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Direct and correlated responses to bi-directional selection on pre-adult development time in *Drosophila montana*

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ABSTRACT

Selection experiments offer an efficient way to study the evolvability of traits that play an important role in insects' reproduction and/or survival and to trace correlations and trade-offs between them. We have exercised bi-directional selection on *Drosophila montana* flies' pre-adult development time under constant light and temperature conditions for 10 generations and traced the indirect effects of this selection on females' diapause induction under different day lengths, as well as on the body weight and cold tolerance of both sexes. Overall, selection was successful towards slow, but not towards fast development. However, all fast selection line replicates showed at the end of selection increased variance in females' photoperiodic diapause response and about one hour increase in the critical day (CDL), where more than 50 % of emerging females enter diapause. Indirect effects of selection on flies' body weight and cold-tolerance were less clear, as the flies of the slow selection line were significantly heavier and less cold-tolerant than the control line flies after five generations of selection, but lighter and more cold-tolerant at the end of selection. Changes in females' diapause induction resulting from selection for fast development could be due to common metabolic pathways underlying these traits, collaboration of circadian clock and photoperiodic timer and / or by the interaction between the endocrine and circadian systems.

Keywords: bi-directional selection experiment, pre-adult developmental time, reproductive diapause, body weight, cold tolerance, critical day length

1. INTRODUCTION

Adaptation to seasonally varying environmental conditions at high latitudes often requires ectotherms, such as insects, to genetically change their reproducing and overwintering strategies. Longer growing seasons, induced by global warming, can change correlations and trade-offs between the life-history traits underlying these strategies, which can significantly alter their evolutionary trajectories (Roff, 1980; Lande, 1982). Higher temperature combined with a longer growing season can also lead to an increase in the number of generations per year (voltinism), which can accelerate population growth and occasionally lead to population out-breaks or phenological mismatches (Altermatt, 2010; Knell & Thackeray, 2016).

Traits like pre-adult development time, critical day length for diapause induction (CDL), body weight and stress tolerance play an important role in insects' life cycle. The evolvability of these traits has been studied by measuring their genetic variation within and between conspecific populations (e.g. Sniegula et al., 2016) and by performing selection experiments in laboratory (e.g. Hard et al., 1993; Chippendal et al., 1997; Davidowitz et al., 2016). Selection experiments in particular have proved to be an effective way to detect mechanisms that may enhance or constrain life history evolution (Scheiner, 2002; Davidowitz et al., 2016) and to find out whether the trade-offs and correlations between the traits are due to linkage disequilibrium, pleiotropy and/or genotype-by-environment interactions (Allen et al., 2008; Bell, 2008). However, selection experiments are also vulnerable to biases and other experimental artefacts that are difficult to control (Harshman & Hoffman, 2000).

Fast pre-adult development time is usually assumed to lead to earlier reproduction and higher fitness in the wild, even though its fitness benefits can be balanced by costs resulting from reduced pre-adult survivorship, marginal larval storage of metabolites and reduced adult size (Chippendal et al., 1997). Bi-directional selection on insects' pre-adult development time can be expected to be more successful towards slow than towards fast development in populations, where natural selection has decreased additive variation towards fast development. This kind of asymmetry has been observed in many (e.g. Takahashi et al., 2013), but not all (e.g. Zwaan et al., 1995; Chippendal et al., 1997) selection experiments. While the relatively high heritability of insects' pre-adult development time makes this trait suitable for selection (Cortese et al., 2002), selection experiments may be complicated by the sensitivity insect development to environmental conditions, including larval density, temperature and day length (Borash et al., 2000; Cortese et al., 2002; Salminen et al., 2012; Fischer et al., 2012). In addition, fast development may involve high fitness costs resulting from reduced preadult survivorship, marginal larval storage of metabolites and reduced adult size (Chippindale et al., 1997).

Selection on one trait can induce changes also in other traits that are controlled by the same developmental / metabolic pathways. The most frequently found correlation in selection experiments for pre-adult development time is the negative correlation between the development time and body size (e.g. Partridge & Fowler, 1992; Chippindale et al., 1997, 2003; Zwaan et al., 1995; Nunney, 1996; Prasad et al., 2000). Davidowitz et al. (2016) have shown in tobacco hornworm, *Manduca sexta*, how simultaneous selection on development time and body size can enable or constrain the trait responses through synergistic or antagonistic changes in growth rate. Furthermore, selection on pre-adult development time has revealed positive correlation with traits involved in the induction of larval diapause in pitcher-plant mosquito, *Wyeomyia smithii* (Hard et al., 1993), and burnet moth, *Zygaena trifolii* (Wipking & Kurz, 2000). Correlated responses of body size and diapause induction during selection on insects' pre-development adult time could at least partly be due to the common metabolic pathways underlying these traits. In *Drosophila* and many other

organisms, the insulin / TOR (target of rapamycin) pathway controls developmental time and growth by regulating environmentally sensitive developmental transitions, which occur in response to the production of the steroid molting hormone, ecdysone and juvenile hormone (Mirth & Shingleton, 2012). On the other hand, the insulin / FOXO (forkhead transcription factor) and ecdysone pathways and juveline hormone have been shown to be major regulators of diapause through their effects on metabolic suppression, fat hypertrophy, and growth control (Richard et al., 2001; Sim & Denlinger, 2013). The connection between flies' pre-adult development time and diapause induction could also be explained by possible collaboration of the two clock mechanisms (Kostal, 2011) and / or by the interaction between the flies' endocrine and circadian systems (Bloch et al., 2013).

In northern Finland, the flies of the *Drosophila virilis* group species (*D. montana*, *Drosophila littoralis* and *Drosophila ezoana*) are practically univoltine (Lumme et al., 1974) and in slightly warmer areas partially bivoltine (Aspi et al., 1993). Females of these species overwinter in reproductive diapause and develop ovaries and mate in late spring or early summer, so that the flies of next generation emerge in July or August when the days are getting shorter (Lumme et al., 1974; Aspi et al., 1993). Our earlier studies have shown that the pre-adult development time of *D. montana* flies is affected by the day length and temperature before emergence, while the induction of female's adult reproductive diapause depends on the same factors during the first days after their emergence (Salminen et al., 2012; Salminen & Hoikkala, 2013). We have also shown that the body size plays an important role in *D. montana* flies' survival over the cold season (Aspi & Hoikkala, 1995). Here we have exercised bi-directional selection on *D. montana* flies' pre-adult development time and studied whether selection on this trait leads to changes in the timing of female diapause response (measured as CDL) and/or in fly body weight and cold tolerance. The fact that the study was done on the flies of a univoltine *D. montana* population offers a unique opportunity to trace the evolution of traits important in adaptation into northern environmental conditions. Our study showed the selection to be successful only towards slower pre-adult development. However, even though selection towards faster development was unsuccessful per se, it led to increased variance in females' photoperiodic response curves (PPRCs) and longer CDL (earlier diapause induction). Effects of selection were less clear on flies' body weight and cold tolerance, largely due to various interactions between the selection regime, sex and generation.

2. MATERIAL AND METHODS

2.1 Base population

Genetically variable *D. montana* base population was established from the progenies of 102 fertilized females collected from Oulanka population in Northern Finland (66.22°N) in summer 2013. Progenies of single females were maintained in separate malt vials in constant light (LL) in 19°C, and in F3 generation 10 virgin sexually mature females and males from each progeny were transferred into a population cage. The cage was 34 cm x 23 cm x 20 cm wide and it had a plexiglass roof and eight malt bottles attached to the holes in the floor and containing malt medium (Lakovaara, 1969). To give time for recombination, the flies were allowed to mate randomly and to produce progeny for three generations before starting selection. The flies of the first population cage generations were kept in LL in 19°C, and the cage was transferred into 16°C just before the flies of the 4th generation started to emerge (Fig. 1). These conditions mimic the environmental conditions in Oulanka (North Finland) in June when *D. montana* flies are reproducing. During selection the flies were kept in malt

vials in wooden light-insulated cabinets in 16°C, so that the three replicates of the same line were in a different cabinet.

