Thermal Plasticity of Stage Specific Development Time in *Drosophila melanogaster* under Shifting Temperatures

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Abstract:

Thermal shifts and fluctuations are inherent aspects of the natural environment. How different developmental stages of the fly respond to thermal fluctuations and shifts are not properly understood. In view of this, we studied how durations of different developmental stages are affected by current and previous stage developmental temperatures when they experience upshifts and downshifts of temperature during developmental transitions. The study revealed a complex interaction of current and prior stage temperatures in shaping development time. The egg-stage temperature is found to exert its effect on both larval and pupal stage. However, the impacts of larva-pupa thermal shifts on pupal and overall development time are greater than that of egg-larva thermal shifts. As one would predict, a prior cold temperature extends subsequent pupal duration, while prior warm condition reduces it. Surprisingly, for larval duration, both prior warm and cold condition had the same effect- both led to significant reductions in subsequent larval stage duration. The warm egg temperature had an accelerating effect on larval development as expected. The other shift from cold egg to warm larval condition that also made development faster than constant warm rearing, was possibly caused by a shift from prior sub-optimal to optimal thermal condition used in the experiment. We also found warm larval stage followed by cold pupal stage makes development time shorter than cold larval followed by warm pupal rearing, suggesting a dominating role of the early-stage temperature in determining overall course of development. And lastly, we also observed two subsequent thermal shifts leave a decelerating effect on pupal development, possibly revealing a difficulty in developmental adjustments in the face of frequent thermal changes.

Keywords: Development time, *Drosophila*, thermal plasticity, thermal shift

Introduction

Temperature influences diverse aspects of biology such as development, physiology, metabolism, behaviour, and life history. Variation in temperature is an inherent aspect of the natural environment and some organisms are affected more by thermal changes than the others. Thermal effects are more pronounced in ectotherms as their body closely tracks the ambient temperature (Addo-Bediako et al., 2000; Angilletta, 2009). Effects of temperature on organisms are manifested through thermal plasticity of different biological traits. Studies on thermal plasticity have gained momentum in past few decades as increasing attention is now being paid on mechanistic and adaptive aspects of plasticity (Trotta et al., 2006; Cooper et al., 2012; McDonald et al., 2018; Lafuente et al., 2024). However, most studies exploring thermal plasticity involve investigating organismal performance at a range of constant temperatures in the laboratory (Trotta et al., 2006; Klepsatel et al., 2019; Lafuente et al., 2024). While such studies offer insights into understanding plastic and adaptive responses of organisms to different thermal environments, how temporal changes in temperature during development and lifetime of an organism influence its biology, is not clearly understood. In view of this, we were interested to study how shifting temperatures during pre-adult stages influence temporal coordination of development. We used the ectotherm *Drosophila melanogaster* for our study.

Drosophila life cycle consists of egg, larva, pupa and the adult. During its developmental cycle, the four life stages of the fly might encounter dramatically varied surroundings. The egg stage is immobile and occurs mostly on the surface of rotting fruits; the larval stage is movable but normally restricted within the fruit; larvae generally wander outside the rotting fruit and pupariate nearby; and the adult stage is not within a substrate but is completely mobile (Sokolowski et al., 1986; Powell, 1997). Consequently, the temperature that each of these four life stages experiences can be dramatically different due to daily temperature fluctuations, heatwaves, and substrate differences. Previous studies reveal thermal tolerance and reaction norms may change between life stages, indicating some stages may be more sensitive to thermal changes than others (e.g. Kingsolver et al., 2011; Austin and Moehring, 2013; Blanckenhorn et al., 2014; van Heerwaarden et al., 2014;). Therefore, we speculate thermal changes during the course of development may have dissimilar impacts on duration of different pre-adult stages. Thermal plasticity of development time has been studied extensively in *Drosophila*, and like majority of ectotherms, cold temperature extends overall

