic and larval stages in *D. pachea* at 25°C.**

661 Embryo duration represents the time from egg laying to the hatching of the larva, based on  
662 time-lapse imaging (dotted line). Larval stages were determined based on mouth hook  
663 morphology of dissected larvae from synchronized cohorts, according to Strasburger (1935).  
664 Black dots indicate single observations (Dataset S2). Numbers correspond to the number of  
665 individuals observed at each stage.

666

667 **Figure 2: Durations of the embryonic development in various *Drosophila* species at  
668 25°C.**

669 The duration of total embryonic development of *D. pachea* (grey) was established based on  
670 time-lapse imaging. The data for the species other than *D. pachea* were extracted from:  
671 blue: Kuntz and Eisen, 2014 (duration up to the trachea filling stage, at 25°C), yellow: David  
672 and Clavel, 1966 (total embryonic development, at 25°C) and green: Powsner, 1935 (total  
673 embryonic development, at 25°C). The data used to establish the cladogram was extracted  
674 from Yassin (2013) and Lang et al. (2014).

675

676 **Figure 3: Progress of pupal development.**

677 A-D: Durations of developmental stages in *D. pachea* pupae at 22°C (A), 25°C (B), 29°C (C)  
678 and 32°C (D), observed at various time points. Temperatures are highlighted in blue colour  
679 tones according to the legend. Black dots indicate single observations (Dataset S3). E:  
680 Overlay of durations from panels A - D. F: Comparison of pupal development at 25°C in *D.*  
681 *pachea* (blue), *D. acanthoptera* (green) and *D. nanoptera* (yellow) based on observations  
682 of synchronized cohorts. The stages 8 to 12 were determined in *D. acanthoptera* by time-  
683 lapse imaging of developing pupae, after removal of the anterior part of the pupal case  
684 (dotted lines). Data of *D. melanogaster* (pink) and *D. guttifera* (purple) were retrieved from  
685 Bainbridge and Bownes (1981) and Fukutomi et al. (2017), respectively. These species were  
686 indicated by stars in the legend.

687

688

689

690 **Supplementary data**

691

692 **Figure S1: Mouth hook morphology at the three different larval instar.**

693 Larval mouth hook from A: first, B: second and C: third larval instar in *Drosophila pachea*.

694 The scale bar is 10  $\mu$ m.

695

696 **Figure S2: Pupal stages in *D. pachea*.**

697 Pupal stages of *D. pachea*, based on the characterization of *D. melanogaster* by Bainbridge

698 and Bones (1983) (Table 2). Pupae of each stage are presented in dorsal (D) and ventral (V)

699 view. The stage (Table 2) is indicated by a number. Arrows point to relevant morphological

700 markers: C: stage 3, dorsal trachea still visible; D: stage 4, bubbles appear and trachea not

701 visible; E: stage 5, distal margins of wings appear; F: stage 6, yellow body visible; G: stage

702 7, non-pigmented eyes visible; H: stage 8, yellow eyes appear; I: stage 9, orange eyes; J:

703 stage 10, red eyes; K: stage 11, thorax bristles visible; L: stage 12, grey wings; M: stage 13,

704 black wings; N: stage 14, meconium visible. The scale is 100  $\mu$ m.

705

706 **Movie S1: Time-lapse of embryonic development of *D. pachea* at 25 °C.**

707 Out of the 28 embryos, 12 completed their development up to the larva hatching. The 16

708 embryos that did not complete their embryonic development were excluded from the

709 analysis.

710

711 **Movie S2: Time-lapse of pupal development of *D. acanthoptera* from 52 h APF up to  
712 the emergence of the adult at 25°C**

713 Out of 5 pupae, 3 completed their development up to adult emergence. The two that died  
714 during the time-lapse were excluded from analysis.

