



Is diapause an ancient adaptation in *Drosophila*?



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ABSTRACT

D. melanogaster enters a state of reproductive arrest when exposed to low temperatures (12 °C) and shorter photoperiods. A number of studies have suggested that diapause has recently evolved in European *D. melanogaster* populations, that it is not present in the sibling species *D. simulans*, that it is non-photoperiodic in American *D. melanogaster* populations, and that it spontaneously terminates after 6–8 weeks. We have studied the overwintering phenotype under different conditions and observe that American, European and, surprisingly, African *D. melanogaster* populations can show photoperiodic diapause, as can European, but not African *D. simulans*. Surprisingly other *Drosophila* species from pan-tropical regions can also show significant levels of photoperiodic diapause. We observe that spontaneous termination of diapause after a few weeks can be largely avoided with a more realistic winter simulation for *D. melanogaster*, but not *D. simulans*. Examining metabolite accumulation during diapause reveals that the shallow diapause of *D. melanogaster* has similar features to that of other more robustly-diapausing species. Our results suggest that diapause may be an ancient character that emerged in the tropics to resist unfavourable seasonal conditions and which has been enhanced during *D. melanogaster*'s colonisation of temperate regions. Our results also highlight how different methodologies to quantify diapause can lead to apparently conflicting results that we believe can now largely be resolved.

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1. Introduction

Organisms living in temperate environments have evolved ways to cope with the rhythmic changes in their surroundings every time the Earth completes a revolution around the Sun: cycling temperature, food and water availability, predation pressure, accessibility to shelters/nests are but some of the challenges associated with changing seasons. The survival strategy of choice for insects is diapause, which allows organisms to escape unfavourable conditions 'in time', as opposed to escaping 'in space' (eg migration). In the wild, diapause is characterised by a preparatory pre-diapause phase, a maintenance (overwintering) phase and a post-diapause phase. These phases are neuroendocrinally controlled, and greatly differ in their metabolic and transcriptomic status (Salminen et al., 2015; Guo et al., 2015; Rozsypal et al., 2013). In particular, *Drosophila melanogaster* experience an adult reproductive winter dormancy triggered by lowered temperature and shortened photoperiod (Saunders and Gilbert, 1990). Dormancy

in *D. melanogaster* shows characteristics of both quiescence (a general metabolic slowing down affecting the development of all cells) and diapause (which is a dynamic, neurohormonally mediated process stimulated by changing environments). Among a number of relevant genetic manipulations, a recent paper (Schiesari et al., 2016) showed that switching on or off the genes encoding the insulin-like peptides *dilp2,3,5* was sufficient to flip diapause from ~0% to ~100% respectively at low temperatures. This was not a general female sterility because these genotypes were fertile at 22 °C. Insulin signalling has also been implicated in diapause of other fly species (reviewed by (Sim and Denlinger, 2013)). Consequently we prefer to use the term 'ovarian diapause', which is prevalent in the *Drosophila* literature and reflects the important neurohormonal component in *D. melanogaster* overwintering.

Ovarian diapause involves secretion of the insulin like peptides (ILP) from the midbrain, binding to the Insulin (like) Receptor (InR) in the ring gland (reviewed in (Schiesari et al., 2011)). This event begins a cascade of phosphorylation that involves CHICO, the phosphatidylinositol 3 kinase (PI3K), and the forkhead transcription factor (FOXO). It has been reported that allelic variations associated with insulin regulated PI3 kinase in *D. melanogaster* correlate with latitudinal difference in levels of ovarian diapause in North America where the incidence of reproductive diapause is higher in northern compared to southern *D. melanogaster* populations

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(Williams and Sokolowski, 1993; Schmidt et al., 2005). However no clear latitudinal cline in diapause inducibility has been detected in European natural lines of *D. melanogaster* (Pegoraro et al., 2017). In addition, a polymorphism in the InR shows a latitudinal cline in both Australia and North America, suggesting a possible seasonal adaptation that could be related to diapause (Paaby et al., 2010). Other genes that have an effect on ovarian diapause are *couch potato* (*cpo*) (Schmidt et al., 2008; Cogni et al., 2014) and the circadian clock gene, *timeless* (*tim*) (Tauber et al., 2007). Schmidt and colleagues suggested that a pair of SNPs in the *cpo* gene (*Ala/Val*³⁴⁷-SNP and 48034(A/T)) correlate with the latitudinal cline in the ovarian phenotype. In Australia, a corresponding latitudinal cline in diapause has also been reported, but the role of the *cpo* polymorphism in the diapause cline could not be confirmed (Lee et al., 2011). However, in Europe, any cline in *cpo* variants is very shallow and is only observed with the *Ala/Val*³⁴⁷ variant, a polymorphism which affects diapause levels, as opposed to the 48034(A/T) variant which neither shows a cline nor any effect on the phenotype (Zonato et al., 2016).