development time of the fly (Davidson and Davidson, 1944; Partridge et al., 1994; Al-Saffar et al., 1995; Ghosh et al., 2013). Developmental changes during embryonic/egg stage are found to be temporally robust across a wide range of temperatures in flies, when tested at various constant temperatures (Chong et al., 2018). Similarly, various stages of pre-adult development like duration of larval instars and pupal stage show proportionate increase with decreasing temperature (Ghosh et al., 2013). The optimal temperature for the model species *Drosophila melanogaster* is 25˚C, while 17˚C stretches the development by two-fold (Ghosh et al., 2013). All pre-adult developmental stages like egg hatching, larval duration, pupal stage are increased by two-fold at 17˚C compared to 25˚C (Ghosh et al., 2013). While there is ample data available for thermal plasticity of development time in constant warm *vs.* constant cold conditions, there is little understanding about what fluctuating temperatures do to *Drosophila* development. The question is relevant given in nature most organisms including flies are frequently subjected to changing temperatures (Angilletta, 2009; Garrity et al., 2010). Changes in temperature can manifest through regular diurnal or seasonal fluctuations, or it can also be evident as sudden rise or fall in temperature. Some studies have employed 12- or 24-hour thermal fluctuations to study how those affect flies (Petavy et al., 2001; Manenti et al., 2015, 2021; Tobler et al., 2015), but we know little about how development adjusts itself when there are instances of abrupt thermal shifts during the course of development. In view of this, we were interested to see how thermal shifts during developmental transitions affect subsequent development time of the fly.

We investigated how thermal changes across different developmental stages like egg, larva and pupa shape duration of subsequent developmental stages and overall development time of flies. What kind of carryover effects are observed when developmental stages of flies are shifted from warm to cold and vice versa? If the current rearing condition is cold but previous stage was warm, does the earlier stage temperature show a carryover effect by shortening the duration of the later stage? Conversely, if a fly was initially developing under cold and then it is brought to warm condition, does it take longer than normal to develop due to carryover effect of the previous cold rearing? To address these questions, we subjected individual *Drosophila melanogaster* flies to thermal shifts at developmental transitions, *e.g.* either upon hatching, or pupariation, or at both transitions. We used two alternating temperatures, 25˚C and 17˚C to upshift or downshift the flies immediately after larval hatching and/or pupariation.

Materials and Methods

Population ancestry

We used a large, outbred laboratory population of *Drosophila melanogaster* named CO-mix for this study. It is a descendant of ancestral CO-populations (Rose et al., 1984; Chippindale et al., 1993, 1997). 5 replicates of CO populations were mixed to create the CO-mix population. This population as well as its ancestors are being maintained at 25˚C. CO-mix flies are maintained on a 14 days discrete generation cycle, under 24 hours light, and ~70% relative humidity on standard cornmeal medium. Rearing density is controlled at ~70 eggs per 6 ml food in a vial. Twenty five such vials are reared every generation for growing the flies, and upon eclosion, all flies are transferred to a population cage, and the eggs are collected after 3 days and incubated in vials, to initiate the next generation.

Experimental protocol

Stage specific development time was assayed at different thermal combinations. *Drosophila* development includes three major developmental transitions, namely egg to larva (hatching), larva to pupa (pupariation), and pupa to adult (eclosion). For different thermal treatments, thermal shifts between the two treatment temperatures, namely, 17˚C and 25˚C were performed either at hatching, or at pupariation, or at both. Total eight thermal combinations were used for the experiment, as shown in Table 1.

Set	Egg	Larva	Pupa
1	25˚C	25˚C	25˚C
2	17˚C	25˚C	25˚C
3	25˚C	25˚C	17˚C
4	17˚C	25˚C	17˚C
5	17˚C	17˚C	17˚C
6	25˚C	17˚C	17˚C
7	17˚C	17˚C	25˚C
8	25˚C	17˚C	25˚C

Table 1: Schematic of the 8 thermal treatments for stage-specific development time assay

For each thermal treatment, 80 microcentrifuge tubes (MCTs) were used, each containing 300 ml of cornmeal food. A small agar piece containing a single egg was placed in each MCT within 2 hours of egg lay from the CO-mix populations. In *D. melanogaster*, eggs appear smooth and swollen when larvae are inside, and after larval hatching, the eggs appear crumpled. This way hatched vs. unhatched eggs can be distinguished clearly under microscope. For treatments in which eggs were reared at 25˚C (Treatments 1, 3, 6, 8), eggs were monitored for larval hatching 12 hours onwards post egg collection, at every 2-hour intervals. The time of larval hatching was noted down for each MCT. Hatching check was continued till 26 hours post egg collection, when no new eggs hatched for 3 consecutive checks. Eggs that failed to hatch and became blackened, were discarded. For treatments 6 and 8, individual tubes were shifted from 25˚C upon hatching, and incubated at 17˚C. For treatments 1 and 3, MCTs were maintained at 25˚C. Similar protocol was followed for eggs reared at 17˚C (Treatments 2, 4, 5, 7), and larval hatching was monitored from 32 hours up to 42 hours post egg collection. For treatments 2 and 4 MCTs were shifted from 17˚C upon hatching, and incubated at 25˚C while for treatments 5 and 7 MCTs were maintained at 17˚C throughout egg and larval stages.

