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

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

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Author contributions

BL and ML designed the experiments. BL generated and analysed the data. BL and ML wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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Abstract

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

Keywords

Drosophila pachea, pupal development, temperature, heterochrony

Highlights

- In poikilotherms, developmental speed usually increases with rearing temperature

- Global pupal development of *D. pachea* is similar at two different rearing temperatures

- Discrete temperature dependent timing differences at specific pupal stages

- *D. pachea* development is longer compared to other Drosophila species

- Temperature-buffering mechanisms may have evolved to ensure a proper development

1. Introduction

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

99   More than 1500 described species of the genus *Drosophila* (Bächli et al., 2021;

100   O'Grady and DeSalle, 2018) occupy a wide range of habitats with various climatic conditions

101   (Markow and O'Grady, 2008). A dozen of species have been reported to be cosmopolitan

102   species (Markow and O'Grady, 2008, 2005), such as *Drosophila melanogaster* (David and

103   Capy, 1988; Li and Stephan, 2006) that potentially dispersed with humans from Africa

104   around the globe (Mansourian et al., 2018). These species may be generalists but were also

105   found to be locally adapted to diverse environments (Kapun et al., 2020; Markow and

106   O'Grady, 2008). In contrast, the vast majority of species are restricted to certain continental

107   ranges or are endemic to a specific geographic region that encompasses a unique habitat

108   with specific food and climate conditions (Markow and O'Grady, 2008, 2005). Because of

109   their inability to disperse outside their habitat, these endemic species may have evolved

110   temperature-buffering mechanisms to ensure a constant developmental timing under

111   variable temperature conditions.

112   *Drosophila pachea* (Diptera : Drosophilidae) is endemic to the Sonoran desert in

113   Mexico and is an obligate specialist on decayed parts, or rot-pockets, of its single host plant,

114   the Senita cactus (*Lophocereus schottii*) (Gibbs et al., 2003; Heed and Kircher, 1965; Lang

115   et al., 2012; Markow and O'Grady, 2005). The micro-climate of the rot-pockets encompasses

116   important changes of temperature all along the year, with a recorded maximum variation

117   from 5°C to 42°C within 24 h (Gibbs et al., 2003). Living in an environment with large daily

118   and annual temperature changes may require a certain temperature robustness with respect

119   to developmental processes in poikiloterm species. We wondered if the developmental

120   timing in *D. pachea* may be sensitive to differing rearing temperatures. To test this, we first

121   determined the overall timing of the *Drosophila pachea* life-cycle. Then, we focussed on

122   pupal development at two different rearing temperatures to investigate differences in the

123   pupal timing. Finally, we compared these durations across closely related sister species

124   *Drosophila acanthoptera* (Diptera : Drosophilidae) and *Drosophila nannoptera* (Diptera :

125   Drosophilidae) to investigate potential species-specific developmental timing differences.

126

2. Materials and methods

2.1. Drosophila stock maintenance

129   Drosophila stocks were retrieved from the San Diego Drosophila Species Stock

130   Center (now The National Drosophila Species Stock Center, College of Agriculture and Life

131   Science, Cornell University, USA). The *D. pachea* stock 15090-1698.01 was established in

132   1997 from individuals caught in Arizona, USA. The *D. nannoptera* stocks 15090-1692.00 and

133   15090-1693.12 were established in 1992 from individuals caught in Oaxaca, Mexico. The *D.*

134   *acanthoptera* stock 15090-1693.00 was established in 1976 from individuals caught in

135   Oaxaca, Mexico (UCSC Drosophila species stock center San Diego, now The National

136   Drosophila Species stock center, Cornell University). These stocks have been kept in good

137   conditions at 25°C in our laboratory since 2012.

138   Flies were maintained in transparent plastic vials (25 x 95 mm, Dutscher) containing

139   about 10 mL of standard Drosophila medium. This medium was composed of 66.6 g/L of

140   cornmeal, 60 g/L of brewer's yeast, 8.6 g/L of agar, 5 g/L of methyl-4-hydroxybenzoate and

141   2.5% v/v ethanol (standard food). We added 40 µL of 5 mg/mL of 7-dehydrocholesterol

142   (7DHC) (Sigma, reference 30800-5G-F) dissolved in ethanol into the food for *D. pachea*, as

143   this species need this sterol for proper development (Heed and Kircher, 1965; Lang et al.,

144   2012; Warren et al., 2001) (standard *D. pachea* food). As a pupariation support, a piece of

145   paper sheet (1 cm x 4 cm, BenchGuard) was added to each vial. Stocks were kept at 25°C

146   or 29°C at a 12 h light:12 h dark photoperiodic cycle with a 30 min transition between light

147   (1080 lm) and dark (0 lm).