A natural polymorphism at the *timeless* (*tim*) locus also has a major effect on diapause induction (Tauber et al., 2007; Sandrelli et al., 2007). *s-tim* encodes a shorter isoform (S-TIM), missing 23N-terminal residues. *ls-tim*, encodes both S-TIM and a L-TIM. The latter is a longer isoform produced when an upstream starting codon is used. It has been shown that *ls-tim* is the most recent variant, and it appeared in southern Italy, at most, a few thousand years ago (Tauber et al., 2007). The new polymorphism has increased in frequency in this region and spread throughout Europe by directional selection, generating an impressive distance cline from the point of origin (Tauber et al., 2007). *ls-tim* increases diapause levels, so would be more adaptive at higher latitudes (Tauber et al., 2007). However, *D. melanogaster* demographic history and the location of origin of the new and young *ls-tim* allele have contributed to the creation of a seemingly counterintuitive European cline in *ls-tim* with high levels in the south and lower levels in the north (Pegoraro et al., 2017).

Some Drosophilids show a very strong and robust diapause response. Most *D. littoralis* natural lines for instance show a clear unimodal short day photoperiodic response curve at 16 °C ((Lankinen, 1986). Three species of the *D. virilis* group (*littoralis*, *montana* and *ezoana*), all show similar photoperiodic response expressing higher diapause levels when exposed to days shorter than 19.5 h of light (the Critical Day Length) (Salminen et al., 2015). However *D. melanogaster* diapause is rather shallow in comparison, as diapause levels are also affected by temperature (Saunders and Gilbert, 1990) and replicates can be rather variable (Saunders et al., 1989).

Emerson and colleagues (Emerson et al., 2009), have reported that American lines do not distinguish between different photoperiods in diapause inducibility. On the other hand, European natural lines present higher diapause levels when reared in shorter, winter-like, photoperiods (Tauber et al., 2007; Saunders, 1973). These experiments were performed in different conditions (after 12 days or 28 days of diapause inducing conditions for the European and American lines respectively). It has also previously been reported that *D. melanogaster* lines exhibit a spontaneous diapause termination after 6–8 weeks of diapause inducing condition (Saunders et al., 1989). Pegoraro et al. (2017) have shown that this termination is significant even after only 4 weeks. It is therefore important to be able to compare the fly lines from the two continents under the same experimental conditions.

Finally, Schmidt and co-workers reported that they were unable to detect any diapause in *D. melanogaster* African lines nor in the sibling species *D. simulans* (Schmidt and Conde, 2006; Schmidt, 2011) suggesting that diapause may have originated upon *D. melanogaster* colonisation of temperate climates after the last glacia-

tion (David and Capy, 1988). Our aims are therefore to revisit diapause in American, European and African lines of *D. melanogaster* and *D. simulans*, to clarify some of these outstanding questions concerning this fundamental survival strategy. We further extend our analysis to other tropical Drosophilids and find, perhaps surprisingly, that they too show evidence for diapause, which can also be photoperiodic. We speculate that diapause may be more deeply rooted in ancestral *Drosophila* than previously believed.

2. Materials and methods

2.1. Ovarian diapause

In order to perform the diapause experiments, flies were reared at 25 °C, in 12 h light, 12 h dark cycles (LD12:12). Virgin females were collected 6 h post eclosion in plastic vials and transferred to the experimental conditions: long (LD16:8) or short (LD8:16) photoperiod at constant 12 °C. These were obtained by placing the vials in light boxes, which were in turn placed inside incubators in order to maintain the experimental temperature. Ovaries were dissected after 12 or 28 days in diapause inducing conditions and diapause was scored according to King (1970). A fly was considered to be in diapause when eggs in both its ovaries were previtellogenic (<stage 8). About 30 females per replicate were analysed, and 6 replicates per condition were performed. Diapause percentage was arcsin transformed before performing statistical analyses.

The populations used are described in S1 Table. Populations in Figs. 1 and 2 were generated by placing 5 fertilised female for each available isofemale line (S1 Table) in 200 ml plastic food bottles. In Figs. 3 and 5, individual isofemale lines are compared.

2.2. Winter/spring simulation

The simulation experiment was performed to mimic a more realistic winter scenario. Adult flies were initially placed at 12 °C LD10:14, and the temperature was reduced by 1 °C and the photoperiod (pp) shortened by 30 min every week. After 4 weeks, when the temperature had reached 8 °C and LD8:16, these conditions were held for 4 weeks (weeks 5–8). In the final 4 weeks (weeks 9–12) temperature and photoperiod were both increased in a symmetrical fashion, to mimic the rise towards spring. Samples were collected at the end of week 2 (11 °C pp 9.5 h), week 4 (9 °C pp 8.5 h) week 8 (8 °C pp 8 h), week 10 (10 °C pp 9 h) and week 12 (12 °C pp 10 h). For controls, samples were collected and scored for diapause at the same time and photoperiod of the winter simulation, but having being maintained at constant 8 or 12 °C (Fig. 4).

2.3. Carbohydrate and protein measurements

Flies were maintained in LD8:16 or LD16:8 at 12 °C (see diapause protocol). After four, 12 or 28 days, flies were transferred to dry ice and separated into males and females.

The fresh weight of 10 females was recorded using a precision balance (Precisa 180A). Glucose, Glycogen, Trehalose and total protein were expressed as µg per fresh weight (Fig. 6). The samples were homogenised in 100 µL of PBS (ice cold) and centrifuged at 13000g for 3 min. Ten µL of the supernatant was separate for protein analysis. The rest of the supernatant was incubated at 70 °C for 5 min and use to measure trehalose, glucose and glycogen.

