Is diapause an ancient adaptation in Drosophila?

Valeria Zonato a,1, Lewis Collins a,1, Mirko Pegoraro a,1, Eran Tauber a,b, Charalambos P. Kyriacou a,4

a Department of Genetics, University of Leicester, Leicester LE1 7RH, UK
b Department of Evolutionary & Environmental Biology, University of Haifa, Haifa 3498838, Israel

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 neuroendocrinologically 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 phos- phatidylinositol 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.
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. Is-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 Is-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). Is-tim increases diapause levels, so would be more adaptive at higher latitudes (Tauber et al., 2007). However, D. melanogaster demographic histoty and the location of origin of the new and young Is-tim allele have contributed to the creation of a seemingly counterintuitive European cline in Is-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 ezaoana), 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 glaciation (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 Table S1. 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 been 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 used to measure trehalose, glucose and glycogen.

The glucose analysis kit from Sigma Aldrich (Glucose GO Assay Kit GAG020) 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
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 trehalose 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 different photoperiods, to investigate the temporal dynamics of the diapause photoperiodic response (Fig. 1). Our overall ANOVA (S2A...
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 × 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 Kenyan 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 Z1-291 shows more than 20% diapause after 28 days). ANOVA (S2C Table) indicates a significant Strain ($F_{1,81} = 25.27, p < 0.0001$) and Days ($F_{1,81} = 275.97, p < 0.0001$) but no Photoperiodic ($F_{1,81} = 0.82, p = 0.368, ns$) 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 × Days $F_{1,81} = 5.68, p < 0.0001$; Strain × Photoperiod $F_{1,81} = 4.09, p < 0.0001$).

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 × constant 12°C, $F_{1,100} = 80.63, p < 0.0001$) and Species ($F_{1,100} = 73.24, p < 0.0001$) main effects. Weeks × Conditions ($F_{4,100} = 6.23, p < 0.0001$), Weeks × Species ($F_{4,100} = 7.85, p < 0.001$) and Conditions × 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 (MBEN 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_{S,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_{S,94} = 3.02, p = 0.0142; Strain \times Photoperiod F_{S,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 ≪ 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 × 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 ≪ 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 ≪ 0.0001, Fig. 6E) and more drastically so in long photoperiods (F_{1,42} = 8.57, p = 0.0055). As a consequence ANOVA revealed a significant Photoperiod × Days interaction (F_{2,42} = 25.01, p ≪ 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 et al. (Parisi et al., 2015; Pegoraro et al., 2013; Heydari and Izadi, 2014). Interestingly, the 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 compared 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., 2008; Su et al., 1994; Sasibhusan 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 (Vanim 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 autumn to winter conditions, as this is likely to significantly condition 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 (e.g. 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. melanogaster* 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.

**References**