2.2 Bi-directional selection for pre-adult development time

Selection for fast and slow development time involved two selection lines and a control line, each of them having 3 replicates (see Fig. 1). To start selection, 200 sexually mature virgin females and males (flies of this species reach sexual maturity in about three weeks in 16°C) of F6 generation were transferred into malt vials (10 flies of both sexes per vial), where they were allowed to mate for 48 hours. After this the flies were transferred for egg laying into six successive malt vials, keeping them in each vial for 3 hours. Flies of the F7 generation were collected in 12-hour intervals as long as they kept emerging and maintained in separate vials until they were used in experiments at age of approximately 25 to 30 days.

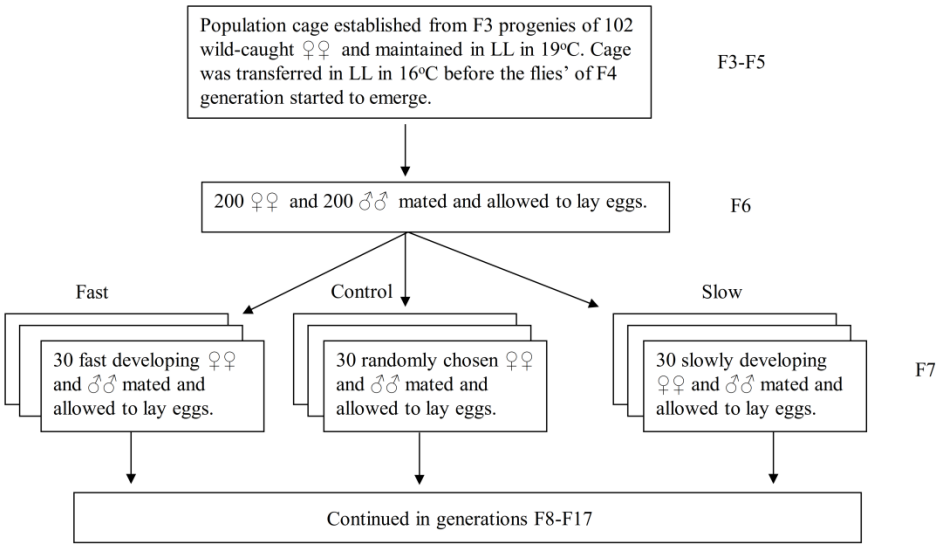

Figure 1. The procedure used in a bi-directional selection experiment for fast and slow pre-adult development time. The flies were phenotyped in F6 – F7 generation (base population) and F12 and F17 generations (selection and control line replicates).

The fast and slow selection line replicates were established by transferring 30 sexually mature virgin F7 generation females and males with the fastest or slowest pre-adult development time in malt vials (10 females and 10 males in each vial; 3 vials per replicate). The control line replicates were started with the same numbers of flies with fast, intermediate and slow development time, taking into account the modal distribution of flies' pre-adult development time (Fig. A1). In the following generations selection was continued along the protocol explained above, except that it was exercised in each selection line only in one direction and that the emerging flies were collected in 24 h intervals, because of extremely high variation in flies' pre-adult development time detected in F7 generation. In F12 and F17 generations the flies were collected at 12 hours intervals for phenotyping.

In generations F7 - F10 adult flies were mated in malt vials and allowed to lay eggs the same way as in generation F6. However, in F8 generation the flies of all replicates produced too few offspring, and nearly all flies had to be used as the parents of the next generation (this was the only generation where selection could not be done). Flies' mating protocol was slightly changed starting from F11 generation to give the flies a chance to choose their mating partner from a larger group of flies and to get malt vials with more equal egg

numbers. In the new protocol all 30 females and males of each replicate were allowed to mate in a plexiglass cage (15 cm x 10 cm x 10.5 cm), which had four malt vials attached to the holes in the walls for egg laying and some dry yeast on the floor. After a 24 hours adjustment period the egg-laying vials were changed in 3 hours intervals during one to three successive days (the same way as earlier). In generation F16 2nd replicate for slow selection line was lost due to detected contamination.

Our aim was to select about 20 % of females and males with the fastest (fast line) or slowest (slow line) pre-adult development time as the parents of the next generation. At the same time the number of parents was kept in 60 individuals (30 females and 30 males) per replicate to avoid excessive inbreeding (see Weber & Diggins, 1990).

2.3 Phenotypic assays

The first set of phenotypic assays was done for the flies of the base population before the selection was started. Photoperiodic response curves (PPRCs) and CDL were measured for the flies of F6 generation and the pre-adult development time for their progeny (F7 generation). In F12 and F17 generations (5 and 10 generations after the start of selection) the flies were phenotyped for pre-adult development time, PPRCs and CDL, body weight and cold tolerance. Phenotyped flies were collected from the whole scale of development times, taking into account the modal distribution of flies' development time in fast, slow and control line replicates, and all assays were performed in 16°C.

Pre-adult development time of the flies of both sexes was measured in LL. Emerging flies were collected in 12 hours intervals and their pre-adult-developmental time was calculated from the midpoint of egg-laying window to the midpoint of eclosion window. In other generations flies' pre-adult development time was measured at 24 hours intervals for restricted time periods (fast line replicates 30-40 days, slow line replicates 34-48 days and control line replicates 32 to 48 days after egg laying) to choose relevant parents for the next generation.

Photoperiodic response curves (PPRCs) and the critical day length for diapause (CDL) were measured by transferring 59 - 98 females per replicate and Light:Dark cycle (LD) within one day after their emergence in five LDs (20:4, 19:5, 18:6, 17:7 and 16:8) in F7 generation and in six LDs (21:3, 20:4, 19:5, 18:6, 17:7 and 16:8) in F12 and F17 generations. After 21 days, females' abdomens were dissected under a microscope to calculate the proportion of diapausing females under each day length. The females were considered sexually mature, if their ovaries contained at least one fully developed egg (Tyukmaeva et al., 2011).

Body weight of the flies of both sexes was estimated by weighting at least 24 females and males per line replicate (see Table A1). The flies were weighted within 15 hours after emergence using Mettler ToledoXS105 Dual range scale (Mettler Toledo™ 30132870).

Cold tolerance of at least 18 flies of both sexes per replicate was studied by placing the flies in individual, sealed vials into a water-glycol bath (Julabo F32-HL, Germany) in 16°C, after which the temperature was decreased 0.5°C/min to check temperature where the flies are not able to stand on their legs (CT_{min}) (Overgaard et al., 2011).

2.4 Statistical analyses

All statistical analyses were performed with R 3.1.1 (R Development Core Team, 2016).

Differences between the flies' pre-adult development time in the control, fast and slow selection regimes were analyzed using a generalized linear mixed model (GLMM), with a gamma distribution and a log link and fitted using maximum likelihood (ML). This was done using the glmer() function in the "lme4" R package (Bates et al., 2015). Here, selection regime (control, fast or slow), generation and sex and their interactions were used as fixed factors and replicate as a random factor. Due to a right skew in the dataset, a gamma distribution appeared to be the best fitting model as validated with model selection.