The glucose analysis kit from Sigma Aldrich (Glucose (GO) Assay Kit GAGO20) was used to measure the concentration of glucose and glycogen. Standards were prepared at 0.01, 0.02, 0.04, 0.08 and 0.16 µg/µL. Five samples were tested in triplicate. For glycogen analysis, amyloglucosidase was added to the glucose reagent to

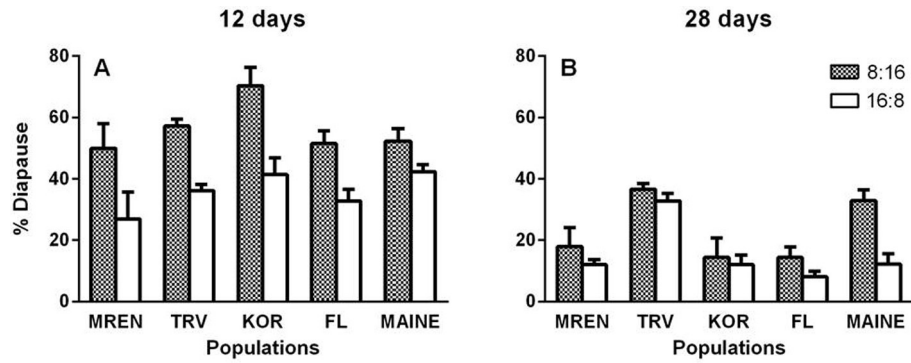


Fig. 1. Diapause induction in European and American lines. Diapause studied at LD8:16 and LD16:8 (filled and white bars respectively) at 12 and 28 days (panel A and B) at 12 °C for a series of natural populations of *D. melanogaster* from the USA and Europe. Means and SEMs are presented. Lines, south of Italy (MREN Lat 39.20N), northern Italy (Treviso, TRV Lat 45.67N), Korpilähti (Finland, KOR, Lat 62.01N), Florida and Maine (FL Lat 28.30N and MAINE Lat 45.17N). 3928 females contributed to these data.

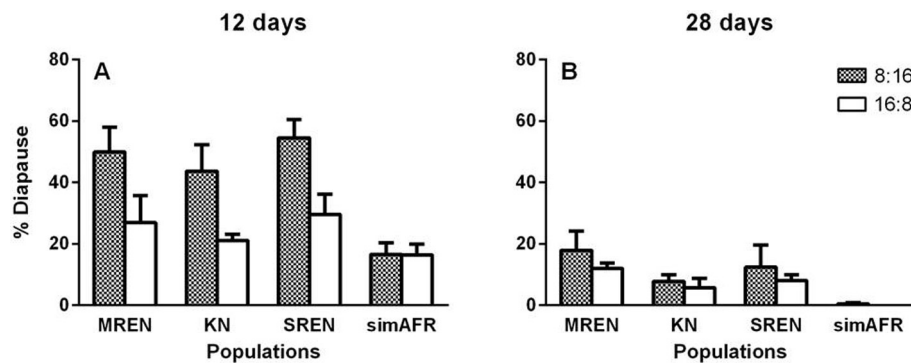


Fig. 2. Diapause induction in European and African *D. melanogaster* and *D. simulans* lines. Female reproductive diapause for natural populations of *D. melanogaster* and *D. simulans* from Europe and Africa. Means and SEMs. The *D. melanogaster* lines (MREN, same data as Fig. 1), and Kenya (KN). *D. simulans* lines south of Italy (SREN) and Africa (simAFR). 3262 females contributed to these data.

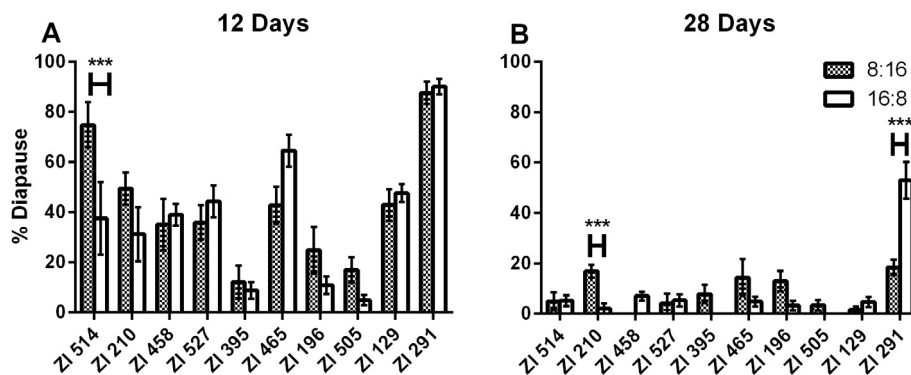


Fig. 3. Diapause induction in Zambian isofemale lines. The level of ovarian diapause of flies maintained either at LD8:16 or LD16:8 at 12 °C was scored after 12 (panel A) and 28 (panel B) days for 10 isofemale lines from Zambia. Means and SEMs are given. 2722 females contributed to these data. ***Indicates Duncan's *post hoc* test $p < 0.001$

breakdown glycogen into glucose. Samples were incubated at 37 °C for 30 min equal volume of sulphuric acid was added in a 96 well clear bottomed plate. The samples were quantified spectrophotometrically at 540 nm.