148

149   **2.2. Cohort synchronisation of *D. pachea* embryos and time-lapse recording of**

150   **embryonic development**

151   For collection of cohorts of synchronised embryos, about 250-500 adult flies were

152   transferred into a 9 x 6 cm plastic cylinder, closed by a net on the top and by a 5.5 cm

153   diameter petri-dish lid at the bottom. The petri-dish contained grape juice agar (24.0 g/L

154   agar, 26.4 g/L saccharose, 20% grape juice, 50% distilled water, 12% Tegosept [1.1 g/mL in

155   ethanol] (Dutscher), 4% 7-DHC (Sigma)) and 50-200 µL fresh baker's yeast as food source

156   and egg laying substrate on top. These plates are named hereinafter "food plates". Female

157   flies were let to lay eggs on the food plates for 1 h, thus embryos had a maximum age

158   difference of 60 min. Then, eggs were retrieved from food plates by filtering the yeast paste

159   through a 100 µm nylon mesh (BS, Falcon 352360).

160   For time-lapse imaging the chorion of embryos was removed by a 90 sec incubation

161   of the embryo-containing filter in 1.3% bleach (BEC Javel) under constant agitation until

162   about half of the embryos were floating at the surface of the bleach bath. Embryos were

163   extensively rinsed with tap water for at least 30 sec. Dechorionated embryos were then

164   gently glued on a cover slip (ThermoFisher) coated with Tesa glue. For coating, 50 cm TESA

165   tape was transferred into 25 mL n-heptane (Merck) and glue was let to dissolve overnight at

166 room temperature. A total of 15 µL of dissolved glues was finally pipetted onto a cover slip to

167 form a 5 x 20 mm rectangular stripe and n-heptane was let to evaporate. Embryos were

168 covered with 40 µL of Voltalef 10S halocarbon oil (VWR) to avoid desiccation. Live-imaging

169 was immediately launched inside a temperature and humidity controlled chamber at 25°C ±

170 0.1°C and 80% ± 1% humidity (Lang and Orgogozo, 2012; Lefèvre et al., 2021; Rhebergen

171 et al., 2016). Time-lapse acquisition was performed at an acquisition rate of 1 picture every

172 7.5 sec using a digital camera (Conrad 9-Megapixel USB digital microscope camera) and

173 Cheese software, version 3.18.1, on a computer with an ubuntu 16.04 linux operating

174 system. Movies were assembled with avconv (libav-tools).

175   Out of 28 embryos monitored, 12 (43%) pursued their development until hatching

176 while the others did not develop at all. Such mortality has been reported previously

177 (Jefferson, 1977; Pitnick, 1993) but potentially also dependent on the above-mentioned

178 bleach treatment. The embryos that died during the experiment were excluded from

179 analysis. Furthermore, the duration of hatching, which is the last stage of embryonic

180 development, has been shown to be more variable in comparison to the other embryonic

181 stages in various Drosophila species (Kuntz and Eisen, 2014). We thus measured both the

182 total embryonic duration, from collection up to larva hatching and the embryonic duration up

183 to the trachea gaz filling stage, which precedes the hatching stage.

184

185 **2.3. Cohort synchronisation of larvae, dissection and imaging of larval mouth hooks**

186   In order to collect cohorts of larvae at a synchronous developmental stage, we first

187 collected embryos from a 2 h egg laying interval (see above) that were placed on a food

188 plate together with fresh yeast. Freshly hatched larvae were retrieved from the yeast paste

189 with fine forceps (Dumont #5, Fine Science Tool) or by filtering the yeast through a nylon

190 mesh (see above). Larvae were transferred into vials containing standard *Drosophila*

191 *pachea* food and were examined once a day until all larvae had turned into pupae.

