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Clinal variation in the temperature and photoperiodic control of reproductive diapause in *Drosophila montana* females

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ABSTRACT

Insect adaptation to climatic conditions at different latitudes has required changes in life-history traits linked with survival and reproduction. Several species, including *Drosophila montana*, show robust latitudinal variation in the critical day length (CDL), below which more than half of the emerging females enter reproductive diapause at a given temperature. Here we used a novel approach to find out whether *D. montana* also shows latitudinal variation in the critical temperature (CTemp), above which the photoperiodic regulation of diapause is disturbed so that the females develop ovaries in daylengths that are far below their CDL. We estimated CTemp for 53 strains from different latitudes on 3 continents after measuring their diapause proportions at a range of temperatures in 12 h daylength (for 29 of the strains also in continuous darkness). In 12 h daylength, CTemp increased towards high latitudes alongside an increase in CDL, and in 3 high-latitude strains diapause proportion exceeded 50% in all temperatures. In continuous darkness, the diapause proportion was above 50% in the lowest temperature(s) in only 9 strains, all of which came from high latitudes. In the second part of the study, we measured changes in CTemp and CDL in a selection experiment favouring reproduction in short daylength (photoperiodic selection) and by exercising selection for females that reproduce in LD12:12 at low temperature (photoperiodic and temperature selection). In both experiments selection induced parallel changes in CDL and CTemp, confirming correlations seen between these traits along latitudinal clines. Overall, our findings suggest that selection towards strong photoperiodic diapause and long CDL at high latitudes has decreased the dependency of *D. montana* diapause on environmental temperature. Accordingly, the prevalence and timing of the diapause of *D. montana* is likely to be less vulnerable to climate warming in high- than low-latitude populations.

1. Introduction

Several insect species living in temperate zones overwinter in diapause either at the embryonic, larval, pupal or adult stage to synchronize the life cycle with appropriate environmental conditions. The onset of diapause can be regulated by a variety of environmental cues, the main ones being photoperiod and temperature. Decreasing day length enables insects to forecast seasonal changes and enter diapause at the time that is optimal at a given latitude, while temperature can affect all levels of the photoperiodic response (Saunders, 2014). Among the best examples of species with photoperiodic reproductive diapause are those of the *Drosophila virilis* group, especially *D. littoralis* and *D. montana*, where the critical day length for diapause induction (CDL; about half of emerging females enter diapause) shows clear latitudinal clines (Lankinen, 1986; Tyukmaeva et al., 2020). In these, and several other insect species, temperature has been found to enhance or inhibit diapause through an interaction with photoperiod and / or to play an important role in diapause maintenance and termination (Gill et al., 2017; Hodek and Hodková, 1988; Koštál, 2006; Pittendrigh and Takamura, 1987; Lankinen, 1986; Tyukmaeva et al., 2020).

Critical daylength (CDL) for diapause induction and the corresponding critical night length (CNL; 24 h – CDL) are typically

Abbreviations: LD, Light:Dark cycle; LL, continuous light; DD, continuous darkness; PPRC, photoperiodic response curve based on female diapause proportions in different photoperiods at a given temperature (here 16°C); CDL, critical day length = midpoint of PPRC (~50% of emerging females enter diapause); TRC, temperature response curve based on female diapause proportions at different temperatures in short daylength (here LD12:12) or continuous darkness; CTemp, critical temperature = midpoint of TRC (~50% of emerging females enter diapause).

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determined as a midpoint of the photoperiodic response curve (PPRC) based on the proportion of diapausing insects in different photoperiods (Light:Dark cycles, LDs) at constant temperature. Effects of temperature on CDL can subsequently be determined from the PPRCs drawn at different temperatures. High temperatures have been found to decrease diapause incidence and / or move the CDL towards shorter day lengths e.g., for adult reproductive diapause in *Drosophila auraria* (Pittendrigh and Takamura, 1987), *D. littoralis* (Lankinen, 1986) and *D. montana* (Tyukmaeva et al., 2020), for maternally determined larval diapause in *Calliphora vicina* (McWatters and Saunders, 1998) and for pupal diapause in *Hyphantria cunea* (Gomi, 1997). Thus, the adaptive adjustment of CDL to latitude can be seen as a change in the temperature dependence of the photoperiodic response (Pittendrigh and Takamura, 1987).

Phenology (the periodic appearance of life-cycle events) currently receives much attention as the effects of global climate change on it are so apparent (Marshall et al. 2020). Increasing temperatures induce changes in environmental pressures maintaining diapause expression within insect populations, which can lead to rapid plastic or genetic changes in their photoperiodism and phenology (Bale & Hayward, 2010; Tougeron et al., 2019). In particular, the photoperiodic responses that regulate diapause must be modified to avoid mismatches between diapause-inducing cues and the increasing growing-season temperatures (Forrest and Miller-Rushing, 2010; Denlinger, 2023). Shifts towards the CDLs that are typical to low-latitude populations have been documented in local populations of several insect species, including pitcher plant mosquito *Wyeomyia smithii* (Bradshaw and Holzapfel 2001) and water strider *Aquarius paludum* (Harada et al., 2005).

While temperature and photoperiod commonly interact to dictate the outcome of diapause, it is not clear, mechanistically, how these two environmental cues interact to program diapause (Denlinger, 2023). We present here a new trait, CTemp (Critical Temperature), which together with CDL helps to determine the effects of temperature and photoperiod and their interaction on the incidence and timing of diapause across the species distribution. CTemp is defined as a temperature, where 50% of the females of a given strain / population enter reproductive diapause in a daylength that induces ~ 100% diapause at low temperatures, and it is estimated from the temperature response curves (TRCs) based on diapause proportions at several temperatures in a photoperiod that is below the CDL of all study populations. A prerequisite for estimating CTemp is that the diapause proportion exceeds 50% in lower temperatures and remains below it in higher temperatures in a diapause-inducing daylength, which may restrict its use in some species / strains. In the present study, we have estimated CTemp for *D. montana* strains from the TRCs drawn for these strains at a broad temperature range (10 °C – 28 °C) in LD12:12 (Light:Dark cycle of 12 h light and 12 h dark) and DD (continuous darkness). In this species CDL varies clinally between 13 and 22 h at 16 °C according to population (Tyukmaeva et al., 2020; see also Table 1).

We have studied the temperature-dependence of photoperiodic reproductive diapause in *D. montana* by measuring latitudinal variation in CTemp and CDL and by examining how these traits change under different selection pressures in laboratory experiments (note that TRC is equivalent to PPRC and CTemp is a quantitative measure comparable to CDL). In the first part of the study, we measured the proportion of diapausing females at several temperatures between 10 °C and 28 °C in LD12:12 for 53 *D. montana* strains and in continuous darkness (DD) for 29 of these strains, drew TRCs for the strains and estimated CTemps when possible. In addition, we drew PPRCs and estimated CDLs for the strains where these data were lacking. In the second part of study, we drew TRCs and PPRCs and estimated CTemp and CDL for selection and control line replicates from a previous selection experiment where we selected for reproduction in short daylength (photoperiodic selection) at 16 °C (Kauranen et al., 2019), and in a new experiment where selection was performed for females that reproduce in LD12:12 in low temperature (photoperiodic and temperature selection). With these studies, we sought answers to the following questions: (1) Do *D. montana* strains show latitudinal variation in the temperature-dependency of females' photoperiodic diapause response in short daylength and / or in continuous darkness? (2) Do CTemp and CDL show a correlation with each other across latitudes and can the possible linkage between these traits be confirmed in laboratory experiments involving photoperiodic and temperature selection? Answering these questions will help understand how *D. montana* populations have adapted to photoperiodic and temperature conditions prevailing on different latitudes and how well they can be predicted to cope with the rising temperatures at their home site during global warming.

