Brief communication

Desiccation stress induces developmental heterochrony in *Drosophila melanogaster*

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Stressful environments are known to perturb developmental patterns in insects. In the purview of desiccation as a stressor, relatively little is known about the developmental consequences linked with desiccation tolerance. In this study, we have particularly focused on the exploration of the temporal profile of postembryonic development in response to desiccation exposure in *Drosophila melanogaster* and the associated trade-offs. We document a correlation between variations in 20-hydroxyecdysone levels and the altered timing of metamorphic events during the life cycle. Following desiccation, we observed an extension in the larval longevity whereas the duration of the pupal and adult stages was significantly shortened. Alternately, feeding of 20-hydroxyecdysone apparently led to the restoration of the normal temporal pattern of development in the desiccated group. In spite of the desiccation-responsive heterochronic shifts in development, the overall lifespan post recovery remained almost unaltered among the desiccated and undesiccated groups suggesting plasticity in developmental control. This observation reminisces 'canalization-like' phenomenon that buffers alterations in the overall lifespan. We thus identified a desiccation-responsive period in the lifespan of *D. melanogaster* during which variations in ecdysone levels are capable to alter the temporal course of development.

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1. Introduction

Temperature coupled with relative humidity forms one of the key determinants of survival, distribution and behaviour of organisms (Addo-Bediako *et al.* 2000; Chown *et al.* 2011; Overgaard *et al.* 2011). Imbalances in these factors lead to the onset of environmental dehydrating conditions which is closely linked to global climate change (Cornelissen 2011; Hadley 1994). Being ectotherms, arthropods in particular, are directly dependent on environmental temperature-humidity interplay and hence are expected to exhibit specific adaptions in order to cope with water deficits mediated by desiccating conditions in their habitats (reviewed in depth by Deutsch *et al.* 2008; Balanyà *et al.* 2006). Insect desiccation tolerance is a widely studied phenomenon encompassing several biochemical and behavioural underpinnings (see Tunnacliffe and Lapinski 2003 for a review; Reyes-DelaTorre *et al.* 2012). However, to date, the basic concerns of the developmental consequences on insect growth and metamorphosis in response to desiccation stress are poorly understood.

In insects, growth and development is characterized by periodic molting which is primarily regulated by ecdysteroids, a type of steroid hormones that include ecdysone, ecdysterone and 20-hydroxyecdysone (20-HE). Ecdysteroids are known for their vital role in growth, differentiation, morphogenesis and metamorphosis (Gilbert *et al.* 2002; Hahn and Denlinger 2011; Tennessen and Thummel 2011; Jindra *et al.* 2013). Ecdysone is the insect molting pro-hormone secreted

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by the prothoracic glands and is converted to 20-HE (active physiological form of ecdysone) in target tissues to initiate the transitions throughout insect development and metamorphosis into adults (Nation 2015). Variations in ecdysone hormonal titres have also been primarily identified in synchronizing developmental changes in response to environmental conditions (reviewed in depth by Shukla *et al.* 2015). This adaptive feature facilitates animals to adopt alternative strategies in order to optimally adjust their life cycle around seasonal/ stochastic fluctuations in their habitats (Oostra *et al.* 2014).

Among insects, the genus *Drosophila* has been extensively used as a model system for investigations concerning desiccation tolerance. The genetic and physiological basis of desiccation tolerance has been established in several *Drosophila* species (Hoffmann *et al.* 2001; Gibbs *et al.* 2003; Thorat *et al.* 2012). However, fundamental knowledge of environmental desiccation bouts on insect growth and metamorphosis are poorly understood. In this study we have explored the temporal pattern of postembryonic developmental consequences upon recovery from desiccation stress in the holometabolous insect, *D. melanogaster*. We also report the developmental trade-offs associated with desiccation tolerance in *D. melanogaster* which have been linked to changes in 20-HE levels. Although the effects of 20-HE-mediated modifications have been widely studied in insects, its involvement in the context of desiccation stress has been reported for the first time in this study.

2. Materials and methods

2.1 *Organisms*

Larvae of *D. melanogaster* (Oregon K strain) maintained in our laboratory for 25 generations were used for all experiments described in this study. Fly culture was reared on standard corn-meal-agar diet at 23±1°C and under photoperiod cycle of 14 h light: 10 h darkness inside a Biological Oxygen Demand chamber (Remi, India Pvt. Ltd.). Larval densities were standardized to ~100 larvae/bottle to avoid overcrowding that might affect the physiology of the growing individuals. Healthy mid-third instar larvae were used for all further experiments.