192   For imaging of the larval teeth, entire larvae were mounted in 20 µL dimethyl-

193 hydantoin formaldehyde (DMHF) medium (Entomopraxis) beneath a cover slip (0.17 mm ±

194 0.01 mm thick, ThermoScientific), which was gently pressed against the microscope slide

195 (ThermoScientific) to orient larval teeth in a flat, lateral orientation to the microscope

196 objective. Larval teeth were imaged at 100 or 400 fold magnification in bright field

197 illumination (Strasburger, 1935) using the microscope IX83 (Olympus). The instar stage of

198 each dissected individual was determined based on tooth morphology (Strasburger, 1935)

199   (Figure S1).

200

201   **2.4. Measurement of the duration of puparium formation in *D. pachea***

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

212

213   **2.5. Characterization of developmental timing in pupae**

214   The developmental duration of *D. pachea*, *D. nannoptera* and *D. acanthoptera* was
215   examined by observation of pupae at different time points after puparium formation (APF).
216   Synchronised pupae were obtained from each species by collecting so-called "white pupae"
217   that had just formed the puparium (Dataset S1). Specimens were collected with a wet brush
218   directly from stock vials. Individuals of the same cohort were placed onto moist Kimtech
219   tissue (Kimberly-Clark) inside a 5 cm diameter petri dish. Petri dishes with pupae were kept
220   at 25°C or 29°C inside plastic boxes, which also contained wet tissue paper. A group of
221   pupae resulting from a single collection event was considered as a synchronised cohort.
222   Developmental progress of synchronised cohorts (Table 1) was examined at various time
223   points by visual examination of the pupae using a stereomicroscope VisiScope SZB 200
224   (VWR) (Dataset S2). Developmental stages were assigned according to morphological
225   markers defined for *D. melanogaster* by Bainbridge and Bownes (1981) (Table 2). The
226   markers used to characterize stages 8 to 12 (eye, wing or body pigmentation, Table 2) were
227   not convenient for the characterization by direct observation of *D. acanthoptera* pupae as
228   these flies develop black eyes, as opposed to most other Drosophila species that have red
229   eyes. In addition, *D. acanthoptera* is generally less pigmented compared to *D. pachea* and
230   *D. nannoptera* (Pitnick and Heed, 1994) and pigmentation changes were not easily
231   detectable through the pupal case. Therefore, we additionally carried out time-lapse imaging

232   of one cohort with five *D. acanthoptera* pupae to investigate the developmental durations of

233   stages 8-12. The anterior part of the pupal case was removed, letting the head and the

234   anterior part of the thorax visible. Image acquisition was done at 25°C ± 0.1°C and 80% ±

235   1% humidity, as previously described. Time-lapse acquisition was performed as previously

236   described and recorded with the VLC media player, version 3.0 at an acquisition rate of 1

237   picture every 13:02 min. Two pupae died during acquisition and were excluded from the

238   analysis.

239

240   **2.6. Data analysis**

241   Data was manually entered into spreadsheets (Datasets S1 and S2) and analysis

242   was performed in R version 3.6 (R Core Team, 2014). Ages expressed in hours after pupa

243   formation were automatically calculated with respect to the time point of white pupa

244   collection.

245

246   **3. Results**

247   **3.1. *D. pachea* embryonic and larval development at 25°C last for about 33 h and 216**

248   **h, respectively**

249   We characterized the duration of embryonic and larval development in *D. pachea* at

250   25°C. The average duration of the total embryonic development in *D. pachea* at 25°C, until

251   hatching of the larva was 32 h 48 min ± 1 h 13 min (mean ± standard deviation ; n = 12)

252   (Figure 1). Embryonic development up to the trachea gas filling stage (see Material and

253   Methods for details) was estimated to be 26 h 48 min ± 1 h 13 min (mean ± standard

254   deviation ; n = 12). These durations appeared to be longer in *D. pachea* compared to those

255   reported for various other Drosophila species, such as *Drosophila melanogaster, Drosophila*

256   *simulans, Drosophila sechellia, Drosophila yakuba, Drosophila pseudoobscura, Drosophila*

257   *mojavensis* (Figure 2) (David and Clavel, 1966; Kuntz and Eisen, 2014; Powsner, 1935).