2. Material and methods

2.1. Study material

D. montana strains used in this study were established from the progenies of single fertilized females collected from a large range of latitudes from Europe, North America and Asia (Table 1). Females that were collected in 2002 or later come from our own collection trips and represent an overwintered generation (43 strains), while information on the establishment conditions of older strains (10 strains) is missing. In the University of Oulu, the strains were maintained in continuous light (LL), 16 ± 1 °C and 60–70% humidity, which prevents the females from entering diapause and is optimal for the flies' survival and progeny production. We have earlier shown that rearing *D. montana* strains in laboratory in these conditions for several years has no effect on strain CDL (43 strains from the same location were maintained in laboratory for 4 to 10 years; Lankinen et al., 2013). In the present study we also show that the length of laboratory maintenance has no significant effect on CTemp measured in LD12:12 or on CDL measured in 16 °C.

Study material also included control and selection lines (3 replicates per line) from our previous selection experiment (Experiment 1) for reproduction in late summer photoperiods (short CDL) performed in the University of Jyväskylä (Kauranen et al., 2019). This experiment was started from a large mass-bred population established from the progenies of 102 fertilized females collected in Oulanka, Finland (Europe 66.40°N) in 2013. After performing selection for 8 generations, the control and selection line replicates were maintained in the University of Oulu at 16 °C in LL and LD15:9, respectively, for 8–9 months (about 4 generations) before scoring their PPRCs and TRCs. About 3 years after finishing selection, we combined the 3 control line replicates to establish a new line (U as United) and allowed the flies to interbreed for 3 generations in LL at 16 °C. This line served as a parental line in Experiment 2, where we selected for females that succeed to reproduce in 12 h daylength in high temperature.

2.2. Experimental procedures and the definition of female reproductive stage

All experiments were performed at the University of Oulu in light-insulated and actively ventilated boxes, which were placed in temperature-controlled rooms (temperature varied<0.5 °C around the mean). The boxes were illuminated with one white fluorescent lamp per chamber (9 W, Megaman, Germany) with a light intensity of 300–1000 lx corresponding to about 5–16 W/m^2 during the photophase.

Flies were transferred into different temperatures in LD12:12 or DD in malt bottles at the pupal stage and maintained in these conditions for 10 to 42 days so that the females had enough time to develop ovaries even in the lowest temperatures. Our previous studies have shown that practically all *D. montana* females develop ovaries by the age of 21 days at 16 °C (Lankinen et al., 2022). Since the speed of ovarian development is sensitive to ambient temperature, we checked the development stage of ovaries after the following number of days at different temperatures: 42 days at 10 °C, 28 days at 13 °C, 21 days at 16 °C, 16 days at 19 °C, 14 days at 22 °C, 12 days at 25 °C and 10 days at 28 °C.

Ovary development of the females of 12 strains that displayed

Table 1
The first three columns show the collecting site, latitude and establishment year of *D. montana* strains used in the present study and the next two columns the codes used for the strains in the present and earlier studies. The last three columns show strains' CTemp measured in LD12:12 and DD and CDL measured at 16 °C. CTemp could be estimated only for the strains where diapause proportion exceeded 50% in the lowest temperature(s) and decreased below this at higher temperatures in LD12:12 and / or DD (temperature-responsive strains). It could not be estimated in LD12:12 for 3 strains whose diapause proportion exceeded 50% at all temperatures (high-diapause strains) and in DD for 20 strains whose diapause was below 50% at all temperatures (low-diapause strains). "NA" means that the diapause responses were not studied for the strains in DD. Strains (strain codes), which displayed diapause at 28 °C and were studied also at 31 °C, are marked with † and the CDLs, which have been determined in our earlier studies, with stars (see the footnotes).

Collecting site	Latitude °N	Year	Strain code	Codes used in earlier papers	CTemp in LD12:12	CTemp in DD	CDL
Europe							
(Finland)							
Pelkosenniemi	67.0	2008	Eu67_1	5PTF	22.96	NA	19.30
Pelkosenniemi	67.0	2008	Eu67_2	18PTF	24.44	NA	18.40
Pelkosenniemi	67.0	2008	Eu67_3†	21PT09	20.73	NA	18.50
Oulanka	66.4	2002	Eu66_1†	Eu1 O33	23.24	12.44	21.12*
Oulanka	66.4	2008	Eu66_2	Eu2 243OJ8	22.76	15.36	19.50*
Oulanka	66.4	2008	Eu66_3	36OJ8	22.01	NA	18.30
Oulanka	66.4	2008	Eu66_4	226OJ8	22.38	NA	19.91
Oulanka	66.4	2008	Eu66_5	O27	16.41	NA	17.10
Kemi	65.7	2002	Eu66_10	Eu3 K15	17.97	Low-diapause	17.44*
Kemi	65.7	2002	Eu66_11†	Eu4 K29	High-diapause	13.21	22.14*
Kemi	65.7	2002	Eu66_12†	K26	23.00	NA	19.88
Kemi	65.7	2002	Eu66_13†	Eu5 K50	24.14	13.36	18.28*
Pudasjärvi	65.4	2009	Eu65_1†	Eu6 29PJ209	22.92	17.84	20.32*
Pudasjärvi	65.4	2009	Eu65-2	11PJF	21.76	NA	18.37
Pudasjärvi	65.4	2009	Eu65_3	1PJ109	22.76	NA	17.96
Paltamo	64.3	2008	Eu64_1†	2KJF	22.58	NA	18.43
Paltamo	64.3	2008	Eu64_2	44KJF	19.17	Low-diapause	18.52
Paltamo	64.3	2008	Eu64_3†	Eu7 1KJF	22.34	Low-diapause	18.27*
Jyväskylä	62.1	2008	Eu62_1†	5SOF	20.49	NA	16.90
Jyväskylä	62.1	2008	Eu62_2	7SOF	21.77	NA	18.33
Jyväskylä	62.1	2008	Eu62_3	10SOF	21.77	NA	19.27
Lahti	61.1	2009	Eu61_1	Eu8 L4	21.50	16.11	18.95*
Lahti	61.1	2009	Eu61_2	Eu9 L6	20.55	Low- diapause	17.49*
Lahti	61.1	2009	Eu61_3	Eu10 L8	19.01	Low-diapause	17.83*
Lahti	61.1	2009	Eu61_4	Eu11 L9	19.05	Low-diapause	17.37*
North America							
Fairbanks, AK, USA	64.9	2013	Am65_1	Am1 FA13F3	26.82	16.39	19.97*
Fairbanks,AK. USA	64.9	2013	Am65_2	FA13F2	25.29	NA	20.89**
Fairbanks. AK, USA	64.9	2013	Am65_3	FA13F9	24.59	NA	20.20
Honolulu Creek, AK, USA	64.1	2013	Am64_1	HO13F3	17.59	NA	18.15
Honolulu Creek, AK, USA	64.1	2013	Am64_2†	Am2 HO13F4	24.86	16.07	18.32*
Seward, AK, USA	60.1	2013	Am60_1	Se13F14	20.60	NA	17.58
Seward, AK, USA	60.1	2013	Am60_2	Se13F16	21.07	NA	18.10
Seward, AK, USA	60.1	2013	Am60_3	Se13F37	High- diapause	NA	18.12
Terrace, BC, Canada	54.5	2014	Am54_1	Ter14F13	19.72	NA	16.73**
Blacksands, ON, Canada	49.5	–	Am50_1	15010–1021.06	15.80	NA	13.55
Vancouver, BC, Canada	49.1	2003	Am49_1	Am3 Can3F20	18.37	Low- diapause	14.74*
Vancouver, BC, Canada	49.1	2003	Am49_2	Am4 Can3F24	21.78	Low- diapause	15.92*
Vancouver, BC, Canada	49.1	2003	Am49_3	Am5 Can3F9	19.96	Low- diapause	16.12*
Ashford, WA, USA	46.8	2013	Am47_1	Am6 1ASH	17.39	Low- diapause	13.98*
Lytton, QC, Canada	46.7	1973	Am47_2	15010–1021.24	19.90	NA	14.90
Grand Teton, WY, USA	43.4	1947	Am43_1	Am7 1510–1021.16	20.39	Low- diapause	15.53*
Afton, WY, USA	42.7	2015	Am43_2	AF12	18.47	NA	15.30**