715

716 **Dataset S1: Observations of embryonic development in *D. pachea* at 25°C**

717

718 **Dataset S2: Observations of larval development in *D. pachea* at 25°C**

719

720 **Dataset S3: Pupae cohorts for developmental timing characterization**

721 **Dataset S4: Observations of pupal development in *D. pachea*, *D. acanthoptera* and *D.*  
722 *nannotpera***

723

724 **Availability of data and material**

725 The movies S1 and S2 supporting the results of this article are available in the DRYAD

726 repository,

727 [https://datadryad.org/stash/share/dfhCAAtgopC4JY6qmkjK6Q\\_UEMmf2WSfc1gdETPPI7gk](https://datadryad.org/stash/share/dfhCAAtgopC4JY6qmkjK6Q_UEMmf2WSfc1gdETPPI7gk).

728

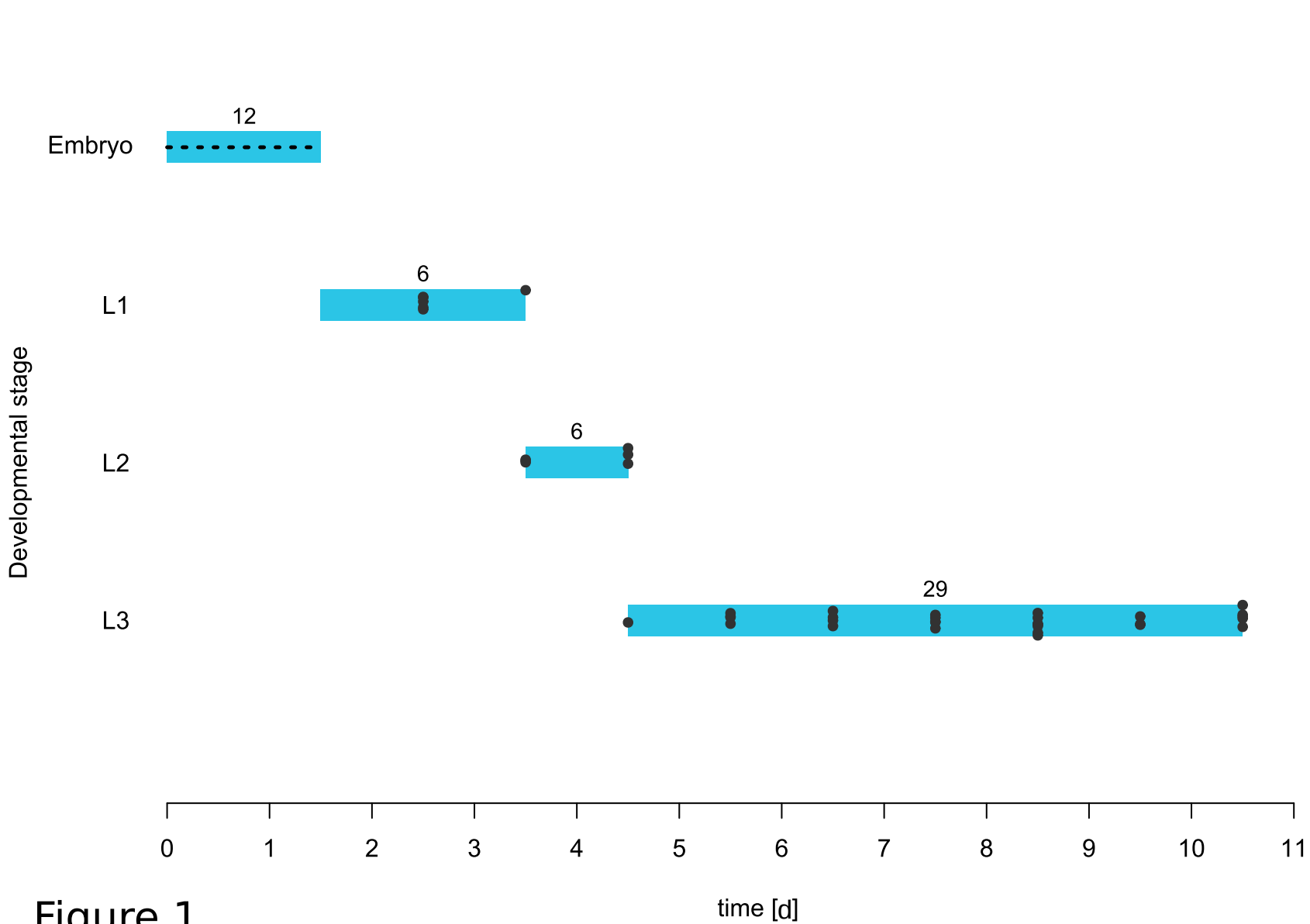


Figure 1

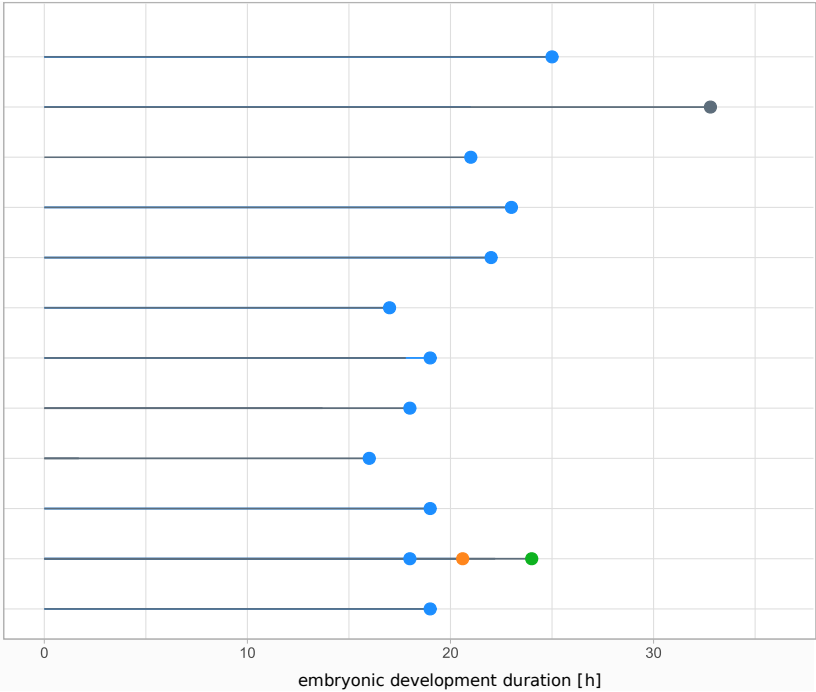
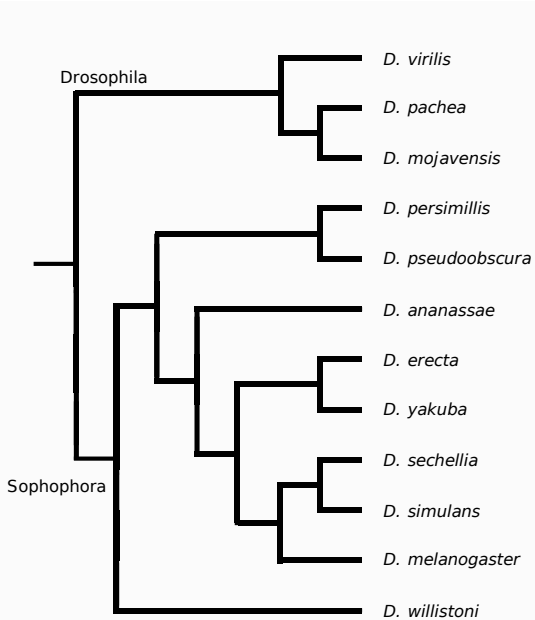


