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.

Abstract

Rearing temperature is correlated with the timing and speed of development in a wide range of poikilotherm 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.

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

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

2. Materials and methods

2.1. *Drosophila* stock maintenance

Drosophila stocks were retrieved from the San Diego *Drosophila* Species Stock Center (now The National *Drosophila* Species Stock Center, College of Agriculture and Life Science, Cornell University, USA). The *D. pachea* stock 15090-1698.01 was established in 1997 from individuals caught in Arizona, USA. The *D. nanoptera* stocks 15090-1692.00 and

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

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

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

For collection of cohorts of synchronised embryos, about 250-500 adult flies were transferred into a 9 x 6 cm plastic cylinder, closed by a net on the top and by a 5.5 cm diameter petri-dish lid at the bottom. The petri-dish contained grape juice agar (24.0 g/L agar, 26.4 g/L saccharose, 20% grape juice, 50% distilled water, 12% Tegosept [1.1 g/mL in ethanol] (Dutscher), 4% 7-DHC (Sigma)) and 50-200 µL fresh baker's yeast as food source and egg laying substrate on top. These plates are named hereinafter "food plates". Female flies were let to lay eggs on the food plates for 1 h, thus embryos had a maximum age difference of 60 min. Then, eggs were retrieved from food plates by filtering the yeast paste through a 100 µm nylon mesh (BS, Falcon 352360).

For time-lapse imaging the chorion of embryos was removed by a 90 sec incubation of the embryo-containing filter in 1.3% bleach (BEC Javel) under constant agitation until about half of the embryos were floating at the surface of the bleach bath. Embryos were extensively rinsed with tap water for at least 30 sec. Dechorionated embryos were then gently glued on a cover slip (ThermoFisher) coated with Tesa glue. For coating, 50 cm TESA tape was transferred into 25 mL n-heptane (Merck) and glue was let to dissolve overnight at

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

Out of 28 embryos monitored, 12 (43%) pursued their development until hatching while the others did not develop at all. Such mortality has been reported previously (Jefferson, 1977; Pitnick, 1993) but potentially also dependent on the above-mentioned bleach treatment. The embryos that died during the experiment were excluded from analysis. Furthermore, the duration of hatching, which is the last stage of embryonic development, has been shown to be more variable in comparison to the other embryonic stages in various *Drosophila* species (Kuntz and Eisen, 2014). We thus measured both the total embryonic duration, from collection up to larva hatching and the embryonic duration up to the trachea gaz filling stage, which precedes the hatching stage.

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

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

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

(Figure S1).

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

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

2.5. Characterization of developmental timing in pupae

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

of one cohort with five *D. acanthoptera* pupae to investigate the developmental durations of stages 8-12. The anterior part of the pupal case was removed, letting the head and the anterior part of the thorax visible. Image acquisition was done at $25^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $80\% \pm 1\%$ humidity, as previously described. Time-lapse acquisition was performed as previously described and recorded with the VLC media player, version 3.0 at an acquisition rate of 1 picture every 13:02 min. Two pupae died during acquisition and were excluded from the analysis.

2.6. Data analysis

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

3. Results

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

We characterized the duration of embryonic and larval development in *D. pachea* at 25°C . The average duration of the total embryonic development in *D. pachea* at 25°C , until hatching of the larva was $32\text{ h }48\text{ min} \pm 1\text{ h }13\text{ min}$ (mean $\pm$ standard deviation ; $n = 12$) (Figure 1). Embryonic development up to the trachea gas filling stage (see Material and Methods for details) was estimated to be $26\text{ h }48\text{ min} \pm 1\text{ h }13\text{ min}$ (mean $\pm$ standard deviation ; $n = 12$). These durations appeared to be longer in *D. pachea* compared to those reported for various other *Drosophila* species, such as *Drosophila melanogaster*, *Drosophila simulans*, *Drosophila sechellia*, *Drosophila yakuba*, *Drosophila pseudoobscura*, *Drosophila mojavensis* (Figure 2) (David and Clavel, 1966; Kuntz and Eisen, 2014; Powsner, 1935).

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

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

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

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).

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*.

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).