(*continued on next page*)

Table 1 (*continued*)

Collecting site	Latitude °N	Year	Strain code	Codes used in earlier papers	CTemp in LD12:12	CTemp in DD	CDL
Cache County, UT, USA	41.7	1999	Am42_1	Am8 BS-3, 1592	17.28	Low- diapause	14.70*
Cache County, UT, USA	41.7	1999	Am42_2	Am9 BS-11, 1593	19.17	Low- diapause	15.17*
Cache County, UT, USA	41.7	1999	Am42_3	Am9-2 BS-4. 1594	14.44	NA	14.75**
Cache County, UT, USA	41.7	1999	Am42_4	Am10 1595	20.02	Low- diapause	14.44*
Verdi, NV, USA	39.5	1949	Am40_1	Am11 15010–1021.17	16.36	Low- diapause	12.83*
Crested Butte, CO, USA	38.9	2003	Am39_1	Am12 C3F2	19.06	Low- diapause	15.15*
Asia							
Kamchatka, Russia	56.2	2013	As56_1†	As1 KR1323	23.86	Low- diapause	17.61*
Kamchatka, Russia	56.2	2013	As56_2	As2 KR1324	20.38	Low- diapause	16.61*
Kamchatka, Russia	56.2	2013	As56_3†	As3 KR1309	High- diapause	14.43	18.10*
Kawasaki, Japan	34.8	–	As35_1	As4 1510–1021.13	21.32	Low- diapause	15.72*
Kawasaki, Japan	34.8	1969	As35_2	As5 1263/20	16.53	Low- diapause	14.14

† Strains which displayed diapause at 28 °C degrees and were studied also at 31 °C.
*CDL determined in Lankinen et al. (2021).
** CDL determined in Lankinen et al. (2022).

diapause at 28 °C (see Table I and Fig. 1) was studied at 31 °C in LD12:12 and LL. As most of the females died in this temperature within a few days, we combined the females that had survived for 9 days at 31 °C and divided them into two groups. Females of the first group (282 females in 42 vials in LD12:12 and 249 females in 42 vials in LL) were dissected to find out whether they had developed ovaries at 31 °C in LD12:12 and / or LL. Females of the second group (18 females in 8 vials in LD12:12 and 28 females in 10 vials in LL) were transferred after 9 days at 31 °C into 19 °C / LL for 3 weeks and then dissected to find out whether they were able to develop ovaries in favourable conditions.

We also drew PPRCs and estimated CDLs for the strains where these data were lacking. For PPRC scoring we maintained groups of flies from the pupal stage to adults in LDs from 22:2 to 12:12 with one hour increments at 16 °C and determined female reproductive stage at the age of 21 days.

Reproductive stage of ether-anaesthetized females from different experiments was defined by dissecting their abdomen and checking whether their ovaries contained at least one fully developed egg (sexually mature females) or whether the females had small and transparent ovaries with no egg yolk (diapausing females). Detailed description on female ovary classification is given in Lankinen et al. (2022).

2.3. Drawing the TRCs and PPRCs and estimating CTemp and CDL

We drew temperature response curves (TRCs), based on female diapause proportions at several temperatures between 10 °C and 28 °C in LD12:12, and estimated CTemps for *D. montana* strains and experimental lines. We have earlier estimated the CDLs of several *D. montana* strains in temperatures between 13 °C and 19 °C (Tyukmaeva et al. 2020), which gave a good basis for choosing the temperatures used for determining CTemp in the present study. Moreover, we have shown LD12:12 to be an effective photoperiod for inducing diapause at 16 °C in *D. montana* strains originating from different latitudes (Lankinen et al. 2022). TRCs could only be drawn for the temperature-responsive strains, i.e., the ones where female diapause proportion exceeded 50% in some temperatures and were below that in others.

CTemp was estimated from TRCs using similar summary statistics as we have earlier used for estimating CDL from the PPRCs (approximately 50% of the females of a given strain enters diapause; Lankinen et al., 2021). For both estimations we used a four-parameter log-logistic model, the only difference between the traits being the upper limit allowed for the statistic: for CTemp this was set to 31 (the temperature at which females failed to develop ovaries) and for CDL to 24 (the maximum number of hours of light in a day). CTemp could be estimated for 50 and 9 temperature-responsive *D. montana* strains in LD12:12 and DD, respectively (see Table 1). CDL has earlier been estimated for 31 strains in 16 °C, using the methods described below (Lankinen et al., 2021, 2022), and here we estimated this trait for the remainder of the strains (22 strains; see Table 1).

CTemp and CDL were estimated with the function drm (with *type* = *'binomial'*) from R package "drc" (v3.0–1; Ritz et al., 2015) using the four-parameter log-logistic model:

$$y = c + \frac{d - c}{1 + exp(b^*(log(x) - log(z)))}$$

y = proportion of diapausing females.

x = the experimental temperature in °C (for TRC) or the number of light hours per day (for PPRC).

c and d = the lower and upper limits (bound between 0 and 1), when × approaches 0 or 31 (for the TRC) or 0 and 24 (for the PPRC).

b = the slope around CTemp or CDL (the point of inflexion of the PPRC, or TRC; bound between 0.001 and 100000).

z = ED50 (middle point between lower and upper limit) = CTemp estimated from TRC or CDL estimated from PPRC.