PPRCs were fitted to sigmoidal model using a dose-response analysis, performed with the drc package in R (Ritz et al., 2015; R Core Team, 2012). This package fits a wide variety of models for data with sigmoid or biphasic distributions as commonly seen in pharmacological or toxicological dose data, and so here we predict the proportion of diapausing females (the response) that occurs per change in hours of light in the LD cycle (the dose). After this, the best fitting models were chosen using the mselect() and modelFit() functions, a lack-of-fit test and AIC scores (see Ritz et al. 2015 for further details). The lack-of-fit test uses an approximate F-test to compare dose-response models with different parameters to a more general ANOVA model to see which the best fitting (Bates and Watts, 1988). In this case the best fitting of the appropriate models was a three-parameter Weibull function, where upper limit of the model is set equal to 1 (coded in the function as "fct = W2.3()").

CDL was determined from the PPRCs as the photoperiod (LD), where 50 % of emerging females enter diapause (see Tyukmaeva et al., 2011). For this analysis the data were made binary, with a value of 0 for diapausing females and a value of 1 for non-diapausing females. To determine the CDLs a model was fitted for the fast, slow and control line replicates in both the F12 and F17 generations. As this analysis can only handle two factors at a time, selection regime (fast, slow or control), and LD cycle were used as fixed factors and then the model was run separately for each generation.

Body weight data were analyzed using linear mixed model (LMM) fitted using a restricted maximum likelihood (REML) estimation. This was done using the lmer() function in the "lme4" R package (Bates et al., 2015). Selection regime, generation and sex, and their interactions, were used as fixed factors and replicate as a random factor.

Cold tolerance data showed no significant differences between replicates (ANOVA: DF = 22.13, F = 0.77, p = 0.765), so after using the model selection criteria describe below the factor "replicates" was dropped from the model entirely (for example models with replicates as random factor the Akaike information criterion (AIC) = 3420, as a nested fixed factor AIC= 3405 and without replicates AIC = 3378). Consequently, the effects of selection regime, generation, sex and their interactions on CTmin data were analyzed using a linear model (LM) and lm() function in base R. All three regression type models mentioned above were set up with control line as an intercept to find out whether fast and/or slow line differ significantly from the control line.

All regression models discussed above were selected using AIC and likelihood ratio tests. In addition, we made standard plots of the residuals to check for homoscedasticity in the model's variance and check visually how well the models fitted. We also checked for over-dispersion in the mixed modes using the "dispersion_glmer()" function from the "nlmeco" package and found it to be low for both development time and body weight (development time GLMM= 0.12, body weight LMM = 0.28).

3. RESULTS

3.1 Selection for fast and slow pre-adult development time

In F7 generation, where the selection was started, flies' pre-adult development time varied from 28.3 to 65 days, the median development time of both sexes being 31.8 days (Table A2). The flies that were chosen as the parents of the fast line replicates developed from egg to juvenile adult in 28.5 – 31.0 days and the ones used to start slow line replicates in 36.5 – 54 days (Fig. A1). The pre-adult development time of the control line flies varied from 28.5 to 53 days (most flies chosen as parents of the next generation developed in 31 to 37 days).

An interaction plot produced from the GLMM model for flies' pre-adult development time is shown in Figure 2, the full model results and test statistics in Table A3, the flies' median pre-adult development time in each control and selection line replicate in Table A2 and the violin plot showing the variation in pre-adult development time between selection and control line replicates in Figure A2. The GLMM analysis showed the random effect (replicate lines nested within the treatments) to explain only 0.9% of variation. Flies' overall pre-adult development time increased from F12 generation to F17 generation ($p < 0.001$; see Table A3), and selection was successful only towards slow development. The difference between the control and fast selection line was non-significant ($p = 0.147$) and showed no significant interactions, while the difference between the control and slow line was significant ($p < 0.001$). The latter comparison also showed a marginally significant selection * sex * generation interaction ($p = 0.033$), as the development time of the slow line males was about 1.03 days longer than that of the females in F17 (Fig. 2). Otherwise sex had little effect on fly pre-adult developmental time ($p = 0.319$).

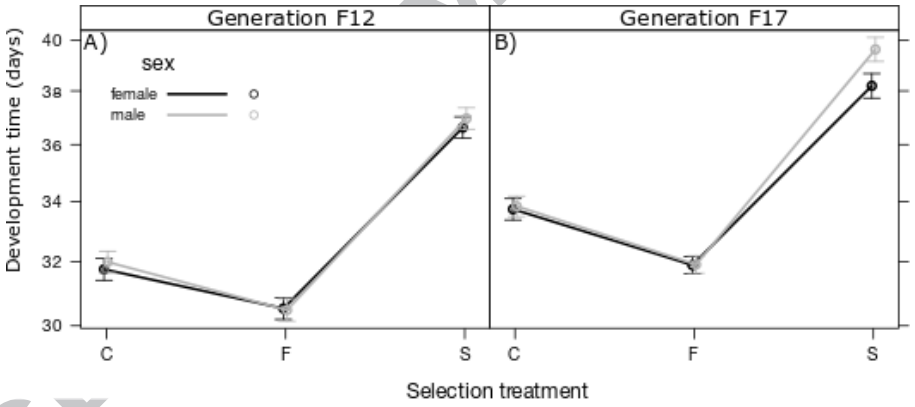

Figure 2. Interaction plot of selection regime (C = control, F = fast and S = slow selection line), sex, and generation for flies' predicted pre-adult developmental time in A) F12 and B) F17 generations from a GLMM model of pre-adult developmental time. Error bars refer to the model predicted standard errors.

3.2 Changes in photoperiodic response curves (PPRCs) and the critical day length (CDL) for diapause

In F17 the PPRC of fast line was much less sharp than that of the other lines (DRM: fast to control; EST=-33.25, SE = 2.94, t = -11.30, $p < 0.001$, fast to slow; EST=-25.42, SE = 3.00, t = -8.45, $p < 0.001$). In contrast, there was no large difference between control and slow flies (DRM: fast to control; EST=-7.83, SE = 4.07, t = 1.92, $p = 0.054$). This slope difference is explained by the high variation in females' diapause response within the line (Fig. 3), which was true for each of the three replicates of the fast line (Fig. 3). PPRC for base population is shown in appendix (Fig. A3).