Hatched larvae were allowed to grow at specific temperatures and subsequently monitored for pupariation after few days. MCTs were monitored for formation of pupae on inner wall of the tube or lower side of the tube's lid every 4 hours. Upon pupariation, the time was noted and tubes were further kept at 25˚C or 17˚C, for treatments that involved no shifts at pupariation. Checks were continued till no new pupa was observed for 3 successive checks. Larvae that failed to reach the wandering stage or pupariate were discarded. For treatments involving shifts at pupariation (Treatments 3, 4, 7, 8), MCTs with pupae were shifted to specified temperature after noting their pupariation time. MCTs containing pupae were incubated at specific temperatures to allow metamorphosis to occur. As metamorphosis ensued over next few days and pupae became dark indicating development of adult flies within pupal cases, individual tubes were monitored for eclosion. As eclosion started, checks were continued every 4 hours and time was noted. Eclosion checks were continued till no new eclosion was observed for 3 successive checks.

Data analysis

For any given MCT, egg duration was calculated by subtracting the midpoint of the 2 hour-long egg collection window from time of larval hatching. Larval duration in a given tube was

calculated by subtracting hatching time from the time of pupariation. Pupal duration was calculated by subtracting pupariation time from the time of eclosion. Total egg to adult development time was also noted for individual flies in all 8 thermal treatments.

ANOVA was performed to compare egg to adult development time across all 8 thermal treatments with treatment taken as fixed factor. For comparing egg duration at 17˚C (N = 151) and 25˚C (N = 116), one way analysis of variance (ANOVA) was performed on individual egg duration data. Thermal treatment was treated as fixed factor. Compared to 25˚C, 17˚C increases duration of developmental stages two-fold in *D. melanogaster*, and therefore it is obvious that an ambient temperature of 25˚C *vs.* 17˚C would significantly affect the duration of any given developmental stage (Ghosh et al., 2013). In this study we rather investigated when the temperature is same for a given developmental stage (*e.g.* larval), how it is affected by contrasting temperature in the preceding stage/s. Therefore, two separate ANOVAs were performed for larval duration, one for comparing treatments in which larval stage was reared at 25˚C (25-25 vs. 17-25), another for treatments in which larvae were reared at 17˚C (17-17 vs. 25-17). Treatment was taken as a fixed factor, and ANOVA was performed for replicate MCT data points. Like larval duration, for pupal duration two separate ANOVAs were performed, one for comparing all thermal treatments in which the ambient pupal rearing temperature was 25˚C, another for comparing duration of all treatments in which pupae were reared at 17˚C. For one pupal duration ANOVA, four groups were compared (25-25-25 vs. 17-25-25 vs. 25-17-25 vs. 17-17-25). In the other pupal duration ANOVA, comparisons were for 17-17-17 vs. 25-17-17 vs.17-25-17 vs. 25-25-17. Post hoc comparisons were done using Tukey's honest significant difference (HSD) test for analysis of pupal duration and total development time. Data analyses were carried out on JMP Pro 17. Homoscedasticity of variance among different groups was verified by Levene's test performed on R.

Results and Discussion

Carryover effect of prior stage temperature on Development is more pronounced when current temperature is 17˚C as opposed to 25˚C

The effect of thermal treatment on total or egg to adult development time was highly significant ($p < 0.0001$). Post hoc comparisons revealed total development time was significantly different across all 8 treatments (Fig. 1). Effect of egg and larval rearing

temperatures on duration of subsequent developmental stages revealed an array of interesting trends, detailed in following paragraphs. It is important to note that the effect of previous stages' temperature on subsequent development time was more pronounced if the later stage temperature was 17˚C instead of 25˚C. Given development is longer at 17˚C, any change caused by thermal environments of preceding stages got magnified at this temperature, while the effects were small at 25˚C because of shorter duration of development at this temperature.