To quantify trehalose, samples were diluted in trehalose buffer for glucose controls or buffer containing 0.3 µl/ml of trehalase for trehalase analysis. The samples were incubated at 37 °C overnight before undergoing the glucose assay as described above.

Total proteins were quantified spectrophotometrically (595 nm) using the Bradford assay reagent (10 µl diluted samples + 200 µl reagent; Sigma-Aldrich, B6916) against a standards

prepared with bovine serum albumin diluted to 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/µl.

3. Results

3.1. Diapause termination and loss of photoperiodic response after 28 days

We compared European (MREN, TRV, KOR) and American (FL, MAINE) populations at both 12 and 28 days, and under two differ-

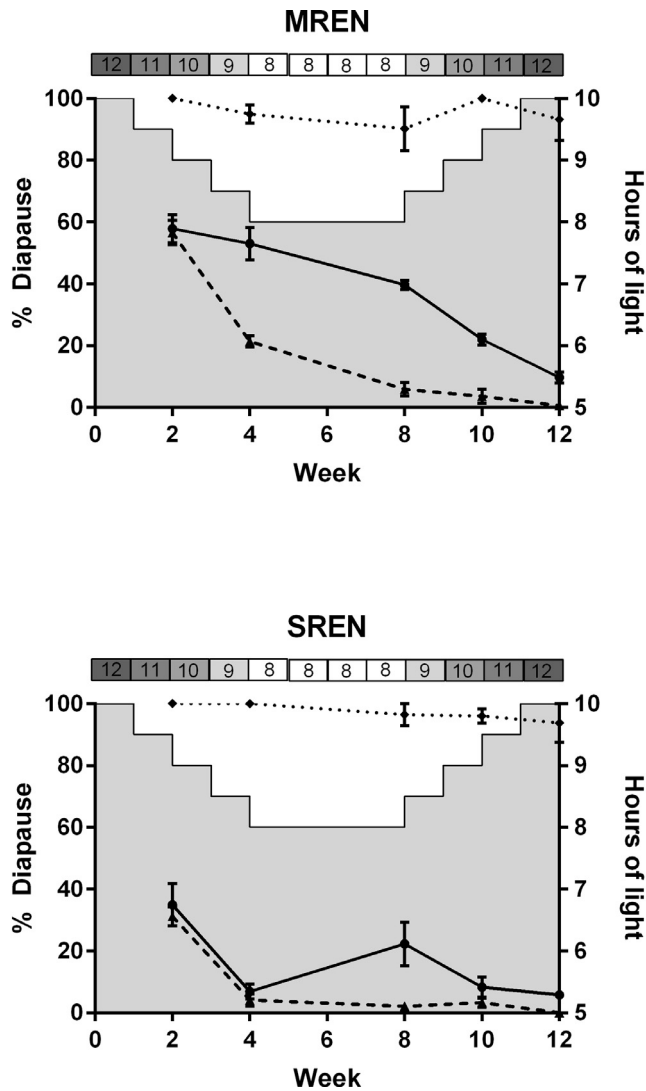


Fig. 4. Diapause levels during winter/spring simulation protocol. Diapause (left Y-axis) was scored in two sympatric *D. melanogaster* (MREN, panel A) and *D. simulans* (SREN, panel B) populations from Rende, south Italy. Samples were collected at the end of week 2 (11 °C; 9.5 h of light), week 4 (9 °C; 8.5 h of light) week 8 (8 °C; 8 h of light), week 10 (10 °C; 9 h of light) and week 12 (12 °C; 10 h of light) during winter simulation protocol (solid line, circles) and two temperature controls at constant 8 °C (dotted line, diamonds) and 12 °C (dashed line, triangles). The right y-axis indicates the number of hours of light (grey area), whereas the blocks on top of the plots indicate the weekly temperature at which the flies were exposed. Means and SEMs are given. 2482 females contributed to these data. As for MREN, diapause in the simulation protocol is significantly different from the controls at 12 °C at weeks 4 (Duncan's *post hoc* test, $p = 0.0001$), week 8 ($p < 0.0001$), week 10 ($p < 0.0001$) and week 12 ($p = 0.003$). In SREN diapause in the simulation protocol is significantly different from the controls at 12 °C only at week 8t (Duncan's *post hoc* test, $p = 0.0001$) and 12 ($p = 0.02$). The controls kept at constant 8 °C were not included in the statistical analysis.

ent photoperiods, to investigate the temporal dynamics of the diapause photoperiodic response (Fig. 1). Our overall ANOVA (S2A Table) highlighted significant Days (12 v 28 days, $F_{1,101} = 118$, $p < 0.0001$), Photoperiod ($F_{1,101} = 30.89$, $p < 0.0001$), and Strain ($F_{4,101} = 5.69$, $p = 0.0004$) main effects. Importantly, a significant interaction between Days \times Strain ($F_{4,101} = 5.31$, $p = 0.0006$) and Days \times Photoperiod ($F_{1,101} = 5.30$, $p = 0.0233$) was also detected. The results suggest that the clear photoperiodic response at 12 days is lost after 28 days when the level of diapause is also dramatically reduced. Importantly, no Photoperiod \times Strain interaction was detected ($F_{4,101} = 0.133$, $p = 0.97$) suggesting that overall, the populations behaved similarly.

