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Geographical analysis of diapause inducibility in European *Drosophila melanogaster* populations

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ABSTRACT

Seasonal overwintering in insects represents an adaptation to stressful environments and in European *Drosophila melanogaster* females, low temperatures and short photoperiods can induce an ovarian diapause. Diapause may represent a recent (<15 Ky) adaptation to the colonisation of temperate Europe by *D. melanogaster* from tropical sub-Saharan Africa, because African *D. melanogaster* and the sibling species *D. simulans*, have been reported to fail to undergo diapause. Over the past few centuries, *D. melanogaster* have also invaded North America and Australia, and eastern populations on both continents show a predictable latitudinal cline in diapause induction. In Europe however, a new diapause-enhancing *timeless* allele, *ls-tim*, is observed at high levels in southern Italy (~80%), where it appears to have arisen and has spread throughout the continent with a frequency of ~20% in Scandinavia. Given the phenotype of *ls-tim* and its geographical distribution, we might predict that it would work against any latitudinal cline in diapause induction within Europe. Indeed we reveal that any latitudinal cline for diapause in Europe is very weak, as predicted by *ls-tim* frequencies. In contrast, we determine *ls-tim* frequencies in North America and observe that they would be expected to strengthen the latitudinal pattern of diapause. Our results reveal how a newly arisen mutation, can, via the stochastic nature of where it initially arose, blur an otherwise adaptive geographical pattern.

1. Introduction

Many organisms resist unfavourable seasonal conditions by inducing a period of dormancy or overwintering, and insects that inhabit temperate zones delay their reproduction and lower their metabolic requirements in a phenomenon termed 'diapause'. Diapause can occur at different developmental stages depending on the insect species and its expression is triggered by environmental cues, usually photoperiod and/or temperature (Kostal, 2011; Schiesari et al., 2011; Williams et al., 2010). Some *Drosophila* species have a reproductive diapause in the adult stage if exposed to short day lengths and low temperatures (Lumme et al., 1974; Saunders et al., 1989, 1990; Ferveur et al., 1995). Diapause is neurohormonally mediated involving many physiological and metabolic changes that provide increased resistance to environmental stress and result in slower aging (Schiesari et al., 2011; Tatar and Yin, 2001; Tauber et al., 1986; Kubrak et al., 2014), and the insulin pathway has been shown to play a key role (Williams et al., 2006; Schiesari et al., 2016). It is thus an excellent model system for studying the neurogenetics and evolutionary biology of a very important life-history trait, with additional implications for understanding longevity.

D. melanogaster overwinter in temperate zones as adults and under colder environments only females have been reported to survive (Izquierdo, 1991). Under winter simulations, adult females exhibit a reproductive diapause that is characterised by a reduction of juvenile hormone and an arrest of oogenesis at the previtellogenic stage (reviewed in (Schiesari et al., 2011)). This ovarian dormancy can be readily induced by exposing females to <14 °C before they are 10 h post-eclosion in short photoperiods <12 h (Saunders et al., 1989). The phenotype in laboratory strains can show a spontaneous reversion after about 6–8 weeks when maintained at these constant conditions, but exposure to higher temperatures stimulates ovarian maturation (Saunders et al., 1989; Kubrak et al., 2014). The photoperiodic modulation of diapause in natural *D. melanogaster* populations is quite shallow (Tauber