Shift at egg-larva transition impacts total development time but the effect is small

Total development time was longest at 17-17-17 (434 h), followed by 25-17-17 (384 h). At 25-25-25 it was the shortest (212 h), followed by 17-25-25 (224 h) (Fig. 1, H *vs.* G). This suggests though a shift upon egg-larva transition (shift 1) significantly affects total egg to adult development time, the effect is small such that overall development time in these treatments remain closest to the development time observed at constant temperatures.

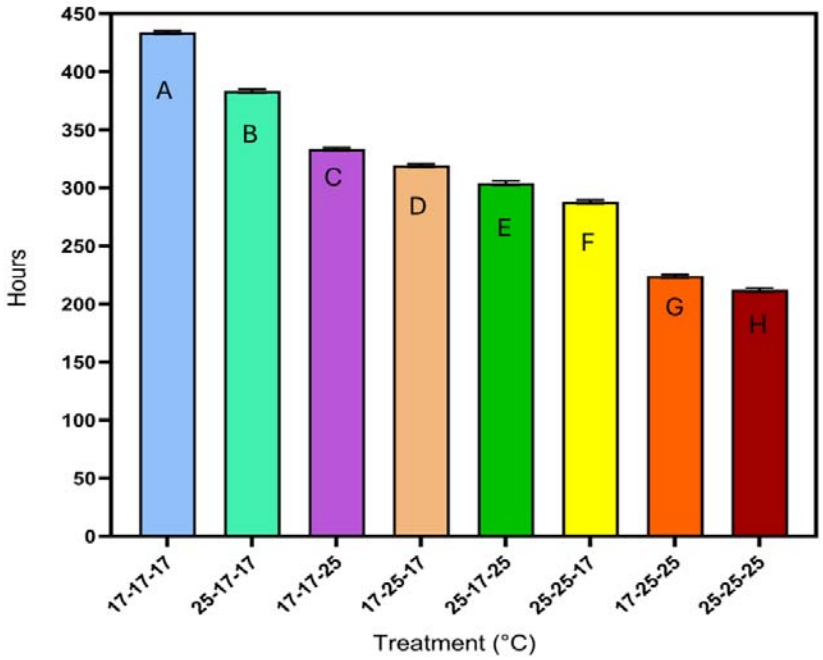

Figure 1: Mean egg to adult development time at different thermal treatments. The error bars represent standard error across replicate MCTs.

Thermal plasticity of egg duration

Egg duration measured by egg to larva hatching time was significantly affected by rearing temperature ($p < 0.0001$). At 25˚C, mean egg hatching time was 21 hours, while at 17˚C, eggs

took 37 hours on average to hatch (Fig. 2.1). This result corroborates findings reported by earlier studies (Powsner, 1935; Chong et al., 2018).

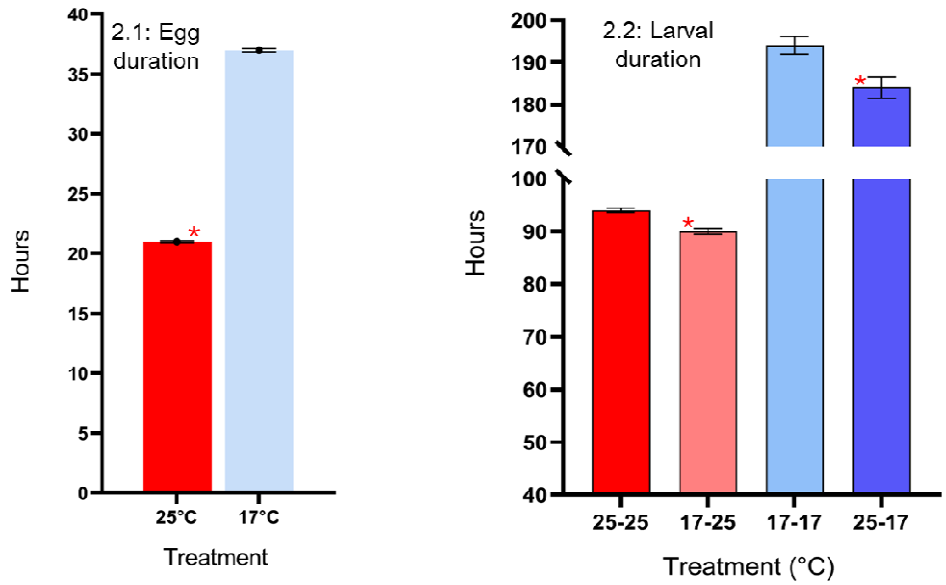

Figure 2: 2.1: Mean egg duration (larval hatching time) at 25˚C and 17˚C. 2.2 Mean larval duration at different thermal treatments. The error bars in both plots represent standard error across replicate MCTs.