258   The total duration of *D. pachea* larval development on standard *D. pachea* food at

259   25°C was 9 days (approximately 216 h). The duration of the first and second instar larva

260   were about 2 days each and the third instar stage lasted for about 5 days (Figure 1). In *D.*

261   *melanogaster,* the total duration of the larval stage was about 5 days for larvae reared on

262   optimal food at 25°C, the first and second instars lasting for 1 day each, and the third instar

263   for three days, according to Strasburger, (1935). The larval development of *D. pachea*

264 appeared thus to be longer compared to those of *D. melanogaster* at 25°C.

265

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

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

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

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

288

3.3. The duration of pupal development in *D. pachea* is not affected by two different rearing temperatures

291   The duration of larval development appears to be sensitive to various environmental factors, such as diet (Matzkin et al., 2011), crowding, or access to food (Vijendravarma et al., 2013). Since pupal development is apparently less affected by such factors, we focussed on the pupal stage to investigate the effect of the rearing temperature on timing of development in *D. pachea*. Furthermore, in laboratory conditions, the life-cycle of *D. pachea*

296   reared at temperature below 25°C is prolonged which favors the accumulation of bacterial

297   infections in the food and decreased survival of the flies. At rearing temperature above 30°C,

298   the food dries out rapidly which causes problems for larvae to feed. We thus chose to

299   compare the development of *D. pachea* at 25°C and 29°C as these two temperatures allow

300   proper survival.

301   At 29°C, *D. pachea* pupae reached the pharate adult stage in less than 55 h, similar

302   to their development at 25°C (Figures 2A, 2C and 2D). However, pupal development is

303   accelerated at 29°C between stages 8 and 13 (beginning of eye pigmentation until the end

304   of body and wing pigmentation) compared to development at 25°C (Figures 2A, 2C and 2D).

305   However, stages 14 and 15 required more time at 29°C and resulted in a similar overall

306   developmental duration of about 100 - 145 h at 29°C compared to 115 - 145 h at 25°C

307   (Figure 3). In comparison, the overall pupal development of *D. melanogaster* lasts about 80

308   h at 29°C and 100 h at 25°C (Powsner, 1935). Thus, in *D. pachea* the rearing temperature

309   influences the relative progress of pupal development at particular stages. However, the

310   overall duration appears to be similar at both temperatures.

311

312   **4.   Discussion**

313

314   **4.1. A possible temperature-buffering mechanism during pupal development**

315   The trend of a decrease of developmental duration when rearing temperature

316   increases was not observed *in D. pachea*, overall pupal development duration being similar

317   at 25°C and 29°C. On the contrary, the duration of the overall pupal development decreases

318   with increasing rearing temperature in *D. melanogaster* (Ashburner and Thompson Jr, 1978;

319   Powsner, 1935). In addition, temperature fluctuations during pupal development of *D.*

320   *melanogaster* are known to either increase or decrease developmental speed (Ludwig and

321   Cable, 1933; Petavy et al., 2001). In this species, the first 24 h of pupal development are

322   more sensitive to temperature changes compared to the rest of the pupal stage (Ludwig and

323   Cable, 1933; Petavy et al., 2001). While *D. melanogaster* is a cosmopolitan species that

324   lives in a wide climate range (David and Capy, 1988), *D. pachea* is a desert species endemic

325   of the Sonora (Heed and Kircher, 1965; Markow and O'Grady, 2005). The mean daily

326   variations of temperature of this habitat are 18°C - 42°C in spring/summer and 6°C - 32°C in

327   fall/winter (Gibbs et al., 2003). *D. pachea* is found in the wild throughout the year but

328   undergoes a strong population decline during August, when the seasonal temperatures are

329   highest (Breitmeyer and Markow, 1998). However, adult *D. pachea* are particularly resistant

330   to high-temperatures and survive up to 44°C, while most other Drosophila species revealed

331   a decreasing survival already at 38°C (Stratman and Markow, 1998). Thus, this species may

332   have developed some heat resistance mechanisms or a certain tolerance to temperature

333   variations that would buffer temperature changes on the developmental progress. This

334   buffering effect could potentially be important for proper development since heat stress has

335   been reported to increase developmental instability in various species (Kristensen et al.,

336   2003; Nishizaki et al., 2015; Polak and Tomkins, 2013). However, the specific mechanism by

337   which temperature affects developmental stability is not well understood (Abrieux et al.,

338   2020; Breuker and Brakefield, 2003; Carvalho et al., 2017; Enriquez et al., 2018).