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et al., 2007) and temperature appears to play the more dominant role (Emerson et al., 2009a). This feature of diapause and the reported absence of a diapause phenotype in African *D. melanogaster* and the sympatric sibling species *D. simulans* (Schmidt et al., 2005a,b; Schmidt and Conde, 2006) suggests that diapause in *D. melanogaster* may be a relatively recent evolutionary adaptation to overwintering, perhaps evolving in response to the colonisation of temperate regions, from its ancestral home in tropical Africa after the last Ice Age ~10–15 Kya (Lachaise et al., 1988). Indeed some authors suggested that diapause may have evolved from an ancestral stress response to the wet-dry season in subtropical populations (Fabian et al., 2015; Denlinger, 1986; Pullin, 1996; Denlinger, 1991; Zhao et al., 2015). *D. melanogaster* has also colonised North America and Australia in the past 2–300 years and during this time both continents have evolved a latitudinal cline in diapause. While in North America elevated levels of ovarian arrest are observed at the higher latitudes, in Australia "dormancy" increases toward both temperate and tropical regions (Lee et al., 2011; Schmidt et al., 2005a; Williams and Sokolowski, 1993). However, a linear cline toward higher latitude was revealed between 25 and 43°S (Lee et al., 2011).

Whether a cline in diapause exists in European *D. melanogaster* populations is not known. However, in Europe, a newly arisen natural polymorphism in the circadian clock gene, *timeless* (*tim*), has a major effect on diapause induction (Tauber et al., 2007; Sandrelli et al., 2007). The ancestral allele, *s-tim* encodes a N-terminal truncated S-TIM isoform, while a recent variant *ls-tim,* has recruited an additional N-terminal 23 residues from an upstream *ATG* codon and so encodes both S-TIM plus a longer isoform, L-TIM. This variant appears to have arisen in south-eastern Italy, at most a few thousand years ago (Tauber et al., 2007), although more recent work suggests it could be as little as 300 years old (Zonato et al., submitted), and has established itself in this region at high levels (~80%). Moving northwards from southern Italy, *ls-tim* frequency falls linearly, so that in Sweden it is at frequencies of ~20%, generating an impressive latitudinal cline between the Mediterranean and Scandinavia (Tauber et al., 2007). Neutrality tests suggest that the *ls/s-tim* polymorphism is maintained by directional selection and at least part of the reason why it may be adaptive, is that it enhances diapause, a phenotype that might be expected to be favoured throughout temperate Europe (Tauber et al., 2007). The molecular basis for this effect appears to be a reduced physical interaction of L-TIM with the circadian photoreceptor, Cryptochrome (CRY), which leads to a more light-stable TIM in *ls-tim* flies (Sandrelli et al., 2007). One consequences of this is that the circadian clock of *ls-tim* flies is less light-sensitive, and in seasonal environments such as Europe, this has been argued to provide an adaptive response to very long summer photoperiods that can otherwise disrupt circadian cycles (Pittendrigh et al., 1991). Similarly, *ls-tim* females may interpret long days as shorter ones and move into diapause prematurely, again a potentially adaptive response in seasonal environments.

The existence of corresponding clines on different continents is often used to support the view that similar selective pressures have shaped the phenotype or genotype (Lee et al., 2011). However, because the diapausing promoting *ls-tim* allele is at higher levels in southern Italy, this would be expected to cloud any latitudinal relationship with the phenotype (Tauber et al., 2007). Thus we have a clear *a priori* expectation that a European cline in diapause, if it exists at all, should be considerably shallower compared to that in North America. If this turns out to be the case, it would provide a novel example of a new adaptive mutation blurring what may have been an established phenotypic cline in Europe simply because of the geography of its origins.

2. Methods

2.1. Latitude experiment

D. melanogaster flies collected from isofemale lines from six geographical locations in Europe between 2007 and 2010 were used in this study (performed in 2011/12) Flies natural *D. melanogaster* populations were collected from southern Spain, various locations in southern and northern Italy, Holland and Finland (Table S1). The long-established laboratory strain, Canton-S, was used for comparison but we did not include it in the analyses. Fertilized females were isolated in single 2 × 10 cm plastic vials containing fly food (4.6% sugar, 4.6% brewer's yeast, 1.25% agar, 0.2% methyl 4-hydroxybenzoate) to establish a number of isofemale lines from each population (Table S1). From the time of collection, flies were maintained at 18 °C in light-dark (LD) 12:12 cycles. From each location, five fertilized females from 10 to 25 isofemale lines (similarly to Lee et al. (2011)) were placed together in 200 ml glass food bottles to establish the six experimental populations used in the study. The time from collection to experimental analysis of *D. melanogaster* lines varied from within 1–3 years (Table S1).