Figure 2



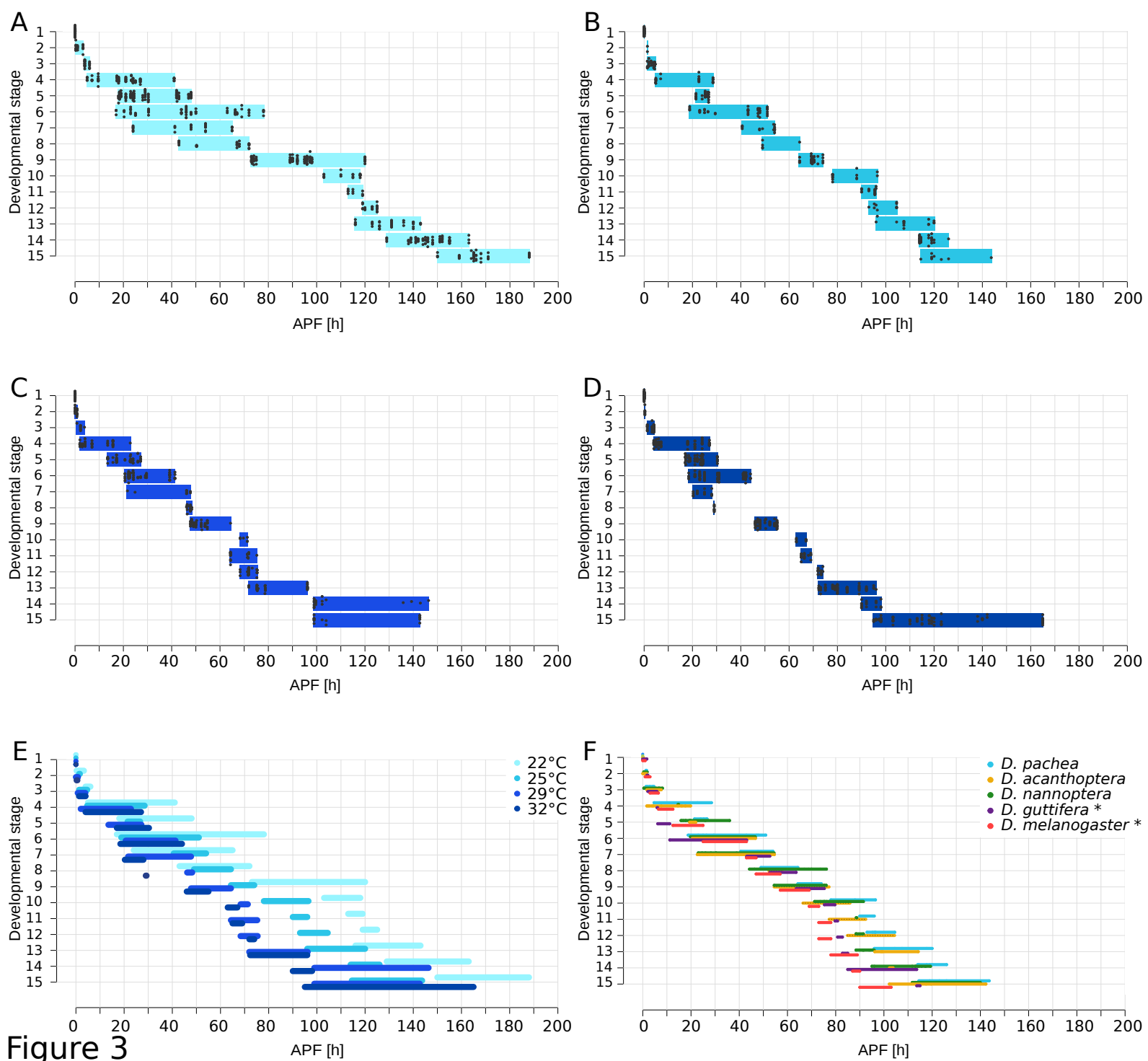


Figure 3

**A**

10  $\mu\text{m}$