339   Alternatively, the observed buffering phenotype may be temperature independent

340   and could perhaps ensure the emergence of the adult fly at a particular moment of the day,

341   such as dawn or dusk, when the environmental temperature might be most suitable for the

342   freshly emerged individual. In the last pupal stage that corresponds to the adult emergence,

343   we observed timing variation between individuals in *D. pachea* (up to 30 h between

344   individuals). This variation could potentially depend on individual differences or on

345   environmental factors that we could not control, such as the light/dark illumination cycle at

346   the moment of adult emergence. Such circadian regulation of adult emergence has been

347   observed in various Drosophila species (Ashburner et al., 2004; Mark et al., 2021; Powsner,

348   1935; Soto et al., 2018). However, the important variation in the last pupal stage is also

349   found among individuals of the same cohort (Datasets S1 and S2). Future monitoring of the

350   emergence of adults from various cohorts collected at different moments of the day will be

351   necessary to test this hypothesis. Future investigations will be needed to further characterize

352   the potential temperature buffering effect during *D. pachea* development and to test the

353   influence of the circadian rhythm in this species. In addition, we must further assess

354   temperature dependent pupal development in a wider range of species that live in distinct

355   climate habitats.

356

357   **4.2. Conservation of the overall developmental progress during early pupal stages**

358   The detailed analysis of the timing of pupal stages revealed that the first stages 1 to

359   7 appear to be rather synchronous among *D. melanogaster* (Bainbridge and Bownes, 1981),

360   *D. guttifera* (Fukutomi et al., 2017), and the three closely related species *D. pachea*, *D.*

361   *acanthoptera* and *D. nannoptera*. Later on, pupal development appears to be more variable

362   between species. This may indicate the existence of some developmental constraints, which

363   are limitations of phenotypic variability due to inherent properties of the developmental

364   system (Smith et al., 1985; Wagner, 2014). Such constraints probably act on outgrowth of

365   adult organs from primordial structures, so-called imaginal discs, that develop throughout

366   larval stages but undergo extensive tissue growth during pupal development up to the

367   pharate adult stage. Thereafter, the timing of development seems to be less constrained and

368   interspecific variations were observed. At least a part of the variation in the pupal

369   developmental timing could be attributed to the developmental marker used. As the

370   coloration markers are qualitative, it is hard to define precise limits of each stage (ie. eyes

371   turn progressively from yellow to red). A solution might be to identify a combination of

372   multiple markers for each stage or to establish gene expression markers that are known to

373   account for specific developmental processes, as it has been recently done for eye

374   development (Escobedo et al., 2021) or male genitalia development (Vincent et al., 2019).

375

376   **4.3. Longer embryonic and larval development durations in *D. pachea* compared to**

377   **other Drosophila species**

378   The embryonic developmental duration at 25°C has been investigated in 11

379   drosophila species other than *D. pachea* (David and Clavel, 1966; Kuntz and Eisen, 2014;

380   Powsner, 1935) (Figure 2) and ranged from 16 h in *D. sechellia* to 25 h in *D. virilis* (Kuntz

381   and Eisen, 2014) (Figure 2), which are shorter compared to embryonic development of *D.*

382   *pachea* at the same temperature. Interspecific variation in the duration of embryonic

383   development might rely on genetic factors, as closely related species tend to have similar

384   embryonic developmental durations compared to those of distantly related ones (Figure 2).

385   Larval development is longer in *D. pachea* compared to those in *D. melanogaster*

386   (Bakker, 1959; Strasburger, 1935). However, the duration of this developmental stage has

387   been shown to be highly variable compared to the other life stages. In particular it has been

388   shown that larvae are very sensitive to food composition and to crowding that affect food

389   quality and food access (Matzkin et al., 2011; Vijendravarma et al., 2013). Food quality and

390   food access in turn prolong the larval developmental duration (Matzkin et al., 2011). This

391   effect of food on developmental duration might also probably affect embryonic and pupal

392   stages indirectly due to nutrient contribution from the adult and larval stages.

393   The slower development observed in *D. pachea* raised in the lab might be due to

394   variations in the ecdysone metabolism. In insects, ecdysone is first provided to the embryo

395   as maternal contribution and then directly produced by the individual (Lafont et al., 2012).