Diapause proportions can be expected to decrease along with an increase in temperature and daylength, or at least remain at the same level. Accordingly, we excluded from the TRC analyses the diapause proportions that were > 10% higher than the ones measured at lower temperatures, or > 10% lower than the ones measured at higher temperatures (see Fig. 1), and from the PPRC analyses the proportions that were > 10% higher or lower than the ones measured in shorter and longer daylengths, respectively. However, we made two exceptions to this rule: (1) we kept the data point at 28 °C in LD12:12 for the strain Eu64_3 despite its high diapause ratio to correct the model inference and (2) we excluded the diapause ratio at 25 °C in LD12:12 for the line Sel 1 (Experiment 1; see section 2.5.) even though it differed <10% from the previous data point to correct inconsistency between the data points at 25 °C and 28 °C. Note that we did not draw TRCs and estimate CTemps

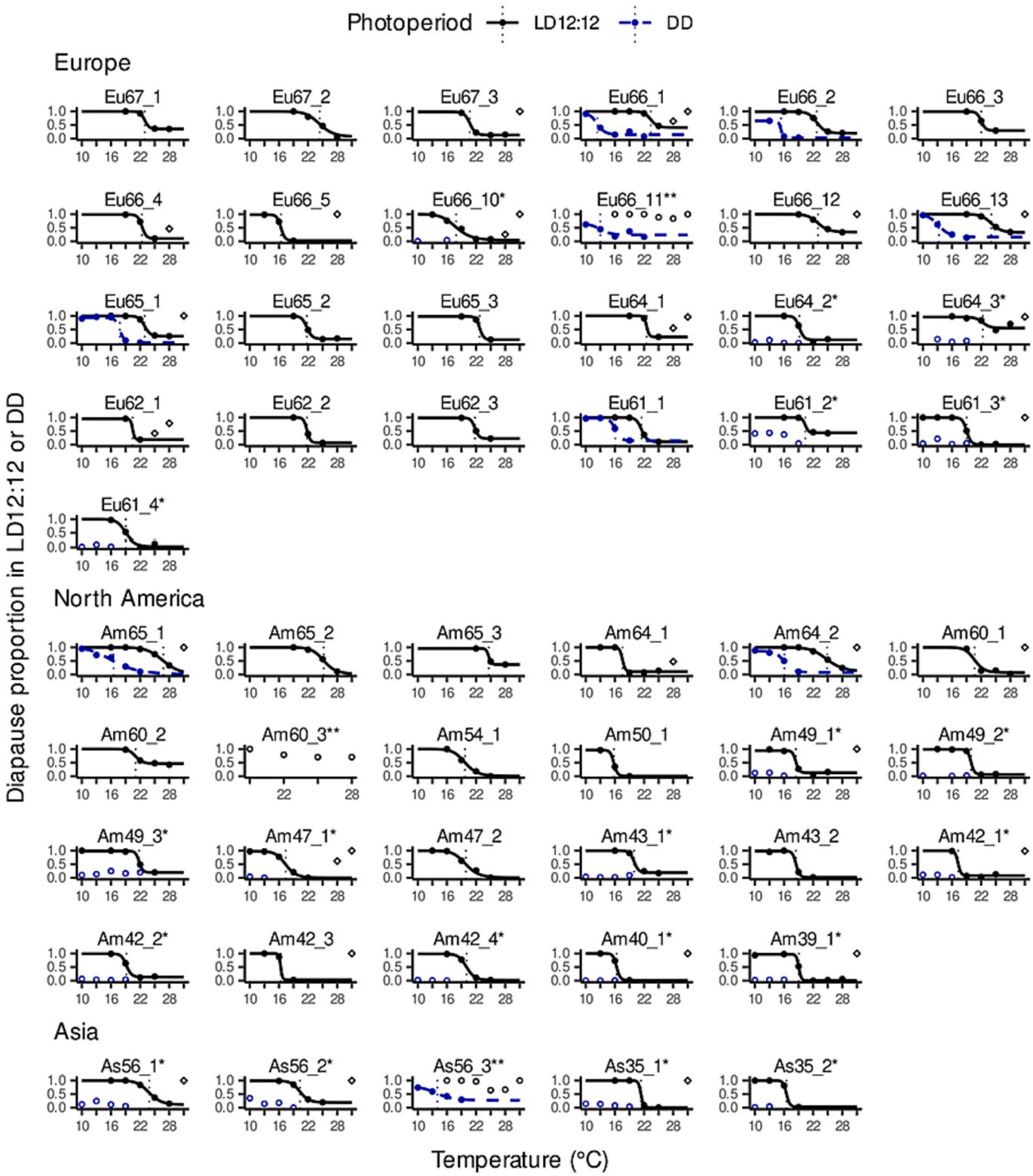

Fig. 1. Diapause proportions (circles or diamonds) at different temperatures and TRCs (lines) and CTemps (intersections of the vertical dashed line and the X-axis) computed with the R package "drc" (Ritz et al. 2015) for *D. montana* strains from high to low latitudes in Europe, North America and Asia in LD12:12 (black line) and DD (blue dashed line). Filled circles represent diapause proportions used for inferring the TRCs and empty diamonds the proportions removed from TRC estimations (outliers). Empty circles show diapause proportions in the strains where diapause proportions were above (*) or below (**) 50% in all temperatures (CTemp could not be calculated; see Section 2.3.).

for the strains where the diapause proportion was above or below 50% in all temperatures (high- and low-diapause strains).

To assess which characteristics could explain variation in the CTemp and CDL of the cline strains, we estimated Pearson's correlation (using "cor.test" in R) between CTemp in LD12:12 and CDL. In addition, we built quasibinomial models with the function "glm" from R package "stats" using the logit link function to assess the effects of the approximate latitude and continent of strains' home site on CTemp and CDL variation. We also added CTemp as a covariate to test its correlation with CDL, and CDL as a covariate to test its correlation with CTemp. Since CTemp and CDL are both bounded variables, we normalized the values by their highest possible value (28 °C for CTemp and 24 h for CDL). We compared models using an Analysis of Deviance and used the likelihood ratio test to identify the parameters that significantly improved the fit of the model (function *anova* with "test = LRT", package stats).

To ensure that strain age did not create a bias in our analyses, we explored the relationship between the number of generations that the strains had been reared in the laboratory and CDL or CTemp using two approaches. First, we estimated the correlation between the number of generations spent in laboratory and CDL or CTemp and, second, we compared GLM models with and without the number of generations as a co-variable with an analysis of deviance.

2.4. Selection experiments

Experiment 1. In our previous selection experiment (Kauranen et al., 2019), we performed selection for *D. montana* females that reproduce in late summer photoperiods (i.e., have short CDL) at 16 °C. Here we drew TRCs and PPRCs, and estimated CTemps and CDLs, for the 3 selection and 3 control line replicates from this experiment about 4 generations after finishing selection to examine whether and how selection for lower CDL had affected CTemp. We performed comparisons between the CDLs and CTemps of the control and selection line replicates (15 and 10 comparisons respectively) using the EDcomp function (package "drc" with the standard errors, confidence intervals and t-values computed with the delta intervals) and applied a Bonferroni correction for multiple comparisons.

Experiment 2. In this experiment we studied how CTemp and CDL change, when the flies are transferred from 22 °C into lower temperatures (19 °C and 16 °C) in 12 h daylength (LD12:12), where the females normally enter diapause at 16 °C (Lankinen et al. 2022). Here we used the flies of the U line, created by mixing the 3 control line replicates from Kauranen et al. (2019; see section 2.1), and maintained this parental line (UPar) in separate generations 16 °C / LL throughout the experiment. It was not possible to establish a control line for selection as both the photoperiod and temperature were changed during the experiment, but CDLs and CTemps estimated for the parental line at the beginning (UParF0) and end (UParF8) of the experiment controlled for the potential effects of drift and unintentional selection on these traits in 16 °C / LL.