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

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*

reared at temperature below 25°C is prolonged which favors the accumulation of bacterial infections in the food and decreased survival of the flies. At rearing temperature above 30°C, the food dries out rapidly which causes problems for larvae to feed. We thus chose to compare the development of *D. pachea* at 25°C and 29°C as these two temperatures allow proper survival.

At 29°C, *D. pachea* pupae reached the pharate adult stage in less than 55 h, similar to their development at 25°C (Figures 2A, 2C and 2D). However, pupal development is accelerated at 29°C between stages 8 and 13 (beginning of eye pigmentation until the end of body and wing pigmentation) compared to development at 25°C (Figures 2A, 2C and 2D). However, stages 14 and 15 required more time at 29°C and resulted in a similar overall developmental duration of about 100 - 145 h at 29°C compared to 115 - 145 h at 25°C (Figure 3). In comparison, the overall pupal development of *D. melanogaster* lasts about 80 h at 29°C and 100 h at 25°C (Powsner, 1935). Thus, in *D. pachea* the rearing temperature influences the relative progress of pupal development at particular stages. However, the overall duration appears to be similar at both temperatures.

4. Discussion

4.1. A possible temperature-buffering mechanism during pupal development

The trend of a decrease of developmental duration when rearing temperature increases was not observed in *D. pachea*, overall pupal development duration being similar at 25°C and 29°C. On the contrary, the duration of the overall pupal development decreases with increasing rearing temperature in *D. melanogaster* (Ashburner and Thompson Jr, 1978; Powsner, 1935). In addition, temperature fluctuations during pupal development of *D. melanogaster* are known to either increase or decrease developmental speed (Ludwig and Cable, 1933; Petavy et al., 2001). In this species, the first 24 h of pupal development are more sensitive to temperature changes compared to the rest of the pupal stage (Ludwig and Cable, 1933; Petavy et al., 2001). While *D. melanogaster* is a cosmopolitan species that lives in a wide climate range (David and Capy, 1988), *D. pachea* is a desert species endemic of the Sonora (Heed and Kircher, 1965; Markow and O'Grady, 2005). The mean daily variations of temperature of this habitat are 18°C - 42°C in spring/summer and 6°C - 32°C in fall/winter (Gibbs et al., 2003). *D. pachea* is found in the wild throughout the year but undergoes a strong population decline during August, when the seasonal temperatures are highest (Breitmeyer and Markow, 1998). However, adult *D. pachea* are particularly resistant to high-temperatures and survive up to 44°C, while most other *Drosophila* species revealed

a decreasing survival already at 38°C (Stratman and Markow, 1998). Thus, this species may have developed some heat resistance mechanisms or a certain tolerance to temperature variations that would buffer temperature changes on the developmental progress. This buffering effect could potentially be important for proper development since heat stress has been reported to increase developmental instability in various species (Kristensen et al., 2003; Nishizaki et al., 2015; Polak and Tomkins, 2013). However, the specific mechanism by which temperature affects developmental stability is not well understood (Abrieux et al., 2020; Breuker and Brakefield, 2003; Carvalho et al., 2017; Enriquez et al., 2018).

Alternatively, the observed buffering phenotype may be temperature independent and could perhaps ensure the emergence of the adult fly at a particular moment of the day, such as dawn or dusk, when the environmental temperature might be most suitable for the freshly emerged individual. In the last pupal stage that corresponds to the adult emergence, we observed timing variation between individuals in *D. pachea* (up to 30 h between individuals). This variation could potentially depend on individual differences or on environmental factors that we could not control, such as the light/dark illumination cycle at the moment of adult emergence. Such circadian regulation of adult emergence has been observed in various *Drosophila* species (Ashburner et al., 2004; Mark et al., 2021; Powsner, 1935; Soto et al., 2018). However, the important variation in the last pupal stage is also found among individuals of the same cohort (Datasets S1 and S2). Future monitoring of the emergence of adults from various cohorts collected at different moments of the day will be necessary to test this hypothesis. Future investigations will be needed to further characterize the potential temperature buffering effect during *D. pachea* development and to test the influence of the circadian rhythm in this species. In addition, we must further assess temperature dependent pupal development in a wider range of species that live in distinct climate habitats.