3.2. Diapause in European and African *D. simulans* and *D. melanogaster*

We tested diapause levels on fly lines from both *D. melanogaster* and *simulans*, collected in Europe and Africa (Fig. 2). For our statistical analysis (S2B Table), the strains were split according to species and continent, both of which significantly affect the diapause incidence ($F_{1,81} = 8.94$, $p = 0.0037$ and $F_{1,81} = 22.76$, $p < 0.0001$ respectively). In addition we found significant Photoperiodic ($F_{1,81} = 10.63$, $p = 0.0016$), and Days ($F_{1,81} = 84.04$, $p < 0.0001$) effects. A significant Species \times Continent interaction was also detected ($F_{1,81} = 4.8$, $p = 0.0313$).

3.3. Photoperiodic diapause is not common in African *D. melanogaster*

We further extended our analysis to include several *D. melanogaster* African lines from Zambia to test whether the photoperiodic response we observed in the Kenya population is a common response in African flies (Fig. 3). As for the Zambian lines we observed a dramatic reduction in the level of diapause between 12 and 28 days (only line ZI 291 shows more than 20% diapause after 28 days). ANOVA (S2C Table) indicates a significant Strain ($F_{9,160} = 25.27$, $p < 0.0001$) and Days ($F_{1,160} = 275.97$, $p < 0.0001$) but no Photoperiodic ($F_{1,160} = 0.82$, $p = 0.368$, ns) main effects. However, lines seem to be affected differently by the length of the experiment and by the photoperiods at which flies were exposed (Strain \times Days $F_{9,160} = 5.68$, $p < 0.0001$; Strain \times Photoperiod $F_{9,160} = 4.09$, $p < 0.0001$).

3.4. Winter simulation strongly affects *D. melanogaster* but has little effect on *D. simulans* diapause

We designed a winter simulation experiment to test whether a combined photoperiod shortening and temperature lowering would result in a sustained diapause expression over time (Fig. 4). Control flies were subjected to the same photoperiods but kept at constant 8 °C and 12 °C. Only the latter were included in the statistical analysis as in the former, diapause approached 100%. In *D. melanogaster* (MREN) we found that during our winter simulation the levels of diapause after 4 weeks was considerably higher than in flies kept at constant 12 °C (Duncan's *post hoc* $p = 0.0001$, Fig. 4). On the contrary, winter simulation has little effect on *D. simulans* (SREN) diapause, which is very low by week 4. Comparing these two sympatric strains from southern Italy, we observed significant Weeks ($F_{1,100} = 63.74$, $p < 0.0001$), Conditions (winter simulation v constant 12 °C, $F_{1,100} = 80.63$, $p < 0.0001$) and Species ($F_{1,100} = 73.24$, $p < 0.0001$) main effects. Weeks \times Conditions ($F_{4,100} = 6.23$, $p = 0.0001$), Weeks \times Species ($F_{4,100} = 7.85$, $p < 0.001$) and Conditions \times Species ($F_{1,100} = 8.00$, $p = 0.006$) interactions were also significant. Taken together these results reveal that *D. melanogaster* integrates both environmental cues to generate a more robust and longer duration diapause phenotype. Furthermore, the *D. melanogaster* response in the simulation is not symmetrical, because at weeks 10 and 12, diapause levels are much lower on the approach to 'spring' than they are for similar environmental conditions as the flies approach 'winter'.

3.5. Diapause in other *Drosophila* species

We extended our diapause protocol to other tropical *Drosophila* species (Fig. 5). All the lines tested (from *D. yakuba*, *D. sechellia*, *D. erecta* and *D. ananassae*) displayed some level of diapause. *D. ananassae* showed ~100% diapause at both 12 and 28 days so we did not include them in the statistical analysis. 3-way ANOVA indicated that each of the factors (Photoperiod, Strain and Days) significantly influenced diapause levels ($F_{1,94} = 38.57$, $p < 0.0001$;

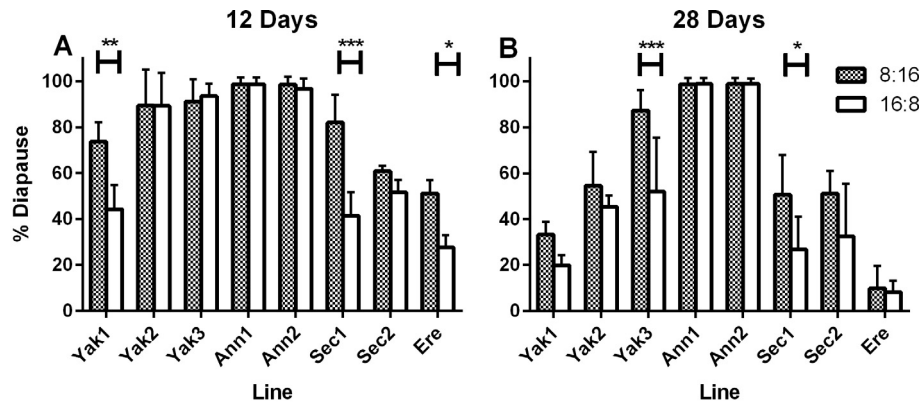


Fig. 5. Photoperiodic diapause induction after 12 or 28 days in isofemale lines of different *Drosophila* species. The level of ovarian diapause of flies maintained either at LD8:16 or LD16:8 at 12 °C was scored after 12 (panel A) and 28 (panel B) days for 8 isofemale lines. Yak: *D. yakuba*, Ann: *D. ananassae*, Sec: *D. sechellia*, Ere: *D. erecta*. Means and SEMs are given. 4913 females contributed to these data. *Indicates Duncan's *post hoc* test $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