Both upshift and downshift of temperature at egg-larva transition accelerate subsequent development

When eggs are reared at 25˚C, average duration of egg stage is 16 hours less compared to 17˚C (Fig. 2.1). The egg rearing temperature of 25˚C not only makes the egg stage develop fast, it also accelerates successive development. This is evident from the finding that the overall shortening of development time observed at 25-17-17 compared to 17-17-17 is 50 hours on average (Fig. 1, B *vs*. A). This clearly shows that the warm egg rearing temperature has a spillover effect, and it accelerates subsequent development of the fly. ANOVA revealed significant impact of egg temperature on larval duration (p = 0.0017) when larvae were reared at 17˚C. Larval duration at 17-17 was 194 h, while at 25-17, it was 184 h (Fig. 2.2), indicating a significant reduction caused by prior egg stage temperature.

Our expectation was in case of the upshift, *i. e*. at 17-25-25, larval and pupal developments would get slower compared to constant developmental temperature of 25˚C, but the results did not meet this expectation. 17˚C hatched larvae upon being shifted to 25˚C (17-25) instead

showed a small but significant reduction of larval duration (90 h) compared to 25-25 larvae (94 h) (Fig. 2.2), and no change in pupal duration (98 h) at 17-25-25 compared to that at 25-25-25 (97 h) (Fig. 3, b *vs*. a). We had speculated a prior warm temperature would leave an accelerating after-effect while a prior cold temperature would do the opposite. However, finding from this experiment suggests it could be more complex than that. In a variable thermal environment, developmental pace of a pre-adult life stage may depend upon previous stage temperature, that is, whether it experienced warmer or colder environment before. In addition, the pace may also depend upon whether the shift was from a sub-optimal to optimal temperature as development may overcome the carry-over decelerating effect of a previous cold temperature and rather may gain some momentum because of sudden attainment of optimal temperature after a sub-optimal one. It is possible that the enzymes driving development at cold and sub-optimal temperature like 17˚C gains momentum upon abrupt shift to the optimal temperature, *i. e.* 25˚C that results in speeding up of larval development. Future research delving into the effect of thermal shifts on efficiency of developmental enzymes may shed more light on this.

Larva-pupa thermal shift: cold larval-warm pupal condition make development longer than warm larval -cold pupal condition

Development time at 17-17-25 was longer (333 h) than that at 17-25-17 (319 h) (Fig. 1, C *vs.* D). Similarly, flies took significantly longer time to develop at 25-17-25 (304 h), compared to that at 25-25-17 (288 h) (Fig. 1, E *vs.* F). This shows colder larval stage followed by warm pupal stage makes overall development longer, compared to warm larval stage followed by cold pupal stage. This is not surprising. Firstly, cold larval rearing makes larval stage long, and it also leaves a carryover effect on pupal stage such that pupal development at warm becomes significantly extended compared to constant warm rearing. The converse also holds true as warm larval rearing not only makes larval development fast but also makes pupal development at cold significantly faster compared to constant cold rearing. Therefore 17˚C larval-25˚C pupal development is longer compared to 25˚C larval-17˚C pupal rearing.