We obtained between 25 and 62 isofemale lines from Maine, Georgia, North Carolina, New Jersey and Florida (Table S1; (Paaby et al., 2010)). These lines were established previously in Paul Schmidt's laboratory and used for the identification of adaptive polymorphism affecting life history traits (Paaby et al., 2010). DNA from a single fly from each isofemale line, was used to determine the frequency of the *ls/s-tim* polymorphism along the east coast of the USA (see Section 2.3; Table S1).

2.2. Ovarian diapause

Male and female flies were collected within a six hour post eclosion window and placed under six photoperiods: LD 8:16, 10:14, 12:12, 14:10, 16:8 and 18:6. They were maintained in 2 × 10 cm plastic vials in 12.5 × 19 × 26 cm light boxes containing a white fluorescent tube (standard T5 F4W/33) with an inbuilt heat sink and an electric fan (220 V, 0.09 A) to control the temperature. Temperature was monitored inside the chambers and maintained at 12.5 ± 0.3 °C within an incubator (Fig. S1A and B). An average of 32 females from each vial was dissected in PBS, 12 or 28 days later, and their ovaries characterized according to (King, 1970). We used a very stringent criterion so that a female was considered to be in reproductive arrest if its most advanced oocyte was pre-vitellogenic (prior to stage 8). The proportion of females in diapause from each vial represented an individual replicate, and ~6 replicates were analysed for each population/photoperiod. Females that were maintained for 28 days were examined only at LD10:14. As a thermal control, ovarian diapause was also tested simultaneously in 'constant darkness (DD)' by covering some additional vials in metal foil even though they were exposed to the same LD cycle as the experimental groups within the same light boxes in the incubators. The diapausing proportion was transformed to arcsin for ANOVA. Females maintained for 12 days were studied at all 6 photoperiods, and as expected, the DD thermal controls which were performed simultaneously with these experiments showed neither consistent nor significant thermoperiodic effects ($F_{5,37} = 1.97$, $p = 0.106$, effect size $\boldsymbol{\eta^2 = 0.09}$).

2.3. Timeless polymorphism genotyping

The genotyping of the American populations was carried out using the same allelic-specific approach as described in Tauber et al. (2007). Two PCRs were performed for each DNA sample using a forward specific for either *ls-tim* or *s-tim*, together with a common reverse primer.

3. Results

3.1. Ovarian diapause in European populations does not show a significant latitudinal cline

Ovarian arrest (eggs < stage 8) for the different populations at the various photoperiods is reported in Fig. 1A. Even though the Canton-S laboratory strain shows a similar pattern to the other natural populations, given its many decades of domestication, we excluded it from further data analysis. Inspection of the natural populations after 12 days in diapausing conditions revealed significant Photoperiod ($F_{5,131} = 12.2$, $p = 1.02\ e^{-09}$, $\boldsymbol{\eta^2 = 0.21}$) and Population ($F_{5,131} = 9.8$, $p = 5.49\ e^{-08}$, $\boldsymbol{\eta^2 = 0.17}$) main effects and a significant Population x Photoperiod interaction ($F_{25,131} = 2.1$, $p = 0.005$, $\boldsymbol{\eta^2 = 0.18}$), which was caused by the 'flatter' profile of the Spanish population, SP22. Generally, a lower level of diapause was observed in southern European populations (MREN, SAL, BOL) compared to the intermediate and more northern ones (HU, KOR). However, the population from southern Spain (SP22) showed very high diapause levels, especially at the extreme photoperiods (LD8:16, 18:6).