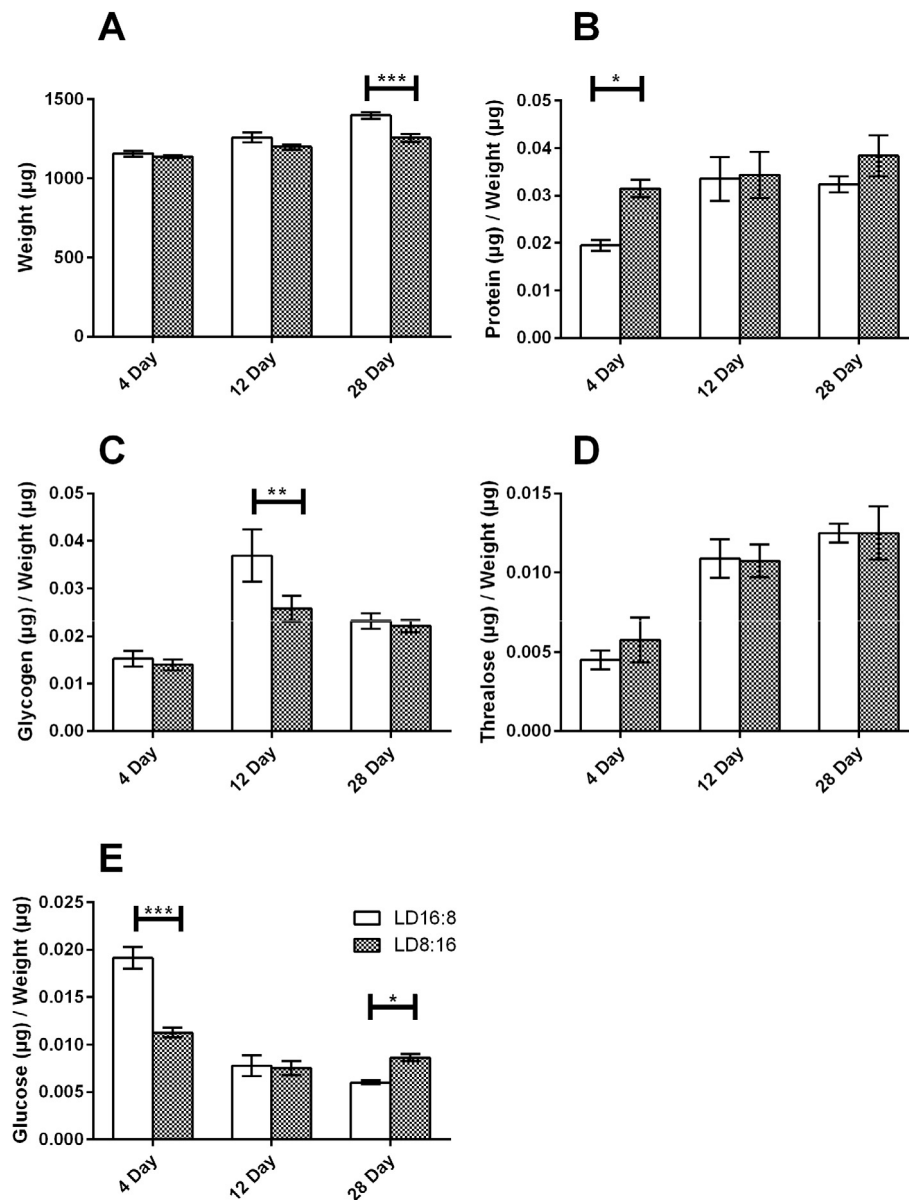


Fig. 6. Photoperiod and time effects on metabolites accumulation. Fresh weight (A) of *D. melanogaster* females (MREN from Rende, south Italy). Samples were collected after 4, 12 and 28 days at 12 °C either at LD8:16 or LD16:8. For the same samples, ratios protein/weight (B), glycogen/weight (C), trehalose/weight (D) and glucose/weight (E) are given. Bars represent means and SEMs. *Indicates Duncan's *post hoc* test $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

$F_{5,94} = 49.74$, $p < 0.0001$; $F_{1,94} = 127.76$, $p < 0.0001$ respectively, S2D Table). The lines are affected by the length of the experiment and by the photoperiod (Strain \times Days $F_{5,94} = 3.02$, $p = 0.0142$; Strain \times Photoperiod $F_{5,94} = 2.38$, $p = 0.0443$).

3.6. Diapausing flies show a dynamic carbohydrate metabolism

We measured the concentration of trehalose, glucose, glycogen, total protein and fresh weight of diapausing *D. melanogaster* females after 4, 12 and 28 days in the two photoperiods (LD8:16 and LD16:8) for the REN strain at 12 °C (Fig. 6). Overall ANOVA (S2E Table) revealed that the total weight of females increased with time ($F_{2,42} = 37.42$, $p \ll 0.0001$) and that small differences between photoperiods became significant after 28 days of induction ($F_{1,42} = 18.54$, $p < 0.0001$, Fig. 6A). A significant Photoperiod \times Days interaction ($F_{2,42} = 4.55$, $p = 0.0162$) suggests that females in long photoperiods gain weight faster than in short photoperiods. Total protein increased mostly between 4 to 12 days ($F_{2,42} = 4.8$, $p = 0.0132$) and accumulated at slightly higher levels in short photoperiods ($F_{1,42} = 5.01$, $p = 0.031$, Fig. 6B). These data suggest that proteins (including those relevant to cold acclimation) build up during diapause induction (as expected), but also that their accumulation is more prominent when short photoperiods correlate with a cold environment.