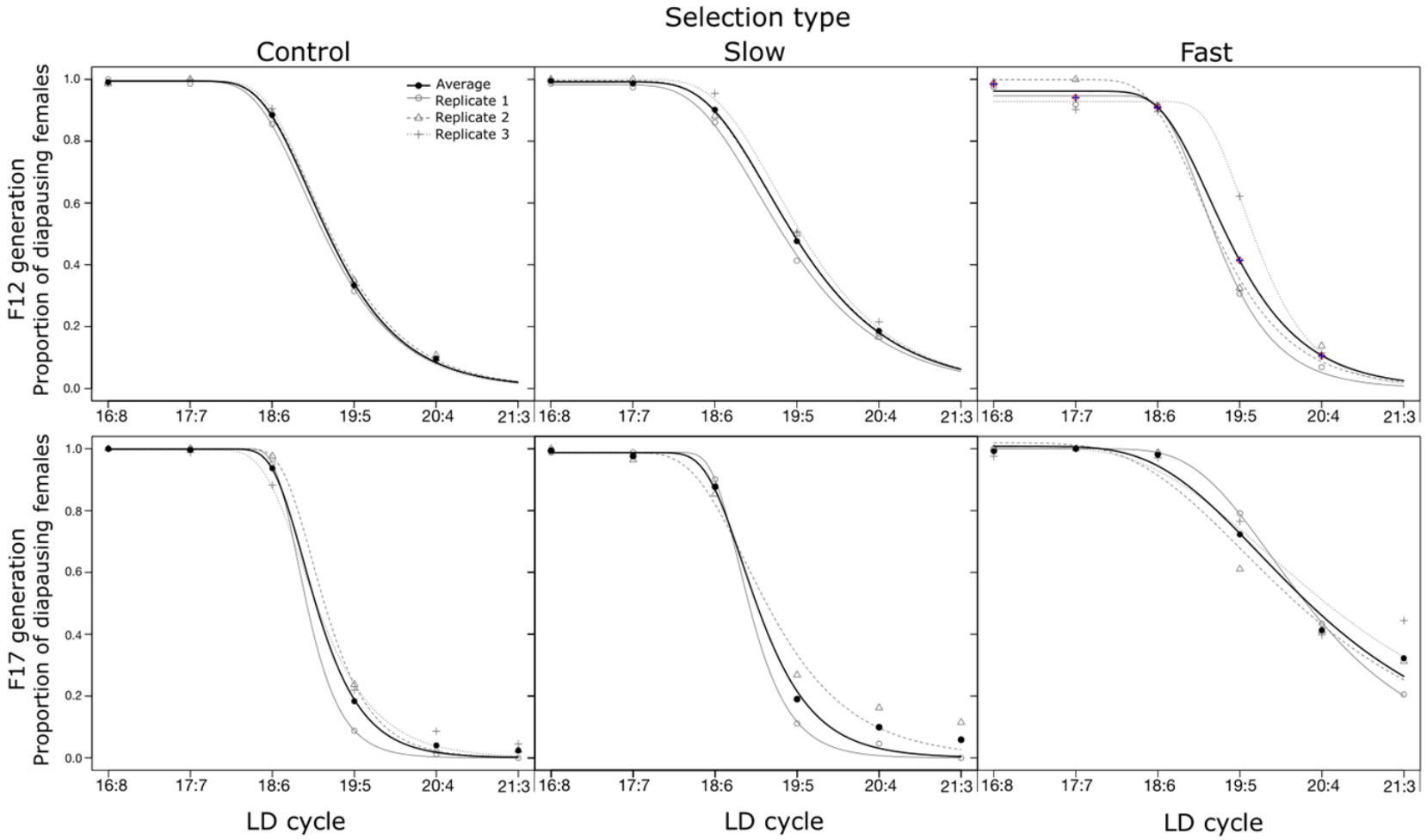

Figure 3. Photoperiodic response curves (PPRCs) of the females of control, slow and fast line replicates and average (black) of replicates of each selection type line measured in different light-dark (LD) cycles in generations F12 and F17. Lines are fitted using dose response model predictions.

CDL of the base population was 19.3:4.7 LD (Fig A3). CDLs of the control, fast and slow line were in F12 generation 18.6:5.4, 18.7:5.3 and 18.8:5.2 and in F17 generation 18.4:5.6, 19.8:4.2 and 18.4:5.6, respectively. In F12 generation the CDLs of fast and slow line differed from that of the control line by 0.1 and 0.2 hours, respectively, both differences being significant (DRM: Control line vs. fast line; EST = 0.18, SE = 0.06, t = -2.95, p = 0.003 and control line vs. slow line; EST= 0.2,SE = 0.062, t = 3.328, p < 0.001). In F17 generation CDL was significantly (1.4 hours) shorter in the control line than in the fast line (DRM: EST= 0.94, SE = 0.06, t = -14.61, p < 0.001), but there was no significant difference between the control and slow lines (DRM; EST= 0.04, SE = 0.05, t = -0.91, p = 0.364).

3.3 Body weight

An interaction plot produced from the LMM model for body weight is shown in Figure 4, full results of this model in Table A4, the mean weight values for the control and selection line replicates in Table A1 and the violin plot showing the variation in body weight between control and both selection line replicates in Figure A4. According to the LMM analyses, 1.07% of variation in body weight was explained by the differences between replicates, and there were significant differences between the sexes (p<0.001) and the two generations (p<0.001). In F12 generation, selection for fast development did not have a uniform effect on body weight when compared to that of the control line (p = 0.51; see details in Table A4), while the flies of the slow line were significantly heavier than those of the control line (p = 0.013). In F17 generation the weight of the control line flies had increased, that of the fast line flies remained constant and that of the slow flies decreased (Fig. 4). There was also a small control vs. slow line * sex interaction (p = 0.029), which can be explained by a larger sex disparity in the control line in F12.

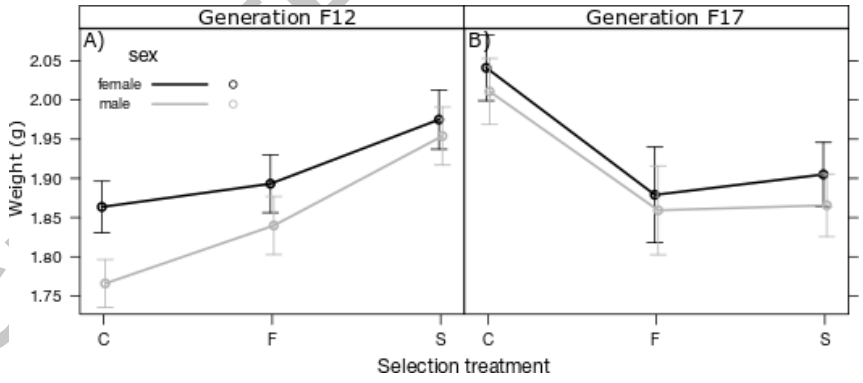

Figure 4. Interaction plot of selection regime (C = control, F = fast and S = slow selection line), sex, and generation for flies' predicted body weight in A) F12 and B) F17 generations from a LMM model of weight. Error bars refer to the model predicted standard errors.

3.4 Cold tolerance

An interaction plot produced from the LM model for flies' CTmin values is shown in Figure 5, the full result of this model in Table A5, the mean CTmin values for each replicate in Table A6 and the violin plot showing the variation in CTmin values between control and both selection line replicates in Figure A5. The test revealed a marginally significant difference between the sexes (p = 0.042), a large significant difference between generations (p < 0.001) and significant interactions between the generations and control vs. fast line (p = 0.006) and control vs. slow line (p = 0.001). As with body weight, selection for fast development did not

have a uniform effect on flies' cold tolerance in F12 generation (p = 0.1; see details in Table A6), while the flies of the slow line were significantly less cold-tolerant than those of the control line (p = 0.01). However, in F17 generation the flies of both selection lines were more cold-tolerant than the control line flies (Fig. 5). Apart of the fast line F17 generation males, the pattern seen in CTmin values reflected that of the body weight. However, interpretation of the CTmin results should be done with caution, as the pattern is less consistent and the level of variance larger than that of the body weight (Fig. 5).

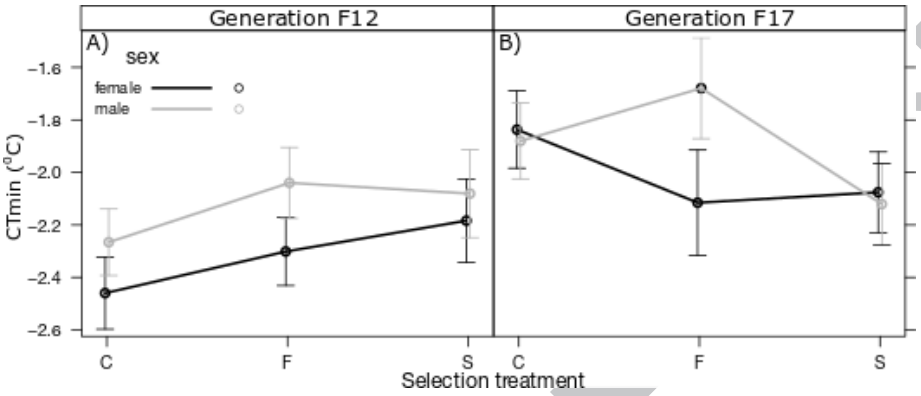

Figure 5. I Interaction plot of selection regime (C = control, F = fast and S = slow selection line), sex, and generation for flies' predicted CTmin values in A) F12 and B) F17 generations from a LM model of CTmin. Error bars refer to the model predicted standard errors.