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

The detailed analysis of the timing of pupal stages revealed that the first stages 1 to 7 appear to be rather synchronous among *D. melanogaster* (Bainbridge and Bownes, 1981), *D. guttifera* (Fukutomi et al., 2017), and the three closely related species *D. pachea*, *D. acanthoptera* and *D. nannopecta*. Later on, pupal development appears to be more variable between species. This may indicate the existence of some developmental constraints, which are limitations of phenotypic variability due to inherent properties of the developmental system (Smith et al., 1985; Wagner, 2014). Such constraints probably act on outgrowth of adult organs from primordial structures, so-called imaginal discs, that develop throughout

larval stages but undergo extensive tissue growth during pupal development up to the pharate adult stage. Thereafter, the timing of development seems to be less constrained and interspecific variations were observed. At least a part of the variation in the pupal developmental timing could be attributed to the developmental marker used. As the coloration markers are qualitative, it is hard to define precise limits of each stage (ie. eyes turn progressively from yellow to red). A solution might be to identify a combination of multiple markers for each stage or to establish gene expression markers that are known to account for specific developmental processes, as it has been recently done for eye development (Escobedo et al., 2021) or male genitalia development (Vincent et al., 2019).

4.3. Longer embryonic and larval development durations in *D. pachea* compared to other *Drosophila* species

The embryonic developmental duration at 25°C has been investigated in 11 drosophila species other than *D. pachea* (David and Clavel, 1966; Kuntz and Eisen, 2014; Powsner, 1935) (Figure 2) and ranged from 16 h in *D. sechellia* to 25 h in *D. virilis* (Kuntz and Eisen, 2014) (Figure 2), which are shorter compared to embryonic development of *D. pachea* at the same temperature. Interspecific variation in the duration of embryonic development might rely on genetic factors, as closely related species tend to have similar embryonic developmental durations compared to those of distantly related ones (Figure 2).

Larval development is longer in *D. pachea* compared to those in *D. melanogaster* (Bakker, 1959; Strasburger, 1935). However, the duration of this developmental stage has been shown to be highly variable compared to the other life stages. In particular it has been shown that larvae are very sensitive to food composition and to crowding that affect food quality and food access (Matzkin et al., 2011; Vijendravarma et al., 2013). Food quality and food access in turn prolong the larval developmental duration (Matzkin et al., 2011). This effect of food on developmental duration might also probably affect embryonic and pupal stages indirectly due to nutrient contribution from the adult and larval stages.

The slower development observed in *D. pachea* raised in the lab might be due to variations in the ecdysone metabolism. In insects, ecdysone is first provided to the embryo as maternal contribution and then directly produced by the individual (Lafont et al., 2012). However, in *D. pachea* the first metabolic step of the ecdysone biosynthesis is different compared to other insect species, the conversion of cholesterol into 7-dehydrocholesterol being abolished (Lang et al., 2012). Instead, *D. pachea* metabolizes sterols produced by the *Senita* cactus on which they feed, such as lathosterol, and potentially campestenol and

schottenol (Heed and Kircher, 1965), into steroid hormones differing in their side residues (Lang et al., 2012). Therefore, in the wild, *D. pachea* may produce different variants of ecdysone that may also differently affect developmental timing compared to the lab conditions that only provide the single ecdysone precursor 7-dehydrocholesterol. Thus, it would be interesting to compare developmental durations of *D. pachea* fed with standard *D. pachea* food used in the lab or with their natural host plant, the Senita cactus. In addition, further investigations would be needed to elucidate how temperature modulates these mechanisms.

4.5. Conclusion

We investigated the effect of temperature on developmental speed in *D. pachea*, a desert species. We characterized the timing of the life-cycle in this species and observed prolonged developmental durations compared to other *Drosophila* species. The global developmental duration at pupal stage is similar at two different rearing temperatures although stage specific timing differences were observed. These observations indicate that *D. pachea* might potentially have evolved mechanisms to buffer the effect of temperature on developmental speed. Such mechanisms might be of importance to preserve the fitness of individuals exposed to extreme temperatures and important temperature variations during their development.

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Tables

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

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

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

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).

Supplementary data

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

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

The scale bar is 10 μ m.

Dataset S1: Pupae cohorts for developmental timing characterization

Dataset S2: Row data of the observations of pupal development in *D. pachea* at 25°C and 29°C, and in *D. acanthoptera* and *D. nannotpera* at 25°C