**Figure S1**

**B****C**

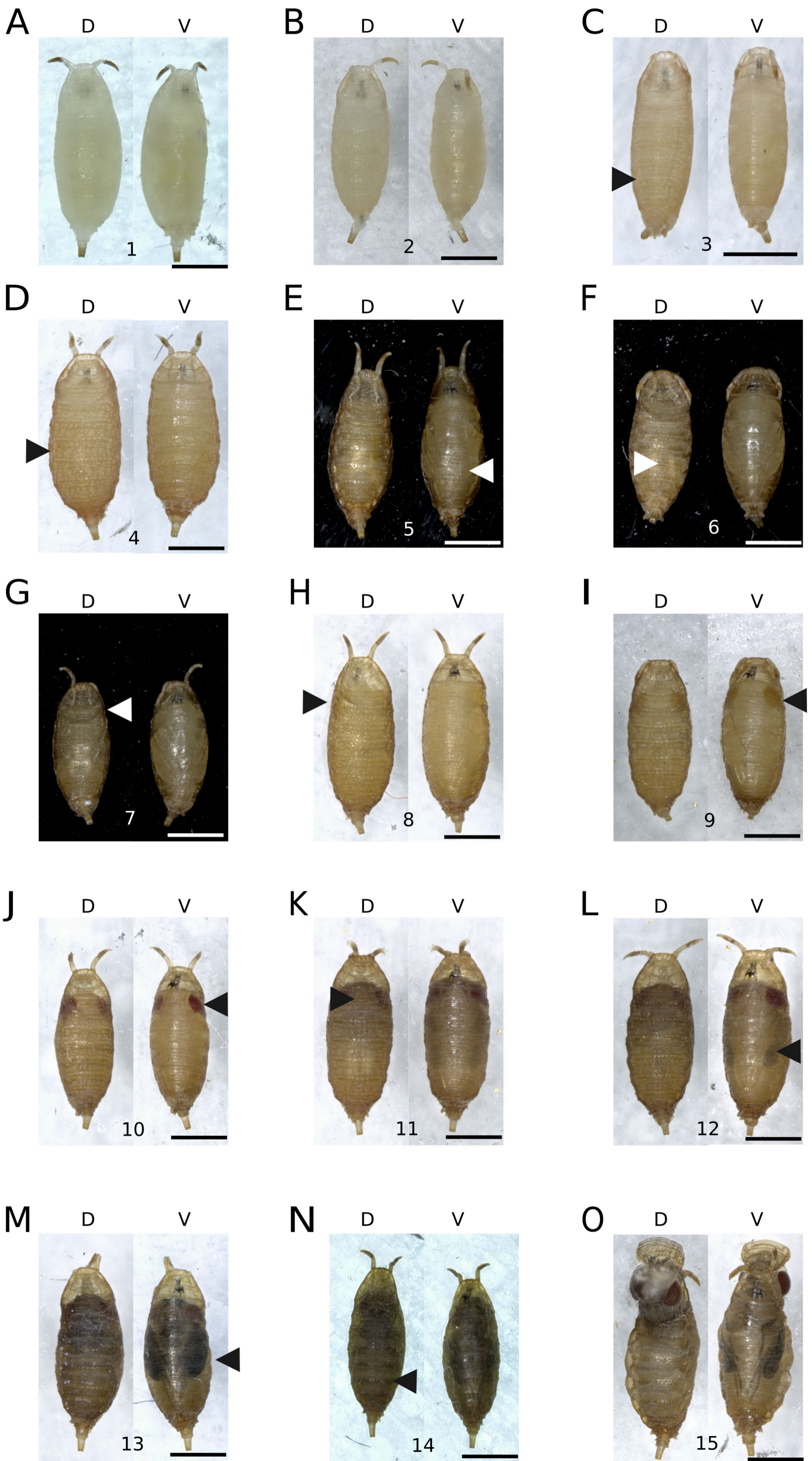


Figure S2