2.2 *Desiccation treatment*

Larvae were exposed to acute desiccation stress at <5% relative humidity (RH) as described before (Thorat *et al.* 2012). In brief, prior to desiccation, groups of 30 larvae were rinsed in tap water to ensure removal of adhering food matter. Larvae were then gently blotted on filter paper and placed on a sterilized glass Petri dish lined with dry tissue paper. The Petri dishes along with the larvae were kept inside the desiccating chamber for 10h based on previously published desiccation assays (Thorat *et al.* 2012). Undesiccated control larvae maintained at standard relative humidity (75 ± 5%) were used as controls. Water content measurements were carried out by gravimetric analysis in the control, desiccated and starved larvae as described previously (Thorat *et al.* 2012).

2.3 *Recovery upon rehydration*

After desiccation treatment, larvae were divided into two groups and transferred to separate Petri dishes (35 × 10 mm) for rehydration: one group was transferred to a Petri dish with water only termed as the 10 h desiccated group without 20-HE feeding while the other group was transferred to a Petri dish with water containing 20-HE (Sigma, USA; 1g 20-HE/ml of water) termed as 10 h desiccated group along with 20-HE feeding following a published protocol (Mirth *et al.* 2005). Larval rehydration with feeding was continued for 12 h during which, at appropriate time points, larvae were picked up for 20-HE measurements and the remainder of the larvae were tracked for their life history. Survival was judged based on the abdominal contractions in response to the gentle stimulus using a blunt needle under a stereo-zoom microscope (Magnus MS 24; Olympus Pvt. Ltd., India).

2.4 *Life history analysis: Larval to adult development post recovery*

Desiccated larvae were allowed to rehydrate under standard conditions and their post-rehydration developmental pattern was tracked in comparison to the undesiccated controls. Larval to pupal transition followed by metamorphosis into adults was monitored. Furthermore, duration of the larval, pupal and adult stages was recorded.

2.5 *Environmental-Scanning Electron Microscopy*

Control, dehydrated and rehydrated larvae were examined under Environmental Scanning Electron Microscope (E-SEM; FEI Quanta 200 3D system, The Netherlands). Analysis was carried out on low vacuum mode and low accelerating voltage at 20 kV. Specimens were then mounted on Peltier cooling stubs covered with carbon black tape. All micrographs were captured using xT Microscope Server (version 1.7.3).

2.6 *Liquid Chromatography-Mass spectrometry analysis*

Crude larval homogenates from the control, desiccated and rehydrated samples were used for 20-HE hormone measurements as described before (Thorat and Nath 2015). In brief, groups of 30 larvae were homogenized in 50 µL of Phosphate-buffered saline (PBS) and mixed with 300 µL of methanol: isooctane solution (1:1). The mixture was centrifuged at 10,000*g* at 5°C for 20 min and the supernatant was evaporated until dry in a water bath maintained at 85°C. The precipitate was reconstituted in 1:1 methanol-water and subjected to LC-MS/MS analysis on an Perkin Elmer (PE 200 series) coupled with API 2000 mass spectrometer (ABS Sciex, Canada) equipped with an electrospray ionization (ESI). The HPLC separation was carried out using Atlantis dC18 (100×2.1 mm, 5µm, Waters India Pvt, Ltd., Bangalore). The mobile phase was composed of (A) methanol: water (90:20, v/v) and (B) methanol: water (90:10, v/v) containing 5 mM ammonium formate (in both). The mobile phase flow rate was 0.4 mL/min with gradient 0–1 min 90% A phase, 1–7 min 98%–5% A phase, 7–8 min 5% A phase, 8–9 min 5%–90% A phase and 9–15 min 90% A phase. Known concentration of 20-HE (Sigma; USA) was used as standard and which eluted at a retention time of 5.9 min. The estimation was performed in positive mode by Multiple Reaction Monitoring (MRM) with mass transition $[M+H]^+$ 481>445 for quantification and 481>165 was used for confirmation.

2.7 *Statistical analysis*

All experiments were carried out in triplicate sets at 23±1°C. Ten replications (10 larvae per replica) were carried out under laboratory conditions. Values (mean ± SD) of the results obtained were subjected to Student's *t*-tests.

3. Results

3.1 *Physiological consequences of desiccation stress*

It was observed that in response to desiccation exposure, *D. melanogaster* larvae exhibited a tolerance threshold of 10 h. Larvae lost water gradually reaching a final water content of 13.56 ± 2.76% at the end of desiccation (figure 1). Upon rehydration, water gain became prominent. Within 3±0.5 h post rehydration, larvae revived and resumed active metabolism. By 6 h post rehydration, larval water content matched with that of the control levels (figure 1).