To establish the selection line from the F0 generation of the parental line (UParF0) of Experiment 2, we collected ~ 200 females and ~ 200 males, divided them evenly into 4 malt bottles and transferred all bottles into 22 °C / LD12:12. In each of the following generations, we collected ~ 200 flies of both sexes in 4 bottles and allowed them to mate and lay eggs at the same or lower temperature than in which they had emerged themselves. The decision on whether to maintain the larvae and pupae in the same temperature as their parents, or whether to transfer them into a lower temperature prior to emergence, was based on the diapause proportion of the parent generation after the females had been given enough time to develop ovaries (about 21 days; females in the bottles with no eggs were given 5 extra days). Progenies were transferred into a lower temperature only if about half of the parent generation females had developed ovaries. Accordingly, the flies of the first 2 generations (F1 and F2) emerged at 22 °C, those of the next 2 generations (F3 and F4) at 19 °C and the flies of the last 4 generations (F5, F6, F7 and F8) at 16 °C in LD12:12.

We drew TRCs and PPRCs, and estimated CTemps and CDLs, for the parental line of *Experiment 2* prior to starting selection (UParF0) and at the 8th generation (UParF8) and for the selection line in F3, F5 and F8 generations (USelF3, USelF5 and USelF8).

3. Results

3.1. Latitudinal variation in the TRCs and CTemps of D. montana strains in LD12:12

The temperatures, where diapause proportions decreased from 100% to 50% (CTemp) and eventually close to 0% in LD12:12, increased towards high latitudes (see Fig. 1 and Table 1), and in 3 high-latitude strains (Eu66_11, Am 60_3 and As56_3) they never declined below 50% (high diapause strains; Fig. 1 and Table 1). Highest temperatures, where practically all females entered diapause in diapause-inducing daylength (LD12:12), varied between 16 °C and 22 °C and the temperatures, where diapause proportion approached 0, between 17 °C and 25 °C. High variation in CTemp within and between the latitudes on different continents, combined with variation in the steepness of TRCs, shows that *D. montana* populations can enter diapause in short daylength within a large temperature window and that the width of this window increases towards higher latitudes.

TRCs of the 50 *D. montana* strains with temperature-responsive diapause in LD12:12 showed robust latitudinal clines on all 3 continents (Fig. 2). Diapause proportions of most of the high-latitude strains (latitudes 55 – 65°N) started to decrease when the temperature increased above 19 °C (or 22 °C), while in some low-latitude strains (latitudes 35 – 40°N) this happened already at 16 °C.

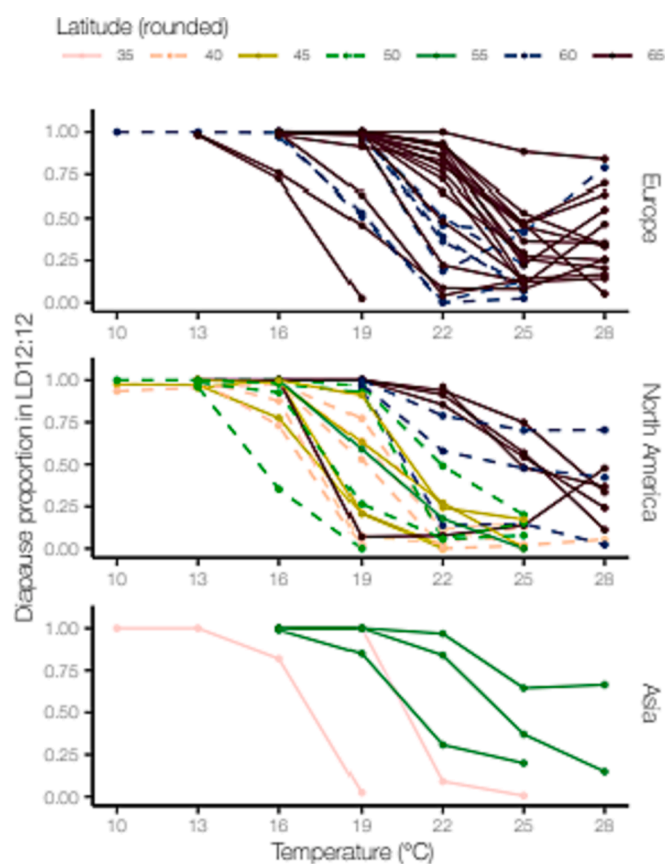

Fig. 2. Diapause proportions across different temperatures between 10°C and 28 °C in LD12:12 in *D. montana* strains from different latitudes in Europe, North America and Asia.

In 12 strains displaying diapause at 28 °C (see Table 1 and Fig. 1), ovarian development was studied also at 31 °C in LD12:12 and LL. In a combined data set for these strains, none of the females that were dissected after 9 days at 31 °C in these photoperiods developed ovaries (group 1; 282 females in LD12:12 and 249 females in LL). However, nearly all females that were transferred after 9 days in 31 °C / LD12:12 or LL into 19 °C / LL for 3 weeks developed ovaries (group 2; 16 out of 18 females transferred from LD 12:12 and all 28 females transferred from LL). This shows that while the females fail to develop ovaries at 31 °C, they retain an ability to develop them in more favourable conditions.

3.2. Correlation between CTemp in LD12:12 and CDL

We created PPRCs and estimated CDLs in 16 °C for 22 *D. montana* strains, for which this information was lacking (see Fig. A2), to be able to trace the correlation between CTemp in LD12:12 and CDL in 16 °C across the latitudes and continents. CTemp and CDL of each strain, whether measured here or in our previous papers (Lankinen et al., 2021, 2022), are presented in Table 1.

CTemp and CDL of 53 *D. montana* strains from latitudinal clines on all continents showed a significant correlation with each other (Pearson's product moment: correlation coefficient = 0.77%, t-value = 8.4335, df = 48, 77%, p-value = $4.9*10^{-11}$), and the correlation between these traits was also significant within the continents (Europe 0.63, North America 0.85 and Asia 0.92, all p-value < 0.05; Fig. 3). We found no significant correlation between the number of generations reared in laboratory and CDL (Spearman's rank correlation test: rho = −0.23, p-value = 0.10) nor CTemp in LD12:12 (rho = −0.24, p-value = 0.1027). We can thus exclude the possibility that the strain age itself has biased our results significantly.

3.3. Model exploration of CTemp in LD12:12 and CDL in latitudinal clines

We used a modeling approach to identify the factors that could explain variation in CTemp detected in our study, as correlation analysis alone has its limitations (such as assuming a linear dependence between the variables or only considering two variables at a time). We also wanted to examine whether and how variation in CTemp explains variation in CDL, and vice versa, across the strains from different latitudes and continents. Therefore, we tested models with CTemp (or CDL) as a response variable and the latitude, the continent of origin and CDL (or CTemp) as dependent variables, using a quasibinomial model with logit link function. Latitude had a significant effect on CTemp and CDL in all tested models (Table A1; p-values < 0.01, estimate 0.025 for CTemp with CDL as a response variable and estimate −0.014 for CDL with CTemp as a response variable). CDL always had a significant effect on CTemp (p-value < 0.01, estimate 5.006), as well as CTemp on CDL (p-value < 0.01, estimate 2.19). The continent of origin had a significant effect on CTemp when CDL or latitude was included as a co-variable, but its effect on CDL was significant only when latitude was not included as a co-variable. Finally, we compared all models for CTemp and CDL variation using an analysis of deviance and a likelihood ratio test. The best models included latitude and CDL for CTemp and latitude and CTemp for CDL as dependent variables, while the continent of origin did not improve the models significantly. Adding the number of generations that the strains had been reared in the laboratory in the final model had no significant effect on the CDL or CTemp, and also an analysis of deviance showed that it did not improve the fit of the model. Overall, our results indicate that the mechanisms determining CTemp and CDL in female diapause induction are at least partly overlapping, that variation in both traits is significantly correlates with latitude and that significant correlation between CTemp and CDL persists after the effect of latitude is factored out.