We also took the data at each photoperiod, and calculated the correlation between diapause and latitude. Only at LD10:14 was the correlation significant ($r = 0.892$, $p < 0.05$, Table 1, Fig. 2B, but

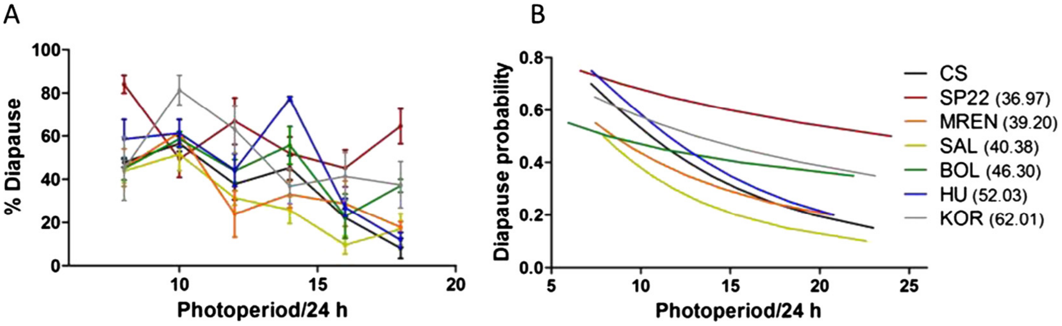

Fig. 1. Photoperiodic reproductive arrest in *D. melanogaster* females. A. Percent diapause (mean and sem) is plotted against photoperiod. B. Logprobit transformation of data in A. The probability of expressing reproductive arrest (Y-axis) is plotted against photoperiod. 9444 individual females were dissected and contributed to these data. Critical Day Length (CDL50) for reproductive arrest in *D. melanogaster* females can be calculated at the 50% point (see Table 1 for fiducial limits). Latitudes are given in brackets next to each population. CS: Canton S, SP22: Nijar, Spain; MREN: Rende, Italy; SAL: Salice, Italy; BOL: Bolzano, Italy; Hu: Houten, Holland; KOR: Korpilahti, Finland. See Table S1 for more details.

Table 1

Critical daylength and latitudinal cline results for European populations. A) The CDL50 and confidence limit are given for each population. B) r^2 values reflecting the correlation between diapause and latitude at each photoperiod for 12 day data, and for 28 day data at LD10:14. $^*p < 0.05$.

A

Population	CDL50 (h)	95% Confidential limits (h)	
KOR	13.09	11.37	15.6
HU	11.54	10.69	13.26
BOL	8.18	1.13	10.64
SAL	7.90	6.74	8.76
MREN	8.48	7.23	9.4
SP22	23.99	17.74	97.58
[CS]	10.96	9.95	11.09

B

Photoperiod (h/24)							
(12d)	8	10	12	14	16	18	10 (28d)
r^2	0.129	0.795*	0.144	0.048	0.058	0.023	0.316