4. DISCUSSION

In the present study, bi-directional selection on *D. montana* flies' pre-adult development time was successful towards slow, but not towards fast development. This kind of asymmetry is a common trend in selection experiments and it reflects the susceptibility of artificial selection to selection pressures prevailing in wild populations. Even though selection for fast development was not successful per se, the last five generations of selection in this direction increased variation in females' diapause propensity under different day lengths and led to longer CDL. Effects of selection on flies' body size and cold-tolerance were more ambiguous. After five generations of selection, the flies of the slow line were significantly heavier and less cold-tolerant than those of the control line, while after another five generations the situation was reversed.

Fast pre-adult development can increase organisms' fitness e.g. through increased survival of larvae under crowded conditions, early reproduction in expanding populations and a reduced risk of parasitism (Boras, 2000; Takahashi et al., 2013). Accordingly, directional selection for fast development time may have reduced additive genetic variation for it in wild populations, which can be seen in asymmetric responses in selection experiments (e.g. Clarke et al., 1961; Bell, 2008). However, fast development may not be maximized in wild populations of some species, like *Drosophila melanogaster*, due to trade-offs with traits like body size and viability (Zwaan et al., 1995; Nunney, 1996; Chippindale et al., 1997; Prasad et al., 2000). For example, Zwaan et al. (1995) selected the flies of this species for fast and slow development for 16 generations and found the mean development time of both the fast (9.5 days) and the slow (13.5 days) line to differ significantly from that of in the control line (11 days). Also, Chippindale et al. (1997) exercised selection for fast development for 125 generations and succeeded to shorten *D. melanogaster* flies' development time by 33 - 37 hours (15 - 17 %). *D. montana* flies' pre-adult development time is much longer than that of *D. melanogaster*, and in this species the selection for fast and slow pre-adult development

time led to clearly asymmetric responses. The median pre-adult development time of the flies was about 32 days in base population and varied around 32 - 34 days, 30 - 32 days and 36 - 40 days in control, fast and slow lines, respectively, only the difference between the control and slow line being significant. *D. montana* flies are practically univoltine in northern Finland (Lumme et al., 1974), and thus selection on pre-adult development time in this species is likely to favour an intermediate development rate.

Asymmetric responses in insects' pre-adult development time can also be due to directional dominance of alleles for fast development or the existence of "developmental barrier" preventing development at a rate appreciably faster than that of the foundation population (Clarke et al., 1961). Furthermore, the outcome of selection can be confounded by inbreeding and/or the accumulation of deleterious alleles (Chippendale et al., 1997), environmental factors like food, larvae density and the level of metabolic wastes (Boras, 2000) cross-generation effects and unconscious selection for other traits (Lintz & Gruwez, 1972) and/or by genetic constraints (Chenoweth et al., 2010). Our *D. montana* base population consisted of the progenies of 102 wild-caught fertilized females and its size was kept between 1000 and 2000 flies in each generation prior to selection to prevent inbreeding. During selection, the parental flies of each replicate involved 30 females and 30 males per generation, which should help to avoid excessive inbreeding (see Weber & Diggins, 1990).

Correlated response of secondary trait may exceed the response of the selected trait, if it possesses higher genetic variation than the selected one and if the two traits are genetically correlated (Bell, 2008). In our experiment, the last 5 generations of selection for faster pre-adult developmental time increased females' CDL, while the selection for slow development had no effect on this trait. Our earlier studies have shown that CDL possesses high genetic variation in *D. montana* Oulanka population (66.22°N), where the study flies come from, and that it decreases along a latitudinal cline from about LD 19:5 in northern Finland (67°N) to LD 16:6 in southern Finland (60°N) (Tyukmaeva et al., 2011; Lankinen et al., 2013). In the present study, selection for fast development increased variation females' tendency to enter diapause under different day lengths and changed CDL towards longer day length (earlier time of summer). The steep PPRCs of the control and slow line at the end of selection show that the change in the proportion of diapausing females from 100 % to 0 % (in calendar time from 0 % to 100 %) occurs within one hour around the CDL. However, the PPRC of the fast line was more gradual, and over 30 % of emerging females entered diapause even at LD 21:3. This kind of curves are usually detected only for mass populations (like the base population of the present study), and only rarely for more inbred lines (Lankinen et al., 2013). CDL of the control and slow line (18.4:5.6 and 18.3:5.7, respectively) correspond July 31st- August 1st and that of the fast line (LD 19.3:4.7) July 24th at flies' home site, which means that the emerging fast line females would enter diapause about one week earlier than the control and slow line females.

High response of CDL to selection on flies' pre-adult development time raises a question on how the two traits are linked with each other. One possibility is that the linkage is due to the pleiotropic effects of insulin and ecdysone signaling pathways and juvenile hormone (JH) (Flatt & Kawecki, 2004; Flatt et al., 2005; Mirth & Shingleton, 2012; Mendes & Mirth, 2016). In *D. melanogaster* insulin / TOR (target-of-rapamycin) pathway interacts with JH and ecdysone to regulate the timing of development and hence the duration of growth (Mirth & Shingleton, 2012; Mirth et al., 2014). Flies' pre-adult development time is affected by changes in insulin signaling that occurs before a larva has reached the critical size, while their body and organ size are affected by changes in insulin signaling after a larva has passed this stage (Shingleton et al., 2005). On the other hand, experiments with adult reproductive diapause in *D. melanogaster* and several other insect species provide strong evidence that

insulin / FOXO (forkhead transcription factor) pathway, JH and ecdysones are important regulators of diapause (Richard et al., 2001; Sim & Denlinger. 2013). Here insulin signaling is suppressed during short day / long night conditions (Sim & Denlinger, 2013), which in turn suppresses JH synthesis and induces diapause.

Another possible link between flies' pre-adult development time and photoperiodically controlled diapause is the time-measuring systems. In *D. melanogaster*, flies' pre-adult development time is regulated by interaction between light regimes and circadian clock, faster clocks speeding up and slower clocks slowing down development (Kyriacou et al., 1990; Klarsfeld & Rouyer, 1998; Kumar et al., 2006; Yadav & Sharma, 2013; Yadav et al. 2014). The timing of flies' diapause, on the other hand, has been shown to be regulated by a photoperiodic timer in several insect species (Saunders, 2002). In *D. montana*, the photoperiodic time measurement is reset after eclosion so that the sensitive period for diapause induction lasts for as few days after emergence. However, the connection between flies' pre-adult development time and diapause induction could be explained by possible collaboration of the two clock mechanisms after eclosion (Kostal, 2011) and / or by the interaction between the flies' endocrine and circadian systems (Bloch et al., 2013).