3.4. Variation in the CTemps of D. montana strains in DD

Diapause proportions of *D. montana* strains were generally lower in DD than in LD12:12 (see Fig. 1). In most strains they remained below 50% in all temperatures in DD (low-diapause strains), and only 9 strains had >50% diapause in the lowest temperature(s) (temperature-responsive strains). Comparisons between the low-diapause strains and the

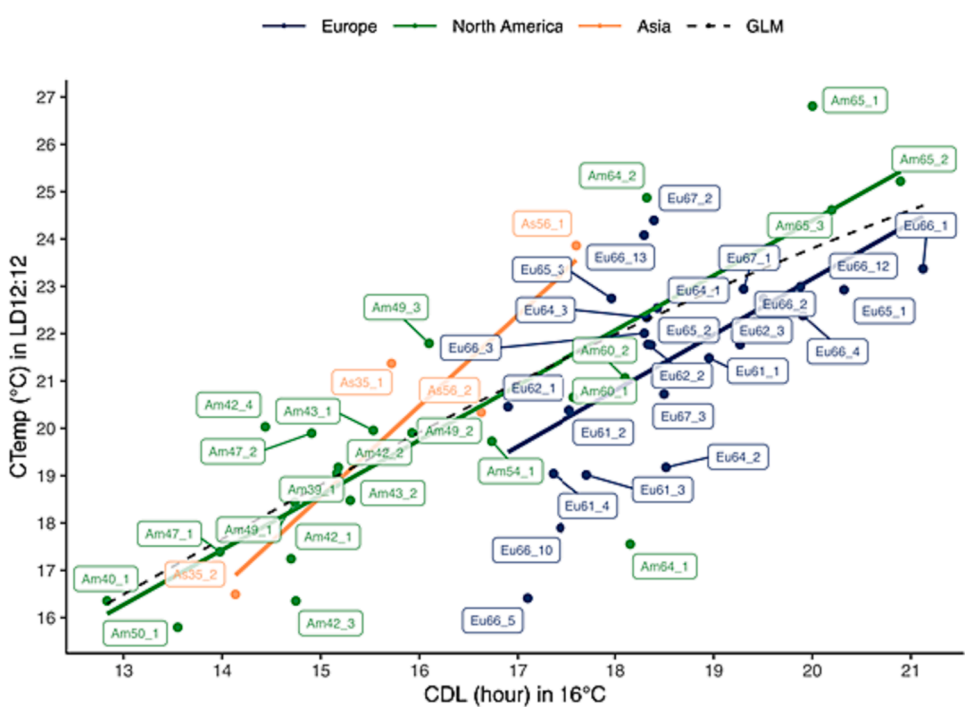

Fig. 3. Correlation between CTemp and CDL values of *D. montana* strains from different continents: Europe (in blue), North America (in green) and Asia (in red) in LD12:12. The black dashed line represents the predicted curve from our retained GLM model with CTemp as a response variable and CDL and Latitude as explanatory variables.

temperature-responsive strains shows that the first ones come from lower latitudes than the latter ones (Mann-Whitney Wilcoxon test, p-value = $6.6x10^{-4}$; Fig. A1). Correlation between the latitude and CTemp among the 9 strains, where CTemp could be measured in DD, was negative and non-significant (Spearman's rank correlation test: rho = -0.48, p-value = 0.19). However, the number of generations that these strains had been maintained in laboratory, showed a correlation with CTemp (rho = -0.68, p-value = 0.046), and thus we cannot exclude an effect of laboratory rearing on variation in CTemp in DD.

3.5. Selection experiments

We also examined the connection between CTemp and CDL in two laboratory experiments. In Experiment 1, selection favoured the females that reproduce in short daylength (photoperiodic selection; Kauranen et al. 2019) and in Experiment 2 the females that reproduce in extremely short daylength at low temperature (photoperiodic and temperature selection).

PPRCs and CDLs of the control and selection line replicates from Experiment 1(Kauranen et al., 2019) differed clearly from each other (Fig. 4A). CDLs of the 3 control line replicates were close to 20 h and those of the 3 selection line replicates around 17 h (see Table A2), the difference between the lines being significant (Est. = 0.866, s.e. = 0.003, t-value = −43.032, p-value < 0.001). Moreover, CTemps of 2 control line replicates (24 °C – 26 °C) were significantly higher than those of the selection line replicates (21 °C – 22 °C; Est. = 0.835, s.e. = 0.015, t-value = −10.99, p-value = 2.04e-28; CTemp could not be estimated for one control line replicate (Cont 3; see Fig. 4B) whose diapause proportion exceeded 50% in all temperatures). In DD, all control line replicates were temperature-responsive, but their CTemps were lower than in LD12:12, while in all selection line replicates diapause proportions remained below 50% at all temperatures (low-diapause strains; Table A2). In this experiment, selection towards short CDL had decreased CTemp in LD12:12 and DD and females' temperature-responsiveness in DD. Changes in CDL and CTemp occurred in the direction of the phenotypes of low-latitude populations, which supports a causal connection between the traits identified already in latitudinal clines.

In Experiment 2, we selected for females that reproduce at low temperatures in extremely short daylength (LD12:12), using the U line created by mixing the 3 control line replicates from Kauranen et al. (2019) as a base line. The idea behind this experiment came from an observation that high temperature (22 °C) can partly compensate the shortness of the light period so that at least some females develop ovaries in LD12:12 (see Fig. 1). The flies of all parental line generations (UParF0 – UParF8) were maintained in 16 °C / LL and the flies of the selection line generations at decreasing temperatures in LD12:12 (USelF1 and USelF2 at 22 °C, USelF3 and USelF4 at 19 °C and USelF5 – UselF8 at 16 °C).

In the first generation of the parental line (UParF0), diapause proportions varied between 80 and 100% at 19 °C, 22 °C and 25 °C and decreased below 50% at 28 °C in LD12:12 (CTemp 27.6 °C), while in the eighth generation (UParF8) diapause proportion did not decrease below 50% in any temperature and CTemp could not be estimated (Fig. 5A; Table A2). On the other hand, transferring the flies of the selection line into 22 °C / LD12:12 and maintaining the next generations in the same or lower temperature as their parents (22 °C, 19 °C and 16 °C) decreased CTemp to 21.8 °C in the F3 generation and to 15.3 °C and 15.6 °C in the F5 and F8 generations (Table A2). In DD, CTemp of UParF0 was very low (~14 °C), and all selection line generations had < 50% diapause in all temperatures (Table A2).

The fact that the PPRCs and CDLs of the parental line did not change during the experiment indicates the stability of female diapause response at 16 °C / LL (CDLs of UParF0 and UParF8 were 20.1 and 20.4 h, respectively). On the other hand, changes detected in CTemp and CDL during selection were clear and even more pronounced than in Experiment 1, as CDL decreased by nearly 3 h compared to UParF0 (CDL was 17.3 h and 17.4 h in the F3 and F5 generations, respectively; Fig. 5B; Table A2). CDL could not be measured in the F8 generation, where diapause proportion was above 0 only in LD12:12. However, an additional PPRC drawn for this generation showed that the females still possess an ability to enter diapause (CDL of this generation was ~ 16 h in 13 °C, but it is not comparable with the ones estimated in 16 °C).