Trehalose levels increased continuously during the 28 days of diapause induction ($F_{2,42} = 22.30$, $p \ll 0.0001$) whereas glycogen increased mostly during the first 12 days ($F_{2,42} = 18.34$, $p < 0.0001$, Fig. 6C and D). In contrast, glucose levels decreased from 4 to 12 days ($F_{2,42} = 67.57$, $p \ll 0.0001$, Fig. 6E) and more dramatically so in long photoperiods ($F_{1,42} = 8.57$, $p = 0.0055$). As a consequence ANOVA revealed a significant Photoperiod \times Days interaction ($F_{2,42} = 25.01$, $p \ll 0.0001$). In addition glycogen accumulates at higher levels in long photoperiods (especially at 12 days), resulting in a marginal photoperiodic effect ($F_{1,42} = 3.93$, $p = 0.054$, Fig. 6C).

4. Discussion

A spontaneous termination from diapause after 6–8 weeks was observed for a laboratory strain and for natural populations of *D. melanogaster* (Saunders et al., 1989; Pegoraro et al., 2017). In this work we extended the protocol to include two photoperiods, and found that the ability of the lines (European and American) to detect different photoperiods, changes with time (Fig. 1). In particular, after 28 days, flies lose the overall ability to discriminate between the two photoperiods (Fig. 1) reflecting a previous study performed with populations from Maine and Florida (Emerson et al., 2009). However at 12 days they behaved similarly to European lines in their response to both photoperiods (Fig. 1). At 12 days, the levels of diapause are quite similar between the two populations, whereas at 28 days, the expected differences in levels between Maine (high) and Florida (low) were confirmed. One difference in our methods was that we examined diapause in shorter photoperiods (LD8:16) rather than LD10:14 as performed by Emerson and co-workers (Emerson et al., 2009) thus likely increasing the probability of obtaining a photoperiodic effect.

In addition, both *D. simulans* populations, one European and one African, show significant levels of diapause at 12 days, the levels in the former being largely indistinguishable from its sympatric Italian *D. melanogaster* equivalent (SREN v MREN) (Fig. 2). Furthermore, the European *D. simulans* population is photoperiodic, whereas the African is not, and at 28 days diapause falls dramatically in the *D. simulans* African population. This could explain why Schmidt and colleagues reported that African *D. melanogaster* and *D. simulans* do not show an ovarian diapause response: in their

experiments females were observed after one month, when, according to our data, the vast majority of diapausing flies had spontaneously reverted to a reproductive state (Schmidt and Conde, 2006).

In light of these results we extended our experiment to *D. melanogaster* lines from Zambia (Fig. 3) and to other *Drosophila* species (Fig. 5). Our results confirmed that diapause is present in all the lines/species tested, but that its levels decrease dramatically over time. Interestingly, the photoperiodic response is not very strong in these lines, not even at 12 days. Our results with African lines appear, at least superficially, to contradict those of Fabian et al. who examined diapause in 119 lines from 10 African populations (Fabian et al., 2015). In their study, only 2/119 lines showed a diapause response after 28 days in inducing conditions at 11 °C. However they defined a diapausing line as one having a majority of individuals in diapause so any line with even 50% diapausing females would be considered as non-diapausing. If we impose that definition on our Zambian lines, only 3/10 would show diapause at 12 days and only 1/10 after 28 days, yet it is clear especially at 12 days, that 7/10 of the lines show substantial (>40%) levels of diapause, and this is at 12 rather than 11 °C. Consequently we suspect that most of the lines studied in Fabian et al. would show some level of diapause, which would be amplified had the observations also been carried out after 12 days.

The spontaneous diapause termination after 28 days might seem something of a puzzle if one extrapolates this to the natural situation, as it implies that the females would become fertile in the middle of winter. However, we should reflect that the laboratory conditions used to study diapause involve a constant temperature, usually at the absolute threshold of inducing ovarian arrest and with a constant photoperiod over the course of the experiment. Perhaps it is not so surprising then that females, on not detecting the expected fall in temperatures that herald oncoming winter, fail to consolidate and stabilise their response. Under more natural conditions, feedback from falling temperatures and shorter photoperiods, as autumn moves into winter, might be expected to generate a more stable phenotype.