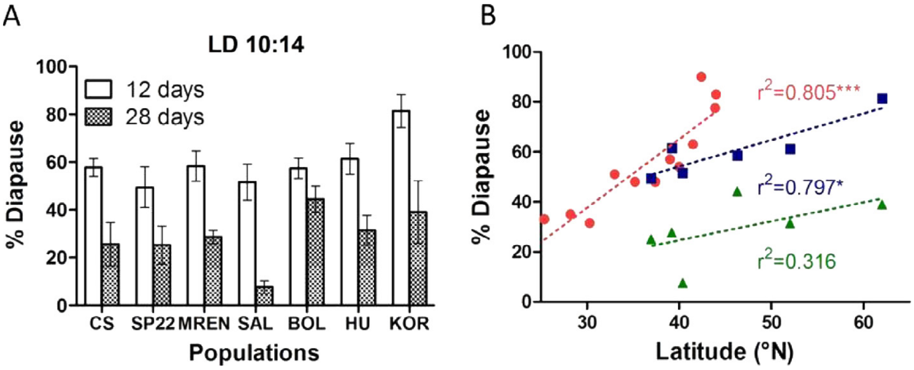

Fig. 2. Diapause for European natural populations at LD10:14 at 12 and 28 days. A) Mean diapause (and sem) are shown for the strains in Fig. 1. The results from 12 days are taken from the LD10:14 data from Fig. 1A. The strains are placed in order of ascending latitude, apart from Canton-S (CS). 2179 individual females were examined. B) Direct comparison of diapause clines in North American (red) (redrawn from (Schmidt et al., 2005a) and European populations in flies maintained for 12 (blue) and 28 (green) days at LD10:14. $^*p < 0.05$, $^{***}p < 0.001$.

not with Bonferroni correction) because only at this photoperiod, does the most southern population, SP22, have marginally the lowest level of diapause compared to the others and the Finnish population have the highest (Figs. 1A, 2A, Table 1B). We also used probit analysis (Finney, 1949) to calculate the critical daylength at which each population shows 50% of individuals in diapause (CDL_{50}; Fig. 1B). The CDL_{50} for the populations from southern Italy was 7.9 h (SAL) and 8.5 h (MREN), northern Italian population (BOL) it was 8.2 h, for the more northern HU population it was 11.5 h, for the extreme northern KOR population it was 13.1 h. A significant correlation of CDL_{50} with latitude is observed ($r = 0.938$, $p < 0.05$), but adding the CDL_{50} of 23.9 h from the southern Spanish population which has a surprisingly high diapause level, severely disrupts this linear pattern ($r = -0.165$, $p = 0.75$, ns). In terms of their 95% fiducial (confidence) limits, SAL, MREN and BOL formed one overlapping group, but were significantly different from HU and KOR, which overlapped with each other (see Table 1A). Thus in spite of the noisy data presented in Fig. 1A, probit analysis of these five populations provides some limited evidence for a possible latitudinal cline in the photoperiodic induction of diapause from southern Europe to Finland, with the two northern population expressing diapause earlier in the year, but only if we exclude the Spanish population SP22 (Table 1).

3.2. Spontaneous ovarian diapause reversion

Studies of diapause in American and Australian populations have dissected females after maintaining them for 25–28 days in constant low temperature and short photoperiods (Emerson et al., 2009a; Lee et al., 2011). We were thus interested in observing whether the levels of diapause at 12 days in inducing conditions would be similar to levels after one month, particularly as it has been reported that flies spontaneously revert to reproductive status after 6–8 weeks (Saunders et al., 1989). The levels of diapause after 12 or 28 days of inducing conditions at LD10:14 were compared for the natural populations (Fig. 2A). In all populations the level of diapause is reduced at 28 compared to 12 days (Day, $F_{1,41} = 64.4$, $p = 6.09\ e^{-10}$, $\boldsymbol{\eta^2 = 0.46}$), with no significant Population x Day interaction ($F_{5,41} = 1.8$, $p = 0.14$, $\boldsymbol{\eta^2 = 0.06}$). These results reveal that as many as 50% of the females that are in diapause at 12 days, will have reverted after one month. A significant Population effect was also observed ($F_{5,41} = 5.3$, $p = 0.0007$, $\boldsymbol{\eta^2 = 0.19}$), but unlike the LD10:14 data from the 12 day experiment reported above, there is no significant relationship between latitude and diapause for the corresponding 28 day, LD10:14 data ($r = 0.562$, $p = 0.25$, ns; Table 1). Consequently at 28 days there is scant evidence for a significant European latitudinal cline, in contrast to the corresponding results obtained under similar conditions in North American (Schmidt et al., 2005a; Emerson et al., 2009b) and Australian populations (Lee et al., 2011). Fig. 2B shows the comparison of the 28 day diapause results from N. America (Schmidt et al., 2005a) with both the 12 and 28 day results from Europe at LD14:10. Recall that the 12 day data from Europe was taken from LD14:10 and all other photoperiods showed no significant correlation between diapause and latitude (Table 1B).