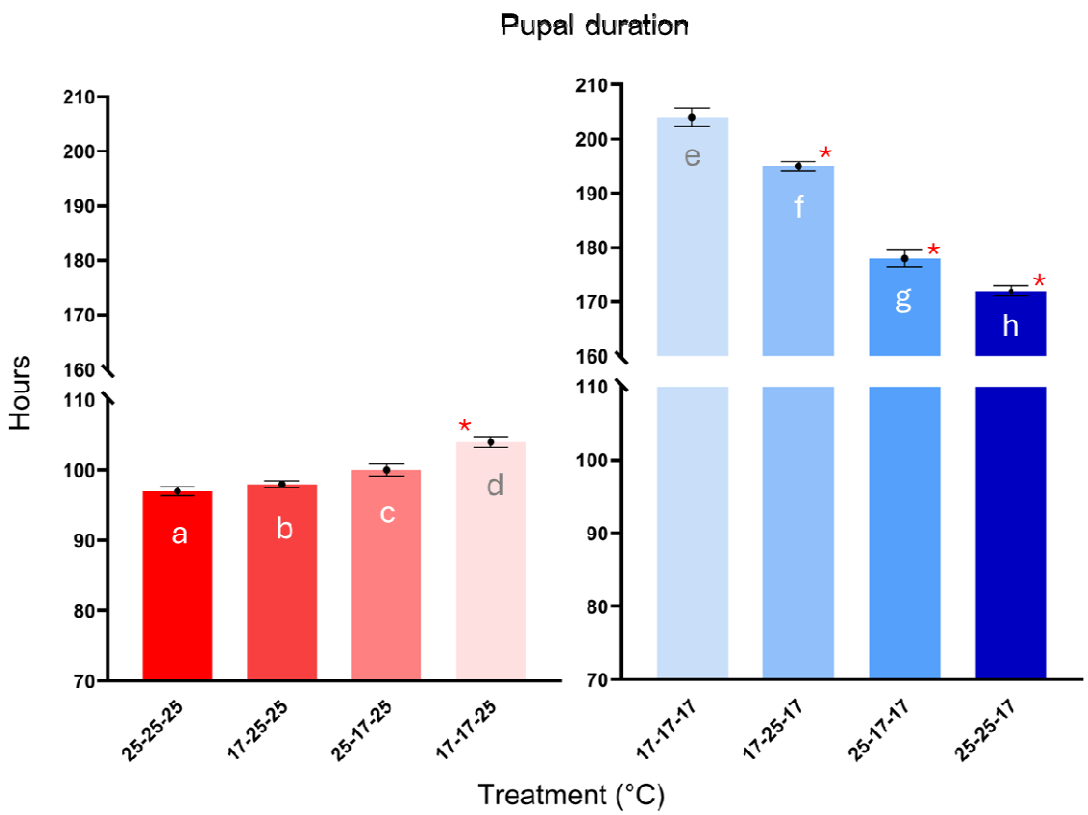

Figure 3: Mean pupal duration at different thermal treatments. The error bars represent standard error across replicate MCTs.

Effects of egg and larval temperatures on pupal duration at 25˚C

When pupae were reared at 25˚C, pupal duration was impacted by the thermal combination experienced during egg and larval stages ($p < 0.0001$). However, at 25-25-25 and 17-25-25, pupal durations were similar, *i. e.* 97 h, and 98 h respectively (Fig. 3, a *vs*. b), indicating egg stage temperature did not have any carryover effect up to the pupal stage when larvae and pupae both experienced 25˚C. At 25-17-25, nonetheless, pupal duration was 100 h (Fig. 3, c), which was significantly longer than the same at 25-25-25, *i. e*. 97 h, showing cold larval temperature has carryover effect on pupal development. At 17-17-25, pupal duration was 104 h (Fig. 3, d), which was significantly longer than the same at three other treatments, indicating combined effect of cold temperature during egg and larval stages have stronger carryover effect in extending pupal duration at 25˚C.

Effects of egg and larval temperatures on pupal duration at 17˚C

Pupal duration at 17˚C too was significantly affected by the temperatures experienced during egg and larval stages ($p < 0.0001$). Post hoc comparisons revealed pupal duration at 17˚C was significantly different across all four different thermal pre-treatments. When eggs were reared at 25˚C, and larvae were downshifted to 17˚C upon hatching and reared at the same temperature till eclosion (25-17-17), both larval (184 h) and pupal developments (178 h) were significantly accelerated compared to development at a constant temperature of 17˚C, revealing a strong carryover effect of warm egg temperature on both larval and pupal stages developing at 17˚C (Fig. 3, g). Moreover, pupae took 204 hours to develop into adults at 17-17-17, which was the longest (Fig. 3, e). At 25-25-17, it was the shortest, as it took 172 hours to complete pupal metamorphosis (Fig. 3, h). The combined effect of warm temperatures during egg and larval stages in accelerating pupal development was clearly evident from this.