Effects of development time selection on flies' body size and cold-tolerance were quite ambiguous. After five generations of selection, the flies of the slow line were significantly heavier and less cold-tolerant than those of the control line, while after another five generations the situation was reversed. Some of these changes in these traits could have been affected by environmental factors in addition to genetic changes. The last emerged flies of the control and slow line may have suffered from the lack of food and high level of metabolic wastes, which could also have affected their cold tolerance. Furthermore, most deleterious mutants delay larval development, and hence the lines selected for slow development will tend to accumulate pathological traits that have little bearing on the evolutionary potential of the species (Nunney, 1996). It should be kept in mind that artificial selection provides no information on the genetic covariance or correlation between two unselected traits (Conner, 2003), and thus one should not draw conclusions on connections between body size and weak cold-resistance.

5. CONCLUSIONS

Fast development and a right timing of diapause are important fitness traits under short growing period, and the selection pressures on these traits may be quite different in populations with different voltinism patters. The lack of additive variation for fast development in *D. montana* suggests that pre-adult development time is in this species under strong directional selection, while the timing of diapause (CDL) is likely to be under stabilizing selection (Lankinen et al., 2013). Strong pleiotropy or tight linkage between these traits could lead to problems in wild populations, as the females that develop fast and enter diapause too early may not survive over the winter. However, as Phelan et al. (2003) have shown, the correlations between functional characters are not necessarily durable features of a species, and short-term evolutionary responses cannot be extrapolated reliably to longer-term evolutionary patterns. Also, flies' developmental time may show high plasticity in insects living in unstable environments, as each preimaginal stage during insect development plays a role in the regulation of time needed for body development (genetic pattern) and in fitting developmental time to environmental conditions (phenotype plasticity) (do Nascimento et al., 2002).

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APPENDIX

Table A1. Mean body weight (mg) ± SEM of the flies (mg) in the three replicates of fast, control and slow lines in F12 and F17 generations. Number of studied flies is given in parenthesis. 2nd replicate of slow developing line was lost in generation F16.

Line	Replicate	F12 generation		F17 generation	
		Females	Males	Females	Males
Fast	1	1,92±0,03 (68)	1,89±0,03 (77)	1,95±0,03 (31)	1,90±0,04 (39)
	2	1,85±0,03 (75)	1,80±0,03 (84)	1,93±0,04 (30)	1,85±0,03 (34)
	3	1,90±0,03 (95)	1,83±0,03 (75)	1,71±0,05 (25)	1,82±0,06 (24)
Control	1	1,90±0,03 (94)	1,81±0,03 (94)	1,98±0,05 (45)	2,01±0,04 (54)
	2	1,85±0,02(106)	1,77±0,03 (128)	2,11±0,04 (65)	2,11±0,04 (55)
	3	1,85±0,03 (93)	1,74±0,03 (122)	2,01±0,04 (67)	1,98±0,03 (67)
Slow	1	2,04±0,03 (63)	2,04±0,03 (63)	1,96±0,03 (93)	1,92±0,03 (99)
	2	1,91±0,03(74)	1,86±0,03 (78)		
	3	1,97±0,03 (81)	1,95±0,03 (89)	1,91±0,03 (93)	1,88±0,03 (96)

Table A2. Median pre-adult developmental time (IQR; interquartile range) of the females and males of the base population in F7 generation and three replicates of fast, slow and control lines in F12 and F17 generations. Number of studied flies is given in parenthesis. 2nd replicate of the slow developing line was lost in generation F16.

	Median (IQR)											
	Generation											
	F7				**F12**				**F17**			
	Females	**N**	**Males**	**N**	**Females**	**N**	**Males**	**N**	**Females**	**N**	**Males**	**N**
Fast					30,27 (2,75)	145	30,46 (2,50)	160	32,77 (1,00)	249	32,90 (1,50)	231
					29,02 (1,38)	169	28,52 (1,94)	149	31,65 (0,88)	315	31,65 (1,13)	283
					30,02 (3,06)	201	29,65 (2,97)	180	30,40 (1,13)	288	30,27 (1,13)	303
Control					30,89 (2,50)	192	30,77 (3,03)	158	32,02 (3,50)	202	31,90 (3,81)	245
	31,79 (4,04)	710	31,79 (4,62)	646	31,40 (4,28)	156	30,90 (5,3)	176	32,46 (4,63)	166	32,21 (5,75)	158
					30,40 (1,75)	165	30,65 (2,63)	184	33,33 (7,81)	170	33,27 (7,87)	179
Slow					34,52 (8,13)	187	35,08 (9,47)	168	34,52 (10,81)	208	37,02 (10,41)	218
					34,15 (6,13)	182	34,08 (6,44)	206				
					36,40 (8,13)	159	37,21 (10,41)	164	39,15 (9,44)	201	42,15 (9,50)	207

IQR = Interquartile range; N = number of individuals tested

Table A3. Generalized linear mixed model with response variable pre-adult developmental time and explanatory variables selection regime, sex and generation as fixed factors and replicate as random factor.

Random effects

	SD	Variation
Replicate	0.012	0.000
Residual	0.124	0.015

Fixed effects

	Estimate	SE	t-value	p-value
(Intercept)	**3.458**	**0.019**	**186.84**	**<0.001**
Selection_Fast	-0.039	0.027	-1.45	0.147
Selection_Slow	**0.143**	**0.025**	**5.61**	**<0.001**
Sex_Male	0.007	0.007	1.00	0.319
Generation_F17	**0.06**	**0.007**	**8.31**	**<0.001**
Selection_Fast: Sex_Male	-0.009	0.010	-0.85	0.396
Selection_Slow: Sex_Male	0.002	0.010	0.18	0.861
Selection_Fast: Generation_F17	-0.017	0.010	-1.72	0.085
Selection_Slow: Generation_F17	-0.019	0.011	-1.69	0.090
Selection_Fast: Sex_Male: Generation_F17	0.007	0.014	0.47	0.640
Selection_Slow: Sex_Male: Generation_F17	**0.032**	**0.015**	**2.13**	**0.033**

Tabble A4. Linear mixed model with response variable body weight and explanatory variables selection regime, sex and generation as fixed factors and replicate as random factor.

Random effects

	SD	Variation
Replicate	0.046	0.002
Residual	0.283	0.080

Fixed effects

	Estimate	SE	t-value	p-value
(Intercept)	**1.864**	**0.031**	**59.81**	**<0.001**
Selection_Fast	0.030	0.045	0.66	0.510
Selection_Slow	**0.111**	**0.045**	**2.47**	**0.013**
Sex_Male	**-0.098**	**0.023**	**-4.30**	**<0.001**
Generation_F17	**0.177**	**0.027**	**6.57**	**<0.001**
Selection_Fast: Sex_Male	0.044	0.035	1.27	0.204
Selection_Slow: Sex_Male	**0.077**	**0.035**	**2.18**	**0.029**
Selection_Fast: Generation_F17	**-0.192**	**0.045**	**-4.25**	**<0.001**
Selection_Slow: Generation_F17	**-0.247**	**0.040**	**-6.19**	**<0.001**
Sex_Male: Generation_F17	0.07	0.038	1.79	0.074
Selection_Fast: Sex_Male: Generation_F17	-0.034	0.062	-0.54	0.588
Selection_Slow: Sex_Male: Generation_F17	-0.086	0.055	-1.57	0.166

Table A5. Linear model with cold tolerance (CTmin) as response variable and explanatory variables selection regime, generation and sex as fixed factors.