In fact when we implemented a simple winter simulation protocol we observed sustained levels of ovarian diapause in *D. melanogaster* for 8 weeks and only a gentle decrease in the following 4 weeks when the temperature was raised and photoperiod extended (Fig. 4A). Furthermore, it appears that the ovarian response is not symmetrical in that the direction of change of the environmental parameters, whether moving into winter or into spring, also has a significant effect on diapause. Consequently the female appears to be sensing the dynamics of environmental changes as well as their absolute values. Interestingly, simulating winter does not result in a sustained level of diapause in *D. simulans* (SREN) (Fig. 4B). If this result is extended to many different *D. simulans* populations it would suggest that in nature, *D. simulans* might not survive winter in temperate regions. Consequently these areas could be repopulated annually by *D. simulans* from warmer clines, consistent with what was concluded by Sedghifar and co-workers (Sedghifar et al., 2016). This could conceivably explain the relative rarity of *D. simulans* clines as compare to *D. melanogaster* (Machado et al., 2016). Traditionally the larger effective population size of *D. simulans* has been invoked to explain why *D. simulans* is less clinal than *D. melanogaster* (Aquadro et al., 1988). The alternative simpler view suggested by our results presents an opportunity to study *D. simulans* cold resistance, physiologically and metabolically.

The observed trehalose accumulation in particular is reminiscent of diapause-associated increase in levels of this sugar in many species (Guo et al., 2015; Rozsypal et al., 2013; Hodkova and Hodek, 2004; Xu et al., 2009; Su et al., 1994; Sasibhushan et al., 2013; Heydari and Izadi, 2014; Lu et al., 2014). Our results largely

overlap with those previously reported by Kubrak et al. (2014) where both glycogen and trehalose levels rose for the first 3 weeks of diapause induction. In our experiment glucose levels decreased initially and then remained at constant levels whereas in Kubrak et al. (2014) glucose increased to a higher level and then remained constant over their 12 week experiment. Trehalose levels correlate with diapause in many species, and it plays a crucial role in the adaptation to many environmental stresses including desiccation, freezing and heat shocks (Tang et al., 2008). Indeed both Denlinger (1986) and Pullin (Denlinger, 1986; Pullin, 1996) suggested that the diapause associated accumulation of carbohydrates could be a primitive feature of ancestral (tropical) insects stress response that has then evolved in relation to overwintering in temperate regions. Our results reveal that trehalose (and glycogen) levels at 12 and 28 days are elevated to similar values, so clearly the stress response had been activated and maintained. However diapause levels fall after 28 days but trehalose and glycogen do not, suggesting an uncoupling of carbohydrate metabolism with diapause *per se*. This suggests that trehalose is at best a very rough biomarker for reproductive dormancy because although egg production is re-initiated after 28 days in many females, their basic locomotor activity level, and hence their overall metabolism, is still very low at such temperatures (Vanin et al., 2012).

African *D. simulans* may indeed not be photoperiodic after 12 days (although we have only tested one composite population) but their European cousins certainly are, so the photoperiodic component may have evolved in this species more recently than in *D. melanogaster*, which were also photoperiodic in high altitude Kenyan populations but not in Zambian lines. We speculate that the relative high level of admixture in Kenya (40%) might contribute to this phenotype (Pool et al., 2012) as this particular population was collected at high altitude (2360 m asl). These more extreme conditions might have selected for more cold adapted variants, favouring European (photoperiodic) alleles over the local African ones.

We were also very surprised to find substantial levels of diapause that was photoperiodic in the lines of the tropical species, *D. yakuba*, *D. ananassae*, *D. sechellia* and rather less in *D. erecta*. While more extensive sampling would be welcomed, we would need to invoke multiple independent evolution of diapause along these lineages to avoid the more parsimonious explanation that diapause might be an ancient adaptation that was present in their common ancestors. Our results certainly would support the view that diapause primarily evolved as stress response in tropical sub-Saharan Africa, possibly to avoid desiccation during the annual wet-dry season cycle and was later adapted to the more dynamic European seasonal environment, albeit somewhat haphazardly, for overwintering in the cosmopolitan *D. melanogaster* and *D. simulans*.

Future laboratory experiments with these *Drosophila* species should consider mimicking seasonal changes more realistically by reducing temperatures and photoperiods gradually from autumnal to winter conditions, as this is likely to significantly consolidate the diapause response. Furthermore, the frequency of diapause in *D. melanogaster* may be quite significantly underestimated in many studies because of the way it is scored (eg (Fabian et al., 2015)). This not only leads to results which are difficult to compare between groups, but also might lead to both type 1 and type 2 errors when comparing populations or conditions characterised by different levels of diapause (Fig. S1). It may be that the *D. melanogaster* diapause response is not really as 'shallow' as it can sometimes appear and this is reflected in our more realistic winter simulation paradigm. The more robust laboratory diapause phenotype that we can generate with this species has important implications for the further genetic dissection of the phenotype, particularly because the molecular genetic toolbox in *D. melanoga-*

ster far exceeds anything else available in other arthropods. Diapause also infiltrates insect life histories, and its evolutionary and ecological flexibility in *Drosophila* will make it an important character for studying selection at the relevant loci which underlie the response.

Competing interests

We have no competing interests.

Authors' contributions

VZ performed the diapause experiments in European and American *D. melanogaster* lines, and in European and African *D. melanogaster* and *D. simulans* lines (Figs. 1 and 2). LC performed the winter simulation experiment, assessed diapause in Zambian *D. melanogaster* lines and in different *Drosophila* species, and performed the metabolites experiment (Figs. 3–6). MP contributed in designing the experiments and analysing the data. MP, VZ and CPK wrote the manuscript. ET and CPK conceived and coordinated the study and obtained the funding. All authors gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2017.01.017>.

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