Two subsequent thermal shifts at egg-larva and larva-pupa make subsequent pupal development slow

The effects of the double thermal shifts on development time were remarkable. At both 25-17-25 and 17-25-17, pupal metamorphosis took substantially long. The lengths of pupal stage in these treatments were second only to treatments in which egg-larva rearing temperature was 17-17. Among treatments involving pupal development at 25˚C, pupal duration at 25-17-25 (100 h) was faster only than 17-17-25 (104 h), and among treatments involving pupal development at 17˚C pupal duration at 17-25-17 (194 h) was slower only compared to17-17-17 (204 h) (Fig. 3, c *vs.* d, f *vs.* e). Pupal duration at 25-17-25 significantly increased to 100 h, from 97 h at 25-25-25, revealing a carryover effect of colder larval rearing environment (17˚C) on pupal development (Fig. 3, c *vs.* a).

It was a little perplexing that pupal duration in the other double shift treatment, the one for 17˚C pupae, *i. e.* 17-25-17 was 194 h, which was significantly slower than 178 h, observed at 25-17-17 (Fig. 3, f *vs.* g). Intuitively, one would expect pupal stage to have greater impact of larval rearing temperature than that of egg temperature, because (a) larval stage immediately precedes the pupal stage, and (b) larval stage is much longer than the egg stage. However, the finding indicates warm temperature during egg rather than larval stage caused bigger reduction of pupal duration in this case. In this regard, we speculate two abrupt thermal shifts at subsequent developmental transitions possibly make developmental adjustments more challenging such that metamorphosis remains slow, and therefore carryover effect of 25˚C larval temperature on pupal development is not found to be as large as expected.

Pupal duration is affected the most when shift is done at larva-pupa transition

For pupae developing at 17˚C, metamorphosis took the shortest time (172 h) in those which were reared at 25˚C during egg and larval stages (25-25-17) (Fig. 3, h). Similarly, for pupae reared at 25˚C, metamorphosis was the longest (104 h) for those reared at 17˚C through egg and larval stages (17-17-25) (Fig. 3, d). This indicates pupal duration at warm temperature is extended the most when both egg and larval rearing were in cold, and conversely, it is accelerated the most when both previous stages were warm.

Conclusion

To summarize, this study reveals several exciting findings about thermal plasticity of development time in *Drosophila melanogaster* under variable thermal conditions. First, it shows thermal effects of preceding developmental stages on later stages are more conspicuous when the later stage temperature is cold. As duration of development is longer in cold, it provides a finer resolution of developmental duration, revealing greater plastic changes caused by previous thermal conditions. Second, thermal shift early during development at egg-larva transition affects subsequent developmental pace, but it has relatively smaller impact on total egg to adult development time compared to thermal shift done at a later stage, *i. e.* larva-pupa transition. This is particularly evident in pupal duration as it is affected more by larva-pupa than egg-larva thermal shift. Third, a downshift from warm egg stage to colder temperature predictably accelerates subsequent larval and pupal development compared to constant cold, due to carry-over effect of prior warm condition. However, a striking finding from this study is an upshift from cold egg temperature to warm conditions also speeds up larval development making it more rapid than that at constant warm, instead of showing a carry-over effect of prior slow pace at cold. We speculate this is a result of gain in momentum presumably due to increased efficiency of developmental enzymes, caused by a shift from sub-optimal to optimal conditions. However, for pupal duration, effect of previous stage temperatures was as expected. Prior stage cold temperature led to lengthening of pupal duration while warm egg or larval temperature led to its reduction. Fourth, cold larval-warm pupal rearing extends development time more than warm-larval-cold pupal rearing, as thermal condition of the earlier stage, that is, larva, and the carry-over effect of that stage on the later pupal stage, play dominant roles in shaping overall development time. Last, but not the least, two subsequent thermal shifts at egg-larva and larva-pupa transitions seem to make developmental adjustments challenging, such that

pupal development remains slow under such conditions. To conclude, this study demonstrates that thermal plasticity of developmental durations in *Drosophila* under shifting temperatures is a complex phenomenon influenced by interaction between previous and ongoing thermal conditions. It seems to depend on whether it involves an upshift *vs.* downshift of temperature, timing and number of thermal shifts, optimality of the temperatures involved and possibly developmental adjustments. This study paves the way for more research delving into roles of gene expression and enzyme activities in coordinating development in thermally variable environments.

Author contributions

SG conceived the idea, designed the experiment, analysed and interpreted the data, wrote the manuscript. AC designed and performed the experiments, analysed data, prepared figures and co-wrote the manuscript. RR designed and performed the experiments and analysed data. PB designed and performed the experiments and collected data. All authors reviewed the manuscript.

Conflict of interest statement

We declare we have no competing interests

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