4. Discussion

Different photoperiodic and climatic conditions experienced by insects in their native regions have created latitudinal clines in the timing and intensity of egg, larval or adult diapause and cold tolerance in several species with a wide geographic range (Bradshaw 1976; Addo-

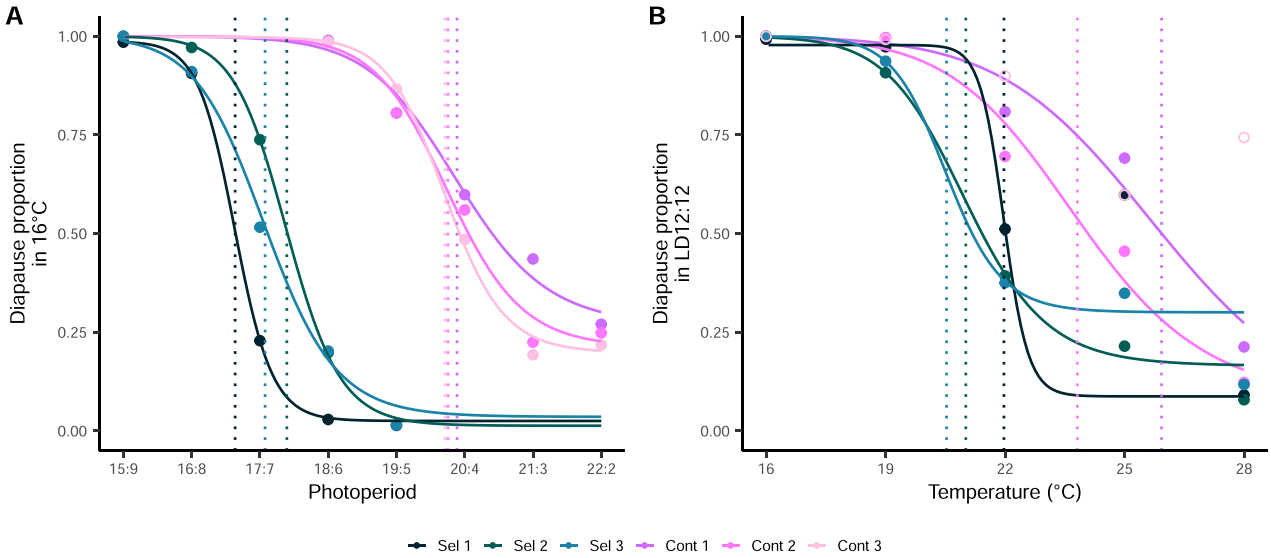

Fig. 4. Diapause proportions and PPRCs (A) or TRCs (B) for the control and selection line replicates from Kauranen et al. (2019), measured in Oulu University (Experiment 1). Interception of the dashed vertical lines and the X axis shows the CDL (in PPRCs) and CTemp (in TRCs) of each replicate. We did not draw TRC and estimate CTemp for Cont 3 since its diapause proportion exceeded 50% at all temperatures (empty circles in b).

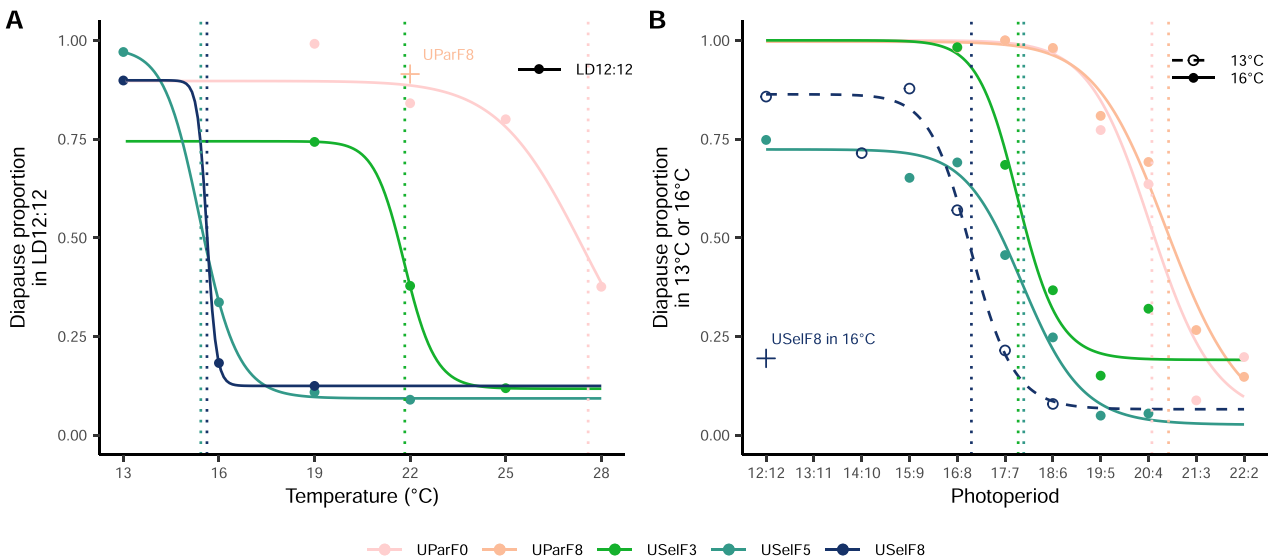

Fig. 5. A. TRCs for the parental line in generations 0 (UParF0) and 8 (UParF8) and for the selection line in generations 3 (USelF3), 5 (USelF5) and 8 (USelF8) drawn in LD12:12. In the UParF8 generation diapause was below 100% only at 22 °C, marked with a cross, and CTemp could not be estimated. B. PPRCs for the same lines and generations drawn at 16 °C. In USelF8, the diapause proportion was above 0 only in the shortest photoperiod (LD12:12, marked with a cross), and for this generation an extra PPRC was drawn at 13 °C (dashed line, empty circles). Intersections of the dashed vertical lines and the X-axis show the CTemps / CDLs of the strain (note that CDL of USelF8 is estimated in 13 °C and is not comparable with the CDLs of other generations).

Bediako et al., 2000; Paolucci et al., 2013). In *D. montana*, clinal variation has been detected in several diapause-linked traits, including CDL and its temperature-sensitivity (Lankinen et al., 2013; Tyukmaeva et al., 2020), females' ability to enter diapause in circadian and non-circadian photoperiods (Lankinen et al., 2021) and cold tolerance (Poikela et al., 2021; Wiberg et al., 2021). Here, we have complemented these studies by measuring latitudinal variation in *D. montana* strains' CTemp in LD12:12 and DD. Like CDL, CTemp in LD12:12 showed latitudinal variation on all continents, being higher in the high than in low latitudes. Moreover, photoperiodic and temperature selection performed in laboratory induced parallel changes in CDL and CTemp, confirming genetic correlation between these traits.