High resolution E-SEM photomicrographs indicated that loss of body water resulted in shrinkage of the larval cuticle in comparison to the undesiccated controls (figure 2A, B). Surface topological changes that were prominent during desiccation were seen to be gradually restored as rehydration progressed (figure 2C). Unlike conventional electron microscopy, E-SEM facilitated examinations without introduction of artifacts resulting from handling and sample preparation.

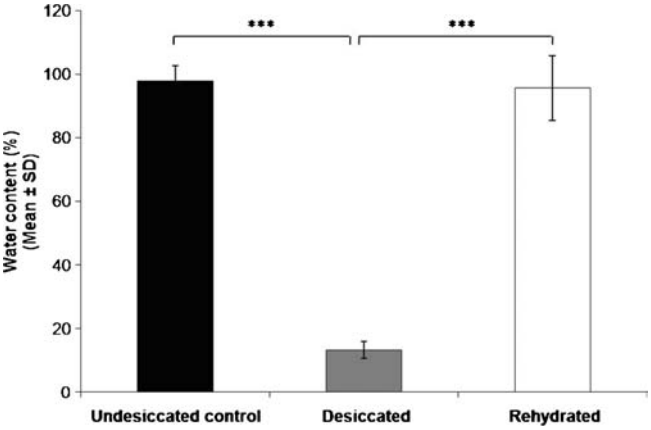


Figure 1. Desiccation-responsive water loss and water gain upon rehydration in the larvae of *D. melanogaster* (Student's *t*-test, $P < 0.0001$).

3.2 *Consequences of desiccation stress on the temporal pattern of development*

Upon rehydration, larvae recovered followed by metamorphosis; albeit delayed. Revived individuals were kept under ambient conditions and their developmental history was recorded. Overall duration (d; in days) of larvae, pupae and adults of the undesiccated control groups was recorded to be 6.2±1.1, 2.1±1.04 and 20.4±0.86 d respectively (figure 3). Larvae desiccated for a maximum of 10 h followed by rehydration with water only, showed extended longevity that averaged to 15.2±0.91 d whereas, there was significant shortening of the pupal and adult stages resulting in an average span of 1.2±0.17 and 11.5±1.15 d respectively. However, upon rehydration with 20-HE, duration of individual life stages seemed to be restored without extension or shortening. Larval, pupal and adult duration was found to be 5.61±0.52, 1.83±0.014 and 21±1.21 respectively. Interestingly, there was no significant change in the overall lifespan starting from the rehydrated larval period up to the adult stage in the undesiccated control and the desiccated groups with or without feeding. Total longevity (sum of larval, pupal and adult lifespans) represented in the pie charts for each group summed up to 28.7±2.24 d, 27.7±1.78 d and 28.44±1.52 d in the control and the unfed and 20-HE fed groups respectively (figure 3). It must also be noted that no apparent phenotypic abnormalities were found in any of the developmental stages.