396   However, in *D. pachea* the first metabolic step of the ecdysone biosynthesis is different

397   compared to other insect species, the conversion of cholesterol into 7-dehydrocholesterol

398   being abolished (Lang et al., 2012). Instead, *D. pachea* metabolizes sterols produced by the

399   Senita cactus on which they feed, such as lathosterol, and potentially campestenol and

13   13

400   schottenol (Heed and Kircher, 1965), into steroid hormones differing in their side residues

401   (Lang et al., 2012). Therefore, in the wild, *D. pachea* may produce different variants of

402   ecdysone that may also differently affect developmental timing compared to the lab

403   conditions that only provide the single ecdysone precursor 7-dehydrocholesterol. Thus, it

404   would be interesting to compare developmental durations of *D. pachea* fed with standard *D.*

405   *pachea* food used in the lab or with their natural host plant, the Senita cactus. In addition,

406   further investigations would be needed to elucidate how temperature modulates these

407   mechanisms.

408

409   **4.5. Conclusion**

410   We investigated the effect of temperature on developmental speed in *D. pachea*, a

411   desert species. We characterized the timing of the life-cycle in this species and observed

412   prolonged developmental durations compared to other Drosophila species. The global

413   developmental duration at pupal stage is similar at two different rearing temperatures

414   although stage specific timing differences were observed. These observations indicate that

415   *D. pachea* might potentially have evolved mechanisms to buffer the effect of temperature on

416   developmental speed. Such mechanisms might be of importance to preserve the fitness of

417   individuals exposed to extreme temperatures and important temperature variations during

418   their development.

419

420   **Acknowledgements**

424   **Funding sources**

432   **References**

14   14

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637 **Tables**

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

639 **characterization**

Species	Temperature (°C)	Total number of pupae	Total number of synchronised cohorts
D. pachea	25	76	11
D. pachea	29	42	5
D. nannoptera	25	40	7
D. acanthoptera	25	61	15

640

641 **Table 2: Summary of morphological markers used to stage pupae, according to**

642 **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 appea, 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.

643

19

Figures

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

Embryo duration represents the time from egg laying to the hatching of the larva, based on time-lapse imaging. Larval stages were determined based on mouth hook morphology of dissected larvae from synchronised cohorts, according to Strasburger (1935). Numbers correspond to the number of individuals observed at each stage.

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

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

Figure 3: Progress of pupal development in *D. pachea*, *D. nannoptera*, *D. acanthoptera*, *D. guttifera* and *D. melanogaster* at 25°C and 29°C.

A: Synchronised cohorts of *D. pachea* pupae at 25°C observed at various time points. The light blue boxes indicate the period of each stage. Grey dots indicate single observations. For each stage, the first number corresponds to the number of pupae observed at that stage, and the second to the number of cohorts from which they originate. B: Comparison of pupal development at 25°C in *D. pachea* (blue), *D. acanthoptera* (green) and *D. nannoptera* (yellow) based on observations of synchronized cohorts. The stages 8 to 12 were determined in *D. acanthoptera* by time-lapse imaging of developing pupae, after removal of the anterior part of the pupal case (dotted lines). Data of *D. melanogaster* (pink) and *D. guttifera* (purple) were retrieved from Bainbridge and Bownes (1981) and Fukutomi et al. (2017), respectively. C: Synchronised cohorts of *D. pachea* pupae at 29°C observed at various time points. The dark blue boxes correspond to the duration of each stage, the grey dots to single observations, and the first and second numbers are the number of observed pupae in each stage and number of cohorts from which they originate. D: Combined observation of developmental progress in *D. pachea* at 25°C (light blue) and 29°C (dark blue).

681 **Supplementary data**

682

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

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

685 The scale bar is 10 µm.

686

687 **Dataset S1: Pupae cohorts for developmental timing characterization**

688

689 **Dataset S2: Row data of the observations of pupal development in *D. pachea* at 25°C**

690 **and 29°C, and in *D. acanthoptera* and *D. nannotpera* at 25°C**

691

Developmental stage

Stage	Value
Embryo	12
L1	6
L2	6
L3	29

Time [days]

Drosophila

Sophophora

D. virilis
D. pachea
D. mojavensis
D. persimillis
D. pseudoobscura
D. ananassae
D. erecta
D. yakuba
D. sechellia
D. simulans
D. melanogaster
D. willistoni

embryonic development duration [h]