Fixed effects

	Estimate	SE	t-value	p-value
(Intercept)	-2.46	0.07	-35.16	<0.001
Selection_Fast	0.16	0.10	1.65	0.100
Selection_Slow	**0.28**	**0.11**	**2.58**	**0.010**
Sex_Male	**0.19**	**0.10**	**2.04**	**0.042**
Generation_F17	**0.62**	**0.10**	**6.07**	**0.000**
Generation_F17: Sex_Male	-0.24	0.14	-1.67	0.095
Selection_Fast: Sex_Male	0.07	0.13	0.50	0.616
Selection_Slow: Sex_Male	-0.09	0.15	-0.60	0.549
Selection_Fast: Generation_F17	**-0.44**	**0.16**	**-2.74**	**0.006**
Selection_Slow: Generation_F17	**-0.51**	**0.15**	**-3.37**	**0.001**
Selection_Fast: Sex_Male: Generation_F17	0.41	0.22	1.85	0.064
Selection_Slow: Sex_Male: Generation_F17	0.09	0.22	0.41	0.679

Table A6. Mean CT min (°C) values for the females and males in the three replicates of fast, slow and control lines in F12 and F17 generations. Number of studied flies is given in parenthesis. 2nd replicate of slow developing line was lost in generation F16.

Line	**Replicate**	**F12 generation**		**F17 generation**	
		Females	**Males**	**Females**	**Males**
Fast	1	-2,24±0,12 (42)	-2,07±0,11 (43)	-2,12±0,25 (25)	-1,66±0,25 (27)
	2	-2,39±0,10 (47)	-2,08±0,11 (50)	-1,97±0,24 (19)	-1,66±0,24 (25)
	3	-2,28±0,10 (64)	1,97±0,12 (49)	-2,25±0,22 (20)	-1,74±0,30 (18)
Control	1	-2,45±0,16 (41)	-2,34±0,08 (42)	-1,94±0,11 (32)	-1,73±0,17 (39)
	2	-2,49±0,06 (51)	-2,40±0,06 (61)	-1,92±0,15 (42)	-1,91±0,13 (37)
	3	-2,43±0,007 (49)	-2,12±0,10 (63)	-1,69±0,17 (45)	-1,98±0,11 (48)
Slow	1	-2,33±0,08 (29)	-2,20±0,10 (23)	-2,10±0,13 (50)	-2,15±0,11 (51)
	2	-2,10±0,15 (35)	-2,27±0,09 (32)		
	3	-2,16±0,07 (38)	-1,85±0,15 (37)	-2,06±0,11 (59)	-2,10±0,12 (56)

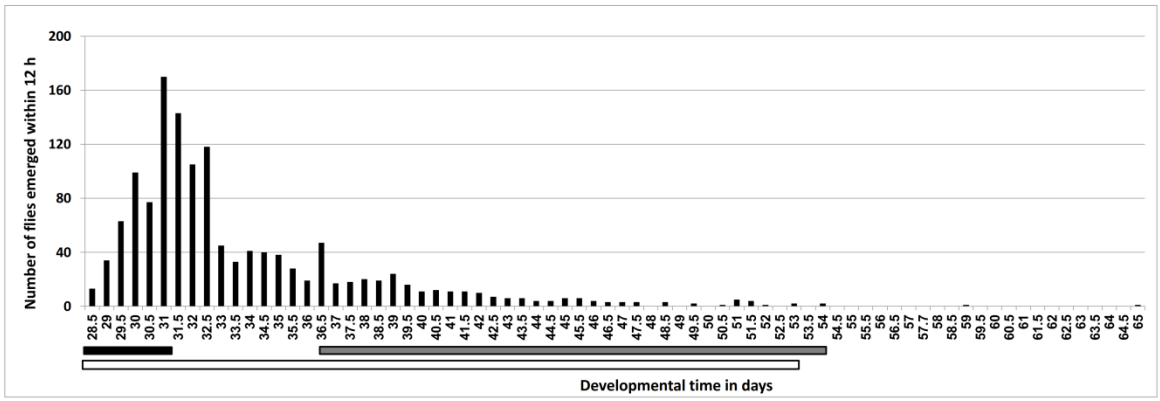

Figure A1. Distribution of the flies' pre-adult development time in F7 generation, where selection was started. Pre-adult development times of the parental flies chosen to establish the three replicates of fast (black) and slow (grey) lines and control (white) lines are shown below.

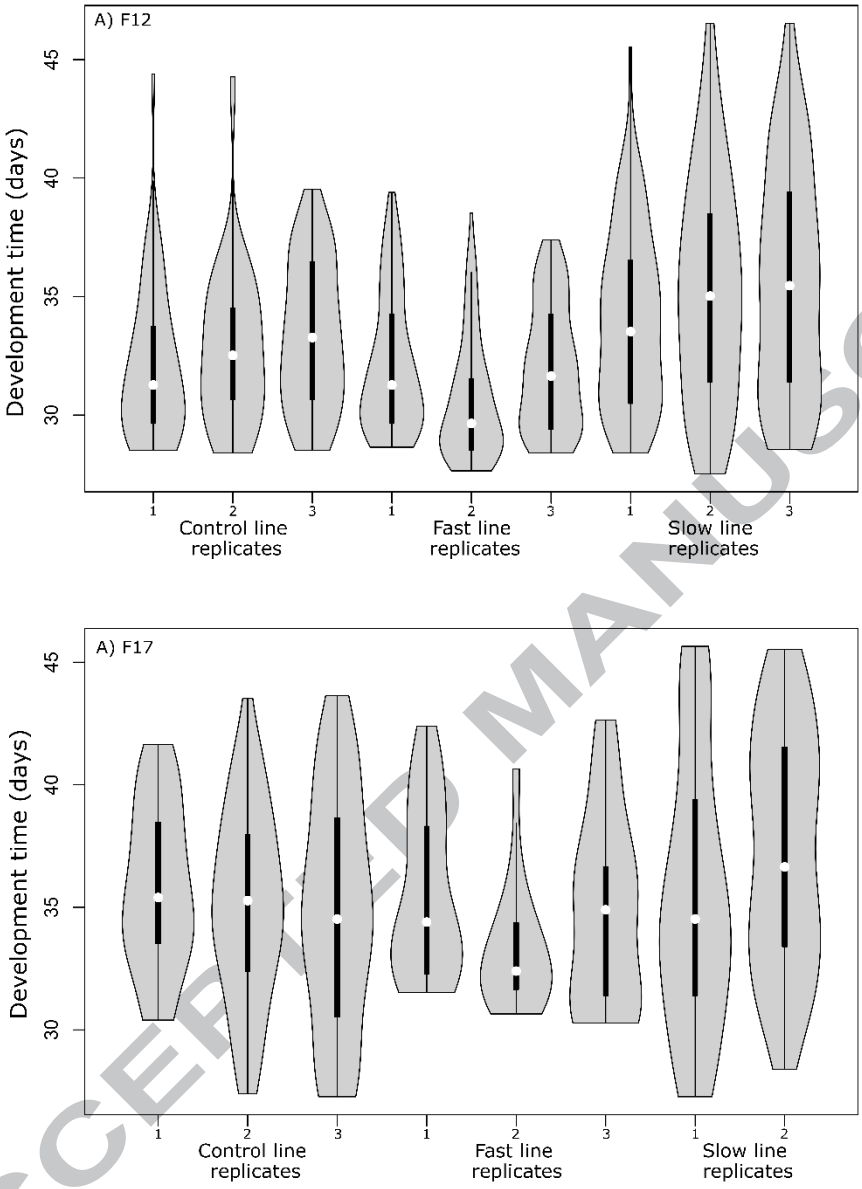

Figure A2. Violin plot showing variation in fly pre-adult development time between control and selection line replicates in A) F12 an B) F17 generations.