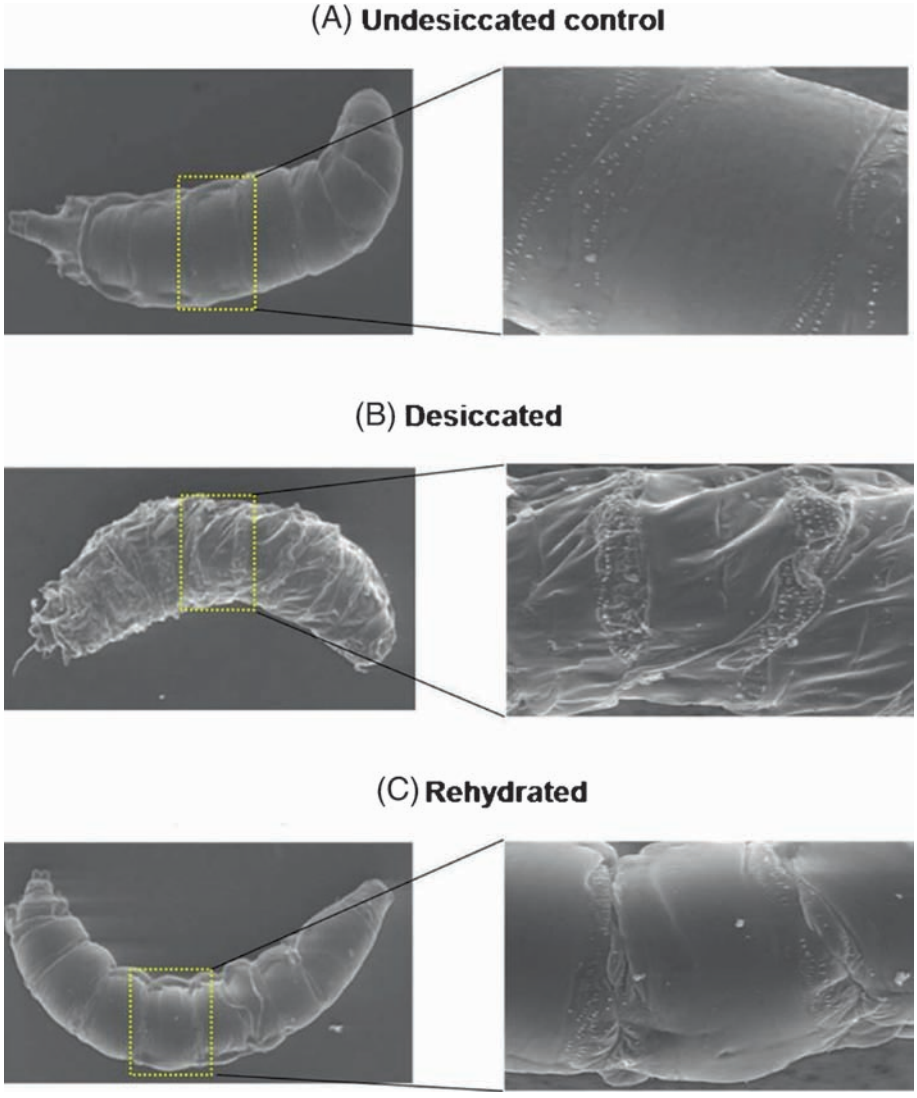


Figure 2. Morphological consequences of desiccation stress in *D. melanogaster* larvae as seen by Environmental-Scanning Electron Microscopy: (**A**) undesiccated control larva, (**B**) larva after 10 h desiccation and (C) larva after 8 h rehydration. As evident, water loss caused altered cuticular integrity in the larva which was restored upon rehydration (whole larva: 100×, magnified view: 500×).

3.3 *Alterations in 20-hydroxyecdyone levels*

Our next goal was to investigate the plausible involvement of ecdysone hormone. Since insect molting is triggered in the presence of high concentrations of 20-HE, we hypothesized that this hormone might be crucial for the desiccation-responsive developmental heterochrony observed in this study. LC-MS analysis revealed decline in 20-HE levels with progressive desiccation (6 h) and reached negligible and undetectable levels by the end of desiccation (10 h) (figure 4A–D). Upon rehydration with water only, levels of 20-HE started rising gradually (6 h) (figure 4E–H), finally matching the controls by 18 h rehydration. Thus desiccation treatment slowed down larval molting with a concomitant decline in 20-HE levels which in turn was responsible for the heterochronic shifts in development. Subsequently, recovery from desiccation stimulated the cascade of metamorphic events marked by a conspicuous rise in 20-HE levels in the larvae. Furthermore, in order to assess whether desiccation directly affected molting or caused developmental delay through independent means, we fed the larvae with 20-HE during rehydration. It was observed that larvae fed with 20-HE showed substantially higher levels of 20-HE right from 1 h rehydration indicating that the exogenously fed 20-HE was present in the larval body (figure 4I–L). Moreover, the fed larvae showed restored levels of 20-HE within 5 h