Reproductive diapause is an essential part of the life cycle of several insect species living at high latitudes and / or altitudes. Temperature can affect all levels of the photoperiodic responses from photoreception to hormonal events triggering the onset of diapause (Saunders, 2014), and thus clinal variation in TRC for diapause induction at short photoperiods detected in *D. montana* is not surprising. In this species, the females of high-latitude populations possess longer CDL than those of the low-latitude ones, which means that they enter diapause at an earlier calendar date (decrease in CDL is about one hour per five degrees decline in latitude; Tyukmaeva et al., 2020). Moreover, a few degrees increase in temperature shortens CDL and postpones the diapause to a later calendar date, especially in low-latitude populations (Tyukmaeva et al., 2020). The present study shows that high temperature can also prevent diapause in a photoperiod that is far below CDL and that the impact of temperature is strongest in low-latitude populations. A second notable feature was high variation in CTemps and CDLs among *D. montana* strains originating from the same latitude. The fact that *D. montana* populations comprise females with different CTemps and CDLs suggests that this species has a high potential to survive over both warm and cold autumns. In addition, temperature-sensitivity of diapause response may help the females to modify their diapause decisions in exceptional years, which can be especially important in *D. montana*, where the females can enter diapause even after reproducing (Lankinen et al., 2022).

Studies of trait variation and correlations along latitudinal clines involve various kinds of pitfalls. According to O'Brien et al. (2011), an inference of a causal relationship between two traits would be reasonable only if (a) variation in each trait is significantly correlated with a third common element like latitude, (b) significant correlation between the two traits persists after the effect of the common element is factored out and (c) the environmental conditions used to show the correlations in (a) and (b) are determined for the same organism under the same conditions. In our study, the first two requirements (a and b) were satisfied, as both CTemp and CDL showed correlation with latitude and were correlated with each other even when the effect of latitude was factored out. Moreover, all trait measurements were performed in controlled conditions for the same strains (CTemp and CDL are characters of strains or populations, not individuals). Selection experiments confirmed linkage between CTemp and CDL, but determining the type and strength of this linkage would require genetic tests. Correlation between CTemp and CDL helps the flies to adapt to warming temperatures; if the diapause would be determined strictly by photoperiodic cues, females would enter diapause at a specific calendar date even in high temperatures.

Quasinatural selection, where the study organisms are transferred into altered environmental conditions over successive generations, offers an efficient way to examine the role of environmental factors in trait evolution and to study trait correlations and trade-offs (Fry, 2003). This type of selection has been used in attempts to create lines that do not enter photoperiodic diapause in diapause-inducing photoperiod e.g., in the heteropteran, *Pyrrhocoris apterus* (Socha and Hodkova 1994), northern corn rootworm, *Diabrotica barberi* (Huynh et al., 2021) and cabbage beetle, *Colaphellus bowringi* (Ma et al., 2011). In the present study, CTemp and CDL appeared to be sensitive to photoperiodic selection and the selection response was even stronger when photoperiodic selection was combined with temperature selection. However, all selection lines maintained an ability to enter diapause.

In *Drosophila*, male gametogenesis is generally regarded to be more sensitive to thermal stress than female gametogenesis (David et al., 2005; Iossa, 2019). Limited evidence in the species of this genus has shown that male sterility under heat stress can vary between locally adapted populations originating from different thermal regimes (Porcelli et al., 2017; Walsh et al., 2019). The detrimental effects of high temperatures on male fertility have been suggested to restrict also the distributions of species of the *D. virilis* group; for example, in *D. montana* 80% of the surviving males are sterile seven days after heat stress at ~ 34 °C (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021). In the

present paper, *D. montana* females failed to develop ovaries in 28 °C or 31 °C, but they were able to retain fertility when brought back to a lower temperature and continuous light (this resembles "hot quiescence" or "hot thermal diapause" with a restricted duration; Liu et al., 2016). Females' failure to develop ovaries high temperatures reminds us that global warming can reduce insect fertility in several ways, not only through disturbances in male gametogenesis.

Pittendrigh and Takamura (1987) have suggested that the photoperiodic control of diapause is only realized within a narrow window of temperatures, and that one consequence of adaptive evolution to new latitudes would be a shift of this window along the temperature scale. Our study shows this to be true for *D. montana*, even though the temperature window for its diapause induction appeared to be broad, especially at high latitudes. The highest temperatures where practically all females entered diapause in LD12:12 varied between 19 °C and 22 °C in high-latitude populations and between 16 °C and 19 °C in low-latitude ones. The larger temperature sensitivity of low-latitude populations allows more flexibility of the photoperiodic response in locations where occasional warm conditions in late summer permit an extension of the reproductive season before winter (Hut et al., 2013).

Over evolutionary time, there has been precise fine-tuning of critical photoperiod and onset / offset of seasonal adaptations in insect populations, and the mismatch between temperature and day length cues can have a profound impact on species fitness and survival (Walker et al., 2019). Understanding the relationship between local climates and population differentiation in phenology will be of the utmost importance in allowing us to anticipate responses to novel selective environments caused by global climate change (Forrest, 2016). Our study shows that even species with a strong photoperiodic diapause, such as *D. montana*, have reasonably high potential to adapt to the rising temperatures during climate warming. The prevalence and timing of diapause is likely to remain unchanged in high-latitude populations, where diapause induction is less dependent on temperature than in low-latitude ones. In low-latitude populations raising temperatures may postpone diapause towards the autumn and, in the worst scenario, prevent diapause induction.

5. Conclusions

Insect populations at high latitudes have repeatedly and rapidly adapted to seasonal changes in daylengths at different latitudes, which indicates that the common use of daylength as a seasonal cue need not strongly limit climate-induced insect range expansions (Bradshaw and Holzapfel, 2010; Ittonen et al., 2022). Whilst the predominant diapause-inducing cue (photoperiod) will be unaffected by global climate change, higher temperatures may modify normal rates of development leading to a decoupling of synchrony between diapause-sensitive life-cycle stages and critical photoperiods for diapause induction (Bale and Hayward, 2010). Pittendrigh and Takamura (1987) have suggested that there is a lower temperature below which all females enter diapause, and a higher temperature above which all females develop ovaries, and thus photoperiodic control is only realized within a narrow window of temperatures. In *D. montana* the width of any such window shows high variation between strains from both similar and different latitudes.

Understanding the evolutionary dynamics of local adaptation for a threshold trait, like diapause, provides not only insights into the adaptive process, but also reveals the adaptive potential of populations (Pruisscher et al., 2018). Pittendrigh and Takamura (1987) have suggested that the temperature dependence of insects' diapause responses forms a major feature controlling the onset and duration of their breeding season and that the adaptive adjustment of CDL / CNL to latitude can be seen as a change in the temperature dependence of the photoperiodic response. This was also found to be true in our study, as the strains with long CDL typically also had high CTemp. The capacity of species to respond ecologically and evolutionarily to the challenges of global thermal change will affect future biodiversity, and thus the identification of key thermally sensitive traits across species, and the quantification of the species' ability to buffer the effects of thermal stress on these traits, is a critical research priority (Moritz and Agudo, 2013; Walsh et al., 2019). Variation detected within and between *D. montana* populations in two traits that play a central role in the timing of reproductive diapause, CTemp and CDL, and a causal correlation between them, suggests that timing of the diapause is less sensitive to climate warming at high than at low latitudes.

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CRediT authorship contribution statement

Pekka Lankinen: Conceptualization, Methodology, Validation, Investigation, Resources. **Chedly Kastally:** Conceptualization, Methodology, Visualization, Software. **Anneli Hoikkala:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Information on data used in this ms is given in Supplementary file.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104556.

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