3.3. *ls-tim* frequencies in Eastern USA show a latitudinal cline

Given our observation that, in contrast to North America, no compelling latitudinal cline for diapause was observed after 12-day, and certainly not after 28-days, we sought to examine whether *ls-tim* frequencies could be contributing to the robust clinal pattern in the USA. We obtained five populations, from Maine, Georgia, North Carolina, New Jersey and Florida (Table S1) and genotyped the *tim* polymorphism. We observed, even with five populations, a significant correlation with latitude, but in contrast to Europe, the levels of *ls-tim* were twofold elevated in the north compared to the south, even though maximum *ls-tim* frequencies were at ~30% (Fig. 3). Thus the diapause-enhancing *ls-tim* variant would be expected to contribute to the strong clinal pattern in overwintering observed on this continent by Schmidt and colleagues (Schmidt et al., 2005a; Cogni et al., 2014).

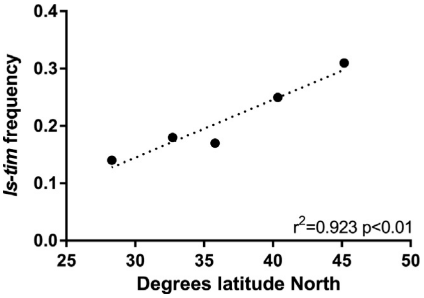

Fig. 3. The frequency of *ls-tim* in natural *D. melanogaster* populations from the eastern USA. Frequency of *ls-tim* in five populations collected along the eastern seaboard of the USA. There is a significant linear relationship between *ls-tim* frequency and latitude ($r^2 = 0.923$, $p < 0.01$).

4. Discussion

We have carried out a full photoperiodic analysis of ovarian arrest in females from several European populations. When the females were maintained in diapausing conditions for 12 days there was little evidence for a latitudinal cline unless we removed our most southern Iberian population. Even then the data was still very noisy. When we treated each photoperiod independently, one significant correlation with latitude was observed with the LD10:14 data, but again, inspection of Fig. 1A shows that the effect was largely caused by the higher diapause levels of the Finnish population – the rest of the populations were clustered together. However, even this modest effect could not be maintained at the more stringent LD8:16 photoperiod. Consequently our use of more than a single short photoperiod in which to study diapause prevented us from making an incorrect conclusion, had we only studied the LD10:14 condition as in previous studies (Lee et al., 2011; Schmidt et al., 2005a). When we used probit analysis to smooth out the 12 day raw data, we did obtain a relationship between critical photoperiods of northern versus southern populations, but only if we removed the Spanish outlier. However, inspecting the corresponding CDL_{50} again reveals that the profiles of the non-Iberian populations cut cross each other, reducing our confidence in the result (Fig. 1B). When we repeated the experiment at the LD10:14 photoperiod, but maintained the females for 28 days in diapausing conditions to match the procedures used by Schmidt et al. (2005a), there was even less evidence for a latitudinal cline.

Lee et al. (2011) have found that diapause increases both toward temperate and tropical regions and suggests that including more low latitude (<25°N) populations from North America could result in a similar trend. These authors speculate that pleiotropy of genes affecting diapause or direct selection for higher resistance to stressful conditions could explain this non-linear trend. It is possible that diapause may have evolved from an ancestral stress response to the wet-dry season in subtropical populations and indeed many tropical insects express dormancy/diapause (Fabian et al., 2015; Denlinger, 1986; Pullin, 1996; Denlinger, 1991; Zhao et al., 2015). However, our populations span between 37°N2°W and 62°N25°E, a geographical distribution that would be expected to produce a robust linear cline of diapause incidence as in Lee et al., (2011) and Schmidt et al. (2005a). We replotted the data from both these studies and reduced the number of populations to 6 with a similar latitude distribution to ours. In both cases these produced highly significant clines (correlation coefficient $r = 0.86$ $p < 0.05$, Australia; $r = 0.94$ $p < 0.01$, North America) suggesting that the number of populations we have tested in Europe should be sufficient to identify a robust latitudinal cline (Table S2).