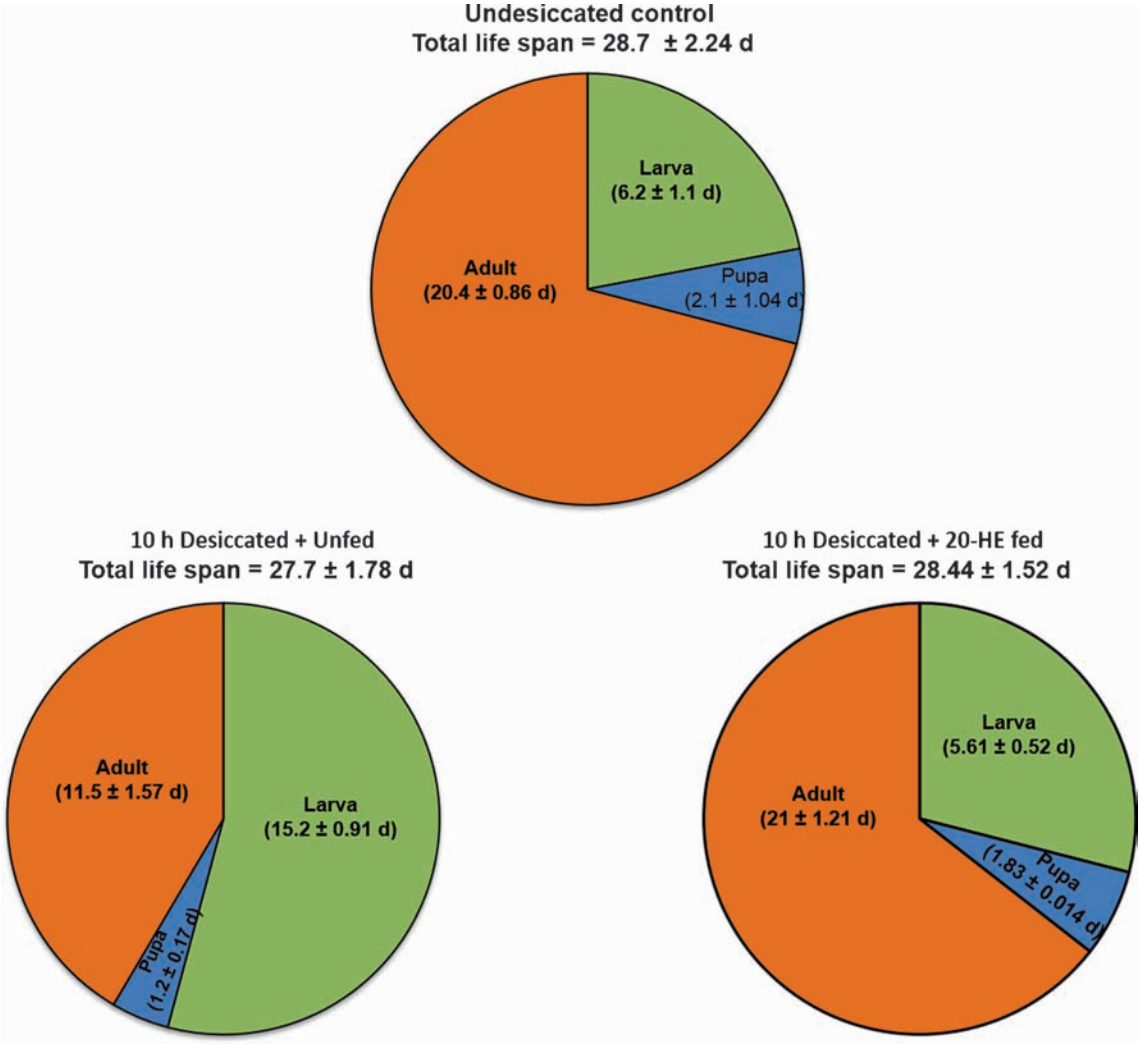


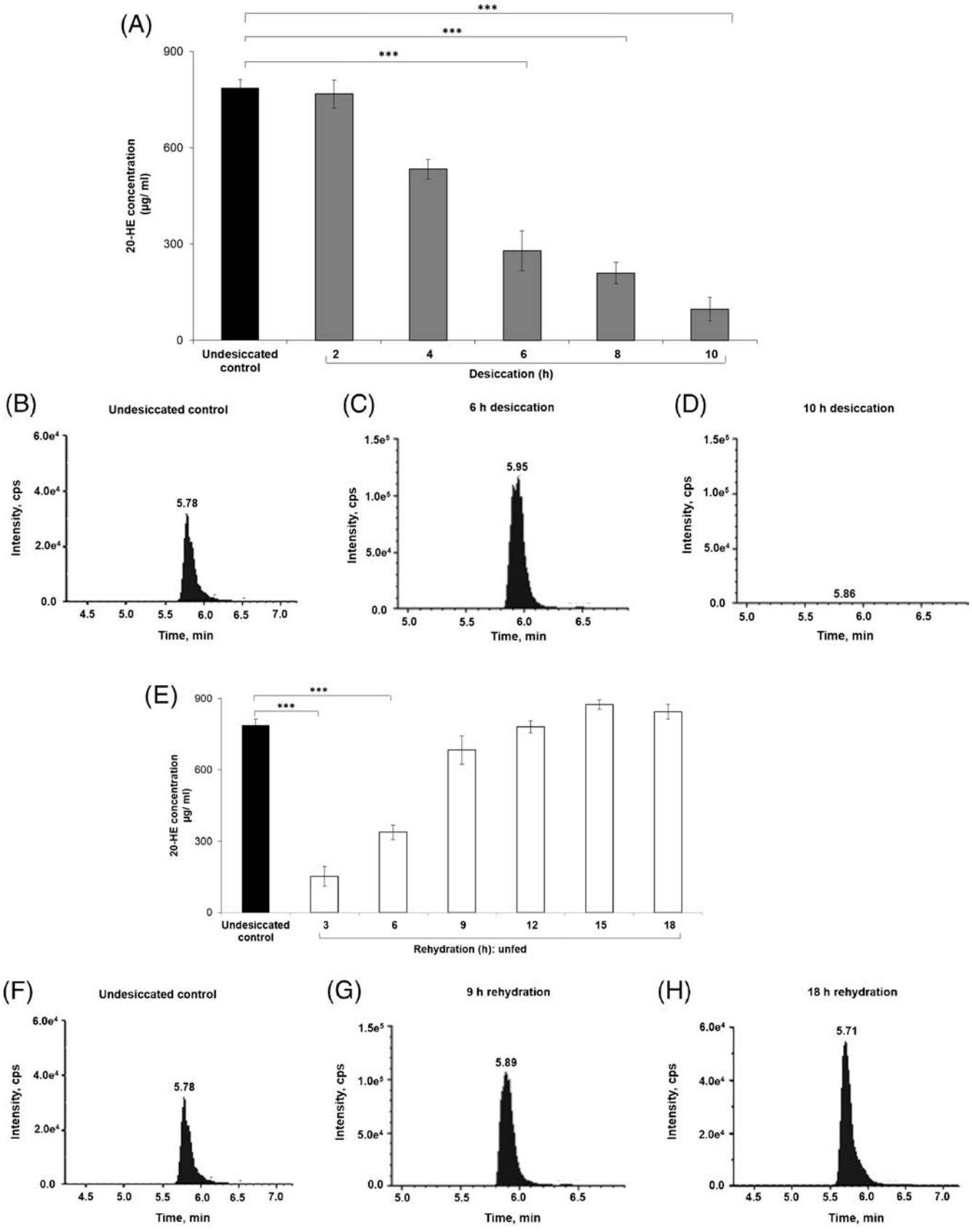
Figure 3. Schematic representation of life history analysis of the undesiccated control and the desiccated groups. The pie charts depict developmental heterochrony in individual metamorphic events. Desiccated larvae rehydrated only with water showed extended longevity of the larvae which was statistically significant (Students *t*-test; $P < 0.005$) in comparison to the undesiccated control larvae. Shortening of the pupal and adult stage was found to be statistically insignificant (Students *t*-test; $P > 0.05$). On the other hand, desiccated larvae rehydrated with 20-HE showed apparent restoration of the temporal pattern of development wherein individual duration of the larval, pupal and adult stages were almost similar to that of the undesiccated control larvae. Nonetheless, in spite of the heterochronic shifts in individual stages in the life cycle, the overall postembryonic life span was more or less the same in the larvae rehydrated with and without 20-HE in comparison to the undesiccated control groups (Students *t*-test; $P < 0.005$).

rehydration to match with those of the control levels unlike the unfed larvae which required almost 12 h recovery period in order to restore normal 20-HE levels.

4. Discussion