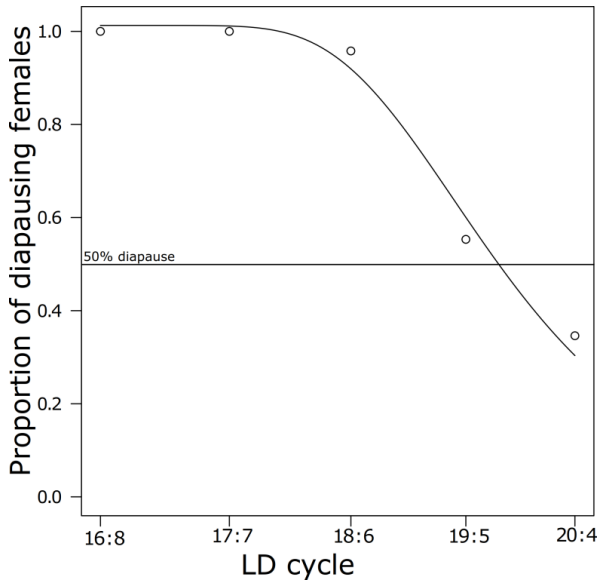

Figure A3. Photoperiodic response curve (PPRC) of the females of base line measured in different light-dark (LD) cycles.

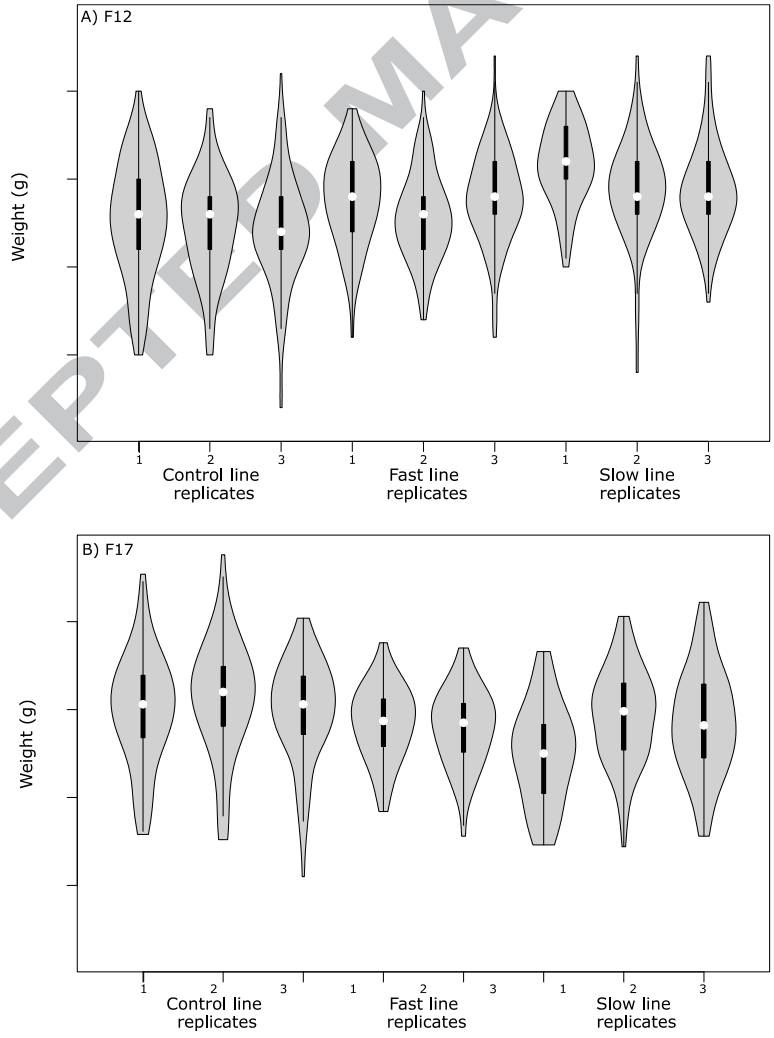

Figure A4. Violin plot showing variation in body weight between control and selection line replicates in A) F12 an B) F17 generations.

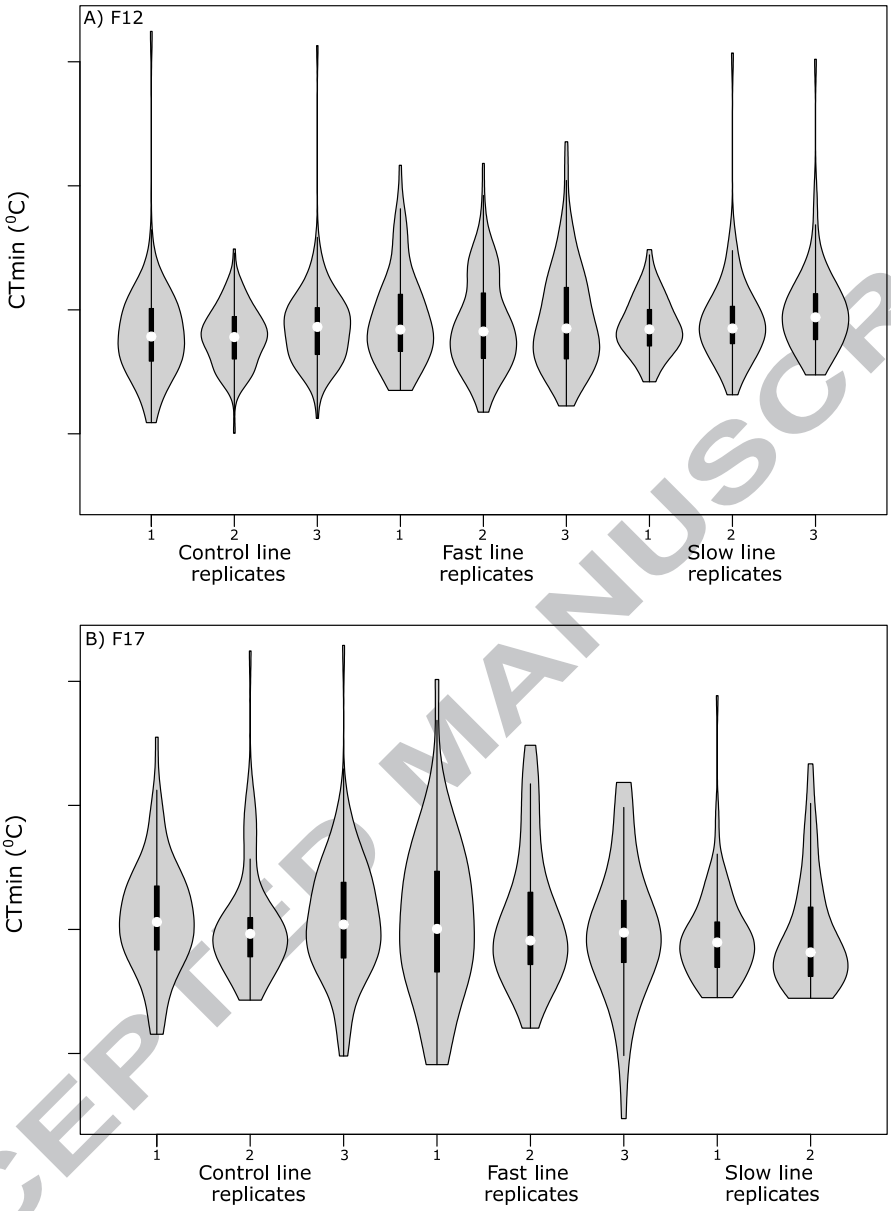

Figure A5. Violin plot showing variation in CTmin values between control and selection line replicates in A) F12 an B) F17 generations.

Highlights

- Selection was successful towards slow, but not towards fast development
- Selection towards fast development led to longer critical day length for diapause
- Effects of selection on body weight and cold-tolerance showed no clear trends