The lack of a photoperiodic response in the population from southern Spain is puzzling. The average January temperature in Nijar is 10.2 °C (min-max 6–14 °C, lat 37° N, data from http://en.climate-data.org) compared to Rende in Italy where the mean is 8.5 °C (min-max 6.1–11 °C, lat 39.2°N), and August average temperatures are very similar (Nijar 24.8 °C, 20–29.7C min-max; Rende 23.8 °C, 19.9–27.7 °C min-max), so SP22 flies from Nijar may not regularly be exposed in the wild to diapause-inducing temperatures. If this scenario is correct, they might be expected to show a more dramatic non-photoperiodic diapause response because average temperatures of <13 °C may be more stressful. In addition, reduced precipitation in Nijar during the summer (average August rain fall 4 mm compared to 29 mm in Rende) might favour higher diapause levels as an escape from desiccation.

African admixture within the Iberian Peninsula is also likely, but as diapause levels in Africa are very low (Fabian et al., 2015), an African contribution should reduce the overall levels of diapause in Spanish population, not increase them. Indeed, admixture in *Drosophila* populations was found to be higher in North America than in Europe (Caracristi and Schlotterer, 2003) especially on the US east coast where admixture itself shows a latitudinal cline (Duchen et al., 2013; Kao et al., 2015; Bergland et al., 2016). This was confirmed by studying the level of linkage disequilibrium (LD) as an indicator of recent admixture (Loh et al., 2013) where it was observed to be higher in North America than in both Europe and Africa (Kao et al., 2015). Bergland et al. (2016) suggested that the clinal variation of many polymorphisms in North America and Australia was generated by recent contact between African and European populations. These demographic effects are less likely to affect clines in Europe given the lower level of admixture and LD. In fact Bozicevic et al. (2016) studying cold adaptation in European and African populations suggested that demographic history could not explain the SNPs distribution associated with the studied traits.

The European cline presented here could be extended by including more populations to test for local adaptation (e.g. the Iberian Peninsula) or for other clines (e.g. altitude). It would be of interest to investigate whether the latitudinal clines in diapause incidence in Australia and America could be confirmed using a full photoperiodic analysis as in this study. In Schmidt et al. (2005a) an isofemale line was scored as diapausing when the most advanced oocyte among three females was pre-vitellogenic. This protocol would be unsuitable for investigating a full photoperiodic response given that a line would be scored as non-diapausing even if 66% of the ovaries were pre-vitellogenic.

Our results are however consistent with the observation that the diapause enhancing *ls-tim* allele is at high frequencies in southern Europe ((Tauber et al., 2007; Sandrelli et al., 2007), Fig. S2), and this would be expected to flatten out any diapause cline. The *ls/s-tim* polymorphism is under directional selection and is a major player in determining diapause variability, in fact across 6 lines, 3 geographical locations and 6 photoperiods there is substantial difference in diapause induction of 24.7% ± 11.2% (mean ± stdev) between *ls-tim* and *s-tim* lines (Tauber et al., 2007). In North America, our survey of *ls-tim* frequencies in five populations, from Maine to Florida revealed the opposite geographical pattern compared to Europe with ~30% *ls-tim* in the northern state compared to ~15% in the southern. As *D. melanogaster* is a human commensal that shadows migrations (Keller, 2007) we speculate that the admixture of predominantly African flies in the southern states (Kao et al., 2015), that do not carry *ls-tim* at any appreciable frequencies, with predominantly European flies which would have been introduced in the northern states in the last ~300 years by northern Europeans initially, could have given rise to this pattern. As seasonal selection would be expected to be stronger in New England compared to Florida, this could conceivably have modulated the initial low colonising frequencies of *ls-tim* to those we see today. Irrespective of how this gradient in *ls-tim* frequency originated, it would favour enhanced diapause in the north and could contribute to the diapause cline that has been reported to be determined by polymorphism in *couch-potato* (*cpo*) (Cogni et al., 2014; Schmidt et al., 2008; Machado et al., 2016).