Episodic exposure to desiccation stress is prominent in the life cycle of terrestrial insects like *Drosophila* (Balanyà *et al.* 2006). Thus, their survival is substantially dependent on their ability to cope with the challenges of body water loss. We have previously reported the desiccation-responsive role of trehalose as one of the physiological mechanisms that enable *D. melanogaster* larvae to sustain the dry state (Thorat *et al.* 2012, 2016). In this study, we have focused our understanding on the temporal pattern of developmental consequences as a result of water deficits in *Drosophila*.

Our results indicated that water loss in the larvae led to shrinkage of the body cuticle which was subsequently restored upon rehydration. Deviation from the normal


(A)
***
***
***
900
600
300
0
20-HE concentration (µg/ ml)
Undesiccated control
2
4
6
8
10
Desiccation (h)
(B)
Undesiccated control
6.0e4
4.0e4
2.0e4
0.0
Intensity, cps
5.78
4.5
5.0
5.5
6.0
6.5
7.0
Time, min
(C)
6 h desiccation
1.5e5
1.0e5
5.0e4
0.0
Intensity, cps
5.95
5.0
5.5
6.0
6.5
Time, min
(D)
10 h desiccation
1.5e5
1.0e5
5.0e4
0.0
Intensity, cps
5.86
5.0
5.5
6.0
6.5
Time, min
(E)
***
***
900
600
300
0
20-HE concentration µg/ ml)
Undesiccated control
3
6
9
12
15
18
Rehydration (h): unfed
(F)
Undesiccated control
6.0e4
4.0e4
2.0e4
0.0
Intensity, cps
5.78
4.5
5.0
5.5
6.0
6.5
7.0
Time, min
(G)
9 h rehydration
1.5e5
1.0e5
5.0e4
0.0
Intensity, cps
5.89
5.0
5.5
6.0
6.5
Time, min
(H)
18 h rehydration
6.0e4
4.0e4
2.0e4
0.0
Intensity, cps
5.71
4.5
5.0
5.5
6.0
6.5
7.0
Time, min