Recently Zonato et al. (2016) have found that SNP $cpo^{Ala347Val}$ shows a weak but significant cline in allele frequency in Europe that appears to be independent from the *In(3R)Payne* chromosomal inversion, in which the *cpo* locus resides. The diapause enhancing variant (cpo^{347Val}) is observed at higher frequencies in northern latitudes. Consequently, the $cpo^{Ala347Val}$ cline and the *ls/s-tim* cline work in opposite directions to each other with the diapause enhancing *cpo* allele in northern and similarly enhancing *ls-tim* allele in southern latitudes. The very weak cline in diapause that we observe in Europe therefore correlates, at least superficially, with the similarly weak cline in $cpo^{Ala347Val}$.

To explain the different patterns of diapause we observe in North America and Europe, we can postulate that the differing intensities of climatic selection such as temperature, that show steeper changes on the eastern coast of America than in mainland Europe, may also be partly responsible (Fig. S3). However, clines in allozymes such as *Adh* have been reported in north America, Europe and Australia and they are all similar in that elevated Adh^F frequencies are associated with higher latitudes (David et al., 1986; Oakeshott et al., 1982; Machado et al., 2016). Other polymorphisms show similar geographical gradients in different continents including in the *Insulin-like-Receptor* (also lies within *In(3R)Payne* (Paaby et al., 2010)), the Thr-Gly repeats in the *period* gene (Costa et al., 1992; Sawyer et al., 2006), as well as other chromosomal inversions (Knibb, 1982). Consequently we suspect that the frequencies of *ls-tim* in Europe and in America are playing significant but opposite roles in generating diapause clines. In America the *cpo* cline is consistent with the *tim* cline while in Europe they are opposed to each other exacerbating the difference in diapause distribution between the two continents. It is therefore intriguing that *ls-tim*, a recently arisen beneficial mutation, by the stochastic nature of its proposed geographical origin and its consequent spread (Sandrelli et al., 2007), has clouded what may have previously been an adaptive latitudinal pattern in a phenotype that is important for overwintering.

In conclusion, our results reveal that inter-continental patterns of diapause can be quite different, with the evidence for a diapause cline in Europe being unconvincing, and unlikely to be the result of any demographic effect given the low admixture and LD in European populations. However, because of the underlying *ls-tim* frequencies in Europe (and North America that we have discovered), we have strong *a priori* expectations for inter-continental differences in diapause. The distribution of *ls-tim* is likely transient, reflecting the history of the new allele, and will change over many generations as *ls-tim* spreads further into the north of Europe, where it is particularly adaptive (Tauber et al., 2007; Sandrelli et al., 2007). This will eventually generate a steeper diapause cline more similar to that in N. America, where the introduction of significant frequencies of *ls-tim* in the north, initially by northern Europeans beginning three centuries ago, will have enhanced the diapause cline that was to develop on that continent. Thus the absence of consistency among continents for diapause, or indeed any phenotypic cline does not necessarily weaken the argument for such clines being adaptive. It may be that differences between continents in the spatial distribution of a trait may be due to the underlying genetic basis and population genetic history of the phenotype, not any differences in the adaptive value of the trait *per se.*

Authors' contributions

MP carried out most of diapause phenotyping (Figs. 1 and 2), participated in data analysis, participated in the design of the study and helped drafting the manuscript; VZ carried out the *ls/s tim* genotyping and statistical analyses (Fig. 3); ERT contributed to the diapause phenotyping (Figs. 1 and 2); GF did the temperature survey (Fig. S3); ET and CPK conceived the study, designed the study, coordinated the study and drafted the manuscript. All authors gave final approval for publication.

Competing interests

We have no competing interests.

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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.2017.01.015.

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