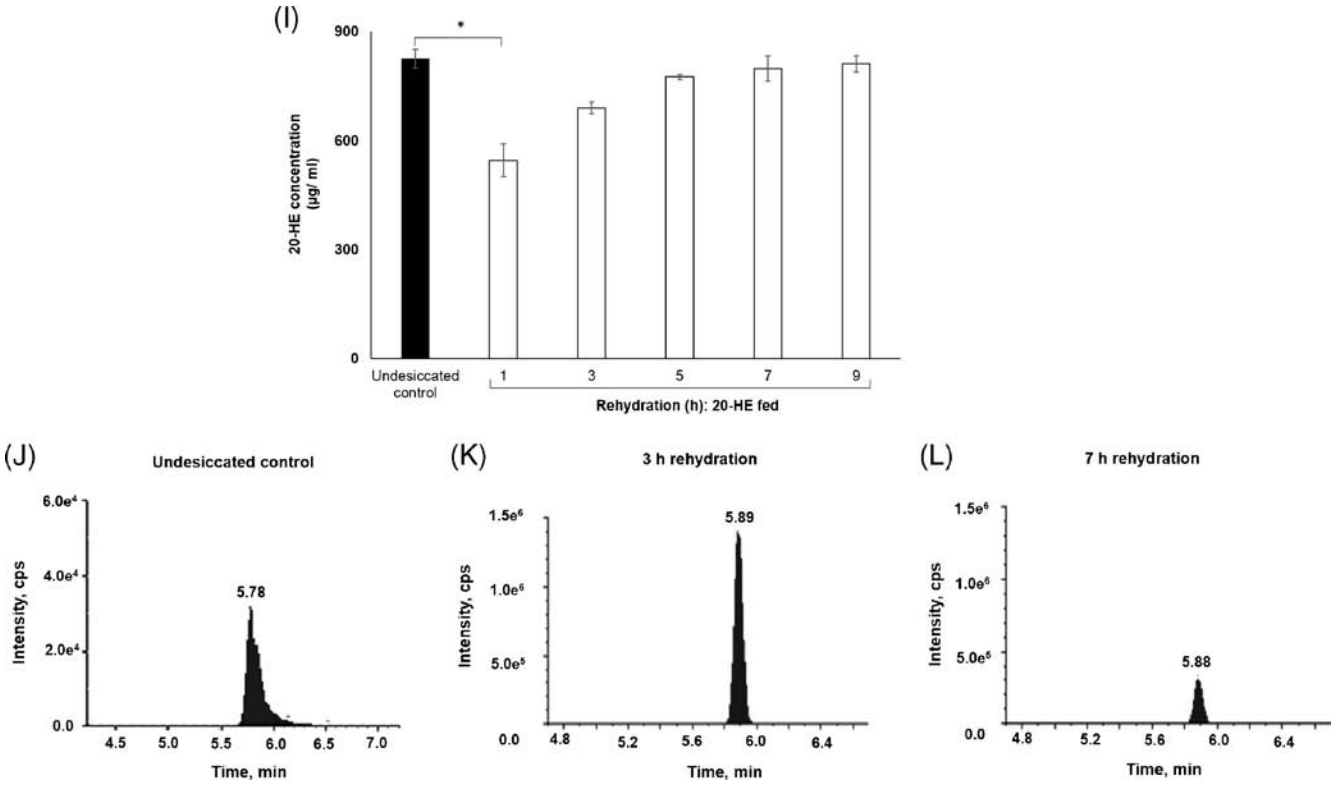


Figure 4. LC-MS analysis for 20-hydroxyecdysone hormone (20-HE) measurements in response to desiccation and rehydration (with and without 20-HE feeding). As seen in (**A**), 20-HE concentration in the larval body was seen to decline with progressive desiccation. The representative chromatograms show basal level of 20-HE peaks in the undesiccated control larvae (**B**), whereas the peaks were seen to fall in the larvae desiccated for 6 h (**C**) and 10 h (**D**). Upon rehydration with water only, an increment in the concentrations of 20- HE was prominent as rehydration progressed (**E**). The representative chromatograms showing 20-HE peaks indicated that in comparison to the undesiccated control larvae (**F**), peak levels of larval samples during early hours of rehydration remained significantly low (**G**) and finally increased to match the control value at 18 h rehydration (**H**). On the other hand, larvae fed with 20-HE during rehydration showed substantially higher levels of 20-HE right from 1 h rehydration which subsequently rose to match the control levels by 5 h rehydration (**I**). The representative chromatograms showing 20-HE peaks indicate that in comparison to the undesiccated control larvae (**J**), peak levels of larval samples during early hours of rehydration were low (**K**) and increased rapidly to match the control peak levels within 7 h rehydration (**L**) (Student's *t*-test, ***$P < 0.001$; *$P < 0.05$).

temporal pattern of post-embryonic development in *D. melanogaster* showed a clear evidence of desiccation stress-induced developmental heterochrony. Apparently, feeding the desiccated larvae with the molting hormone (20-HE) during rehydration showed restoration of the temporal pattern of development. In other words, the extended larval duration (developmental arrest in the larval stage), could be 'rescued' by 20-HE feeding. This suggested that heterochronic shifts observed in individual developmental stages were indeed induced due to desiccation stress exposure. Thus, desiccation-induced developmental heterochrony could be strongly correlated with the levels of the insect molting hormone.

Heterochrony refers to the change in the timing or rate of events involved in growth and development (Smith 2003). In the case of desiccation tolerant animals, life cycle and aging can be categorized into three hypothetical models (see Schill 2010 for details). First, wherein the organism completely disregards the entire time spent in the dry state, the second, in which there is partial discount of the time spent in the dry state and the third, wherein the organism registers the exact time spent in the dry state exhibiting non-extended longevity. Based on our results, *D. melanogaster* seems to fit into the first category, also known as the 'Sleeping Beauty' model which is characterized by extended longevity in the duration of the stage that experiences desiccation exposure. Other desiccation tolerant organisms namely, the tardigrade *Milnesium tardigradum* has also been shown to follow the 'Sleeping Beauty' model. The nematode, *Panagrolaimus rigidus* has been demonstrated to age while in the dry state whereas rotifers do not appear to age in the desiccated state (Schill 2010). In spite of the heterochrony in development observed in our study, the overall duration of postembryonic development of the life cycle remained almost unaltered. This

'compensatory effect' could be a kind of an adaptive buffering response of this species to adjust their life histories around optimal seasonal conditions reminiscing Waddington's canalization (Waddington 1942). The high degree of uniformity in terms of overall lifespan of *D. melanogaster* suggests canalization-like phenomenon of this trait.

Stressors like hypoxia, high temperatures, starvation and sleep deprivation are known to modify the 20-HE-mediated molting cycle with consequent effects on life cycle in *D. melanogaster* (David *et al.* 2004; Ishimoto and Kitamoto 2010; Schwedes and Carney 2012). This study thus adds to the yet unexplored understanding of the altered temporal programme of the developmental calendar in the life cycle of *D. melanogaster* induced by desiccation stress. It must be noted that during desiccation, animals are also under starvation stress. In other words, desiccation tolerance refers to survival without food in dry conditions while starvation tolerance indicates survival without food in humid conditions. Hence, it will be interesting to gain a comparative understanding of the similarities and differences in post-embryonic developmental effects in *Drosophila* as a result of a combinatorial stress treatment of desiccation and starvation. Recent work from our laboratory using another dipteran insect model, *Chironomus ramosus* has demonstrated heterochronic shifts in development in response to desiccation and lack of nutrition (Nath and Babrekar 2011; Thorat and Nath 2015). Another chironomid midge, *Diamesa mendotae* showed extended longevity during periods of below-freezing temperatures (Mazack *et al.* 2015). Molting in the Arctic springtail, *Cryptopygus antarcticus* was also shown to increase sharply upon recovery from prolonged exposure to freezing (Clark *et al.* 2007). Thus, fluctuations in a variety of extrinsic factors can have a profound impact on insect growth, development and metamorphosis *via* modulations in ecdysteroid titres (Yamashita *et al*. 2001).

Heterochronic shifts in the developmental calendar can have ecological ramifications because uncoordinated trends of life history in nature could be detrimental for survival and perpetuation of insect species as well as other biological communities in the food chain (Thorat and Nath 2015). For instance, a prolonged developmental stage might encounter unanticipated seasonal conditions and might eventually succumb to death if it fails to cope with the adverse environments. On the other hand, in the case of a shortened developmental stage, it is quite likely that the organism might miss the optimal weather conditions required for survival and reproduction.

Developmental switches in numerous other animals have been displayed under stressful environments (Hartfelder and Emlen 2012). Being desiccation tolerant, *Drosophila* serves as a useful system for the understanding of the physiological and developmental consequences underlying desiccation tolerance. However, to the best of the authors' knowledge, there have been no previous reports on desiccation-stress induced changes in developmental timings in any insect model. In a broader perspective, insect developmental heterochrony triggered by desiccation stress provides important insights into the ecological dynamics, plant-insect interactions and ecological energetics of the food web where insects are involved. Our study thus adds to the growing body of literature demonstrating the phenotypic plasticity of juveniles that facilitates their escape from desiccating environmental bouts by inducing developmental heterochrony. Selection experiments and genetic analysis with offspring obtained from the mating between desiccated and undesiccated parents are underway.

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